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Tissue-Specific Regulation of Mouse Hepatocyte Nuclear Factor 4 Expression

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Hepatocyte nuclear factor 4 (HNF-4) is a liver-enriched transcription factor and a member of the steroid hormone receptor superfamily. HNF-4 is required for the hepatoma-specific expression of HNF-1α, another liver-enriched transcription factor, suggesting the early participation of HNF-4 in development. To prepare for further study of HNF-4 in development, the tissue-specific expression of the mouse HNF-4 gene was studied by analyzing the promoter region for required DNA elements. DNase-hypersensitive sites in the gene in liver and kidney tissues were found in regions both distal and proximal to the RNA start site that were absent in tissues in which HNF-4 expression did not occur. By use of reporter constructs in transient-transfection assays and with transgenic mice, a region sufficient to drive liver-specific expression of HNF-4 was identified. While an HNF-1 binding site between bp −98 and −68 played an important role in the hepatoma-specific promoter activity of HNF-4 in transient-transfection assays, it was not sufficient for the liver-specific expression of a reporter gene in transgenic mice. Distal enhancer elements indicated by the presence of DNase I-hypersensitive sites at kb −5.5 and −6.5, while not functional in transient-transfection assays, were required for the correct expression of the mouse HNF-4 gene in animals.

Several liver-enriched transcription factors, proteins that are apparently important in liver-specific gene expression, are known: hepatocyte nuclear factor 1 (HNF-1), HNF-3, and HNF-4, as well as C/EBP or variants of this group of proteins (for a review, see references 12 and 21). Genes expressed only or mainly in the liver have repeatedly been found to have binding sites for these transcription factors, suggesting that cell-specific gene expression at least in adult hepatocytes relies on the combinatorial use of this group of transcription factors. While these factors are expressed only in a limited number of adult tissues, none appears to be liver specific. Cell-specific transcription of these factors is an important element of their distribution in specific cell types (24). Determination of how this adult pattern of expression is established requires knowledge of the regulation of the genes for these factors during embryogenesis.

In this paper, we describe the analysis of the tissue-specific expression of the mouse HNF-4 gene both in transient-transfection assays and in transgenic mice. DNase I-hypersensitive sites in the presumptive HNF-4 promoter and distant upstream regions (up to kb −6.8) were present in the chromatin only in tissues in which the gene was expressed. Transient-transfection assays with hepatic and nonhepatic tumor cell lines identified a region (bp −228 to +182) sufficient to drive hepatoma-specific expression of a reporter gene. HNF-1α appeared to be a major protein binding to this region (bp −98 to −68) in crude liver nuclear extracts. However, the promoter-proximal region alone was not sufficient to drive liver-specific expression of a reporter gene in transgenic mice. A segment of the upstream region containing the DNase I-hypersensitive sites, while not active in transient-transfection assays, was required for the correct expression of the HNF-4 gene in mice.

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MATERIALS AND METHODS

HNF-4 genomic DNA clone screening. HNF-4 genomic DNA clones were obtained by screening a mouse λEMBL4 genomic DNA library (a gift from N. Heintz) with a 0.5-kb rat HNF-4 cDNA fragment coding the N-terminal HNF-4 protein. The screening method was as described elsewhere (22), with hybridization and final washing at 65°C. The resultant genomic clone (c7II) was analyzed by restriction mapping, probe hybridization, and subcloning. All the intron-exon junctions and the ~1 kb of the immediate 5′ upstream region in c7II were sequenced by the dideoxyl method (18).

Primer extension. For primer extension, 1 ng of ^{32}P-labeled oligonucleotide probe was hybridized with 30 μg of total RNA or 5 μg of mRNA at 60°C (0.4 M NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.4]–1 mM EDTA, in 20 μl) for 3 h. Transcription with avian myeloblastosis virus reverse transcriptase (50 U/50 μl) was carried out at 43°C for 45 min in a solution of 150 mM KCl, 6 mM $MgCl_2$, 50 mM Tris-Cl (pH 8.3 at 43°C), 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphates, and 0.75 U of RNasin per μl. The primer extension products were electrophoresed in denaturing 8% polyacrylamide gels.

DNase I-hypersensitive site analysis. Two probes, the 5′ *Bgl*II-*Pst*I fragment (~0.15 kb) and the 5′ *Eco*RV-*Pst*I fragment (~0.2 kb) from c7II (see Fig. 1), were used to map the DNase I-hypersensitive sites in the 1.9-kb *Bgl*II fragment (kb −0.8 to +1.1, containing the RNA start site) and the ~10-kb *Eco*RV fragment (kb −10 to −0.5), respectively. Nucleus isolation and digestion, as well as DNase I-hypersensitive site mapping, were as described elsewhere (16).

HNF-4 promoter-reporter constructs and transient-transfection assays. HNF-4 enhancer/promoter-reporter plasmids

were constructed by inserting HNF-4 5′ upstream fragments (from various 5′ deletions to bp +182) into a plasmid vector containing the promoterless luciferase reporter gene. 5′ deletions of the HNF-4 upstream region were achieved either by restriction enzyme digestion (kb −6.8, *Bgl*II; bp −228, *Sty*I; and bp +27, *Dra*II) or by mung bean nuclease over digestion after *Bam*HI (bp −363) or *Hph*I (bp −34) digestion. These fragments were cloned into the promoterless pZLUC (15), between the poly(A) trimer and the promoterless luciferase reporter gene. The −363, −228, −34, and +27 fragments were cloned between *Sma*I and *Hin*dIII sites, while the fragment from kb −6.8 to −1.4 was cloned between the *Bam*HI and *Hin*dIII sites. Internal deletion constructs were made by cloning the respective blunted restriction fragments (*Sal*I-*Xba*I [kb −7.5 to −5.1], *Bgl*II-*Xba*I [kb −6.8 to −5.1], and *Sty*I-*Bst*NI [bp −228 to −159]) into the blunted *Bam*HI site of respective constructs (−1.4 kb and −34 bp). Three extra nucleotides (5′-ATC) exist between bp +182 and the pZLUC *Hin*dIII site. The HNF-4 initiation codon is not in frame with that of the luciferase gene, with a stop codon 1 bp 5′ to the luciferase ATG.

HepG2 cells were cultured in a solution of 50% Dulbecco modified Eagle medium, 50% Ham F12 medium, and 1× nonessential and essential amino acids (GIBCO), supplemented with 10% fetal calf serum. C2 cells were cultured in Dulbecco modified Eagle medium with 10% fetal calf serum. DNA transfections were performed by the method of Maniatis et al. (14), with modifications (22). For each construct, the molar equivalent of 5 μg of pzLUC per transfection was used. All transfections were repeated several times. Cellular extracts were in 0.1 M KPO_4 (pH 7.8) and 1 mM dithiothreitol. Luciferase assays were performed by adding 100 μl of luciferin (1 mM in 25 mM Gly-Gly (pH 7.8), 75 mM Mg_2SO_4, 4 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]) into 200 μl of extract mixture (50 μl of extract, 10 mM $MgCl_2$, 0.1 M KPO_4, 5 mM ATP) and counted for 10 s (6). The results were normalized to β-galactosidase (β-Gal) internal control values (13).

Crude nuclear extract preparation, DNase I footprinting, and gel mobility shift assay. Crude nuclear extract preparation and mobility shift assay were by the method described elsewhere (22). Oligonucleotide probes used for gel shift experiments are as follows:

4 FP1, GATCACGGGGGTGATTAACCATTAACTCCTACC
TGCCCCCACTAATTGGTAATTGAGGATGGGATC

β28, as described by Baumhaueter et al. (2).

The probes were labeled by filling in with Klenow enzyme.

For DNase I footprinting, the DNA template (bp −363 to +49) was generated by PCR, using ^{32}P-labeled oligonucleotide probes (by polynucleotide kinase) at either end. The labeled probe (10^4 cpm) was incubated with protein extracts (4 μg) at room temperature for 20 min in a solution of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 60 mM KCl, 4 mM $MgCl_2$, 0.5 mM dithiothreitol, and 10% glycerol. The reaction mixture was then digested with DNase I by adding an equal volume of a solution of 5 mM $CaCl_2$, 1 mM EDTA (pH 8.0), and ~24 μg of DNase I per ml. The digested DNA fragments were electrophoresed in a 6% denaturing acrylamide gel.

Transgenic mice. The parent β-Gal construct was made by ligating at the *Eco*RV site the 5′ *Sal*I (5′)-*Eco*RV β-Gal fragment (promoterless), which also contained the 5′ cloning sites from pMC1871 (19) with the *Eco*RV (5′)-*Hin*dIII (blunted) fragment from pCMVβ-gal (13), which contained the 3′ β-Gal fragment and the simian virus 40 (SV40) polyadenylation sites (nucleotides 2533 to 2729) immediately 3′ to the β-Gal gene. The resultant β-Gal–SV40–polyadenylation site fragment was cloned into pBluescript (*Sal*I-*Xba*I [blunted]). The HNF-4 enhancer/promoter elements were then cloned 5′ to the β-Gal gene, either at the *Sma*I site (in the pMC 1871 polycloning sites, for the bp −363 to +182 fragment [EV-1]) or between the *Xho*I (on pBluescript) and *Sma*I sites (for the kb −7.5 to +182 fragment [S-1]). The HNF-4 translation initiation codon (+169 to +171) was in frame with the β-Gal gene (starting from amino acid 9). As a result, an extra 14 amino acids (5 from HNF-4 and 9 from pBluescript polycloning sites when HNF-4 fragments were cut out for the cloning: MDMAESSLSIPGDP) were attached to the N terminus of the β-Gal protein.

For transgenic mice, the above constructs were cut with *Sal*I (on the EV-1 construct) or with *Eco*RI (in the HNF-4 5′ upstream region, at kb −7.2) and *Sal*I (S-1 construct). The HNF-4–β-Gal–SV40 fragments were isolated from low-melting-point agarose gels and further purified by being passed through Elutip columns (Schleicher & Schuell). The DNA was resuspended in TM buffer (5 mM Tris-Cl [pH 7.5], 0.1 mM EDTA; 1.5 μg/ml) for the microinjection.

Transgenic animals were generated by the method of Hogan et al. (9). The donor mice were FVB/N, and the recipient mice were CD-1.

Mice carrying the transgene were determined by genomic Southern blot analysis with DNA isolated from tail tissue and probed with β-Gal and HNF-4 probes.

RNA isolation, poly(A) selection, and Northern (RNA) blot analysis were carried out as described elsewhere (22). An RNase T_2 protection assay was performed by the method of Costa et al. (3) with minor modifications. The antisense probe was the *Bss*HII and *Eco*RV fragments of the β-Gal gene.

For X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining, the embryos were fixed with 2% formaldehyde and 0.2% glutaraldehyde for 1 to 2 h and stained with X-Gal at 37°C for up to 24 h in a solution of 100 mM sodium phosphate (pH 7.3), 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, and 1 mg of X-Gal per ml. The embryos were dehydrated in methanol and cleared in benzyl alcohol–benzyl benzoate (2:1).

RESULTS

The mouse HNF-4 gene. HNF-4 genomic clones were obtained by screening a λEMBL4 mouse genomic DNA library. One clone, c7II, was obtained after ~4×10^5 recombinants were screened with a cDNA probe containing the N-terminal coding region including part of the DNA-binding domain. This clone (Fig. 1), with a 16-kb insert, contained three exons encoding the first 526 bp (including amino acids 1 to 120) of the mouse HNF-4 cDNA. Further restriction mapping of the clone revealed that the restriction fragments of *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, and *Xba*I could account for every band on the genomic Southern blot (data not shown), in accordance with the earlier finding that mouse HNF-4 might be a single-copy gene residing on chromosome 2 (1).

The presumptive translation initiation codon was on exon 1, which encodes nucleotides 1 to 256 (nucleotide 1 is defined by primer extension as described below). The HNF-4 DNA-binding domain was encoded by exons 2 and 3, which comprise nucleotides 257 to 431 (encoding amino acids 30 to 88) and nucleotides 432 to 526 (encoding amino acids 88 to 120), respectively. Of the eight cysteines that make up the two zinc fingers, the first five are encoded by exon 2 and the other three

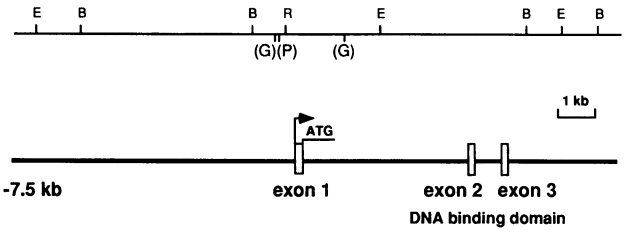


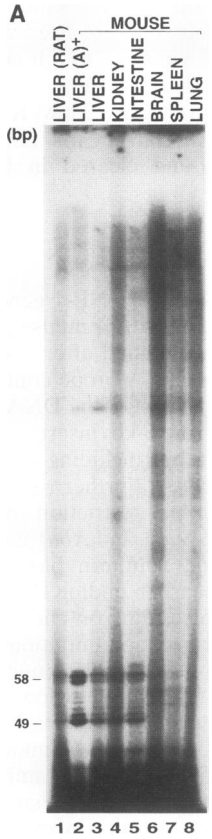
FIG. 1. Partial genomic structure of the mouse HNF-4 gene. The structure is based on that of clone c7II. (Top panel) Partial restriction enzyme map. E, *Eco*RI; B, *Bam*HI; R, *Eco*RV; G, *Bgl*II; P, *Pst*I. (The restriction sites in parentheses are not the only sites for these enzymes.) (Bottom panel) Partial genomic structure of mouse HNF-4. It contains the first three exons and introns and 7.5 kb of the 5′ upstream region. Arrowhead represents the RNA start site. ATG is the putative translation start site.

cysteines are encoded by exon 3. Of interest is the conservation of this unusual coding pattern between *Drosophila melanogaster* and mammals (25). Most steroid-thyroid superfamily members divide the eight cysteines, with four in each of two exons. The first intron is ~4.2 kb, and the second intron is ~0.7 kb. The third intron, which was not complete in the λ clone, is more than 3 kb. The upstream region present in this clone was ~7.5 kb.

Mouse HNF-4 transcription start site. In order to determine the transcriptional start site of the HNF-4 gene, a primer extension assay with RNA isolated from several different tissues was performed (Fig. 2A). A 24-nucleotide primer generated 10 primer-extended bands (of between 48 and 57 bp, with the 48-, 56-, and 57-bp bands stronger than the others). These bands were present only when the template RNA was from tissues (liver, kidney, and intestine) in which HNF-4 is expressed and not from the tissues (brain, spleen, and lung) in which HNF-4 is not expressed (22). No differences between poly(A)$^+$ RNA and total RNA (liver) were observed. Similar results were observed with total rat liver RNA, even though the signal is much weaker, presumably because the primer has a few mismatches (3 of 24) with the rat sequence. The rat bands were consistent with the bands observed by using primers further downstream for the primer extension assays (data not shown). By using anchor PCR, the primer extension products from mouse liver poly(A)$^+$ RNA were cloned and sequenced, and the sequence matched that of the genomic clone up to the nucleotide shown as +1 (data not shown). Furthermore, RNase T_2 protection analysis with an antisense RNA probe corresponding to the genomic sequences around the presumptive RNA start site (bp +182 to −560) generated protected bands that were consistent with those expected from the primer extension results with mouse liver, kidney, and intestine RNAs (data not shown). Thus, there appear to be 10 clustered HNF-4 start sites, 1 bp apart within a 10-bp region. The 3′-most start site (corresponding to the 48-bp band) was designated +1.

The sequence up to bp −1125 from the start site region was obtained from the c7II clone (Fig. 2B, and data not shown). No apparent TATA box, CAAT box, or Sp1 sites were found near the RNA start site. The absence of these conserved sequences may be related to the multiple initiating nucleotides (Fig. 2A).

Proximal and distal DNase I-hypersensitive sites in the mouse HNF-4 upstream region. To detect possible tissue-specific protein binding sites, DNase I hypersensitivities in the



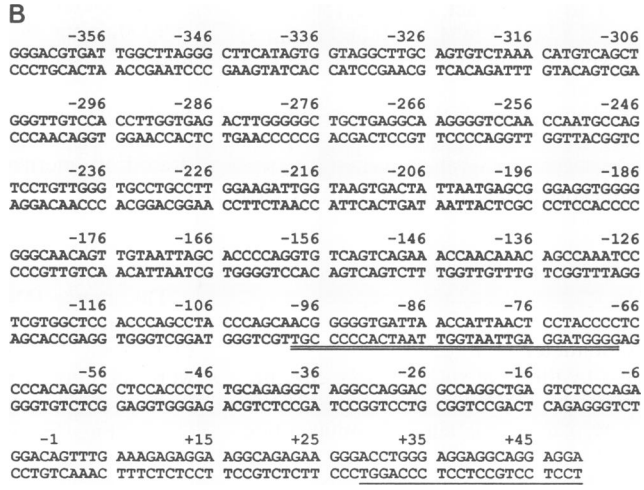


FIG. 2. Mouse HNF-4 RNA start site. (A) Primer extension analysis of mouse HNF-4 RNA start site. An oligonucleotide probe (24-mer [singly underlined sequence in panel B]) was labeled with ^{32}P. Lane 1, adult rat liver total RNA (30 μg); lane 2, poly(A)$^+$ RNA from mouse liver (5 μg); lanes 3 to 8, total RNA (30 μg per lane) from six different adult mouse tissues: liver, kidney, intestine, brain, spleen, and lung, respectively. (B) Nucleotide sequence of 5′ upstream region of mouse HNF-4. The sequence (bp −365 to +49) was based on that of the c7II clone. The designated RNA start site is +1 (see text). The singly underlined nucleotides represent the probe used for primer extension (Fig. 2A). The doubly underlined sequences represent the liver-specific footprint (Fig. 5A).

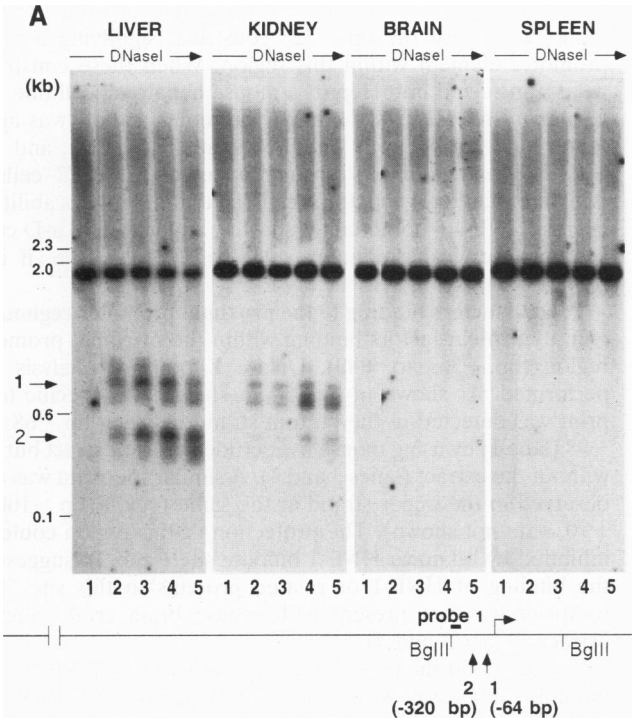
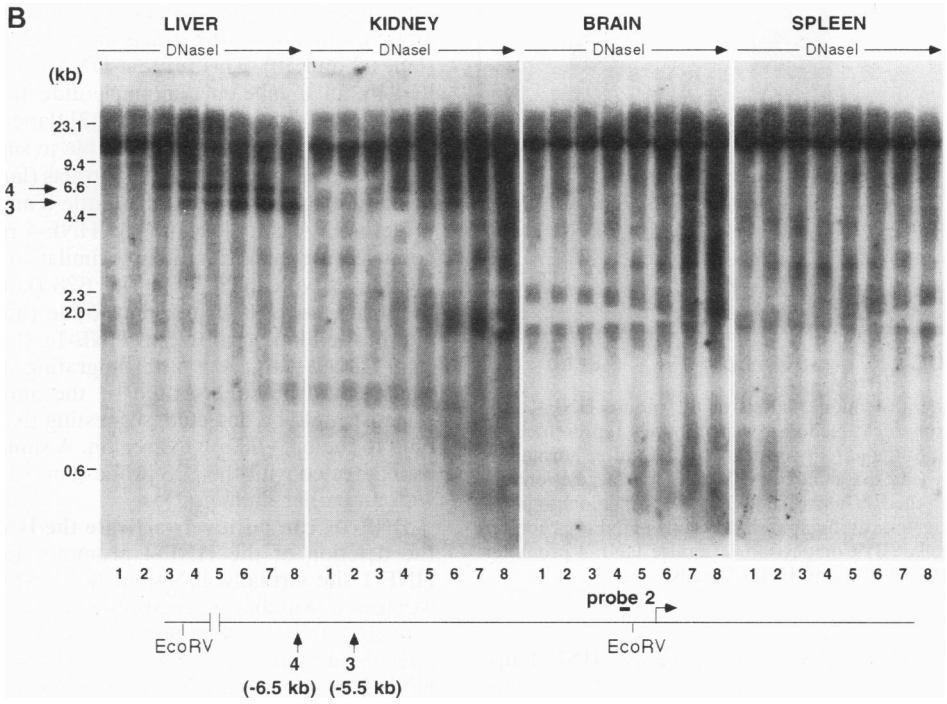


FIG. 3. DNase I hypersensitivity in the mouse HNF-4 5′ upstream region. (A) Proximal DNase I-hypersensitive sites. DNase I treatment was 12.5, 100, 200, 400, and 800 μg/ml (lanes 1 to 5, respectively). Genomic DNA was digested with *Bgl*II and run on a 1.5% agarose gel. Hypersensitive sites were revealed by indirect end labeling with a ^{32}P-labeled 5′ *Bgl*II-*Pst*I DNA fragment (Fig. 1) from c7II. (B) Distal DNase I-hypersensitive sites. DNase I treatment was 0, 12.5, 25, 50, 100, 200, 400, and 800 μg/ml (lanes 1 to 8, respectively). Purified DNA was digested with *Eco*RV and run on a 0.8% agarose gel. Hypersensitive sites were detected by probing with a *Pst*I-*Eco*RV fragment from c7II (Fig. 1).



presumptive enhancer/promoter region of the mouse HNF-4 locus from four different mouse tissues (liver, kidney, brain, and spleen) were examined. Two sets of DNase I-hypersensitive sites were found between the *Bgl*II sites around the RNA start site (bp −788 to kb +1.1 [Fig. 3A]). These hypersensitive sites were present only in liver and kidney tissues (in which HNF-4 is expressed), but not in brain and spleen tissues (in which HNF-4 is not expressed). The first set of hypersensitive sites was around bp −64 (between bp −171 and +82). The second set of hypersensitive sites was around bp −320 (between bp −425 and −287).

Two apparent hypersensitive sites were detected within the upstream *Eco*RV fragment (which extended from kb −10 to bp −368) and kb −5.5 and −6.5 upstream (Fig. 3B). Again, these distal hypersensitive sites were present only in liver and kidney tissues, in which HNF-4 is expressed.

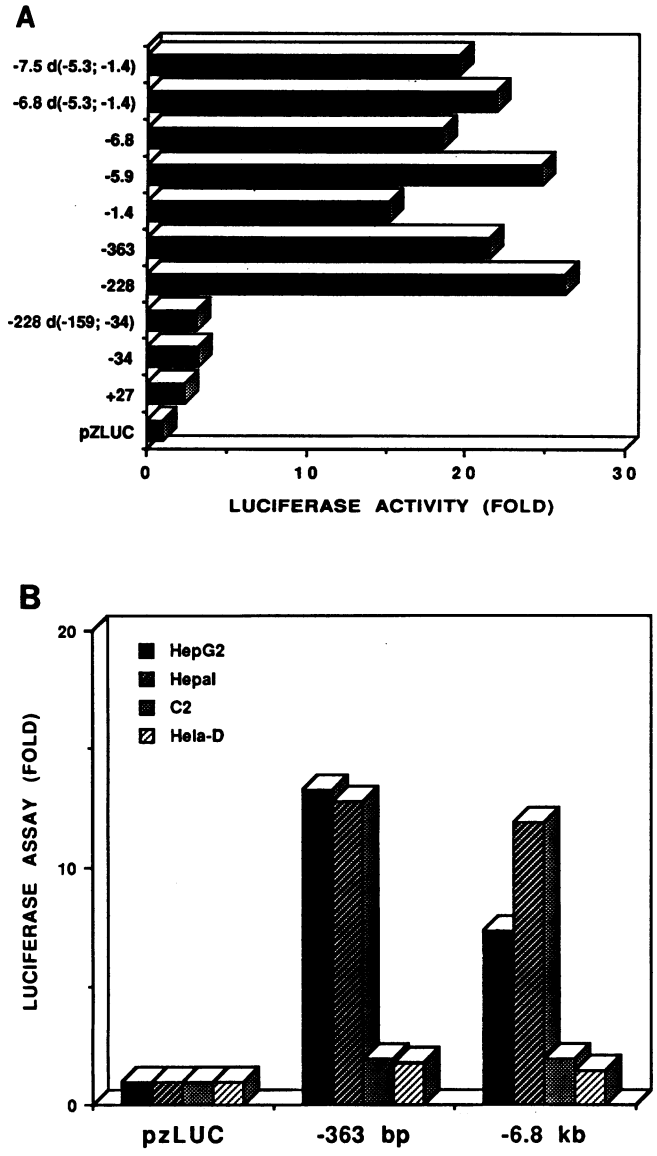


FIG. 4. Transient-transfection analysis of the mouse HNF-4 promoter. For each construct, luciferase activity was represented in comparison with that of the parent construct pzLUC, after normalization to β-Gal activity (internal control). +27 to −363, 5′ deletions (in base pairs) relative to the RNA start site; −1.4 to −7.5, kilobase pairs. d, internal deletions as indicated in parentheses. (A) HNF-4 promoter activity in HepG2 cells. (B) Comparison of mouse HNF-4 promoter activity in HepG2, Hepa1, C2, and HeLa-D cells.

Transient-transfection analysis of the mouse HNF-4 upstream region. HepG2, a human hepatoma cell line (10) that expresses HNF-4, was used for transient-transfection analysis to identify potential liver-specific elements. To decrease the chance of transcription that might contribute to the reporter gene activity starting within the vector, a trimer containing repeated polyadenylation signals was placed 5′ to the presumptive HNF-4 promoter region in these constructs (see Material and Methods). As shown in Fig. 4A, 5′ deletions from kb −7.5 to bp −228 had little effect on the activity of the presumptive HNF-4 enhancer/promoter, judging from the reporter gene activity. However, further 5′ deletion to bp −34 abolished over 95% of the promoter activity. Furthermore, an internal deletion between bp −159 and −34 virtually destroyed the promoter activity of the bp −228 construct, signifying a major promoter element within this region. When these constructs were transfected into Hepa1, a mouse hepatoma cell line that also expresses HNF-4, the major promoter activity was again found between bp −159 and −34 (data not shown, and Fig. 4B). However, similar transfection assays with C2 cells, a dedifferentiated rat hepatoma cell line that has lost its ability to express HNF-4 (4, 5; data not shown), and with HeLa-D cells, a human cervical carcinoma cell line, showed little, if any, reporter activity.

Protein factors binding to the proximal promoter region. To identify protein factors binding within the proximal promoter region (bp −363 to +49), DNase I footprint analysis was performed. As shown in Fig. 5A, a strong liver-specific footprint was detected in the bottom strand between bp −68 and −98 (lane 1) by using mouse liver crude nuclear extract but not without the extract (lanes 3 and 4). A similar footprint was also observed in the upper strand at the same region (bp −100 to −70; data not shown). The protection of this region could be inhibited by a known HNF-1 binding site (lane 2), suggesting the binding of HNF-1 or related proteins to this site. This footprint was not present with mouse brain crude nuclear extracts (lanes 3 and 4).

To verify that the protein binding to this site is HNF-1, a gel mobility shift assay was performed (Fig. 5B). A ^{32}P-labeled DNA fragment (4FP1) corresponding to the bp −98 to −68 footprinted region formed a prominent complex with crude nuclear extracts from HepG2 cells and mouse liver (lanes 1 and 5), but not with extracts from C2 cells or mouse brain (lanes 3 and 10). This protein-DNA complex could be inhibited by an unlabeled oligonucleotide fragment containing a known HNF-1 binding site (β28 [2] [lane 7]). Furthermore, an antiserum against HNF-1α was able to supershift this complex in the HepG2 and mouse liver extracts (lanes 2 and 8), while an antiserum against HNF-1β had little if any effect (lane 9). This protein-DNA complex with the HNF-4 promoter-derived oligonucleotide had a mobility similar to those of HNF-1α complexes with the HNF-1 site β28 (lane 13). Furthermore, the upstream HNF-4 oligonucleotide (bp −98 to −68) could inhibit the binding of β28 to HNF-1α (lane 15).

In C2 cell extracts, a faster-migrating shift complex (lane 3) which could be supershifted by the anti-HNF-1β antiserum (lane 4) could be detected, suggesting that HNF-1β could also bind to the bp −98 to −68 region. A similar shift complex was also detected with the β28 probe (lanes 18 and 19), consistent with an earlier finding (2).

HNF-1α can positively activate the HNF-4 promoter. Since the deletion of the HNF-4 promoter region containing the HNF-1 site virtually abolished the HNF-4 promoter activity, we tested whether overexpression of HNF-1α can further activate HNF-4 promoter activity in transient-transfection analysis in HepG2 cells. As shown in Fig. 5C, when a mouse HNF-4 promoter-reporter construct (bp −228) was cotransfected with an HNF-1α expression vector, a threefold increase in the reporter gene activity could be observed. On the contrary, an HNF-1β expression vector had little, if any, effect on the reporter gene activity. In addition, transfection was also carried out with C33 cells, a human cervical carcinoma cell line that has neither HNF-4 nor HNF-1 DNA-binding activity. Again, the reporter gene construct (bp −228 of the HNF-4 promoter series) could be stimulated about sixfold by cotransfection of the HNF-1α expression vector and about two- to threefold by the HNF-1β expression vector. While these cotransfection experiments don't necessarily invoke an in vivo

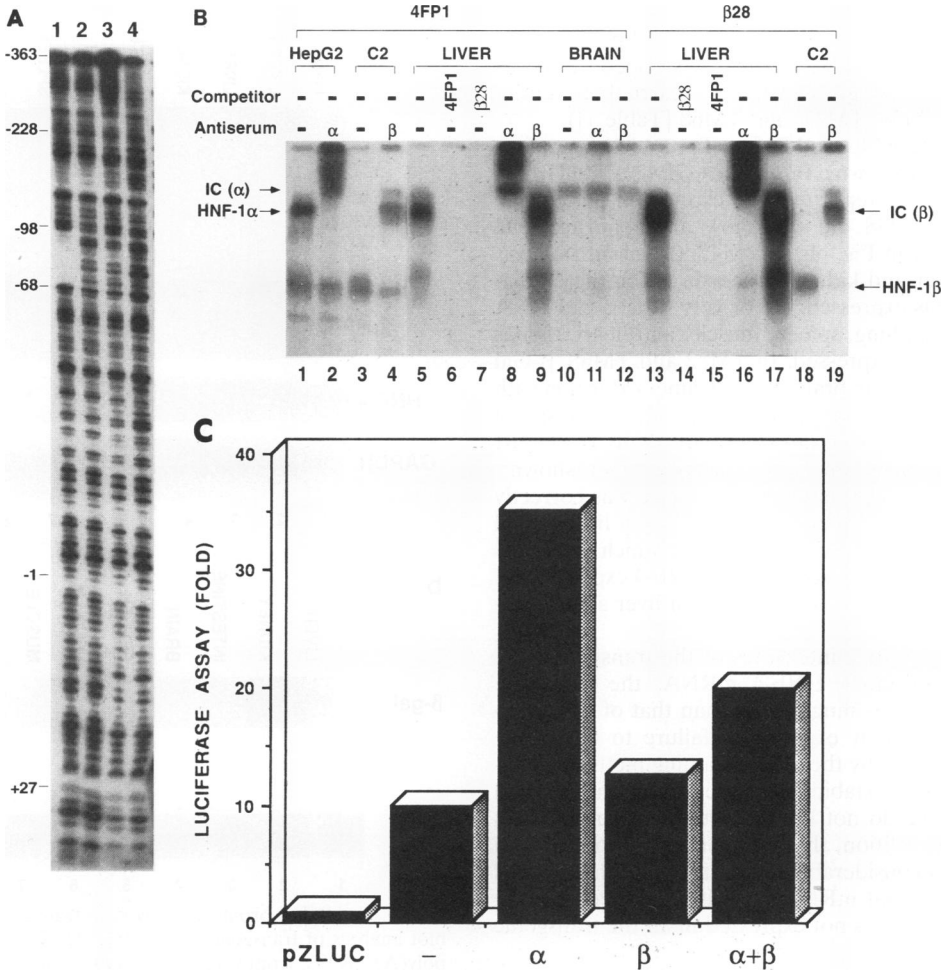


FIG. 5. Protein factors binding to the proximal mouse HNF-4 promoter region. (A) DNase I footprint analysis of the bp −363 to +49 region. The DNA fragment was labeled with ^{32}P at the 5′ end of the lower strand. Lanes 1 and 2, crude mouse liver nuclear extracts in the absence (lane 1) or presence (lane 2) of excess oligonucleotide containing the HNF-1 binding site β28; lane 3, crude mouse brain nuclear extracts; lane 4, bovine serum albumin. (B) Electrophoretic mobility shift analysis. ^{32}P-labeled DNA probes containing either the bp −98 to −68 sequence of the HNF-4 promoter (4FP1; lanes 1 to 12) or the known HNF-1 binding site β28 (2) (lanes 14 to 19) was used. Crude nuclear extracts from various cell lines or mouse tissues, unlabeled DNA probes (100-fold) as specific competitors, and various antisera were added as indicated. α, HNF-1α; β, HNF-1β; IC, immune complex. (C) HNF-1α positively activates the HNF-4 promoter. The mouse HNF-4 promoter-reporter construct, bp −228 (5 μg), was cotransfected with 2.5 μg of either an HNF-1α-expressing vector (α) or an HNF-1β-expressing vector (β), or both (α+β). For each transfection, the luciferase activity was compared with that of the parent construct pZLUC, after normalization by β-Gal activity (internal control).

action of the HNF-1 proteins, they do suggest the bp −98 to −68 region as having importance for hepatocyte function.

The mouse HNF-4 enhancer/promoter elements in transgenic mice: bp −363 to +182 and reporter gene expression. Since the bp −363 to +182 region of the mouse HNF-4 gene contained all the demonstrable hepatocyte-specific promoter activity in transient-transfection assays, we examined the ability of this region to drive liver-specific expression of a reporter gene in transgenic mice. The HNF-4 enhancer/promoter (bp −363 to +182) fused to a promoterless β-Gal gene was prepared and injected into fertilized eggs. Three founder animals (TM1, TM7, and TM37) from a total of 38 contained the construct as a transgene.

Two of the transgenic mouse lines carrying this transgene (TM1 and TM37) were analyzed for reporter gene expression by Northern blot analysis. (We were unable to obtain convincing β-Gal staining in adult tissues of any of our transgenic mice, including those described below.) The mRNAs from several adult tissues of F1 mice were probed with a ^{32}P-labeled β-Gal DNA fragment. In the TM1 line, no reporter gene expression was detected in the tissues tested (liver, kidney, intestine, brain, lung, spleen, muscle and heart [data not shown]). In the TM37 line (Fig. 6a), no β-Gal mRNA was detected in either liver or intestine, two tissues in which HNF-4 is expressed. The reporter gene expression was detected in lung and brain tissues, in which HNF-4 is normally not expressed. Only minimal β-Gal mRNA was detected in the kidney tissue. The size of the reporter mRNA in kidney and lung tissues was as expected (about 4 kb). Brain tissue seemed to have several different forms of the message, some of which were considerably larger than the expected size. The transgene in the F1 mice of TM7 appeared to have rearranged inserts and thus was not analyzed for expression.

Reporter gene expression and the kb −7.2 to bp +182 region. The failure of the bp −363 to +182 region of the HNF-4 promoter to drive liver-specific expression of the transgene prompted us to test the ability of the entire kb −7.2 to bp +182 region of the promoter in transgenic mice. This

larger region contained all the observed liver- and kidney-specific hypersensitive sites (kb −6.5, kb −5.5, bp −320, and bp −64 [Fig. 3A and B]). Of the 146 mice developed from eggs microinjected with the larger construct, 4 carried the entire transgene (TM22, TM38, TM47, and TM68 [Table 1]).

As tested by Northern blot and RNase T_2 protection analysis (Fig. 6b, and data not shown), three lines (TM22, TM47, and TM68) had very similar reporter gene expression patterns in the tissues tested. TM38 did not show any reporter gene expression. As shown in Fig. 6b, in TM47, β-Gal mRNA was detected only in liver and kidney, tissues in which the endogenous HNF-4 gene is expressed. No or very little β-Gal RNA was detected in brain, lung, spleen, muscle, and heart, tissues in which HNF-4 is not expressed. In TM22 and TM68, β-Gal mRNA was also detected in brain tissue. Primer extension with an oligonucleotide primer from within the β-Gal gene resulted in primer-extended products consistent with the correct start site from the HNF-4 promoter region (data not shown), implying that the transcription of the transgene was correctly initiated. One interesting observation is that very little β-Gal mRNA was detected in intestine, a tissue in which HNF-4 is expressed, implying that the regulation of HNF-4 expression in intestine tissue may be different from that of liver and kidney tissues.

Judging from the relative intensities of the transgene transcript and the endogenous HNF-4 mRNA, the transgene mRNA concentration was much lower than that of the endogenous HNF-4, which may explain the failure to detect the reporter gene expression by the X-Gal staining method. Without knowing the relative stabilities of the HNF-4 mRNA and the β-Gal mRNA, we do not know whether the promoter is normally active. In addition, the reporter gene expression in intestinal tissue was considerably lower than that in liver and kidney tissues, and β-Gal mRNA was also detected in brain tissue, in which HNF-4 was not expressed in all the transgenic animals tested.

Transgene expression during embryogenesis. To test whether the kb −7.2 to bp +182 region is also sufficient to drive liver-specific expression of the reporter gene during embryogenesis, X-Gal staining of the whole-mount 9.5-day (postconception [p.c.]) transgenic embryos was performed. Reporter β-Gal activity was detected in the embryonic liver and intestine (Fig. 6c-A) in a pattern similar to that of endogenous HNF-4 at this stage (17a). At day 11.5 (p.c.), X-Gal staining was also observed in the mesonephric tubules, the anlage of the kidney (Fig. 6c-B). Most other tissues show little, if any, β-Gal staining at this stage. However, reporter gene activity was observed in the embryonic pancreas and certain embryonic ribs.

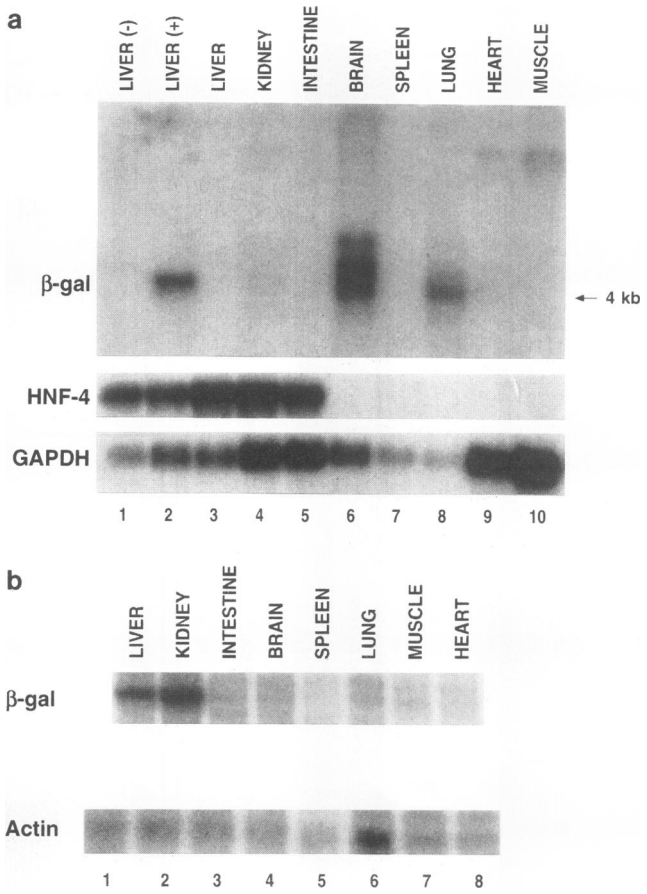
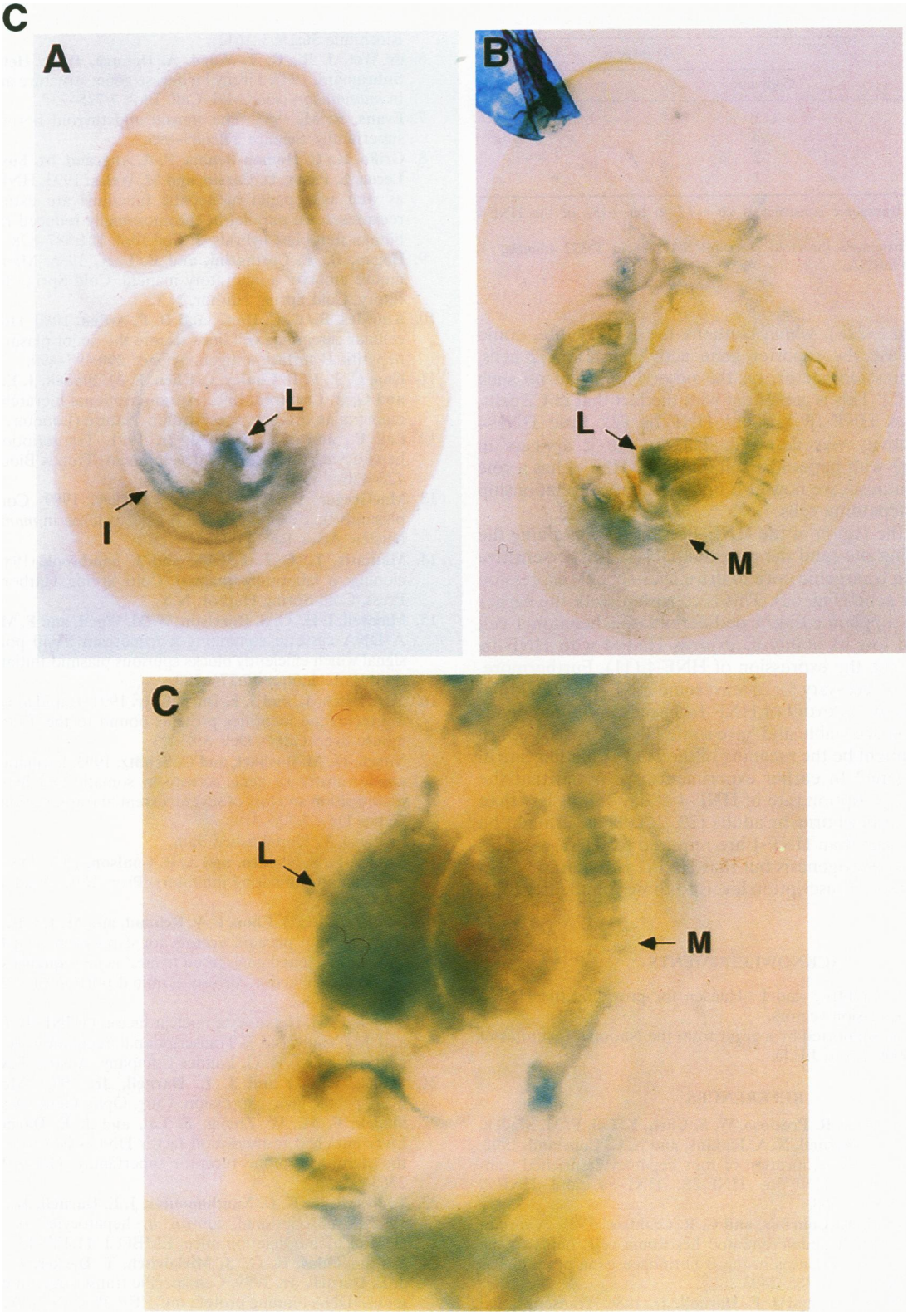


FIG. 6. HNF-4 promoter activity in transgenic mice. (a) Northern blot analysis of transgenic mouse line 22 (TM22). A total of 5 μg of poly(A)$^+$ RNA, from various F1 adult tissues (liver, kidney, intestine, brain, spleen, lung, heart, and muscle [lanes 3 to 10, respectively]), was separated on a 1% formaldehyde agarose gel and transferred to an Immobilon-N filter (Millipore). The filter was probed with a ^{32}P-labeled β-Gal cDNA fragment. Liver poly(A)$^+$ RNAs from mice without the transgene (lane 1) and from mice carrying and expressing the transgene (lane 2) were used as the negative and positive controls, respectively, for β-gal expression. A mouse HNF-4 cDNA probe and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe served as controls (the two bottom panels). Autoradiography was performed at −70°C with two intensifying screens for 6 days (β-Gal) and 6 h (HNF-4 and GAPDH). Approximately 10 times less GAPDH probe compared with HNF-4 or β-Gal was used (10^5 cpm/ml) for hybridization. (b) RNase T_2 protection analysis of transgenic mice line 47 (TM47). A ^{32}P-labeled antisense RNA probe corresponding to the β-Gal sequence was used. An actin antisense probe was used as a control. (c) X-Gal staining of TM22 embryos (heterozygotes). (A) 9.5 days (p.c.); (B and C) 11.5 days (p.c.). L, liver; I, intestine; M, mesonephric duct.

DISCUSSION

HNF-4 has proved to be a very commonly employed transcription factor in adult tissues (20), and several findings suggested it might also prove to be important early in development: (i) a deletion on chromosome 7 (mouse) that leaves genes for HNF-1 and HNF-4 intact results in loss of HNF-4 and HNF-1 mRNA (23); (ii) cell lines often lose both HNF-1 and HNF-4 (8, 17); (iii) HNF-4 can drive HNF-1α transcription in dedifferentiated hepatoma cells (while the reverse is not true), suggesting a hierarchy with HNF-4 upstream of HNF-1α (11); and (iv) there is a *Drosophila* gene of high homology to HNF-4 that is expressed very early in gut, fat bodies, and Malpighian tubules, the insect counterparts to tissues in which HNF-4 mRNA is found in mammals. Because of these results, we undertook to characterize the mouse HNF-4 gene and its functional promoter in more detail.

The structure of the mouse HNF-4 gene proved of interest. Of the eight cysteines in the zinc finger of this steroid-thyroid receptor family member, five are encoded in exon 2 and three are encoded in exon 3. This is the same distribution as in the fly gene and is different from those of all other family members, in which four cysteines are encoded in each exon (7, 25). This result and the highly conserved protein speak in favor of structural conservation throughout evolution. The start site of transcription was defined. No immediate upstream hall-



marks (TATA box or CCAAT box) were observed, but a cluster of start sites was identified. Further, DNase I-hypersensitive site analysis suggests that the mouse HNF-4 gene is active only in tissues in which the hypersensitive sites were observed.

When the potential regulatory sequences in the gene were explored in transient-transfection assays, we uncovered a required site between bp −98 and −68 to which HNF-1 can clearly bind (Fig. 5A and B). Furthermore, cotransfection of an HNF-1α expression vector with an HNF-4 promoter-reporter

TABLE 1. Transgene copy numbers and mRNA levels in various transgenic founder lines carrying the larger transgenic construct[a]

Founder line	Transgene	
	Copy no.	mRNA level (liver)
TM22	1	+
TM38	>3	−
TM47	2	+++
TM68	1	+

[a] The larger transgenic construct is kb −7.2 to bp +182 of the HNF-4 promoter.

[b] +++, approximately threefold more mRNA than in TM22 animals; −, failure to detect mRNA.

construct (bp −228), which contains the HNF-1 site, could further increase the reporter gene activity in HepG2 cells. HNF-1β expression vector, on the other hand, had no such effect (Fig. 5C). This is consistent with the fact that in C2 cells, which express HNF-1β but not HNF-1α (2), little HNF-4 promoter activity was detected. Thus, while it appears in earlier studies with hepatoma cell lines that HNF-4 has a role in HNF-1 synthesis, we now also find a reciprocal relationship in cultured hepatoma cells.

However, the region of the HNF-4 promoter containing the HNF-1 binding site (and the proximal DNase I-hypersensitive sites) failed in transgenic mice to drive liver-specific expression of a reporter gene (Fig. 6a). This is consistent with the earlier finding that supplementing a dedifferentiated hepatoma cell line that had lost its ability to express HNF-4 with HNF-1α could not rescue the expression of HNF-4 (11). Furthermore, HNF-1α is not necessary for at least some HNF-4 expression in mice. Mutant mice devoid of HNF-1α because of homologous recombination are viable and have some HNF-4 mRNA (24a). What, then, might be the meaning of the HNF-1 binding site in the HNF-4 gene? In earlier experiments, we noted that the embryonic transcription rate of HNF-4 at day 15 was less than at day 18 or in newborns or adults (22, 24). Thus, it might be that factors other than HNF-1 are required for HNF-4 activation early in embryogenesis but that HNF-1 is required late in gestation to raise transcription levels to those found after birth and in adults.

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