The embryonic expression of the tissue-specific transcription factor HNF1α in *Xenopus*: rapid activation by HNF4 and delayed induction by mesoderm inducers

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ABSTRACT

The tissue-specific transcription factor HNF1α is expressed in kidney, liver, intestine and stomach of *Xenopus*. We show that the HNF1α gene is transcriptionally activated at the onset of zygotic gene transcription and that this transcription is maintained throughout development. Ectodermal explants of blastulae (animal caps) express HNF1α mRNA upon stimulation with the mesoderm inducers activin A and BMP4 as well as on overexpression of Smad2 and Smad1, the corresponding members of the intracellular TGF-β signal transducers, respectively. Beside these factors that mediate their response through serine/threonine kinase receptors, bFGF, which acts via tyrosine kinase receptors, leads to HNF1α expression, too. These embryonic inducers result in a delayed appearance of HNF1α mRNA, excluding a direct activation of HNF1α. In contrast, the maternally expressed nuclear receptors HNF4α and HNF4β activate the initial HNF1α transcription, since overexpression of HNF4 leads to a rapid expression of HNF1α mRNA in animal caps. Similarly, in entire neurulae HNF4 overexpression results in increased HNF1α transcription. Therefore, we assume that the initial activation is dependent on maternal HNF4α and HNF4β transcription factors whereas HNF1α induction by growth factors reflects the property of these factors to induce the differentiation of mesodermal and endodermal cell types expressing HNF1α.

INTRODUCTION

The differentiation of tissue-specific cell types during development of a multicellular organism is initiated in the fertilized egg by maternal components laid down during oogenesis. Some of the most important maternal determinants regulating these early events include cell surface signalling molecules and their receptors as well as transcription factors. The interplay of growth factors and transcription factors, which is best analyzed in *Drosophila* (1), is assumed to define the spatial activation of specific genes in development.

In *Xenopus*, it is well established that maternal inductive signals emanating from the vegetal region of the cleaving embryo lead to a distinct pattern of gene expression in the equatorial region and thereby determine the prospective mesodermal tissues of the embryo (2). These inductive factors are fibroblast growth factors (FGF) and members of the transforming growth factor β (TGF-β) family, with bFGF and activin A or Vg1 as the most significant members (2–6). Whereas the FGF signal is received by membrane-bound tyrosine kinase receptors, TGF-β act exclusively through serine/threonine kinases on the cell surface (7). Intracellular TGF-β signalling involves a class of proteins referred to as Smad proteins (7–10). In *Xenopus* embryos, Smad1 and Smad2 overexpression in the animal cap mimics the effect of the TGF-β family members BMP4 (bone morphogenetic protein) and activin A, respectively (11,12). In general, the Smad proteins seem to be phosphorylated at C-terminal serine residues by the activated type I receptor (13,14), form heteromeric complexes with Smad4 (15) and migrate into the nucleus, where they regulate the expression of TGF-β-responsive genes by direct interaction with specific transcription factors (16). Based on all these data Smad proteins are considered as essential intracellular components in TGF-β signalling.

In an approach to link maternal components with the transcriptional activation of a tissue-specific transcription factor we have investigated the expression of HNF1α in *Xenopus* (17–19). This tissue-specific transcription factor found in all vertebrates is expressed in endodermal tissues such as liver, gut and stomach but also in the kidney, a mesodermal derivative (17,20). As binding sites for HNF1α are present in several genes specifically expressed in these tissues, it is assumed that HNF1α participates in the establishment of the differentiated state. Previously, we showed by RNase protection analysis that the first HNF1α transcripts appear at the gastrula stage and therefore HNF1α gene transcription is zygotically activated (17). To reveal the regulatory elements involved in HNF1α gene activation we injected promoter constructs into fertilized eggs. We identified a minimal promoter fragment of the *Xenopus* gene sufficient for proper activation of the reporter gene in swimming larvae (18,19). The

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promoter elements mediating gene activation appear to be conserved in vertebrate evolution as the injected rat HNF1α promoter is also regulated in Xenopus. By mutational analysis of the minimal regulatory unit of the Xenopus HNF1α promoter, an OZ element, two HNF1 binding sites and one binding site for HNF4 were characterized as regulatory elements (18,19). The HNF4 binding site seems to play a predominant role, as it is not only sufficient but also essential for activation of the reporter gene (18). HNF4 is encoded in a small gene family, with HNF4α and HNF4β identified in Xenopus (21,22). Both HNF4 proteins are expressed maternally and are present in a gradient from the animal to the vegetal pole (22). Overexpression of rat HNF4α in Xenopus leads to the ectopic appearance of HNF1α protein in the head and tail of swimming larvae (18). These data support the assumption that HNF4 resides on the top of a transcriptional cascade leading to activation of the HNF1α gene during embryogenesis. Since ectopic HNF1α expression was analyzed only in swimming larvae, when organogenesis is already established, it is not known whether HNF4 is able to activate initial HNF1α transcription in embryogenesis.

We have recently shown that activin A induces HNF1α protein expression in animal caps that differentiate into mesodermal and endodermal cell types (23). By injecting various HNF1α promoter constructs, we could define the HNF4 binding site as an apparent activin A-responsive element in the HNF1α promoter (23). However, since HNF1α induction in animal caps was measured at the protein level after 3 days of culture (23), it is an open question whether activin A or its related factor Vg1, both expressed maternally and are present in a gradient from the β head and tail of swimming larvae (18). These data support the assumption that HNF4 resides on the top of a transcriptional cascade leading to activation of the HNF1α gene during embryogenesis. Since ectopic HNF1α expression was analyzed only in swimming larvae, when organogenesis is already established, it is not known whether HNF4 is able to activate initial HNF1α transcription in embryogenesis.

Animal cap assay
Animal pole explants of non-injected and mRNA-injected stage 9 embryos were prepared as described by Weber et al. (23) and incubated at 23°C for 3, 6, 24, 48 or 72 h. These incubation periods correspond to the developmental stages 11, 13, 27, 33 and 42, respectively. The human recombinant activin A was kindly provided by Yuzuru Eto (Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan) and used at a concentration of 10 ng/ml. Recombinant human bFGF was obtained from Gibco BRL and used at the given concentrations.

Western blotting
Entire and animal cap dissected embryos were homogenized as described (18). Aliquots of 10 µg of the protein extract were separated on a 12.5% SDS gel and transferred to nitrocellulose filters (30). Flag tagged Smad2 was detected using the monoclonal antibody M2 (Kodak) directed against the N-terminal Flag tag according to the manufacturer’s instructions.

Reverse transcription–PCR (RT–PCR)
Total RNA was prepared using the RNA clean system (AGS, Heidelberg, Germany) following the instructions of the manufacturer. RT–PCR of whole embryos and animal caps was performed as previously described (22). For the detection of specific RNAs the following primers, annealing temperatures and cycle numbers were used. HNF1α: upstream 5'-CTGAGAGGGCAGTCACTCAG-3', downstream 5'-GTCCTGACAGAAATGCAGG-3', 54°C, 35 cycles. Brachyury: upstream 5'-GGATGGTATCACCTCTG-3', downstream 5'-GGGATGGTATCACCTCTG-3', 54°C, 30 cycles. Gooseoid: upstream 5'-ACAAGTGGAGTGCTACTCGA-3', downstream 5'-CAGCCGGTGGCTGCATCG-3', 54°C, 30 cycles. Globin: upstream 5'-GCTGACAGCAAGAGGACAG-3', downstream 5'-CATGACGGGAGCCGGCTG-3', 54°C, 30 cycles. Muscle actin: upstream 5'-GCCCTGACATTGAGAGGACAG-3', downstream 5'-GTGACAGCAAGAGGACAG-3', 54°C, 30 cycles. Ornithine decarboxylase (ODC): upstream 5'-AATGGATTTCAAGAGCACTGCACCA-3', downstream 5'-CCAGGCTAAAGTTGCTG-3', 44°C, 30 cycles.

RESULTS
HNF1α expression starts with the onset of zygotic gene transcription at the mid blastula transition (MBT)
To identify the factors involved in embryonic activation of the HNF1α gene in the developing Xenopus embryo it was a prerequisite to determine the temporal expression pattern of HNF1α mRNA by a more sensitive technique than previously used (17). By RT–PCR (Fig. 1) we revealed that HNF1α transcripts are absent in the early cleaving embryo between the fertilized egg (stage 1) and the mid blastula (stage 8) stages, whereas the first transcripts are detectable at the late blastula (stage 9) stage corresponding to the initiation of zygotic gene transcription in Xenopus (31). The amount of transcript remains constant up to the late gastrula (stage 12) stage and increases by ∼100-fold further development up to the swimming larva (stage 42) stage. The amount of ODC mRNA, known to remain constant in Xenopus development (32), was in all RNA samples.

MATERIALS AND METHODS
Microinjections of synthetic capped mRNAs into Xenopus eggs
The cDNAs for Xenopus Smad1 and human Smad2, kindly provided by Gerald Thomsen and Jeff Wrana, were cloned into the pCS2+ expression vector (27) with the N-terminus fused to a Myc or a Flag epitope, respectively. For in vitro mRNA synthesis both clones were linearized with NosiI and transcribed with SP6 polymerase. The BMP4 cDNA was a gift from Walter Knöchel (28) and was cloned into the pSP64T vector. To synthesize the BMP4 mRNA the vector was cut with BamHI and transcription was driven by SP6 polymerase (29). The green fluorescent protein (GFP2) cDNA cloned into the pCS2 vector was kindly provided by Enrique Amaya and synthetic mRNA transcription was driven by SP6 polymerase after linearization with PvuII. The rat HNF4α (19) and Xenopus HNF4β (22) cDNAs were cloned into the Rc/CMV vector (Invitrogen). After linearization with Ndel we used T7 polymerase for the in vitro transcription. Synthetic capped mRNAs were microinjected (1 ng of Smad1, Smad2, BMP4, HNF4α or HNF4β and 200 pg of GFP2) into the animal pole of fertilized Xenopus eggs at the one cell stage and the embryos were allowed to develop until the late blastula or early neurula stages.
Temporal expression pattern of the HNF1α gene in Xenopus embryogenesis. At the indicated developmental stages as defined (46) total RNA from five embryos was isolated and analyzed by RT–PCR for the presence of HNF1α and ODC transcripts. The time point of the mid blastula transition (MBT) when zygotic gene transcription starts is indicated and hours after fertilization are given. Control RT–PCR reactions containing embryonic RNA of stage 42 (+cont.), no RNA (H2O) or HeLa cell RNA are shown on the left.

Therefore, HNF1α is zygotically expressed in Xenopus and transcription of this gene is maintained during all developmental stages. Obviously, initial HNF1α transcription at the onset of zygotic gene transcription is initiated by regulatory components of maternal origin.

The activin A signalling pathway leads to HNF1α induction

To analyze whether the maternal inducer activin A might initiate HNF1α expression we investigated at what time point activin A treatment leads to HNF1α gene transcription in explants of the animal pole of the blastula (animal caps). Using RT–PCR we detected HNF1α mRNA in animal pole explants at 72 h after treatment with activin A (data not shown). This late appearance of HNF1α mRNA is very similar to the induction we have seen previously when analyzing the effect at the protein level (23). As treatment of animal pole explants with activin A in conjunction with retinoic acid enhances activin A-dependent HNF1α protein induction (23), we determined the time point of HNF1α gene activation by activin A in the presence of retinoic acid. As indicated in Figure 2 the first HNF1α transcripts are detectable after 24 h of incubation (lane 4). Since under these conditions both goosecoid and brachyury transcripts are visible already after 3 h of treatment (lane 2), HNF1α expression cannot be an immediate response to activin A signalling. As Smad2 is known to be an intracellular mediator of activin A signalling (7,33), we analyzed whether the introduction of this downstream effector might also activate HNF1α gene transcription. Using an mRNA encoding a Flag tagged Smad2 protein we could verify in a western blot using a Flag tag-specific monoclonal antibody that Smad2 protein was present in the injected embryos at the time of animal cap explantation (Fig. 3, lanes 3–8). To analyze expression of HNF1α mRNA in these Smad2-injected animal caps, total RNA from the explants was isolated at different time points of culturing. Based on RT–PCR analysis HNF1α transcripts were absent in animal caps derived from Smad2 mRNA-injected embryos after 0, 3 and 6 h of incubation (Fig. 3, lanes 3–5). The first few HNF1α transcripts appeared after 24 h (lane 6). Their amount rose to a maximum (lane 7, 48 h) and remained at this level after 72 h of culture (lane 8). The same level of HNF1α induction was observed in uninjected animal caps treated with activin A for 72 h (lane 2).

In contrast, brachyury and goosecoid, both known to be activated as an early response to activin A/Smad2 signalling (34), were expressed at the time point of dissection (lane 3), indicating a successful initial mesodermal induction caused by the translated Flag tagged Smad2 mRNA. As expected, the expression of both marker genes declined with longer culture periods and was replaced after 24 h by expression of muscle actin (lane 6), indicating late dorsal mesoderm, and after 48 h by the late ventral mesodermal marker globin (lane 7), as reported by Graff et al. (34). These data show that the activated activin A/Smad2 pathway leads to a late induction of HNF1α gene expression.

The BMP4/Smad1 pathway leads to HNF1α mRNA expression

Signalling by BMPs that are also members of the TGF-β family is another important maternal pathway in the early Xenopus embryo leading to activation of genes involved in mesoderm induction (reviewed in 35,36). To investigate its potential role in HNF1α induction, we injected Xenopus eggs with RNA encoding
Figure 3. Kinetics of HNF1α gene induction by overexpression of Smad2. Animal caps dissected from Smad2 mRNA-injected embryos were incubated for 0, 3, 6, 24, 48 and 72 h in buffer (lanes 3–8). For comparison animal caps derived from un.injected embryos were either cultured in buffer alone (lane 1) or in the presence of 10 ng/ml activin A (lane 2). The leftover dissected blastulae of each group were used for protein extract preparation and analyzed by western blot analysis for expression of the Flag tagged Smad2 protein. At the indicated time points the expression of HNF1α, brachyury (Xbra), goosecoid (gsc), globin, muscle actin and ODC were measured by RT–PCR.

BMP4 that leads to the translation of functional BMP4 protein in the early cleavage stages (37). To monitor successful expression of the introduced RNA we co-injected RNA encoding GFP. At the blastula stage animal caps were explanted exclusively from GFP-positive embryos and cultured for different time periods prior to RNA extraction. Figure 4 demonstrates that HNF1α transcripts were detectable after 48 h of culture (lane 8), whereas the brachyury gene (Xbra), an immediate response gene of BMP4 signalling (38), was transcribed already at the time of animal cap explantation (lane 4). The late appearance of HNF1α transcripts was also confirmed in an experiment where we injected RNA encoding Smad1 (data not shown), the mediator specific for BMP4 signalling (34).

**bFGF induces the appearance of HNF1α transcripts**

The mesoderm inducer bFGF is another maternal component of the FGF family (39) and exerts its effect by binding to corresponding tyrosine kinase receptors (40). To explore whether this pathway is able to activate HNF1α gene expression, we incubated animal caps in increasing concentrations of bFGF and analyzed the level of HNF1α mRNA. After 72 h of incubation abundant HNF1α transcripts were found in caps treated with between 3 and 150 ng/ml bFGF (data not shown). Using the highest concentration of bFGF we determined the kinetics of HNF1α mRNA appearance in explanted animal caps. Figure 5 reveals that HNF1α mRNA was present after 48 h (lane 5) and 72 h (lane 6) of bFGF treatment. A similar late appearance was also observed for globin transcripts, whereas brachyury transcripts (Xbra) were already seen 3 h after bFGF addition (lane 2), as previously described (41). This delayed induction of HNF1α excludes the possibility that bFGF signalling leads to direct activation of the HNF1α gene.

**HNF4 overexpression in animal caps leads to rapid activation of HNF1α transcription**

Since it is known that HNF4 activates HNF1α protein expression in swimming larvae and HNF4 protein is expressed maternally (18,22), we explored whether HNF4 is able to activate the initial HNF1α transcription. Therefore, we microinjected synthetic mRNA encoding rat HNF4α into fertilized Xenopus eggs. Animal caps of injected blastulae were explanted and total RNA was either isolated immediately or after culturing in buffer for
Figure 6. Kinetics of HNF1α gene activation in animal caps by overexpression of rat HNF4α. Fertilized eggs were injected with rat HNF4α mRNA and animal caps dissected from a stage 9 blastula. After culturing for the given time period RNA was isolated from the animal caps and analyzed by RT–PCR for expression of HNF1α and ODC. As controls animal caps of non-injected embryos were cultured for 72 h either in buffer (lane 1) or in the presence of 10 ng/ml activin A (lane 2) and analyzed as above.

3–72 h. By RT–PCR we detected HNF1α transcripts at the time of explantation of the animal caps in the HNF4α-injected embryos (Fig. 6, compare lane 3 with lane 1). HNF1α mRNA accumulated in injected animal pole explants between 3 and 72 h of culture in the absence of any external inducer (lanes 4–6). Neither eggs nor brachyury, both marker genes for early mesoderm, were activated by HNF4α overexpression (data not shown). Analyzing older caps we found no transcripts of the dorsal or ventral mesodermal markers muscle actin or globin, respectively (data not shown).

Our data clearly establish that the tissue-specific transcription factor HNF4 activates HNF1α gene transcription without delay in animal caps and that this event is independent of mesoderm induction, since none of the mesodermal markers analyzed was affected.

HNF4 but not Smad2 increases HNF1α expression in the entire embryo
To explore whether HNF4 acts as an early activator of HNF1α gene transcription also in the entire embryo, we overexpressed HNF4 by microinjection of synthetic mRNA encoding *Xenopus* HNF4β into fertilized eggs. As shown in Figure 7A, there is a much higher level of HNF1α transcripts in HNF4β-injected embryos (lanes 3 and 4) than in un.injected (lane 1) or GFP2 mRNA-injected control larvae (lane 2). We conclude that overexpression of the *Xenopus* HNF4β protein leads to increased expression of the endogenous HNF1α gene. A similar induction was observed by injecting rat HNF4α mRNA (data not shown). Clearly, these data establish that HNF4α as well as HNF4β can dramatically up-regulate early expression of the endogenous HNF1α gene.

To investigate whether Smad2 overexpression is able to activate early HNF1α transcription in the whole embryo as shown for HNF4 overexpression, we injected Flag tagged Smad2 mRNA into fertilized eggs and prepared extracts at the neurula stage from injected and un.injected stage 14 embryos. By western blot analysis using a monoclonal antibody directed against the Flag epitope we can detect overexpressed Flag tagged Smad2 protein in two independent batches of mRNA-injected embryos (Fig. 7B, lanes 2 and 3). RT–PCR of non-injected control and Smad2-injected early neurula embryos could detect no significant difference in HNF1α transcription (Fig. 7B, lanes 1–3). This establishes that Smad2, unlike HNF4, is not able to increase early HNF1α gene transcription in the entire embryo and this finding correlates with the late induction found in animal caps (Fig. 3).

DISCUSSION

HNF1α expression during embryogenesis
Our data show that HNF1α transcripts appear at stage 9 of *Xenopus* development, immediately after the onset of zygotic gene transcription at the MBT (Fig. 1). This finding extends our previous RNase protection analysis showing transcripts of the HNF1α gene only at stage 11 (17) and reflects the higher sensitivity of the RT–PCR method. Even using this most sensitive detection system we failed to detect any maternal HNF1α mRNA. This establishes that HNF1α expression depends entirely on zygotic transcription and thus on activation by maternal components.

Transcription of HNF1α is initiated prior to organogenesis but as the low amounts of transcripts do not yield significant protein levels (17), we doubt that HNF1α has a regulatory role in these early stages. This is reminiscent of the early expression pattern of *Xenopus* MyoD, where the initial weak and unrestricted expression in the mid blastula stage is without significance for muscle differentiation (42).
HNF1α is a late response gene to the mesoderm inducers

The present data show that HNF1α induction by activin A, BMP4 or bFGF is a late response and thus differs from the early responses seen for the goosecoid and brachyury genes under the same conditions. The much delayed response of HNF1α expression upon addition of the mesoderm inducers implies that the appearance of HNF1α mRNA reflects the differentiation of mesodermal and endodermal cells that express HNF1α. This interpretation is also valid for preloading of the animal caps with Smad2 or Smad1, essential intermediate components of TGF-β signalling, that fails to produce an early response of the HNF1α gene, too (Fig. 3 and data not shown).

Since we have shown previously that in animal caps the activin A signal is received by the HNF4 binding site of injected HNF1α promoter constructs (23), we assume that HNF4 is the major factor acting in the mesodermal and endodermal cells differentiated upon activin A treatment. Further evidence that activin A signalling and HNF4-induced activation of the HNF1α gene are separate events in the early embryo is provided by our observation that in gel retardation experiments using protein extracts from Xenopus gastrulae with combined overexpression of Smad2 and HNF4 proteins, no Smad2/HNF4 interaction could be identified (data not shown). In contrast, such complexes have been found between the transcription factor FAST1 and the TGF-β signalling molecule Smad2 that were bound to the activin response element of the Mix.2 promoter (16). Consistent with this direct interaction on the induced promoter it has also been established that activin A stimulation of the Mix.2 promoter is an early response (43).

HNF4 initiates HNF1α gene transcription

The main focus of our work was to analyze the mechanisms involved in the initial embryonic activation of the HNF1α gene. We revealed that HNF4 is able to initiate HNF1α expression at the onset of zygotic gene transcription in blastula embryos whereas the mesoderm inducers activin A, BMP4 and bFGF fail to activate HNF1α gene expression in these early embryonic stages.

Clearly, the HNF4α and HNF4β transcription factors are good candidates for initial activators, since they are present as maternal components (18,22) and the HNF1α promoter contains a functional HNF4 binding site (18,19). This potential link can be verified by overexpression of rat HNF4α or endogenously expressed Xenopus HNF4β in both cases we found an increase in HNF1α gene transcription at the early neurula stage (Fig. 7 and data not shown). This induction is very rapid, as animal caps injected with HNF4α mRNA contained induced HNF1α mRNA already at the blastula stage, when they were explanted (Fig. 6). This implies that HNF4 translated on the injected mRNA during early cleavage stages prior to the MBT had accumulated and activated HNF1α transcription rapidly at the MBT. From this observation we conclude that the cells of the animal caps are competent for efficient HNF1α gene transcription if triggered with the appropriate signal. The induction of HNF1α by HNF4 is a specific effect and independent of mesoderm induction, as other mesoderm markers are not expressed in HNF4-injected animal caps (data not shown).

All these findings support the idea that maternal HNF4 is the initial activator of the HNF1α gene in Xenopus. A loss-of-function study to test this assumption is difficult to perform, since HNF4 protein is already present in the egg and thus RNA depletion experiments are not possible. Therefore, we introduced a fusion protein containing the HNF4 DNA binding domain and the putative ligand binding domain linked to the repressor domain of Drosophila into fertilized eggs. Although this HNF4 repressor acted efficiently in hepatoma cells and was expressed at high levels in the injected embryos, we could not detect a significant reduction in endogenous HNF1α mRNA accumulation (data not shown). We assume that the level of HNF4 repressor translated prior to the MBT is not sufficient to compete with the amount of maternal HNF4α and β proteins (18,22) that had accumulated during several months of oocyte maturation. In contrast, in all experiments where dominant negative variants of transcription factors have been successfully used to repress endogenous factors, e.g. brachyury or eomesodermin (44,45), the inhibition involved zygotically accumulated transcription factors. In these cases inhibition is probably facilitated as the embryo is preloaded with the repressors prior to expression of the zygotic factor to be inhibited.

Overexpression of either rat or Xenopus HNF4 leads to increased transcription of the HNF1α gene in the early stages of embryogenesis (Fig. 7A and data not shown). This is in contrast to experiments done with the myogenic transcription factor MyoD: in this case only murine MyoD activated ectopic expression of myogenic markers in Xenopus embryos, whereas overexpressed Xenopus MyoD was hardly active (27). Thus, in contrast to MyoD, the activity of HNF4 is species independent, indicating the high degree of functional conservation of the HNF4 family members in vertebrates (18,21,22).

Our data show no differences in the activating potential between distinct HNF4 isoforms in the early stages of development, as the injected β isoform increases HNF1α transcription (Fig. 7) as efficiently as the α isoform of HNF4 (Fig. 6 and data not shown). This finding possibly reflects the identical expression patterns of HNF4α and HNF4β we have seen in whole-mount immunostaining in early cleavage stages (22).

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