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*HNF4*β, a New Gene of the *HNF4* Family with Distinct Activation and Expression Profiles in Oogenesis and Embryogenesis of *Xenopus laevis*

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The transcription factor hepatocyte nuclear factor 4 (HNF4) is an orphan member of the nuclear receptor superfamily expressed in mammals in liver, kidney, and the digestive tract. Recently, we isolated the *Xenopus* homolog of mammalian HNF4 and revealed that it is not only a tissue-specific transcription factor but also a maternal component of the *Xenopus* egg and distributed within an animal-to-vegetal gradient. We speculate that this gradient cooperates with the vegetally localized embryonic induction factor activin A to activate expression of HNF1α, a tissue-specific transcription factor with an expression pattern overlapping that of HNF4. We have now identified a second *Xenopus HNF4* gene, which is more distantly related to mammalian *HNF4* than the previously isolated gene. This new gene was named *HNF4*β to distinguish it from the known *HNF4* gene, which is now called *HNF4*α. By reverse transcription-PCR, we detected within the 5′ untranslated region of *HNF4*β two splice variants (*HNF4*β2 and *HNF4*β3) with additional exons, which seem to affect RNA stability. HNF4β is a functional transcription factor acting sequence specifically on HNF4 binding sites known for HNF4α, but it seems to have a lower DNA binding activity and is a weaker transactivator than the α isoform. Furthermore, the two factors differ with respect to tissue distribution in adult frogs: whereas HNF4α is expressed in liver and kidney, HNF4β is expressed in addition in stomach, intestine, lung, ovary, and testis. Both factors are maternal proteins and present at constant levels throughout embryogenesis. However, using reverse transcription-PCR, we found the RNA levels to change substantially: whereas HNF4α is expressed early during oogenesis and is absent in the egg, HNF4β is first detected in the latest stage of oogenesis, and transcripts are present in the egg and early cleavage stages. Furthermore, zygotic *HNF4*α transcripts appear in early gastrula and accumulate during further embryogenesis, whereas *HNF4*β mRNA transiently appears during gastrulation before it accumulates again at the tail bud stage. All of these distinct characteristics of the newly identified HNF4 protein imply that the α and β isoform have different functions in development and in adult tissues.

A central event in early development is the establishment of different cell types from a single cell, the fertilized egg. This cell type is highly specialized, as it contains maternal components that define embryonic induction processes and the initiation of transcriptional cascades. This unique feature of the egg is especially evident in amphibians such as *Xenopus laevis*. In this species, no gene transcription occurs in early cleavage stages and all early events are regulated by preexisting transcripts and translational products made during oogenesis. Many of these maternal components are transcription factors that can most easily be analyzed in *Xenopus* (e.g., fos [13], B-myb [3], c-ets [27], and Xrel [22]). Recently, we revealed that hepatocyte nuclear factor 4 (HNF4), previously known as a tissue-specific transcription factor mainly expressed in the liver, kidney, and digestive tract, represents such a maternal factor in *Xenopus* and is responsible for the embryonic activation of a transcriptional cascade (11). The presence of HNF4 early in development is evolutionarily conserved, since the mRNA for the *Drosophila* homolog of HNF4 also is maternal (32). Within this species HNF4 is essential during embryogenesis, since a chromosomal deletion spanning the *HNF4* locus results in flies with defects in tissues homologous to vertebrate kidney, liver, and gut (32). Also in mammalian embryogenesis, an early function of HNF4 has been deduced, as mice with homozygous knockout of the *HNF4* gene show severe gastrulation defects and die in utero (5). However, in the mammalian system, the presence of maternal HNF4 has not been established.

One target gene of HNF4 is the gene encoding the tissue-specific transcription factor HNF1α (reviewed in reference 4), which is transcriptionally activated early in *Xenopus* development (1). Upon treatment of animal cap explants with the embryonic induction factor activin A, activation of the *HNF1*α promoter, which is dependent on the HNF4 binding site, can be observed (29). Since the maternal HNF4 protein is present in an animal-to-vegetal gradient (11) and the activin signal is thought to emanate from the vegetal part of the cleaving egg (reviewed in references 23 and 26), we assume that the cooperation of these two signals is involved in determining the HNF1α expression pattern (29). The interplay of HNF4 and HNF1α is not restricted to embryogenesis, since in adults these two factors are expressed in the same tissues, and upon differentiation and dedifferentiation of hepatoma cell lines, their coexpression is correlated with the differentiated phenotype (reviewed in reference 4). HNF4 and HNF1α are also functionally connected due to the fact that several target genes

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(e.g., the α_1-antitrypsin, phosphoenolpyruvate carboxykinase, pyruvate kinase, and transthyretin genes) bind both factors within their promoters (reviewed in reference 4).

Based on the amino acid sequence, HNF4 is a member of the steroid-thyroid-hormone receptor superfamily (reviewed in reference 19), which is characterized by the presence of a zinc finger DNA binding domain and a hydrophobic region involved in ligand binding and dimerization. Since a ligand has not been identified for HNF4, it belongs to the group of orphan receptors (25). The six-amino-acid sequence of the P box (CDGCKG), located in the second knuckle of the first zinc finger of the DNA binding region, is a unique feature of the HNF4 protein among the nuclear receptor superfamily and has been considered a characteristic specific for HNF4 (16).

Here, we demonstrate in *Xenopus* the presence of a new HNF4 protein (HNF4β) that is distinct from the previously identified *Xenopus* protein (HNF4α), which represents the homolog of the mammalian protein. As HNF4β is also clearly different from the human isoform HNF4γ that we have recently cloned (9), we conclude that HNF4 is encoded in a gene family with at least three members in vertebrates. Furthermore, we demonstrate that HNF4β is a maternal component of the *Xenopus* egg and shows a tissue-specific expression pattern distinct from that of HNF4α. More importantly, the genes encoding these two transcription factors are differentially expressed in oogenesis and embryogenesis, implying that these related regulatory factors have distinct functions.

MATERIALS AND METHODS

Isolation of *Xenopus* HNF4β cDNA clones. A partial cDNA clone representing HNF4β was isolated from a *Xenopus* liver cDNA library (1), using experimental conditions as described previously (11). The missing 5′ part of the cDNA was cloned in a PCR approach using the cDNA library as a template. As primers, the HNF4β-specific oligonucleotide H4βPCR1 (5′-GAGTAAACCCGGGTGGCA G-3′, position +152 with respect to the translational start site) and a primer specific for λgt10 (5′-AGCAAGTTCAGCCTGGTTAAGT-3′) were applied. A continuous cDNA sequence was generated in the vector pBluescript SK^+ by combining the partial λ clone and the PCR fragment (HNF4β-SK^+).

Reverse transcription-PCR (RT-PCR). RNA was prepared by using the RNA clean system (AGS) as instructed by the manufacturer. The reverse transcription into cDNA was performed with the Superscript II reverse transcriptase (Gibco BRL) under conditions recommended by the supplier. The reaction was incubated at 42°C for 1 h in a mixture containing the RNA equivalent of one embryo or oocyte and 300 ng of random hexanucleotide primer. As a control for plasmid contaminations, each reverse transcription was accomplished in duplicate with and without the addition of reverse transcriptase. The PCR was performed in a 50-μl reaction with 1× Goldstar reaction buffer (Eurogentec, Seraing, Belgium), 125 nM deoxynucleoside triphosphate, 1 mM $MgCl_2$, 50 pmol of primer, 0.1 U of Goldstar-Taq polymerase (Eurogentec), and 0.1 μCi of [α-^{32}P]dCTP, using either 5 μl of the reverse transcription reaction as a template for the amplification of *HNF4*α and -β cDNAs or 1 μl for the detection of ornithine decarboxylase cDNA. The HNF4β-specific PCR was performed with the primers H4βPCR2 (5′-GTTGGTTTAGTCGAGGCCTTAT-3′), position −102 with respect to the translational start site) and H4βPCR1 (see above). For the detection of HNF4α, the primer H4αPCR1 (5′-ATGGATATGGCGGATTATAC-3′, priming on the translational start site) and H4αPCR2 (5′-AACTCGACCTCCTCGTAC-3′, position +402 with respect to the translational start site) were used. PCR for ornithine decarboxylase was done with primers as described previously (14). The PCR conditions for HNF4α were 2 min at 95°C; 18 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1.5 min; 13 cycles of 94°C for 45 s, 45°C for 1 min, and 72°C 1.5 min; and 72°C for 7 min. For HNF4β, the conditions used were the same as for HNF4α but with 20 cycles followed by 15 cycles. The ornithine decarboxylase PCR conditions were as follows: 94°C for 2 min; 27 cycles of 94°C for 45 s, 44°C for 1 min, and 72°C for 1.5 min; and 72°C for 7 min.

The PCR fragments were separated on 6% polyacrylamide gels and dried, and the products were visualized by autoradiography.

Expression of recombinant HNF4α and HNF4β in the baculovirus expression system. The whole coding region starting with the initiator methionine and ending with the translational stop codon of *HNF4*α and *HNF4*β was amplified by using specific primers carrying *Bgl*II adapters. These fragments were cloned into the *Bgl*II site of the baculovirus transfer vector pBlueBacHisB (Invitrogen). Within this construct, the open reading frame of both clones is preceded by 37 amino acids coded by the vector which include a six-histidine tag. The expression of the histidine-tagged proteins in the baculovirus expression system and purification of the proteins by using Ni-nitrilotriacetic acid-agarose (Qiagen) were performed as described previously (31).

Gel retardation assays. Gel retardation assays were performed as described previously (15) in a reaction mixture containing 300 ng of salmon sperm DNA, 10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 4% Ficoll 400, using either 50 or 200 ng of the purified HNF4α or HNF4β protein, respectively. The sequence of the oligonucleotide H4, representing the HNF4 binding site of the HNF1α promoter, was as reported previously (31). The complementary strands of the oligonucleotide Apo B, spanning the HNF4 binding site of the human apolipoprotein B promoter (20), have the sequences 5′-GATCCACTTTGTGGGGTCCAAAGGGCGGTAA-3′ and 5′-CAAGTTA CCGCCCTTTGGACCCCACAAAGTG-3′, whereas the strands of the oligonucleotide Apo CIII, which represent the HNF4 binding site of the apolipoprotein CIII promoter (24), have the sequences 5′-GATCCACTTTGTGGTGGGCAA AGGTCAGTAA-3′ and 5′-CAAGTTACTGACCTTTGCCCACCACAAAGT G-3′. As an unrelated oligonucleotide, the HNF3 binding site of the mouse transthyretin promoter (6) (5′-AGCTTGTTGACTAAGTCAATAATCAGAAT CAGG-3′ and 5′-GATCCCTGATTCTGATTATTGACTTAGTCAACA-3′) was used. Competition was performed by including 5, 10, or 20 ng of the oligonucleotides.

Transient transfection assays. Transient transfection assays using the dedifferentiated hepatoma cell line C2 (8) were performed by the calcium phosphate coprecipitation method (31). As reporter constructs, 2 μg of each of the luciferase plasmids −594/−207 luc (29), containing a fragment of the *Xenopus* HNF1α promoter, and H4-tk-luc (8), carrying four HNF4 binding sites in front of the thymidine kinase promoter, was transfected. Preparation of cell extracts and the luciferase assay were done as described previously (8).

The expression vector HNF4α/CMV was constructed by using the *Hin*dIII and *Spe*I sites (positions −14 and +2200, respectively, with respect to the translational start, [11]) of the HNF4α cDNA for cloning into the same sites of the eukaryotic expression vector Rc/CMV (Invitrogen). To clone the expression vector HNF4β/CMV, the whole coding region of HNF4β1 (positions −85 to +1702) was inserted into the vector Rc/CMV. This was done by using the *Stu*I site of the clone HNF4β-SK^+ (see above) and the *Xba*I site of the cloning vector pBluescript SK^+.

Production of HNF4α- and HNF4β-specific antisera and Western blotting. The HNF4α-specific antiserum XH4α was generated against the C-terminal 14 amino acids of HNF4α (CTSIPQSTITKQEAM). A cysteine was added at the N terminus of the peptide for coupling. This peptide was produced by Eurogentec and used for the immunization of two rabbits according to their standard protocol.

The antiserum XH4β was generated against a part of the F domain (amino acids 376 to 455) of HNF4β. The coding sequence for amino acids 368 to 455 and about 370 bp of the 3′ nontranslated region were excised from clone HNF4β-SK^+ (see above) by restriction with *Pst*I and *Hin*dIII and inserted into the prokaryotic expression vector pQE31 (Qiagen). This resulted in an HNF4β fusion protein in which an N-terminal six-histidine tag is followed by 12 amino acids encoded by the vector. The fusion protein was expressed in *Escherichia coli* and purified by using Ni-nitrilotriacetic acid-agarose (Qiagen) as recommended by the manufacturer. This protein was used by Eurogentec for the immunization of one rabbit according their standard protocol. The immune serum was affinity purified with the same protein as used for the immunization immobilized on tresyl-activated agarose (Schleicher & Schuell).

Western blots were prepared as described previously (11). For the detection of HNF4α or HNF4β, the blots were incubated with the specific antiserum XH4α or the affinity-purified serum XH4β overnight at 4°C in a 5,000- or 200-fold dilution, respectively. Horseradish peroxidase-conjugated goat anti-rabbit antibodies were used to detect the bound primary antibodies, using the Amersham ECL system.

Nucleotide sequence accession number. The sequence of HNF4β1 was deposited in the EMBL data bank (accession number Z49827).

RESULTS

Two members of the HNF4 family of transcription factors are expressed in *Xenopus* liver. By screening a *Xenopus* liver cDNA library with a probe specific for rat HNF4, we isolated not only cDNA clones that are highly homologous to this gene (11) but also cDNA clones that are clearly distinct. As the open reading frame of each of these cDNAs encodes a protein that differs substantially from HNF4, we named this newly identified protein HNF4β and the previously described protein HNF4α. We exclude the possibility that these two proteins are derived from pseudoalleles which are generated by evolutionary genome duplication of the *Xenopus* genus, since the overall identity between HNF4α and HNF4β is just 67% and for pseudoalleles, a conservation of greater than 90% is expected (e.g., *Xenopus* HNF1α [1]).

A

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                10        20        30        40        50
XHNF4β   MKLCEAMMDMDMPDYTETLDSSYTMLEFDSIRVLPSNTEIITVETASPGLLNN-GINSFC
XHNF4α   .R.SK.LI....A....A..PA..T...ENMQ..SIG.DTS.SDVT.LSAS.SI....L.

          60        70        80        90       100       110
XHNF4β   AICGDRATGKHYGASSCDGCKGFFRRSVRKNHVYACRFSRQCIVDKDKRNQCRYCRLRKC
XHNF4α   ..............................M.S.......V............K....

         120       130       140       150       160       170
XHNF4β   FRAGMKKEAVQNERDRISMRRSSYEDNGSLSINVLTQAEAMVHQYSPVSPVHSSDISMKK
XHNF4α   ..................T.......SSLP....I...VLSQ.ITSSVG.LNT..RG..

         180       190       200       210       220       230
XHNF4β   VASISDVCESMKQQLLLLVEWAKYIPAFCELPLDDQVALLRAHAGAHLLLGVAKRSLPYK
XHNF4α   I.C.I...D.......V.............................E.....AT...MMF.

         240       250       260       270       280       290
XHNF4β   DFLLLGNDFIMPMHCPELEIARVPCRILDELVKPLREIQIDDNEYVCLKAIIFFDPDCKG
XHNF4α   .I......RLI.RN.....VG..AV.......L.FQ.L.......A...........A..

         300       310       320       330       340       350
XHNF4β   LSDQTKVKNMRFQVQVNLEDYINDRQFDSRGRFSDILLLLPPLQSITWQMIEQVQFAKLF
XHNF4α   ...P..I.R..Y....S.........Y......GEL.....T...........I..V...

         360       370       380       390       400       410
XHNF4β   GVARIDSLLQELLLGGTTMDGGQYINSGHSSLNLDLLPGPTVHSHN-LHSVIHTVSSLSP
XHNF4α   .M.K..N....M....SANEASHTHHHL.PH.VQ.H.ATNVIVAN.T.P.QL.NGQMST.

          420       430       440       450
XHNF4β   ET---SPPTNSTSEDYKMNTATVSSIPLMQRTVIAKKEIL
XHNF4α   ..PQP...AG.GA.Q..IVHG.IA..N-K.P.S.PQST.TKQEAM
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B

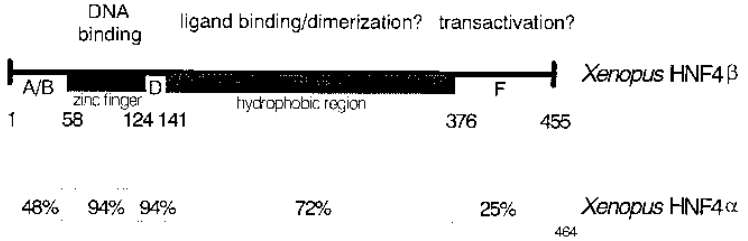

FIG. 1. HNF4β cloned from *Xenopus* liver is a new member of the HNF4 family. (A) The protein sequences of *Xenopus* HNF4β and HNF4α are aligned. Amino acid differences are shown; dots indicate identical amino acids, and dashes indicate missing amino acids. The DNA binding region (amino acids 59 to 125) and the potential dimerization/ligand binding region (amino acids 142 to 376) are presented in grey boxes, and the conserved eight cysteines of the zinc finger are underlined. (B) The domain structure of HNF4β is shown schematically (24), and the percent amino acid identity to *Xenopus* HNF4α is given.

The amino acid sequences of both proteins are aligned in Fig. 1A, and a schematic comparison is made in Fig. 1B. From the comparison of the overall sequences of HNF4α and -β, it is evident that the different protein domains display variant degrees of conservation (Fig. 1B). It is obvious that the DNA binding region (domain C) and the hinge region (domain D) are most conserved between the two proteins, displaying a homology of 94%. Nevertheless, this high degree of homology is lower than that between the *Xenopus* and rat HNF4 proteins, which are completely identical in this region (11). The potential ligand binding and dimerization domain (domain E) is also well conserved between the α and β isoforms (72%), whereas the N-terminal transactivation region (domain A/B) shows a lower degree of identity (48%). In contrast, the C-terminal region encoding another potential activation domain (domain F) shows hardly any homology, and it is in this region that HNF4β is eight amino acids shorter than HNF4α (Fig. 1B). The abrupt change in the homology to the HNF4α protein at amino acid 377 (Fig. 1B) occurs at the position of an intron in the mouse *HNF4*α gene (28), and thus the F domain may be encoded by an exon specific for the *HNF4*β gene.

Analyzing HNF4β expression by RT-PCR of RNA derived from *Xenopus* liver, using primers spanning the 5′ untranslated part and the region coding for the N-terminal 42 amino acids, we identified three distinct transcripts for *HNF4*β (see Fig. 7, lane 16), two of which (*HNF4*β2 and *HNF4*β3) were larger than expected. Cloning and sequencing of the PCR fragments revealed that the fragment of the expected size (*HNF4*β1) contains the sequence found in the cDNA clones, whereas the *HNF4*β2 and *HNF4*β3 fragments contain 17 nucleotides up-

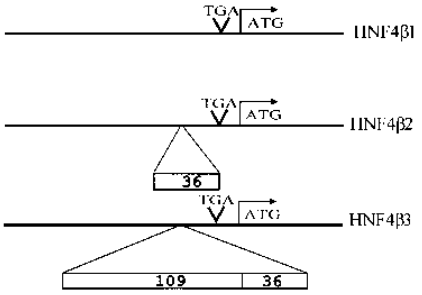

FIG. 2. HNF4β is expressed in *Xenopus* liver in three splice variants. A schematic representation of the three HNF4β splice variants is shown. HNF4β1 corresponds to the sequence found originally, whereas HNF4β2 and HNF4β3 contain insertions of 36 and 145 bp, respectively, at position −17. The 36-bp additional exon is common in HNF4β2 and HNF4β3. The in-frame stop codon TGA preceding the open reading frame is indicated.

stream of the translational start codon inserts of 36 and 147 bp, respectively (Fig. 2). As the 36 bp of *HNF4*β2 are included in the *HNF4*β3 transcript, and all of the insert boundaries match the splicing junctions, we assume *HNF4*β1, -2, and -3 represent splice variants and that an intron exists upstream of the open reading frame. No intron has been found at this position in the mouse *HNF4*α genomic sequence (28, 33), and it is therefore probable that this intron is *HNF4*β specific. Since the additional exons of the *HNF4*β gene are located upstream of the translation initiation site and the in-frame stop codon (Fig. 2), they cannot readily influence the sequence of the HNF4β protein (see Discussion).

HNF4β binds to known HNF4 binding sites and encodes a functional transcriptional activator. The sequence of the zinc finger DNA binding region of the nuclear receptor superfamily is known to determine the target sequence selection. Since the DNA binding region of HNF4β exhibits four amino acid differences from HNF4α proteins (Fig. 1A), we examined whether this isoform nevertheless binds to known HNF4 binding sites. Therefore, we expressed both HNF4 transcription factors in the baculovirus expression system to obtain pure proteins, which were used in gel retardation assays. Figure 3A demonstrates the binding of recombinant HNF4α to the labeled oligonucleotide H4, which includes the HNF4 binding site of the *Xenopus* HNF1α promoter (lane 1). Upon addition of increasing amounts of the unlabeled homologous oligonucleotide, a competition of protein-DNA complex formation was observed (lanes 2 to 4). Efficient competition was also visible in assays using the HNF4 binding site of the apolipoprotein B promoter (lanes 5 to 7) and of the apolipoprotein CIII promoter (lanes 8 to 10). In contrast, an unrelated oligonucleotide representing a binding site for the transcription factor HNF3 did not affect complex formation (lanes 11 to 13), thus demonstrating the sequence specificity of DNA binding.

Recombinant HNF4β was also able to bind to the H4 oligonucleotide (Fig. 3B, lane 1), and all examined HNF4 binding sites competed for the binding of HNF4β in a dose-dependent manner (lanes 2 to 11), whereas an unrelated oligonucleotide (HNF3) had no effect (lanes 11 to 13). This finding establishes that HNF4β also binds to DNA in a highly specific manner. Only minor differences between the sequence specificities of HNF4α and HNF4β were visible: whereas the binding of HNF4α was best abolished by the HNF4 binding site of the apolipoprotein B promoter, HNF4β was competed most efficiently by the HNF4 binding site of the apolipoprotein CIII promoter. However, as shown in Fig. 3, HNF4α formed about threefold more complex compared to HNF4β, although one-fourth of the amount of HNF4β was included in the assay (compare lanes 1 in Fig. 3A and B). This finding suggests that recombinant HNF4β has a considerably lower DNA binding activity than recombinant HNF4α.

To analyze whether HNF4β was able to transactivate the transcription of a promoter containing functional HNF4β binding sites, we cloned the cDNA coding for HNF4β1 into the eukaryotic expression vector Rc/CMV (HNF4β/CMV) and performed transient transfection assays using the dedifferentiated rat hepatoma cell line C2, which lacks endogenous HNF4 (8). As an HNF4-dependent reporter, we used a construct which contains a 287-bp fragment of the *Xenopus* HNF1α promoter in front of the luciferase gene (−594/−207 luc [29]). The cotransfection of increasing amounts of HNF4β/CMV led to an eightfold activation of the reporter gene (Fig. 4). Since cotransfection of the expression vector Rc/CMV alone did not result in transactivation of the promoter (data not shown), we conclude that HNF4β is a functional transcription factor. Using the same amounts of the expression vector encoding HNF4α, a similar extent of transactivation was obtained, but in contrast to HNF4β, lower amounts of the HNF4α expression vector were needed to induce nearly saturating activation of the promoter. Similarly, using the synthetic reporter construct H4-tk-luc, which carries four HNF4 binding sites of the human α_1-antitrypsin promoter in front of the thymidine kinase pro-

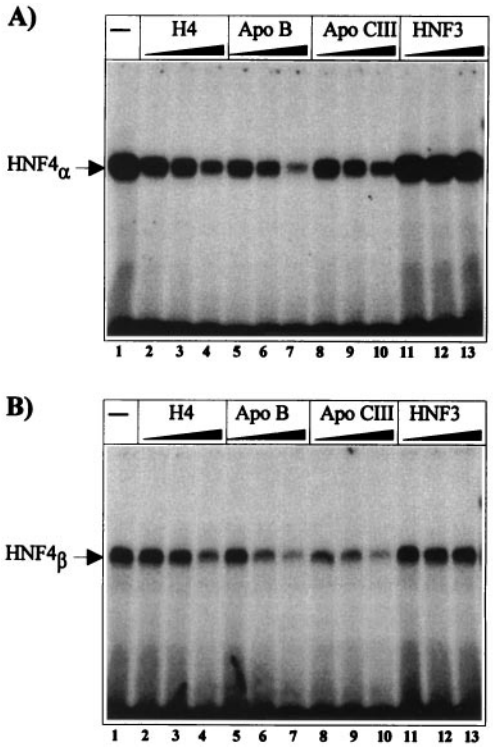

FIG. 3. HNF4α and HNF4β are able to bind highly sequence specifically to DNA in vitro. (A) Fifty nanograms of recombinant HNF4α made in the baculovirus expression system was included in a gel retardation assay using the HNF4 binding site of the *Xenopus* HNF1α promoter (H4) as a radiolabeled oligonucleotide (lane 1). The arrow indicates the retarded HNF4α-DNA complex. In lanes 2 to 4, increasing amounts of the homologous oligonucleotide (5, 10, and 20 ng) were added. A competition was performed with the HNF4 binding site of the human apolipoprotein B promoter (Apo B; lanes 5 to 7), the apolipoprotein CIII promoter (apo CIII; lanes 8 to 10), and as an unrelated oligonucleotide, the HNF3 binding site in the mouse transthyretin promoter (HNF3; lanes 11 to 13). (B) Two hundred nanograms of baculovirus-expressed recombinant HNF4β protein was used in the same assay as described for panel A.

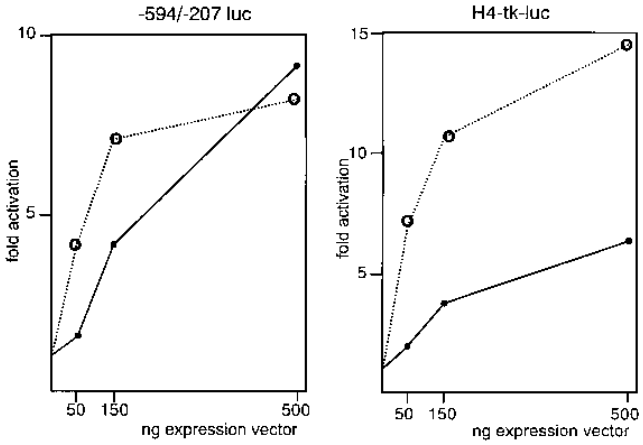

FIG. 4. HNF4α and HNF4β are functional transcription factors. Transient transfections were performed in the dedifferentiated hepatoma cell line C2, using HNF4-responsive promoter luciferase gene constructs. Construct −594/−207 luc represents a fragment of the *Xenopus HNF1α* promoter, and H4-tk-luc is composed of four HNF4 binding sites in front of the thymidine kinase promoter. The luciferase activity achieved without cotransfection of expression vectors was set to 1. The transactivation obtained by the cotransfection of the indicated amounts of the expression vectors for HNF4α (open circles) and HNF4β (closed circles) is given.

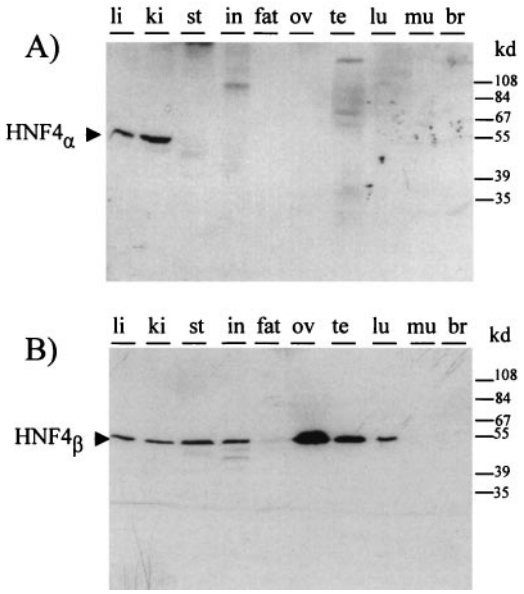

FIG. 5. HNF4α and HNF4β display different tissue distributions in adult frogs. (A) The antiserum XH4α, specific for HNF4α, was used in a Western blot analysis where protein extracts prepared from different tissues of adult *Xenopus* frogs were applied. HNF4α protein is marked with an arrow, and the migration of a protein size marker is given. li, liver; ki, kidney; st, stomach; in, intestine; ov, ovary; te, testis; lu, lung; mu, muscle; br, brain. (B) The HNF4β-specific antiserum was used in a Western blot analysis as for panel A.

moter (8), we confirmed that HNF4β1 is a less potent transactivator than HNF4α (Fig. 4).

HNF4β and HNF4α show distinct expression patterns. To reveal potentially different functions of HNF4α and HNF4β, we wanted to analyze the expression patterns of both factors in different tissues. Therefore, we raised antisera against the F domains of the two proteins, which show hardly any homology (Fig. 1B), and demonstrated the specificity on Western blots with the recombinant proteins (data not shown). Using these antisera in Western blot analyses with extracts prepared from different tissues of adult *Xenopus* frogs, we could detect HNF4α protein in the liver and kidney (Fig. 5A). HNF4β is colocalized in these organs, but it is also expressed in similar amounts in the stomach, intestine, testis, and lung (Fig. 5B). In addition, HNF4β is highly expressed in the ovary, whereas fat, muscle, and brain do not contain significant amounts of HNF4α or -β. Thus, HNF4α and -β exhibit distinct tissue distributions, arguing for different roles in the adult frog.

HNF4β is a maternal factor in *Xenopus* embryogenesis. Previously we have shown that HNF4α is a maternal factor and a start point of a transcriptional cascade during embryogenesis, leading to the localized activation of the transcription factor HNF1α (11). Therefore, we were interested in determining whether also HNF4β is present as a maternal protein. Using the HNF4β-specific antiserum, we demonstrated in Western blots (Fig. 6) that HNF4β is present from the egg stage onward and that the amount remains constant throughout development. Thus, HNF4β expression is similar to that observed for the HNF4α protein (11). In whole-mount immunostaining, HNF4β was detected in an animal-to-vegetal gradient (data not shown) as we have previously reported for the HNF4α protein (11). Furthermore, HNF4β translocates as the α form (11) into the nuclei prior to the midblastula transition, suggesting that it is involved in the early events of zygotic gene transcription in *Xenopus* (data not shown).

HNF4α and HNF4β differ in appearance during oogenesis and embryogenesis. In our previous experiments, we had shown that HNF4α is present as a maternal protein in the *Xenopus* egg, but we were unable to detect the corresponding mRNA at this developmental stage by RNase protection assays (11). Using the more sensitive technique of RT-PCR, we have now compared the appearance of the mRNA encoding HNF4β with the appearance of the mRNA coding for HNF4α during oogenesis and embryogenesis. As shown in Fig. 7, *HNF4*β mRNA accumulates during the last stage of oogenesis (stage VI [lane 5]), where just the HNF4β3 isoform is expressed. During the maturation of the oocytes into eggs, the other two isoforms are also expressed, so that the egg contains HNF4β-1, -2, and -3 (lane 6). In early cleavage stages, the *HNF4*β1 RNA persists as the predominant species (four-cell stage [lane 8] and early blastula [lane 9]) and is lost in midblastula (lane 10), when zygotic transcription starts. Since the RNAs encoding HNF4β3 and -β2 decline more rapidly than the RNA coding

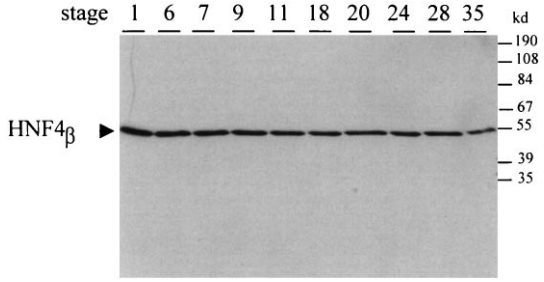

FIG. 6. HNF4β is a maternal protein and present in nearly equal amounts during *Xenopus* embryogenesis. Using the HNF4β-specific antiserum XH4β (Fig. 5), Western blotting was performed with protein extracts prepared from different stages of *Xenopus* embryogenesis. The migration of a protein molecular weight marker is indicated, and the protein band representing HNF4β is marked with an arrow. Stage 1 represents a *Xenopus* egg, stage 6, 7, and 9 are blastula embryos, and stage 11 is an early gastrula embryo. Neurula embryos were from stage 18 (neural groove) and stage 20 (neural tube). The latest embryonic stages analyzed were early tail bud (stage 24) and swimming larvae (stage 35). Staging was performed as described by Nieuwkoop and Faber (21).

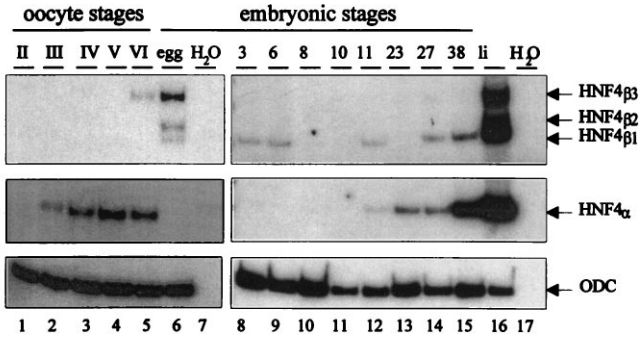

FIG. 7. HNF4α and HNF4β mRNA are expressed differentially during *Xenopus* oogenesis and early embryogenesis. RT-PCR was performed with RNA prepared from the indicated oocyte and embryonic stages. The primers were specific for HNF4β (upper panel), HNF4α (middle panel), and ornithine decarboxylase (ODC; bottom panel). An RT-PCR performed with adult *Xenopus* liver RNA (lane 16) was used as a positive control. RT-PCR done without RNA (lane 7 and 17) as well as parallel assays without addition of reverse transcriptase (data not shown) yielded no amplified fragments.

for HNF4β1 at a time period when no zygotic gene transcription occurs, we assume that the additional exons in the transcripts are involved in the RNA stability during early cleavage stages.

In early gastrula (stage 11 [lane 12]), transcripts of the *HNF4*β gene appear, but at neurula (stage 23 [lane 13]), the level of *HNF4*β mRNA drops again. Later, newly synthesized *HNF4*β RNA can be detected from tail bud stage onward (stage 27 [lane 14]) and accumulates until the swimming-larva stage (stage 38 [lane 15]). This transient expression pattern of *HNF4*β mRNA at the gastrula stage could be confirmed in RNase protection experiments (data not shown).

Analyzing the expression of the *HNF4*α gene, we observed that this mRNA accumulates much earlier in oogenesis, as the first transcripts can be detected in oocyte stage III (lane 2). The RNA level peaks at stage V (lane 4) and declines at stage VI (lane 5), and no *HNF4*α RNA is detected within the fertilized egg (lane 6). During embryogenesis, the first zygotic *HNF4*α mRNA expression is visible in early gastrula (stage 11), and the level increases until the swimming-larva stage, where the highest expression is found (lane 15). The amount and quality of the RNA were controlled by performing RT-PCR with primers specific for the gene encoding ornithine decarboxylase (Fig. 7, bottom panel), whose expression is constant throughout oogenesis and embryogenesis (2, 12).

DISCUSSION

HNF4, a newly identified family of transcription factors. Analyzing a *Xenopus* liver cDNA library, we identified a cDNA encoding a new *HNF4* gene that was named *HNF4*β to distinguish it from the previously isolated *Xenopus HNF4* gene (*HNF4*α [11]). Whereas *Xenopus* HNF4α shows a high homology to the rat HNF4α protein (11), HNF4β is more distantly related to *Xenopus* and rat HNF4α (Fig. 1A). Nevertheless, the homology is high enough to clearly classify HNF4β as an HNF4 protein. This is most easily seen in the DNA binding region including the two zinc fingers. As illustrated in Fig. 8, this domain contains only four amino acid differences between the α and β proteins, whereas in this region retinoic acid receptor RXRα, the most closely related member of the nuclear receptor superfamily, is different from HNF4α at 26 positions. Figure 8 also includes the amino acid sequences of the HNF4γ protein that we have recently identified in human kidney (9) and of the HNF4 protein found in *Drosophila melanogaster* (32). These two proteins are highly homologous but clearly distinct from HNF4β, as each member has a unique set of amino acid differences in the zinc finger domain. Since the HNF4α proteins identified so far in *Xenopus* (11), rat (24), mouse (28), and human (9) contain the same sequence in the DNA binding domain, we assume that the amino acid sequences of the β and γ proteins will also turn out to be highly conserved in the DNA binding domains of different species. This is reminiscent of the retinoic acid receptor families RAR and RXR, which are encoded from amphibians to mammals in three isoforms each, with the homologies being more extensive between different species of a given isoform than within the isoforms of one species (reviewed in reference 18).

The newly identified HNF4β protein contains in the second knuckle of the first zinc finger the P-box sequence CDGCKG (Fig. 8). The same sequence is also present in the human HNF4γ protein that we have recently identified (9) as well as in the *Drosophila* protein (32). We therefore consider this P-box sequence a hallmark of the members of the HNF4 family.

Sequencing of chromosome III of *Caenorhabditis elegans* has revealed a gene similar to *HNF4* (30). However, the sequence homology in the zinc finger region of the gene product is rather low (Fig. 8, 30 amino acid differences from HNF4α), and the P-box sequence differs from the one common to the HNF4 proteins. Therefore, we consider the *C. elegans* protein a distinct protein that does not belong to the HNF4 family.

With respect to the DNA binding region, we cannot classify the *Drosophila* HNF4 homolog into one of the three isoforms, as this protein is equally homologous to all three vertebrate isoforms (Fig. 8). We speculate that they are the product of a gene duplication event after the separation of vertebrates and invertebrates and that the *Drosophila* protein is the homolog to this ancestral HNF4 protein.

HNF4β is a functional transcription factor. Gel retardation assays with the recombinant HNF4 proteins show that both HNF4α and HNF4β are able to bind highly sequence specifically to the various known HNF4 binding sites (Fig. 3). The very similar DNA binding specificities of the two HNF4 isoforms may reflect the highly conserved DNA binding regions of both proteins (Fig. 8). However, the recombinant HNF4β protein made in baculovirus-infected cells displays a DNA binding affinity about an order of magnitude lower than that of HNF4α (Fig. 3). A similar lower binding affinity was seen in assays using recombinant HNF4β protein synthesized in the reticulocyte lysate (data not shown). Although we cannot prove that the recombinant proteins are identical to the endogenous gene products, the transfection experiments in mammalian cells are consistent with the different binding activities observed with

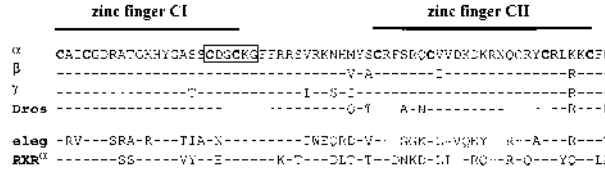

FIG. 8. Classification of the three HNF4 isoforms with respect to the sequences of their DNA binding regions. The amino acid sequences of the DNA binding regions of vertebrate HNF4α (rat [24], mouse [28], *Xenopus* [11], and human [9]), *Xenopus* HNF4β (this report), and human HNF4γ (9) are aligned. The location of the P box and the sequences of the two zinc fingers are marked. The sequences of *Drosophila* HNF4 (Dros) (32), of a potential HNF4 homolog from *C. elegans* (eleg) (30), and of the human retinoic acid receptor RXRα (17) are included for comparison.

the recombinant proteins: in assays using two different reporter constructs, higher amounts of the expression vectors coding for HNF4β were needed to get transactivation levels similar to that observed with the HNF4α constructs (Fig. 4). Since the human HNF4γ protein also exhibits a DNA binding activity lower than that of human HNF4α (10), the various isoforms seem to show a particular affinity to DNA. Therefore, we speculate that the balance between the different HNF4 family members present within a cell contributes to the overall transactivation of a promoter. This may be influenced further by the differential interactions of the HNF4 isoforms with other transcription factors, cofactors, or potential ligands. Furthermore, one has to consider that splice variants may alter the transactivation potential, as recently demonstrated in human cells for the HNF4α variants that encode distinct proteins (9). Concerning the three splice variants identified for the *Xenopus HNF4*β gene (Fig. 2), we would not expect a change in the encoded protein, as the additional sequences are upstream of the translation initiation site. However, one has to keep in mind that alternative 5′ untranslated regions may influence the translation efficiency. Such mechanisms are believed to be especially important during early embryogenesis prior to zygotic transcription (reviewed in reference 7).

To address the question of whether HNF4 isoforms can heterodimerize, we analyzed liver nuclear extracts containing HNF4α and HNF4β or extracts prepared from baculovirus-infected cells expressing HNF4α and HNF4β simultaneously. Using antibodies specific for the isoforms, we failed to demonstrate any heterodimerization of HNF4 in gel retardation assays (data not shown). In transient transfection assays where both HNF4 proteins were cotransfected simultaneously in nonsaturating concentrations, the resulting transactivation was additive compared to the transfection of the particular factor alone (data not shown). Therefore, even if heterodimers were formed, they seemed to exhibit characteristics expected for the homodimers. Thus, it is not the heterodimerization between HNF4α and HNF4β that creates a new regulatory control within a cell but rather the balance between the amounts of HNF4α and HNF4β.

HNF4α and HNF4β show different expression patterns in adult frogs. Using a specific antiserum for HNF4α, we revealed that this isoform is expressed in livers and kidneys of adult *Xenopus* frogs (Fig. 5A). This is in partial agreement with the expression data for rat *HNF4* mRNA, which was detected in the liver, kidney, and intestine (24). The absence of HNF4α in the adult intestine seems to be *Xenopus* specific, since the mRNA for mouse *HNF4* was also detected in this organ (28). In contrast, HNF4β protein was found in the liver, kidney, digestive tract (stomach, intestine), gonads (ovary and testis), and lung but not in fat, muscle, and brain (Fig. 5B). Thus, HNF4β exhibits a much broader tissue distribution than HNF4α. These different expression patterns of HNF4α and -β suggest that the two proteins have different functions in the adult frog. Similarly, the human HNF4γ protein displays an overlapping but distinct tissue distribution compared to its HNF4α counterpart (9). Therefore, we speculate that each member of the HNF4 family has its own role in the various tissues and that this function has been largely conserved during vertebrate evolution. A comparable situation seems to exist for the members of the RAR and RXR families of transcription factors, which also show overlapping but distinct expression patterns in mammals (reviewed in reference 18).

The different expression patterns of HNF4α and HNF4β during oogenesis and embryogenesis imply distinct functions. During embryogenesis in *Xenopus*, HNF4α (11) and HNF4β (Fig. 6) are maternal transcription factors, arguing that both play essential roles during early development. Furthermore, both HNF4α (11) and HNF4β (data not shown) are localized within an animal-to-vegetal gradient in early cleavage stages, implying that during this period, both HNF4 proteins may act in the same cells. As the *Drosophila* HNF4 homolog is also a maternal factor (32), we conclude that this function during early development has been evolutionarily conserved. In *Drosophila*, this role seems to involve functions in the establishment of organs homologous to vertebrate kidney, liver, and gut. In mammals, the homozygous loss of HNF4α leads to early embryonic death at day 10.5 postcoitum, showing the essential role of this isoform (5). Thus, the potential homologs of HNF4β and HNF4γ are not able to complement the lack of HNF4α in mammalian development, which would further indicate distinct roles for the two HNF4 isoforms.

In addition to the unique spatial distribution of both HNF4 isoforms, we observed significant differences between the temporal expression patterns of HNF4α and HNF4β during oogenesis and development (Fig. 7). Using the sensitive technique of RT-PCR, we detect transcripts of the *HNF4*α gene in the growing oocyte at stage III, and this level increases gradually, but the mRNA is lost in the fertilized egg. In contrast, *HNF4*β transcripts can be detected only in the latest stage of oogenesis and in the fertilized egg (Fig. 7). As we know that these transcripts are translated into proteins in the oocytes (data not shown), we speculate that HNF4α and HNF4β may already function as transcription factors during oogenesis. However, the genes that might be regulated by HNF4 in these cells are not known.

Based on the RT-PCR assay, no *HNF4*α transcripts are present in the fertilized egg and during the early stages of embryogenesis (Fig. 7). Nevertheless, HNF4α protein is a maternal component in the egg, as shown by Western blots and whole-mount immunocytochemistry (11). This finding implies that HNF4α is a rather stable protein compared to its mRNA and that it is the store of HNF4α made during oogenesis that persists in early embryogenesis. Based on this quite unexpected finding, we would like to stress that the absence of a specific mRNA in the fertilized egg does not exclude the presence of the protein as a maternal factor. Furthermore, one should be aware that the identification of an mRNA as a maternal component does not necessarily prove the presence of the corresponding protein. Obviously, the only reliable conclusion for the presence of a maternal transcription factor can be obtained by using specific antibodies; therefore, many data concerning maternal factors should be reevaluated.

One of the target genes activated by maternal HNF4 encodes the tissue-specific transcription factor HNF1α (11, 31), which is activated shortly after the midblastula transition (1). We have recently demonstrated that the embryonic inducer activin A exerts its effect via the HNF4 binding site in the HNF1α promoter to activate HNF1α gene transcription (29). Therefore, HNF4 is one of the first factors identified which connects embryonic induction processes with transcription and contributes to the complex expression pattern of genes that are induced in endodermal tissues such as the liver and the gut as well as in the mesodermally derived pronephros of the developing larvae (29). It is an attractive hypothesis that this process is influenced by the balance of the two HNF4 isoforms within a cell.

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