The mutated human gene encoding hepatocyte nuclear factor 1β inhibits kidney formation in developing *Xenopus* embryos

Wiltrud Wild*, Elke Pogge von Strandmann*, Aristotelis Nastos*, Sabine Senkel*, Anja Lingott-Frieg*, Michael Bulman†, Coralie Bingham†, Sian Ellard†, Andrew T. Hattersley†, and Gerhart U. Ryffel*‡

*Institut für Zellbiologie (Tumorforschung), Universitätsklinikum Essen, Essen, Germany; and †Department of Diabetes and Vascular Medicine, School of Postgraduate Medicine and Health Sciences, University of Exeter, Exeter, United Kingdom

Edited by Donald D. Brown, Carnegie Institution of Washington, Baltimore, MD, and approved February 22, 2000 (received for review January 10, 2000)

The transcription factor hepatocyte nuclear factor 1β (HNF1β) is a tissue-specific regulator that also plays an essential role in early development of vertebrates. In humans, four heterozygous mutations in the HNF1β gene have been identified that lead to early onset of diabetes and severe primary renal defects. The degree and type of renal defects seem to depend on the specific mutation. We show that the frameshift mutant P328L329fsdelCCTCT associated with nephron agenesis retains its DNA-binding properties and acts as a gain-of-function mutation with increased transactivation potential in transfection experiments. Expression of this mutated factor in the *Xenopus* embryo leads to defective development and agenesis of the pronephros, the first kidney form of amphibians. Very similar defects are generated by overexpressing in *Xenopus* the wild-type HNF1β, which is consistent with the gain-of-function property of the mutant. In contrast, introduction of the human HNF1β mutant R137-K161del, which is associated with a reduced number of nephrons with hypertrophy of the remaining ones and which has an impaired DNA binding, shows only a minor effect on pronephros development in *Xenopus*. Thus, the overexpression of both human mutants has a different effect on renal development in *Xenopus*, reflecting the variation in renal phenotype seen with these mutations. We conclude that mutations in human HNF1β can be functionally characterized in *Xenopus*. Our findings imply that HNF1β not only is an early marker of kidney development but also is functionally involved in morphogenetic events, and these processes can be investigated in lower vertebrates.

The tissue-specific transcription factors HNF1α and HNF1β are two closely related homeodomain factors that initially were identified in hepatocytes but are also present in various other cell types of endodermal and mesodermal origin (1–4). In vertebrate development, both transcription factors are expressed in defined embryonic regions including the developing kidney. In mammals (5–7) as well as in *Xenopus* (8, 9), the expression of HNF1β precedes the appearance of HNF1α. HNF1β-deficient mouse embryos die soon after implantation with poorly organized ectoderm and no discernible visceral or parietal endoderm (10, 11). This embryonic lethality so far has precluded the analysis of later functions of HNF1β in mammalian development. Heterozygous mutations in the human HNF1β gene have been detected in patients with maturity onset diabetes of the young (MODY) (12–15), but in contrast to patients with mutations in other MODY genes (16, 17), mutated HNF1β is also associated with severe nondiabetic renal defects (12–15). In the case of the R137-K161del mutant, two of four females, in addition, had genital malformation with vaginal aplasia and rudimentary uterus (14). The mutation P328L329fsdelCCTCT (P328L329) associated with nephron agenesis encodes a truncated protein retaining the DNA-binding domain (15) and, thus, is distinct from the three mutants that all lack part of the DNA-binding domain (12–14).

Here we show that the HNF1β mutant P328L329 has an increased transactivation potential and that its overexpression in *Xenopus* embryos leads to defective development and agenesis of the pronephros, the first kidney form of amphibians. In contrast, introduction of the HNF1β mutant R137-K161del with impaired DNA binding has a minor effect on pronephros development.

Materials and Methods

Constructions of HNF1 Expression Plasmids. The cDNA clone encoding human HNF1β [vHNF1-A in Bach *et al.* (18)] kindly provided by Moshe Yaniv (Institut Pasteur, Paris) was used to amplify the ORF by PCR using as forward and reverse primers the oligonucleotides 5′-GGCAAAGCTTCCATGGTGTCCAAGCTCACG-3′ and 5′-CCATCTAGACGTCCGTCAGGTAAGC-3′, respectively. The amplified fragment was digested with *Hin*dIII and *Xba*I and inserted into the expression vector myc-Rc/CMV. This plasmid is derived from the expression vector Rc/CMV (Invitrogen) by inserting into the *Hin*dIII site a *Cla*I-*Xho*I fragment containing the six myc epitope tags of the Mt + pCS2 (19). The expression vector encoding the mutant P328L329fsdelCCTCT was generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the oligonucleotides 5′-CAGCCTGAACGCTCTCCCAC-3′ and 5′-GTGGGAGAGCGTTCAGGCTG-3′ as primers. The expression vector encoding the mutant R137-K161del was generated by replacing the *Hin*dIII-*Bsr*GI fragment of the HNF1β expression vector with the *Hin*dIII-*Bsr*GI fragment of a PCR product made with the forward primer 5′-ATTCAAGGCCTCTCGAAGCTTCCATGGTGTCCAAGCTCAC-3′ and the reverse primer containing complementary sequences upstream and downstream of the deletion (14). The expression vector encoding human HNF1α was generated by inserting a *Hin*dIII-*Nsi*I fragment containing amino acids 1–235 and the *Nsi*I fragment containing the remaining part of the ORF into the myc-Rc/CMV vector. The *Hin*dIII-*Nsi*I segment was derived from a PCR amplification product made with the forward primer 5′-GGCAAAGCTTCCATGGTTTCTAAACTGAGCCAG-3′ and a reverse primer downstream of the *Nsi*I site at amino acid 241. Thus, the entire ORF of the human HNF1α as cloned in HCL16 (20) was linked to the six myc epitope tags. The integrity of the ORFs in all of the expression vectors was verified by sequencing the inserted DNA sequences.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: HNF, hepatocyte nuclear factor; MODY, maturity onset of the young; GFP, green fluorescence protein.

‡To whom reprint requests should be addressed at: Universitätsklinikum Essen, Institut für Zellbiologie (Tumorforschung), Hufelandstrasse 55, D-45122 Essen, Germany. E-mail: gerhart.ryffel@uni-essen.de.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.080010897. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.080010897

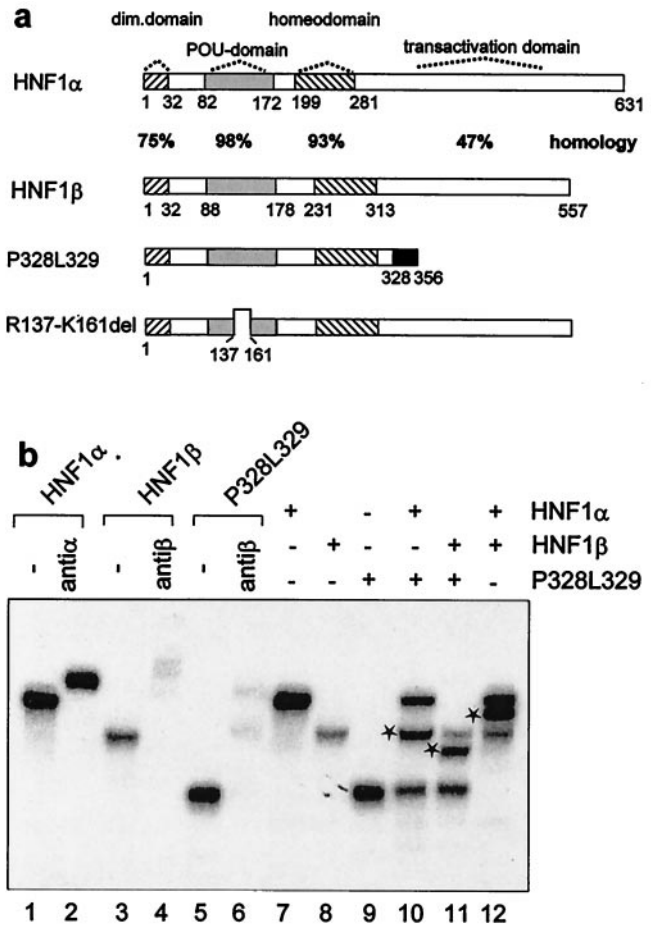

Fig. 1. DNA binding and dimerization of the P328L329 mutant. (*a*) Scheme of the related human transcription factor HNF1α and HNF1β with the frameshift mutations P328L329fsdelCCTCT (P328L329) and R137-K161del. The various domains are indicated, and the numbers refer to the amino acid positions. The homology between HNF1α and HNF1β is given (2). (*b*) Gel-retardation assay using the ^{32}P-labeled HNF1-binding site as a probe. The additions of the various *in vitro* translation products and of the antibodies are given. The heterodimers are marked with an asterisk. The methods used are described (21).

Results

We investigated the functional characteristics of the HNF1β mutation that was associated with absence of nephrons in an 18-week-old fetus (15). Because the deletion in P328L329 is 14 aa downstream of the homeodomain of the HNF1β DNA-binding domain (Fig. 1*a*), we explored whether the mutated factor retains DNA binding. Using *in vitro* translation to generate the truncated protein P328L329, we tested the DNA-binding properties in gel retardation assays with the HP1 oligonucleotide containing an HNF1-binding site (22). Fig. 1*b* demonstrates that P328L329 binds to the HP1 oligonucleotide by forming a complex (lane 5) that is abolished by the addition of an antibody (lane 6) raised against full-length HNF1β (21). As expected, the complex containing P328L329 migrates faster than the complexes formed with full-length HNF1β (Fig. 1*b*, lane 3) and HNF1α (lane 1), which interact specifically with antibodies against HNF1β and HNF1α, respectively. By mixing the translational product of P328L329 with the wild-type factors, we could show that the mutant heterodimerizes with HNF1α or HNF1β, because in each mixed probe a complex with intermediate mobility could be detected (Fig. 1*b*, lanes 10 and 11). In conclusion, these binding studies reveal that P328L329 retains its DNA-binding activity and its ability to form heterodimers. This is in clear contrast to the mutant R137-K161del that contains a 25-aa deletion in the POU domain involved in DNA binding (Fig. 1*a*) and that has been shown to lack DNA binding (14). Thus, these two mutated factors have distinct properties and this correlates with the different clinical phenotype: the P328L329 mutant is associated with the absence of nephrons (15), whereas the mutant R137-K161del is characterized by oligomeganephronia, a condition characterized by a reduced number of nephrons with hypertrophy of the remaining ones (14).

To define the function of the P328L329 protein we determined its transactivation potential in transfection experiments in HeLa cells by using a reporter construct containing four binding sites for HNF1 in front of the thymidine kinase promoter (23). As shown in Fig. 2*a*, the addition of increasing amounts of an expression vector encoding P328L329 gradually activates the reporter without reaching saturation under the conditions used. In contrast, corresponding transactivation assays with wild-type HNF1β reached saturation at least 2-fold below the value attained by P328L329. Very similar data were obtained by using a reporter construct driven by the albumin promoter containing a single HNF1-binding site (data not shown). Because only one allele is mutated in the patients and the mutated HNF1β factor heterodimerizes with wild-type HNF1β (Fig. 1*b*), we cotransfected expression vectors encoding mutated and wild-type HNF1β to mimic homozygous and heterozygous situations. Under all conditions tested, the mutant P328L329 affects the activity of the wild-type factor as expected from the contribution of its own activity (data not shown). In conclusion, the transfection data establish that P328L329 behaves as a factor with an increased transactivation potential compared with the wild type. In contrast, the mutant R137-K161 lacking part of the DNA-binding domain shows no transactivation in transfection assays and does not affect the activity of the wild-type factor (14).

The development of the amphibian species *Xenopus* laevis is a most valuable system with which to analyze early embryonic events in vertebrates. Using this model we have shown previously that the *Xenopus* HNF1α promoter contains an HNF1-binding site (26). By injecting synthetic RNA encoding human HNF1α or HNF1β into fertilized *Xenopus* eggs, we observed in the late gastrula an activation of the endogenous HNF1α gene (Fig. 2*b*, lanes 5 and 6). When RNA encoding P328L329 was injected, a similar increase in HNF1α transcripts was observed (lanes 7 and 8). Clearly, these injection experiments establish that both human HNF1 proteins as well as the mutated protein have the potential to activate the endogenous HNF1α gene in developing *Xenopus*.

In vertebrates, three distinct kidney forms are made sequentially, with the pronephros as the first and most simple version containing only one nephron. This first kidney has a similar functional organization as the mesonephros and metanephros, which have 10–50 and about a million nephrons, respectively. In addition, the same factors seem to control the genesis of these three different types of kidneys (27, 28). To explore whether the truncated HNF1β interferes with nephrogenesis, we injected the RNA encoding P328L329 into a single cell of the two-cell-stage embryo, coinjecting RNA encoding GFP (green fluorescence protein) as a tracer. Some of the embryos did not develop properly through gastrulation and neurulation, but that was quite variable from one experiment to the next, implying rather technical inconsistencies than a specific effect of P328L329 expression. At the swimming larval stage we selected all normal animals, with green fluorescence restricted to either the right or left side. As exemplified in the animal shown in Fig. 3*a*, the left side marked by GFP lacks the coiled tubular body of the pronephros (Fig. 3*b*), whereas the pronephros is normally developed on the right side.

Because our transfection data have shown that P328L329 has a higher transactivation potential than the wild-type factor (Fig. 2*a*) and HNF1β is known to appear early in *Xenopus* develop-

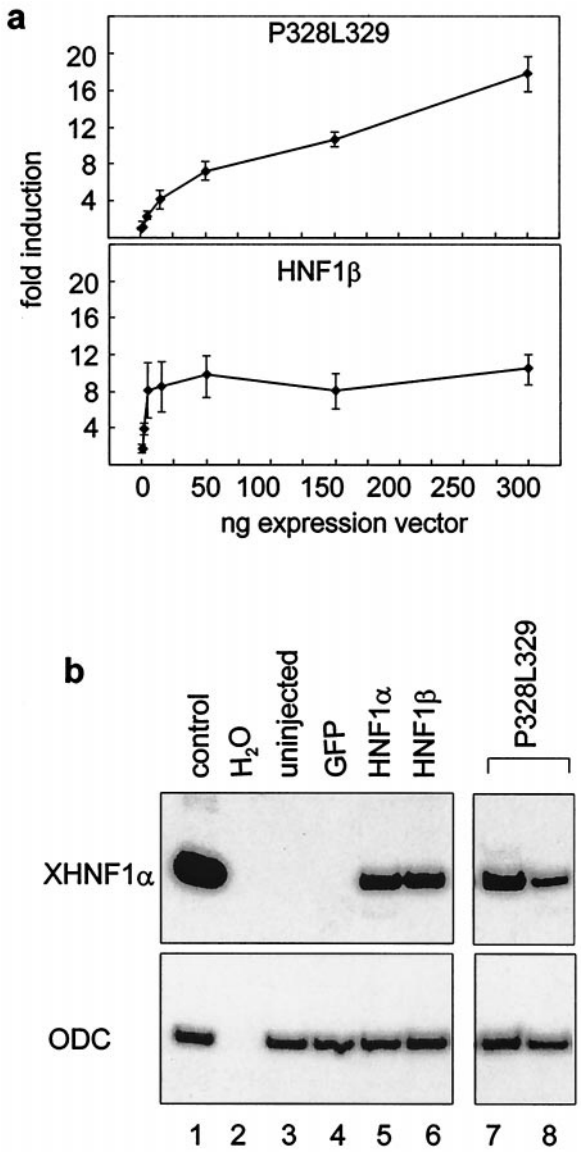

Fig. 2. Transactivation potential of the P328L329 mutant. (*a*) Increasing amounts of expression vector encoding P328L329 or HNF1β were cotransfected into HeLa cells with a luciferase reporter containing four HNF1-binding sites (23). The fold activation compared with transfections lacking any transfected factor is given. The SDs from at least six determinations are presented, and the methods used have been described (23). (*b*) RNA encoding HNF1α, HNF1β, or P328L329 was injected into fertilized *Xenopus* eggs, and the RNA was extracted at the late gastrulae stage (stage 12.5). Endogenous HNF1α and ODC (ornithine desoxycarboxylase) transcripts were determined by reverse transcription–PCR as described (24). The control is RNA of a stage 20 embryo. Stages are as given in ref. 25.

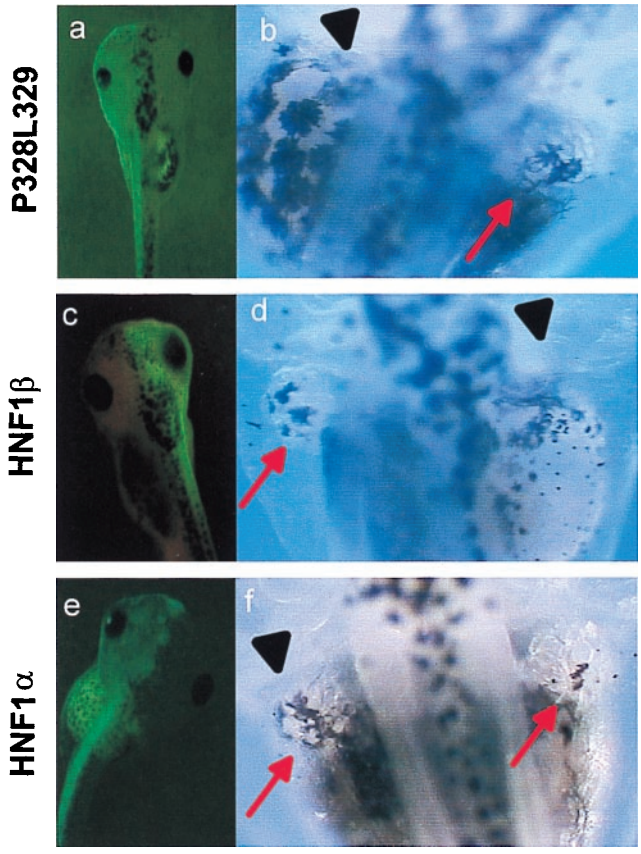

Fig. 3. Phenotypic changes in pronephros formation observed in living *Xenopus* larvae (stages 45–48). Two-cell-stage embryos were injected with 0.25 ng of GFP RNA together with 1 ng of RNA encoding P328L329 (*a* and *b*), HNF1β (*c* and *d*), or HNF1α (*e* and *f*). Larvae expressing green fluorescence on the left or right side are given in *a*, *c*, and *e*. The same animals were examined for pronephros formation at higher magnification (*b*, *d*, and *f*). The injected side and the pronephros are marked by black arrowheads and by red arrows, respectively. The techniques have been described (24).

ment (4, 9), we assumed that overexpression of the wild-type HNF1β in *Xenopus* embryos has a similar effect. Fig. 3 *c* and *d* document that overexpression of the wild-type factor leads to impaired pronephros development on the injected side. This is a specific effect because injecting the related HNF1α transcription factor into the embryos does not interfere with pronephros development (Fig. 3 *e* and *f*).

A quantification of the developmental defects (Fig. 4) in 95% of the animals had a significant effect on overexpression of P328L329. In the majority of these affected animals, a complete lack of the pronephros was obtained. A similar high extent in defective pronephros development was observed in the larvae derived from HNF1β-injected embryos. However, only 6% of larvae derived from embryos injected with the mutation R137-K161del contained either a reduction or a complete agenesis of the pronephros. Thus, the mutation R137K161del affects pronephros development at a much lower level than the mutant P329L329. The effect of R137K161del is clearly above the level observed upon HNF1α injection (Fig. 4, where no effect was seen) and the reported 1.5% spontaneous abnormal pronephros development (29).

Fig. 5 illustrates the range of developmental defects observed in P328L329 and HNF1β RNA injected larvae by using antibodies recognizing terminal differentiation markers of the pronephric tubules and duct (30, 31). In stage 36–38 embryos, where the appearance of the pronephric tubule has occurred, a clear reduction (Fig. 5*a*) or even a complete absence of pronephric differentiation (Fig. 5*b*) was observed in the injected side of the larvae. In contrast, normal pronephric tubules could be identified in larvae derived from HNF1α-injected, two-cell-stage embryos (Fig. 5*c*). In feeding larvae of stages 45–48 pronephros development has progressed further by an extensive coiling of the tubules as well as the appearance of the Wolffian duct extending in its anterior region as a coil underneath the convoluted pronephric tubules and reaching the cloaca at the posterior end (see Fig. 5*i* and ref. 30). Analyzing injected larvae at this later stage revealed complete agenesis of the pronephros in some larvae (Fig. 5*d*) or a substantial reduction of the pronephros in

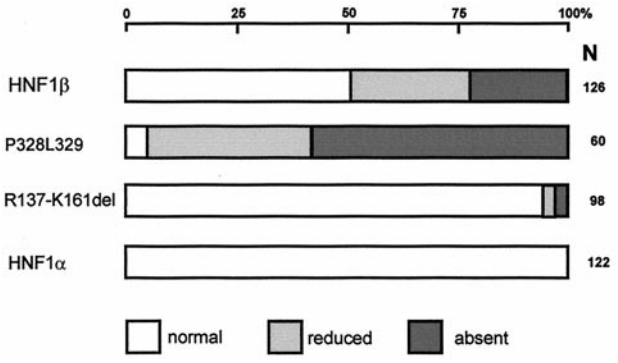

Fig. 4. Quantification of the phenotypic changes in pronephros formation observed in living *Xenopus* larvae. Injected normal larvae that showed green fluorescence on either the right or left side were scored at stage 45–48 for pronephros development on the injected side. The percent distribution of pronephros of normal or reduced size as well as the absence is given for the various mutants analyzed. Reduction in size of at least one-fourth was used as criteria to classify as reduced whereas in cases defined as absent, no tubular structures were visible. The number of analyzed larvae is given (N). Larvae that had unusual pigmentation, differences in the size of the eyes, or a narrower head structure were included. These minor phenotypes approximated to about 20% and were not correlated to the type of transcription factor injected.

other animals (Fig. 5 *e*– *g*). Quite frequently, the Wolffian duct was connected to cyst-like structures (Fig. 5*e*) or even completely absent (Fig. 5*g*). Such defects were never seen in larvae derived from HNF1α-injected embryos (Fig. 5 *h* and *i*) or in untreated animals (data not shown). The cyst-like structures found in the affected *Xenopus* pronephros are reminiscent of the cysts observed in human patients (12–15). Immunostaining the larvae derived from R137-K161del-injected embryos in general revealed a completely normal morphology (data not shown) except in the few cases in which a reduced or deficient pronephric structure was seen that was identical to the one observed in the P329L329-injected larvae.

The transcription factor Xlim-1, the *Xenopus* homologue of the mammalian factor lim-1 (32), is one of the earliest markers known to define the pronephros anlage (27, 28, 33). Using an antisense probe for Xlim-1 in whole-mount *in situ* hybridization experiments of tail-bud embryos, we observed Xlim-1 expression in the dorsolateral region developing into pronephros and pronephric duct (Fig. 6). Most significantly, this pattern of Xlim-1 expression shows no reduction in larvae derived from injected two-cell-stage embryos (compare left and right side in the HNF1β- and HNF1α-injected embryos in Fig. 6 *a* and *b*, respectively). Thus, we conclude that the effect of HNF1β on pronephros development is exerted downstream of the expression of Xlim-1.

Discussion

Our results establish a distinct property between the two related transcription factors HNF1α and HNF1β in the early development of *Xenopus*, and this difference correlates with their known defects in humans: mutations in both human HNF1 genes cause MODY by altering β cell function (16, 17), but they lead to very different renal phenotypes. Mutations in the human HNF1α gene (reviewed in ref. 16) do not affect kidney morphogenesis but may alter renal glucose reabsorption (34), whereas mutations in the human HNF1β gene can lead to severe renal malformations that are present early in fetal development (12–15). A clear functional distinction between the two members of the HNF1 family also is observed in knock-out mice, where the homozygous inactivation of the HNF1α gene affects only postnatal development, including hepatic dysfunction, a renal Fanconi

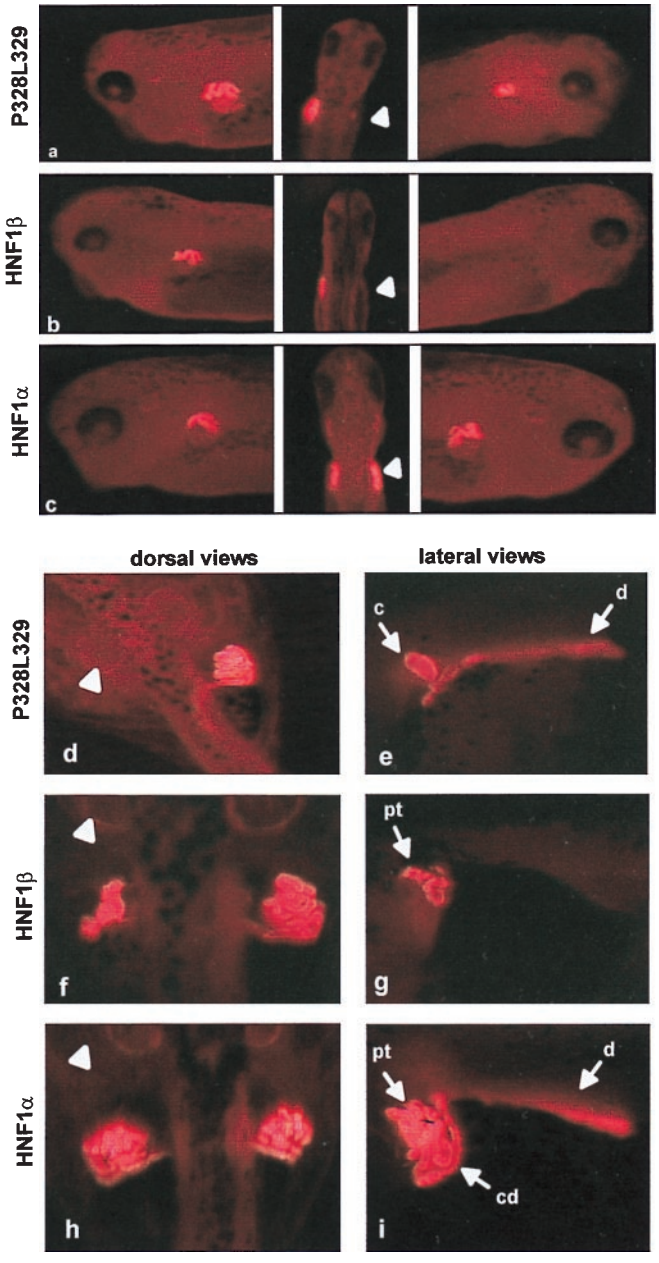

Fig. 5. Whole-mount staining of the pronephros of larvae derived from injected embryos. (*a*– *i*) Whole-mount immunostainings with a mixture of the mAbs 3G8 and 4A6, which recognize terminal differentiation markers of the pronephric tubules and duct, respectively (30). A Cy-3-conjugated secondary anti-mouse antibody was used to get red fluorescence (31). (*a*– *c*) Stage 37–39 larvae obtained from P328L329-, HNF1β-, and HNF1α-injected embryos. Each image shows an individual animal with its lateral and dorsal views. The injected side is marked by a white arrowhead. (*d*– *i*) Individual stage 45–48 larvae that had received the indicated constructs. The injected sides are marked by white arrowheads in the dorsal views, and the lateral views represent the injected sides. Pronephric tubules (pt), ducts (d), coiled ducts (cd), and cysts (c) are indicated in the lateral views.

syndrome, defective pancreatic insulin secretion, and dwarfism (35–37), whereas the corresponding knock-out of the HNF1β gene leads to embryonic lethality by impaired ectoderm and endoderm differentiation (10, 11). A corresponding distinction between HNF1α and HNF1β function is seen in our experiments in *Xenopus*, where only HNF1β overexpression affects pronephros development (Fig. 4). All these *in vivo* defined differences

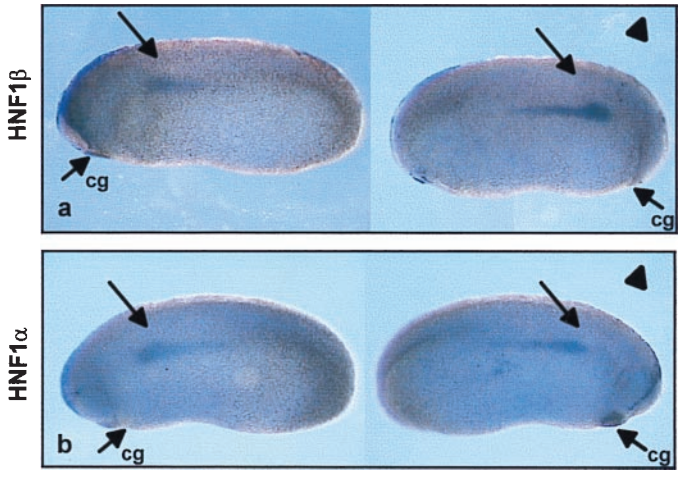

Fig. 6. Whole-mount staining of the pronephros anlage by Xlim-1. (*a* and *b*) Whole-mount *in situ* hybridizations using an Xlim-1 antisense probe on HNF1β- and HNF1α-injected embryos. The left and right side of the same animal is shown with the cement gland (cg) marked to indicate the anterior part of the stage 20 tail-bud embryos. The arrow points to the pronephros anlage stained by Xlim-1 hybridization. The staining involved 8, 9, or 4 embryos injected with HNF1α, HNF1β, or P329L329 (not shown) and was indistinguishable from 10 uninjected embryos.

between HNF1α and HNF1β document the differential function of these two related transcription factors that show only minor differences when assayed *in vitro* by transfection assays and DNA-binding studies (2, 3).

Our results show that, consistent with the transfection data, the P328L329 mutant seems to act as a gain-of-function mutation in *Xenopus*, because it generates in the developing embryo the same phenotype as the overexpression of the wild-type factor that is known to be expressed in the pronephros anlage (9). The R137-K161del mutant, which is, in contrast, a loss-of-function mutation in DNA-binding and transfection assays (14), affects pronephros development in *Xenopus* as well, although to a much lower degree (Fig. 4). Therefore, based on the *Xenopus* assay system the R137-K161del mutant cannot be classified as a loss-of-function mutation. This finding agrees with the observations that humans containing this type of mutation show renal defects (12–14). The interpretation that the R137-K161del mutant is not a loss-of-function mutation agrees with the recent finding that mice heterozygous for HNF1β gene deficiency are completely normal (10, 11), because in this case one allele is completely inactivated whereas the mutated factors found in humans retain an activity leading to disturbed nephrogenesis.

Based on our observation that the HNF1α RNA in the *Xenopus* embryo is increased upon HNF1β expression (Fig. 2*b*), the HNF1α promoter is a potential target of HNF1β *in vivo* in the developing pronephros. Consistent with this interpretation, HNF1β expression already is seen in the early gastrula (stage 10) preceding the appearance of HNF1α in developing *Xenopus* (4, 8, 9). Using a myc-specific antibody to detect the proteins made on the injected RNA, the human HNF1β constructs are abundantly present at stage 13 (data not shown). This demonstrates that the introduced factor may exert its action at the time normal HNF1β function occurs. However, it is unlikely that the developmental defect generated by the mutated HNF1β protein as well as by the wild-type HNF1β protein acts via HNF1α expression, because HNF1α overexpression fails to induce any developmental defects (Figs. 3*f* and 5 *c*, *h*, and *i*). Because the expression of Xlim-1 is not influenced by overexpression of wild-type HNF1β (Fig. 6) or the mutant P328L329 (data not shown), we conclude that HNF1β acts downstream of the expression of Xlim-1. Hence, the observed agenesis of the pronephros depends on a mechanism distinct from the reduction of pronephros formation observed upon overexpression of the tumor suppressor WT1, which is correlated with a decrease in Xlim-1 expression (29). Our findings imply that HNF1β not only is an early marker of pronephros development but also is functionally involved in morphogenetic events. In addition to HNF1β, the transcription factors Xlim-1 and XPax-8 are present in the pronephros anlage of the *Xenopus* neurula (reviewed in ref. 28). Overexpression of either of these two factors leads to a moderate enlargement of the embryonic kidney, whereas the coexpression of both factors generates a synergistic effect with pronephric structures of up to five times the normal size and even some ectopic pronephric tubules (38). Because these two transcription factors influence pronephros development in the opposite way as HNF1β, we speculate that the concerted action of HNF1β, Xlim-1, and XPax-8 is a crucial event for normal renal organogenesis.

The attractiveness of the *Xenopus* pronephros as a simple model for the genesis of the vertebrate excretory system relies mainly on the fact that it represents a single nephron and, thus, the basic unit of the successive renal vertebrate organs, i.e., mesonephros and metanephros (27, 39). Furthermore, the developing pronephros easily can be monitored in *Xenopus* as exemplified in our study. In addition, pronephros development not only can be dissected in the entire *Xenopus* embryo, but also can be induced in embryonic explants (40, 41) with the potential of organ engineering (42). Our finding that HNF1β plays an essential role in pronephros morphogenesis in *Xenopus* opens up new approaches to dissect the molecular mechanisms involved in these processes. Furthermore, this lower vertebrate species is a most relevant model for mammalian development as well.

We are most grateful to M. Yaniv and I. Dawid for the human HNF1 and Xlim-1 cDNAs, respectively, and E. A. Jones for antibodies 3G8 and 4A6. This work was supported by the Deutsche Forschungsgemeinschaft, the British Diabetic Association, and the Exeter Kidney Unit Development Fund.

1. Mendel, D. B. & Crabtree, G. R. (1991) *J. Biol. Chem.* **266,** 677–680.
2. Tronche, F. & Yaniv, M. (1992) *BioEssays* **14,** 579–587.
3. Cereghini, S. (1996) *FASEB J.* **10,** 267–282.
4. Pogge v. Strandmann, E., Nastos, A., Holewa, B., Senkel, S., Weber, H. & Ryffel, G. U. (1997) *Mech. Dev.* **64,** 7–17.
5. Lazzaro, D., De Simone, V., De Magistris, L., Lehtonen, E. & Cortese, R. (1992) *Development* **114,** 469–479.
6. Cereghini, S., Ott, M. O., Power, S. & Maury, M. (1992) *Development* **116,** 783–797.
7. Coffinier, C., Barra, J., Babinet, C. & Yaniv, M. (1999) *Mech. Dev.* **89,** 211–213.
8. Bartkowski, S., Zapp, D., Weber, H., Eberle, G., Zoidl, C., Senkel, S., Klein-Hitpass, L. & Ryffel, G. U. (1993) *Mol. Cell Biol.* **13,** 421–431.
9. Demartis, A., Maffei, M., Vignali, R., Barsacchi, G. & De Simone, V. (1994) *Mech. Dev.* **47,** 19–28.
10. Barbacci, E., Reber, M., Ott, M., Breillat, C., Huetz, F. & Cereghini, S. (1999) *Development* **126,** 4795–4805.
11. Coffinier, C., Thépot, D., Babinet, C., Yaniv, M. & Barra, J. (1999) *Development* **126,** 4785–4794.
12. Horikawa, Y., Iwasaki, N., Hara, M., Furuta, H., Hinokio, Y., Cockburn, B. N., Lindner, T., Yamagata, K., Ogata, M., Tomonaga, O., *et al.* (1997) *Nat. Genet.* **17,** 384–385.
13. Nishigori, H., Yamada, S., Kohama, T., Tomura, H., Sho, K., Horikawa, Y., Bell, G. I., Takeuchi, T. & Takeda, J. (1998) *Diabetes* **47,** 1354–1355.
14. Lindner, T. H., Nj, Horikawa, Y., Bostad, L., Bell, G. I. & Sovik, O. (1999) *Hum. Mol. Genet.* **8,** 2001–2008.
15. Bingham, C., Ellard, S., Allen, L., Bulman, M., Shephard, M., Frayling, T., Berry, P. J., Clark, P. M., Lindner, T., Bell, G. I., *et al.* (2000) *Kidney Int.* **57,** 898–907.
16. Hattersley, A. T. (1998) *Diabetes Med.* **15,** 15–24.
17. Frayling, T., Beards, F. & Hattersley, A. T. (1999) in *Type 2 Diabetes: Predictions and Prevention*, ed. Hitman. G. A. (Wiley, New York), pp. 107–126.

18. Bach, I., Mattei, M. G., Cereghini, S. & Yaniv, M. (1991) *Nucleic Acids Res.* **19,** 3553–3559.
19. Rupp, R. A., Snider, L. & Weintraub, H. (1994) *Genes Dev.* **8,** 1311–1323.
20. Bach, I., Galcheva-Gargova, Z., Mattei, M. G., Simon-Chazottes, D., Guenet, J. L., Cereghini, S. & Yaniv, M. (1990) *Genomics* **8,** 155–164.
21. Lemm, I., Lingott, A., Pogge v. Strandmann, E., Zoidl, C., Bulman, M. P., Hattersley, A. T., Schulz, W. A., Ebert, T. & Ryffel, G. U. (1999) *Mol. Carcinogen.* **24,** 305–314.
22. Clairmont, A., Ebert, T., Weber, H., Zoidl, C., Eickelmann, P., Schulz, W. A., Sies, H. & Ryffel, G. U. (1994) *Cancer Res.* **54,** 1319–1323.
23. Drewes, T., Clairmont, A., Klein-Hitpass, L. & Ryffel, G. U. (1994) *Eur. J. Biochem.* **225,** 441–448.
24. Nastos, A., Pogge v. Strandmann, E., Weber, H. & Ryffel, G. U. (1998) *Nucleic Acids Res.* **26,** 5602–5608.
25. Nieuwkoop, P. & Faber, J. (1975) *Normal Table of Xenopus laevis (Daudin)* (Elsevier/North–Holland, New York).
26. Holewa, B., Pogge v. Strandmann, E., Zapp, D., Lorenz, P. & Ryffel, G. U. (1996) *Mech. Dev.* **54,** 45–57.
27. Carroll, T., Wallingford, J., Seufert, D. & Vize, P. D. (1999) *Curr. Top. Dev. Biol.* **44,** 67–100.
28. Brändli, A. W. (1999) *Int. J. Dev. Biol.* **43,** 381–395.
29. Wallingford, J. B., Carroll, T. J. & Vize, P. D. (1998) *Dev. Biol.* **202,** 103–112.
30. Vize, P. D., Jones, E. A. & Pfister, R. (1995) *Dev. Biol.* **171,** 531–540.
31. Brennan, H. C., Nijjar, S. & Jones, E. A. (1998) *Mech. Dev.* **75,** 127–137.
32. Shawlot, W. & Behringer, R. R. (1995) *Nature (London)* **374,** 425–430.
33. Taira, M., Otani, H., Jamrich, M. & Dawid, I. B. (1994) *Development* **120,** 1525–1536.
34. Menzel, R., Kaisaki, P. J., Rjasanowski, I., Heinke, P., Kerner, W. & Menzel, S. (1998) *Diabetes Med.* **15,** 816–820.
35. Pontoglio, M., Barra, J., Hadchouel, M., Doyen, A., Kress, C., Bach, J. P., Babinet, C. & Yaniv, M. (1996) *Cell* **84,** 575–585.
36. Pontoglio, M., Sreenan, S., Roe, M., Pugh, W., Ostrega, D., Doyen, A., Pick, A. J., Baldwin, A., Velho, G., Froguel, P., *et al.* (1998) *J. Clin. Invest.* **101,** 2215–2222.
37. Lee, Y. H., Sauer, B. & Gonzalez, F. J. (1998) *Mol. Cell Biol.* **18,** 3059–3068.
38. Carroll, T. J. & Vize, P. D. (1999) *Dev. Biol.* **214,** 46–59.
39. Vize, P. D., Seufert, D. W., Carroll, T. J. & Wallingford, J. B. (1997) *Dev. Biol.* **188,** 189–204.
40. Moriya, H., Uchiyama, H. & Asashima, M. (1993) *Dev. Growth Differ.* **35,** 123–128.
41. Weber, H., Holewa, B., Jones, E. A. & Ryffel, G. U. (1996) *Development* **122,** 1975–1984.
42. Chan, T. C., Ariizumi, T. & Asashima, M. (1999) *Naturwissenschaften* **86,** 224–227.