Development 122, 1975-1984 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV2065

Mesoderm and endoderm differentiation in animal cap explants: identification of the HNF4-binding site as an activin A responsive element in the *Xenopus* HNF1α promoter

Heike Weber, Beatrix Holewa, Elizabeth A. Jones* and Gerhart U. Ryffel†

Universitätsklinikum Essen, Institut für Zellbiologie (Tumorforschung), Hufelandstraße 55, D-45122 Essen, Germany

*Author for correspondence (e-mail: gerhart.ryffel@uni-essen.de)
†Present address: Department of Biological Sciences, Warwick University, Coventry, CV4 7AL, UK

SUMMARY

The gene encoding the tissue-specific transcription factor HNF1α (LFB1) is transcriptionally activated shortly after mid-blastula transition in *Xenopus* embryos. We have now shown that the HNF1α protein is localized in the nuclei of the liver, gall bladder, gut and pronephros of the developing larvae. In animal cap explants treated with activin A together with retinoic acid, we induced HNF1α in pronephric tubules and epithelial gut cells, i.e. in mesodermal as well as in endodermal tissues. HNF1α can also be induced by activin A, but not by retinoic acid alone. To define the promoter element responding to the activin A signal, we injected various HNF1α promoter luciferase constructs into fertilized eggs and cultured the isolated animal caps in the presence of activin A. From the activity profiles of the promoter mutants used, we identified the HNF4-binding site as an activin-A-responsive element. As HNF4 is a maternal protein in *Xenopus* and localized in an animal-to-vegetal gradient in the cleaving embryo, we speculate that the activin A signal emanating from the vegetal pole cooperates with the maternal transcription factor HNF4 to define the embryonic regions expressing HNF1α.

Key words: tissue-specific transcription, mesoderm-inducing factors, retinoic acid, pronephros, *Xenopus*, endoderm, animal cap, HNF4, activin A

INTRODUCTION

Specification of the body plan during early embryogenesis is dependent on the cooperation of inductive interactions and transcriptional hierarchies. Induction is a process where a signal emitted from one cell population determines the developmental fate of other cells. This developmental fate is largely executed by transcriptional regulation. It is assumed that transcriptional cascades initiated by maternal transcription factors regulate the expression of downstream transcription factors and thus establish a cell-type-specific regulatory network. In this paper, we investigated the effect of defined embryonic inducers on a specific transcriptional casacade in *Xenopus*.

During *Xenopus* development, mesoderm is induced in the equatorial region of the blastula embryo by signals originating in the underlying vegetal pole cells. These inductive processes result in a region-specific activation of gene expression at mid-blastula transition, when zygotic transcription starts in the embryo. Nieuwkoop (1973) demonstrated that the explanted animal region of the blastula embryo (animal cap), which forms ectodermal tissues during normal development, can be converted to a mesodermal fate when recombined with vegetal pole cells. Using the mesoderm induction assay, where animal cap explants are incubated with soluble proteins, several factors have been identified that mimick the mesoderm-inducing capacity of vegetal pole cells. These include members of the fibroblast growth factor family such as basic (b)FGF as well as multiple members of the transforming growth factor (TGF)-β family including activin A and Vg-1, all regulating gene expression in their target cells by activating transmembrane receptors (reviewed in Hogan et al., 1994; Dawid, 1994; Slack, 1994; Fukui and Asashima, 1994; Smith, 1995). In addition to their mesoderm-inducing capacities, both groups of factors are also competent to induce endoderm differentiation in isolated animal caps (Jones et al., 1993).

The receptors for bFGF and activin have been cloned and encode protein tyrosine kinases and serine/threonine kinases, respectively (reviewed in Heldin, 1995). Several molecules identified in mammalian cell culture systems as important components of bFGF-mediated receptor tyrosine kinase signalling are also involved in mesoderm induction in *Xenopus*. These include the GTPase $p21^{ras}$ (Whitman and Melton, 1992) as well as the cytosolic kinase raf-1 (MacNicol et al., 1993) and the mitogen-activated protein (MAP) kinase (LaBonne et al., 1995; Gotoh et al., 1995). In contrast, signal transduction mediated via TGF-β receptor serine/threonine kinases is largely unknown. However, as specific blocking of the bFGF signal transduction pathway also prevents animal cap explants from expressing several mesodermal markers in response to activin A (LaBonne and Whitman, 1994; LaBonne et al., 1995;

Gotoh et al., 1995), it seems that bFGF signaling is also required for activin-A-mediated mesoderm induction. Although many distinct genes have been identified that are transcriptionally activated by these inducers (reviewed in Smith, 1993; Asashima, 1994; Dawid, 1994), so far no promoter element has been identified that might receive the inductive signal.

HNF1α (also referred to as HNF1 or LFB1) is a tissue-specific transcription factor found in liver, stomach, gut and kidney; i.e. it is found in endodermal as well as mesodermal derivatives (Blumenfeld et al., 1991; De Simone et al., 1991; Mendel et al., 1991; Bartkowski et al., 1993). As binding sites for HNF1α are present in several genes specifically expressed in liver, kidney and the digestive tract (reviewed in Tronche and Yaniv, 1992), HNF1α is assumed to play an important role in the differentiation of these tissues. During *Xenopus* development, the gene is activated in the early gastrula and thus belongs to the first genes that are transcribed zygotically in the embryo (reviewed in Weber et al., 1996). To identify the transcription factors involved in embryonic activation of the *HNF1α* gene, we injected various HNF1α promoter mutants into fertilized eggs. From the activity of these constructs in the developing larvae, we deduced that an OZ-element, two HNF1-binding sites and an HNF4-binding site are involved in promoter activation (Zapp et al., 1993; Holewa et al., 1996). The OZ-element interacts with the maternal factor OZ-1, which was initially described to bind to elements within the promoters of the *GS17* and the *N-CAM* genes, both of which are activated early in embryogenesis (Ovsenek et al., 1992). The factor interacting with the HNF1-binding site might be HNF1α itself, acting in an autoregulatory mode, or the related factor HNF1β (also refered to as vHNF1 or LFB3), which is characterized by a closely similar DNA-binding specifity (Mendel et al., 1991; De Simone et al. 1991; Demartis et al., 1994). The third regulatory element involved in embryonic HNF1α activation is the binding site of HNF4, a member of the steroid hormone receptor superfamily, which has been reported as a key regulator of the *HNF1α* gene in mammalian cell cultures (reviewed in Sladek, 1994). We could further document the importance of HNF4 in *Xenopus* embryogenesis by the fact that injection of HNF4 mRNA into fertilized *Xenopus* eggs causes ectopic expression of the *HNF1α* gene in the developing larvae (Holewa et al., 1996). As HNF4 is present as a maternal protein in the fertilized *Xenopus* egg (Holewa et al., 1996), we assume that HNF4 is on top of a transcriptional cascade, leading to the activation of the *HNF1α* gene and thereby establishing tissue-specific gene expression. However, as the HNF4 protein is present in an animal-to-vegetal gradient in the cleaving embryo and thus is also localized in embryonic regions that do not develop into tissues containing HNF1α (Holewa et al., 1996), we assume that other factors contribute to the activity of the maternal HNF4.

We demonstrate here that HNF1α is found in all the larval tissues (including the pronephros) that contain this transcription factor in the adult. More importantly, we report that HNF1α is induced in isolated animal caps treated with activin A together with retinoic acid, a treatment known to induce pronephric tubules (Moriya et al., 1993). Analyzing the activation of injected HNF1α promoter constructs in this mesoderm induction assay, we defined the HNF4-binding site as an activin-A-responsive element, implying that the maternal HNF4 protein is activated by activin A signaling.

MATERIALS AND METHODS

Tissue fixation and immunochemistry

Xenopus larvae and animal cap explants were fixed in periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974) for 2 hours at 4°C. They were rinsed in 0.1 M phosphate buffer (pH 7.0), incubated in 30% sucrose overnight at 4°C, embedded in tissue tek (Miles) and stored at –20°C. Cryosections of 8-10 μm were mounted onto glass slides coated with 0.5% gelatine/0.05% chromium potassium sulfate. For immunofluorescence analysis, sections were rinsed with PBS, blocked with 10% goat serum in PBS for 1 hour at 23°C and incubated with the primary antibody overnight at 4°C (XAD5, Bartkowski et al., 1993; undiluted or 15-fold diluted after purification using protein G-Sepharose from Pharmacia). Subsequently, the glass slides were rinsed with PBS, incubated with the Cy3-conjugated secondary antibody ($F(ab')_2$ rat anti-mouse IgG; Jackson Immunoresearch), rinsed with PBS and then mounted in phenylendiamine glycerol (Johnson and Nogueira Aranjo, 1981). To amplify fluorescence intensity, sections were incubated subsequently with Cy3-conjugated $F(ab')_2$ rat anti-mouse IgG, mouse anti-rat IgG $F(ab')_2$ (Jackson Immunoresearch) and $F(ab')_2$ rat anti-mouse IgG for 1.5 hours at 23°C. For double immunostaining, incubation with the secondary antibody was followed by incubation with the pronephros (3G8, Vize et al., 1995) or endoderm-specific (4G6, Jones et al., 1993) antibody overnight at 4°C. These antibodies were visualized using a FITC-conjugated rabbit anti-mouse IgG antibody (Dako).

Animal cap assay

Fertilized eggs were dejellied by treatment with 2% cysteine-HCl (pH 8.0) for 1 minute, washed thoroughly with H_2O and cultured in 0.1× MBS (Peng, 1991). At stage 9 (according to Nieuwkoop and Faber, 1975), the vitelline membrane was removed and animal caps of about 0.5×0.5 mm were dissected using tungsten needles. Isolated animal caps were immediately transferred to the test solutions in 96-well multititer plates coated with 1% agarose and cultured for several hours or days at 23°C. Test solutions were prepared as described by Moriya et al. (1993) using human recombinant activin A (kindly provided by Dr Yuzuru Eto, Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan). The activin A and retinoic acid concentrations used in our experiments were 10 ng/ml and 10^{-5} M, respectivly.

Western blot analysis

For extract preparation, animal cap explants were homogenized in SDS loading buffer (Sambrook et al., 1989), boiled, cleared by centrifugation (10 minutes, 10°C, 100,000 ***g***) and stored at –80°C. *Xenopus* liver extract was prepared as described previously (Pogge v. Strandmann and Ryffel, 1995). Extracts were separated on 10% SDS gels and electrotransferred to a nitrocellulose membrane (Sambrook et al., 1989). The blots were incubated with 2.5% blocking reagent (Boehringer) for 1 hour at 23°C, the primary antibody XAD5 (5-fold diluted in blocking reagent) and a horse-radish-peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Dianova). Bound antibodies were visualized using the ECL system (Amersham).

Plasmid construction

To generate *Xenopus* HNF1α promoter luciferase constructs, we used the plasmids pXP1 and pXP2, characterized by different orientations of the multiple cloning sites (Nordeen, 1988). The promoter fragment –886/–46 (Zapp et al., 1993) was introduced in the vector pXP2 using *Hin*dIII and *Sst*I sites. The other HNF1α promoter fragments were fused to the *luciferase* gene by cloning into the vector pXP1. The constructs –594/–57 and –574/–57 (Zapp et al., 1993) were introduced

into *Bam*HI and *Hin*dIII sites. The fragments spanning the sequence from −594 to −207 with intact HNF4 and HNF1α-binding sites, mutated HNF4-binding site (h4 mut 1) or mutated HNF1α-binding sites (b1 mut, see Holewa et al., 1996) were cloned using *Sma*I and *Hin*dIII sites. To generate −276/−207 HNF1α promoter-luciferase constructs with an intact or mutated HNF4-binding site, the constructs OZ-H4 and OZ-H4 mut (Holewa et al., 1996) were introduced in the vector pXP1 using *Sma*I and *Hin*dIII sites. Subsequently, the plasmid was digested with *Bam*HI to delete the OZ-element. The plasmids containing the thymidine kinase promoter (−81 to +52) and the cytomegalo virus promoter are as described by Nordeen (1988).

Microinjection procedure and luciferase assay

Fertilized eggs were dejellied and injected in the animal half with 0.5 ng, 1 ng or 2 ng of each plasmid in NAM/12 (Peng, 1991). Variation of the DNA concentration in these ranges did not significantly influence the relative luciferase activities. Injection was performed in 0.1 M MBS (Peng, 1991) and embryos were cultured in this buffer. At stage 9 (according to Nieuwkoop and Faber, 1975), animal caps were isolated and explants from the same pool of injected embryos were treated in the absence or presence of inducers as described in the text. After a culture period of 3 days, animal caps were rinsed in Ca^{2+}/Mg^{2+}-free PBS and luciferase activity of individual animal caps was determined as described (Drewes et al., 1994).

RESULTS

HNF1α is expressed in the liver, gall bladder, gut and pronephros of the hatched larvae

In western blots, HNF1α protein can first be detected in the 2-day-old hatched larvae and accumulates during further development (Bartkowski et al., 1993). To determine the tissues expressing HNF1α in the larvae, we performed immunostainings on sections using the monoclonal antibody XAD5 raised against recombinant *Xenopus* HNF1α (Bartkowski et al., 1993). At stage 42, the morphological differentiation of endodermal tissues is well advanced and intense HNF1α-specific staining is found in the liver and the gall bladder (Fig. 1A) as well as in the epithelial cells of the gut (Fig. 1B). Besides these endodermal tissues HNF1α is clearly detectable in the pronephric tubules (Fig. 1B) originating from the mesoderm. In all these tissues, HNF1α is restricted to the nuclei as expected for a functional transcription factor.

HNF1α is induced in animal caps treated with activin A and retinoic acid

Since the differentiation of mesodermal and endodermal tissues is dependent on the action of embryonic inducing factors, we were interested to define factors leading to *HNF1α* gene activation. As the treatment of isolated animal caps with activin A in combination with retinoic acid results in frequent induction of pronephric tubules (Moriya et al., 1993), we wondered whether HNF1α is found in this tissue and thus can be induced in animal cap explants by activin A and retinoic acid. Therefore, we dissected ectoderm explants from stage 9 blastulae and incubated them without inducing factors or with 10 ng/ml activin A together with 10^{-5} M retinoic acid. After 4 days culture, cryosections of the explants were analyzed by immunostaining to detect HNF1α. Animal caps cultured in the absence of inducing factors form atypical epidermis lacking HNF1α-specific staining (Fig. 2A). In contrast, incubation of explants with activin A together with retinoic acid leads to activation of HNF1α in about 80% (44/55) of the specimens analyzed. Immunostaining reveals that HNF1α is localized in tissues resembling pronephric tubules (Fig. 2B; arrow) as well as in other cell types frequently localized at the periphery of the animal cap (Fig. 2B; arrowhead). To confirm the pronephric expression of HNF1α, we performed immunostainings using an antibody specifically recognizing the pronephric tubules and nephrostomes, i.e., the anterior part of the pronephros (3G8; Vize et al., 1995). In larvae, HNF1α is colocalized in cells expressing the anterior pronephric marker, whereas posterior pronephric cells stained by an antibody recognizing the Wolffian duct and the nephrostomes (4A6; Vize et al., 1995) lack HNF1α (data not shown). The pronephric tubules induced in animal caps treated with activin A and retinoic acid are visualized by the antibody against anterior pronephros that characteristically decorates the apical surface of the tubular epithelium (Fig. 2C). In all of these pronephric cells, HNF1α is found in the nuclei (compare Fig. 2C and D). To analyze whether other cell types expressing HNF1α are of endodermal character, we performed immunostainings using a gut-specific antibody (4G6; Jones et al., 1993). As shown in Fig. 2, HNF1α is frequently expressed in the nuclei of cells expressing the 4G6 gut marker (compare Fig. 2E and F). These results demonstrate that HNF1α can be induced in animal cap

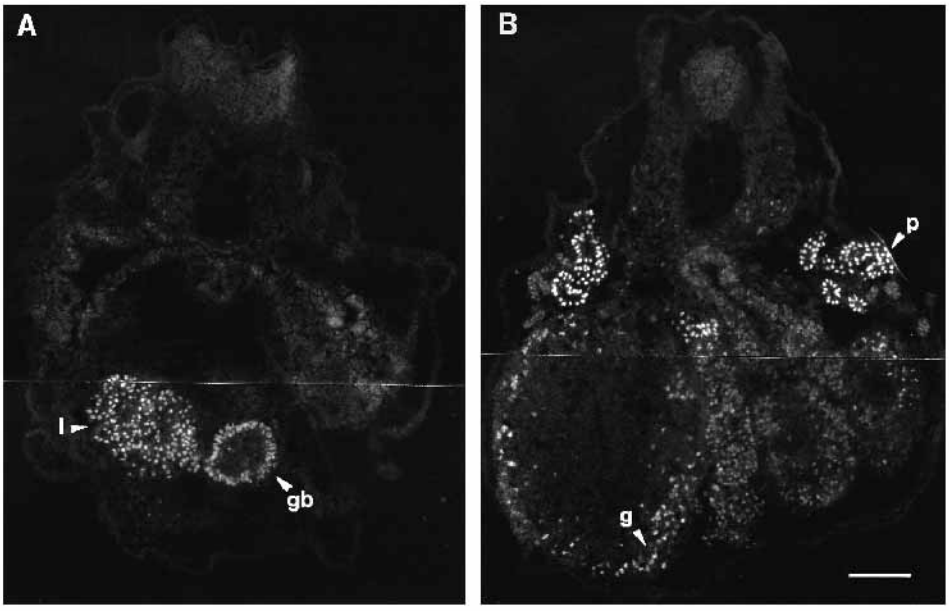

Fig. 1. Localization of the HNF1α protein in the hatched *Xenopus* larvae. HNF1α was detected on cryosections of a stage 42 larvae by indirect immunofluorescence using the monoclonal antibody XAD5 (Bartkowski et al., 1993). (A,B) Transversal sections demonstrating HNF1α-specific staining in the liver (l), gall bladder (gb), pronephric tubules (p) and the epithelial cells of the gut (g). Bar, 100 μm.

explants by activin A and retinoic acid in mesodermal as well as endodermal tissues thus reflecting the expression pattern of HNF1α in the larvae.

To analyze at what time point HNF1α appears during differentiation of the animal cap, explants treated with activin A and retinoic acid were harvested at various stages for western blot analysis using the XAD5 monoclonal antibody. No HNF1α protein was detected in the freshly dissected explants (data not shown) and in animal caps cultured without inducers (see Fig. 6, lane 2). However, traces of HNF1α are present 6 hours after induction (Fig. 3, lane 1) and, at 24 hours after induction, a significant level of HNF1α is present (lane 2), which accumulates further within the following 2 days (lanes 3 and 4).

The microinjected HNF1α promoter is activated in animal cap explants upon induced differentiation

To investigate the molecular mechanisms leading to the activation of the *HNF1α* gene by activin A and retinoic acid, we next questioned whether a microinjected HNF1α promoter construct extending from position −886 to −46 relative to the translation start site is activated by these factors in ectoderm explants. In contrast to our previous experiments where promoter fragments were fused to the *chloramphenicol acetyltransferase* (*CAT*) gene as a reporter (Zapp et al., 1993; Holewa et al., 1996), we now injected a plasmid containing the *luciferase* reporter gene, as the assay for luciferase activity is far more sensitive. The luciferase reporter driven by the HNF1α promoter is properly activated in the developing larvae as previously observed for the CAT constructs (data not shown). To analyze the activation in animal cap explants, the promoter construct was injected into the fertilized egg and animal cap explants of the developing blastulae were incubated in the absence or presence of inducing factors. After 3 days of culture, luciferase activity was determined in individual animal caps. Luciferase activity driven by the HNF1α promoter is clearly increased in ectoderm explants treated with activin A together with retinoic acid relative to the uninduced controls (Fig.4A). This difference is statistically significant using the Wilcoxon statistics test that applies for samples lacking a normal distribution. However, the activity observed in individual caps is quite different and probably reflects to some extent the uneven distribution of the injected plasmid in distinct explants. In addition, in treated explants, HNF1α is only found in about 80% of the explants (see above) and the morphological differentiation in individual animal caps is very heterogenous, thus most likely contributing to an uneven response. Therefore, to readily compare the luciferase activity

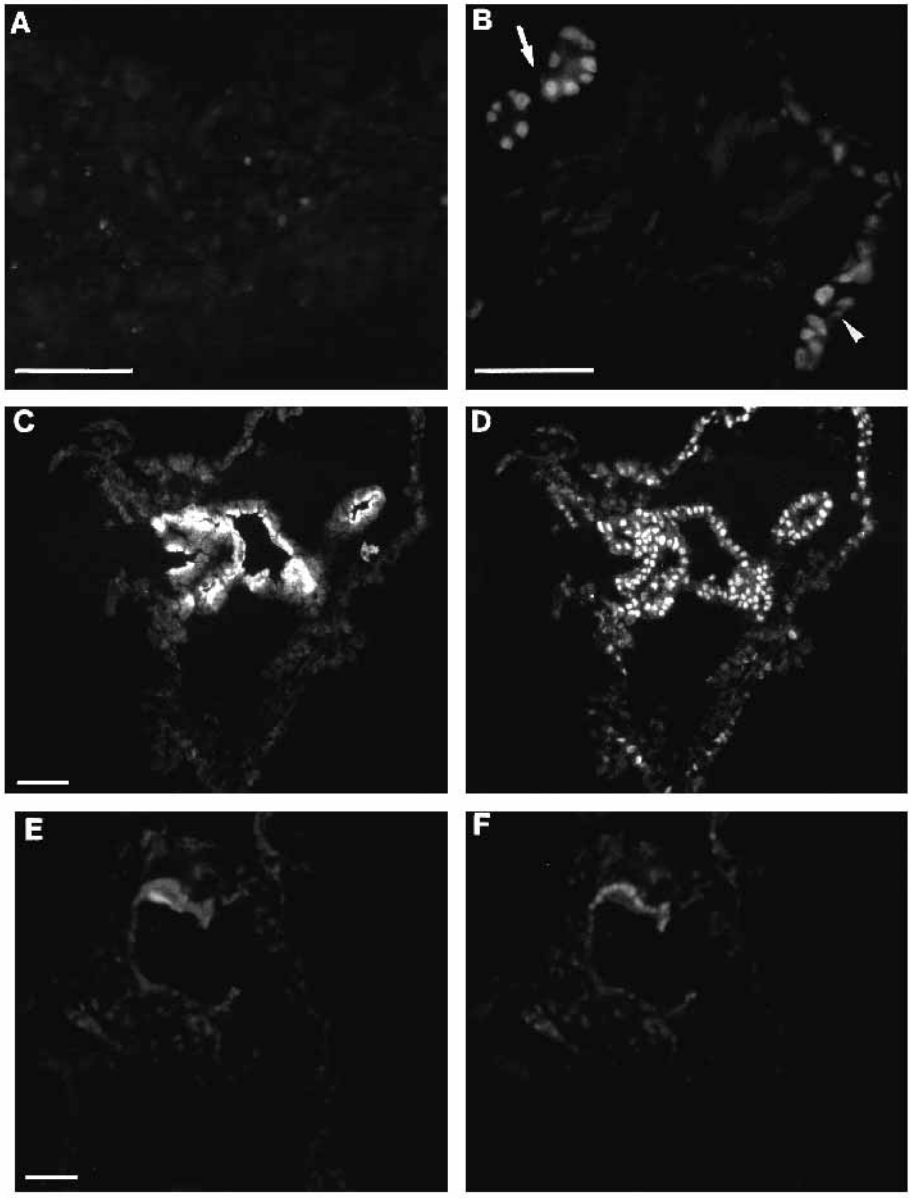

Fig. 2. Localization of the HNF1α protein in animal cap explants upon induced differentiation. Explants were incubated in the absence (A) or presence (B-F) of activin A together with retinoic acid and harvested for indirect immunofluorescence analysis after a culture period of four days. Cryosections of explants were incubated with (A,B,D,F) the HNF1α-specific antibody XAD5 (Bartkowski et al., 1993), (C) an antibody specifically recognizing pronephric tubules and nephrostomes (3G8; Vize et al.,1995) and (E) an antibody recognizing the gut (4G8; Jones et al., 1993). (A) Atypical epidermis lacking HNF1α-specific staining. (B) HNF1α-specific staining in pronephric tubules (arrow) and other cell types (arrowhead) induced by activin A and retinoic acid. (C) Pronephric tubules stained by the 3G8 antibody. (D) Same section as in C demonstrating that HNF1α is localized in the nuclei of pronephric tubules and in other cell types lacking the 3G8 pronephric antigen. (E) Epithelial gut cells detected by the 4G6 antibody. (F) Same section as in E demonstrating HNF1α-specific staining in the nuclei of gut cells. Bar, 50 μm.

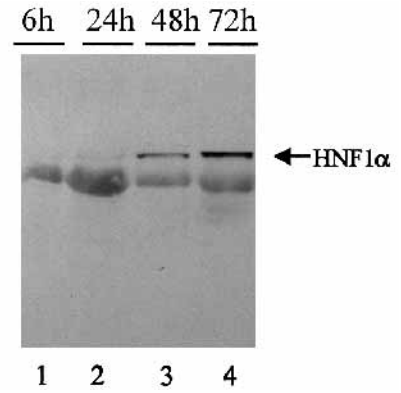

Fig. 3. Appearance of the HNF1α protein during differentiation of the animal cap. Animal caps were incubated with activin A together with retinoic acid and harvested at the time points indicated. Under the temperature conditions used, embryos developed to stage 12, 31, 37/38 and 42 after 6, 24, 48 and 72 hours, respectively. For each time point, extracts of 21 animal caps were analyzed in a western blot using the monoclonal antibody XAD5 specific for HNF1α. Migration of the hepatic HNF1α is marked (compare Fig. 6B) and the identity of the broad band with higher mobility found in all samples is unknown.

obtained from the treated and the untreated explants, the values are given as relative stimulation compared to the mean activity found in untreated explants. To visualize the distribution of luciferase activities of individual animal caps in a given pool of explants, we defined four groups that represent explants that are either not activated (less than 2-fold), weakly activated (2- to 6-fold), considerably activated (6- to 18-fold) or strongly activated (more than 18-fold). The percentage of animal caps scored into these groups is shown in Fig. 4B, which clearly shows the stimulation of the HNF1α promoter by activin A together with retinoic acid.

To determine whether the HNF1α promoter is specifically activated, we studied the effect of these factors on the activity of the thymidine kinase (tk) promoter as well as the cytomegalo virus (CMV) promoter. A tk promoter construct extending from position −81 to +52 was used, as its basal activity in explants incubated in the absence of inducing factors is similar to that of the HNF1α promoter. As Fig. 4 illustrates, the activity of this tk promoter construct is not increased when activin A and retinoic acid are added. Similarly the CMV promoter that is characterized by an extremely high activity in the untreated explants is not activated in explants incubated in the presence of activin A and retinoic acid (Fig. 4). Therefore, we conclude that activin A together with retinoic acid specifically stimulates the HNF1α promoter.

The HNF4-binding site is essential and sufficient for activation of the HNF1α promoter in animal cap explants

To define the promoter elements required for proper activation of the HNF1α promoter by activin A and retinoic acid, we analyzed the stimulation of various promoter mutants in animal cap explants. A promoter construct extending from position −594 to −57 characterized by the OZ-element at its 5′ border is clearly activated by activin A and retinoic acid compared to the uninduced controls (Fig. 5A). Deletion of the OZ-element does not reduce promoter activation (Fig. 5B) suggesting that the OZ-element is not required for activation of the gene in animal cap explants. A promoter fragment extending from position −594 to −207, which was shown to be the minimal promoter region driving appropriate reporter gene expression in the embryo (Holewa et al., 1996), is also clearly stimulated by activin A and retinoic acid in animal pole explants (Fig. 5C). To test whether the HNF1-binding sites (pos. −523 and −497) and the HNF4-binding site (pos. −264) are required for the activation, specific mutations were introduced into these elements abolishing the binding of the corresponding factors. Mutations of the HNF1-binding sites do not alter the activation pattern (Fig. 5D), whereas mutation of the HNF4-binding site results in a complete loss of

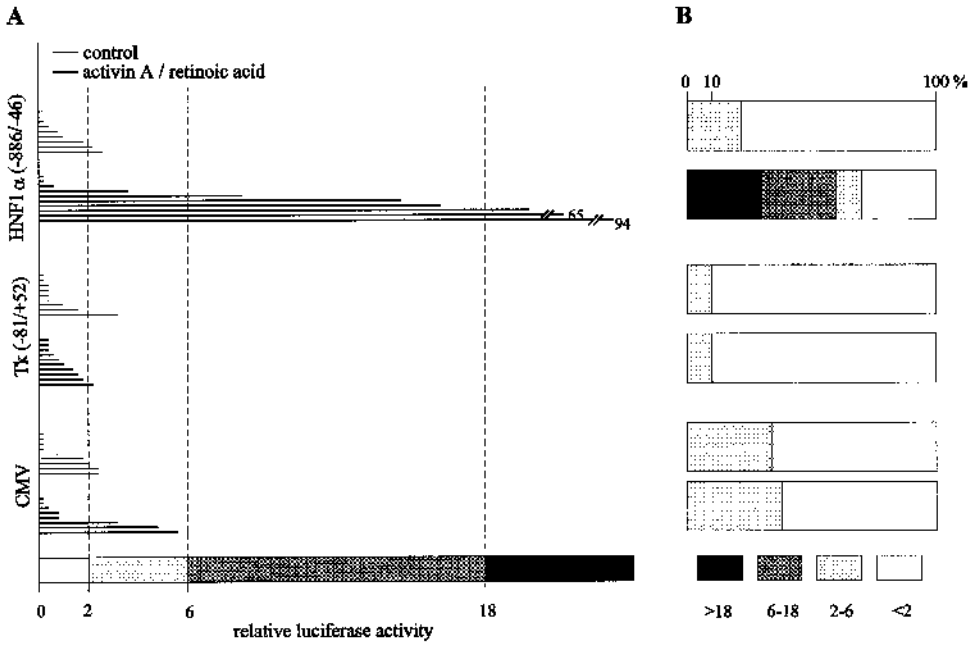

Fig. 4. Specific activation of the luciferase gene driven by the HNF1α promoter (−886/−46) in ectoderm explants treated with activin A and retinoic acid. (A) Luciferase activities in individual animal caps incubated in the absence or presence of inducers are given as relative values compared to the mean activity in untreated explants. The mean value was 2100, 3300 and 1200000 light units for the HNF1α, the tk and the cytomegalo virus promoter, respectively. Relative activities are scored into four groups beginning with less than 2-fold activated (white colour) up to more than 18-fold activated (black colour) as indicated by the dotted lines. (B) Diagram showing the percentage of animal caps scored into the groups as defined in (A).

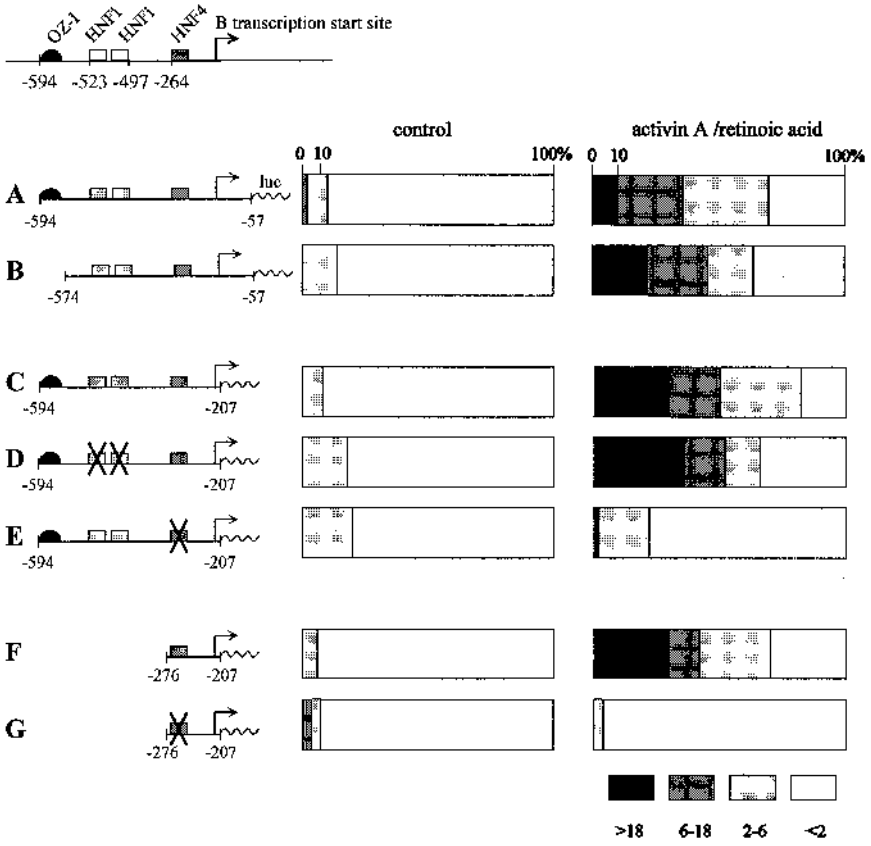

Fig. 5. Activation of the *luciferase* gene driven by HNF1α promoter mutants in ectoderm explants treated with activin A and retinoic acid. Shown are the HNF1α promoter constructs used for injection experiments. The numbers indicate the distance to the translation initiation site and the locations of the binding sites for OZ-1, HNF1α and HNF4 are given (Zapp et al., 1993). Mutated binding sites are marked by a cross. The percentage of animal caps scored into the groups as defined in Fig. 4A is shown for explants incubated in the absence or presence of inducers. Each bar corresponds to 30-40 explants analyzed.

promoter activation (Fig. 5E). These data imply an important role for the HNF4-binding site in mediating the activin A/retinoic acid signal. To test whether the HNF4-binding site is sufficient to drive proper expression of the luciferase gene, the activation of a 69 bp promoter fragment containing the HNF4-binding site and the transcription start site was analyzed. Fig. 5F documents significant activation of this promoter fragment by the inducers and this activation is due to the HNF4-binding site, as stimulation is abolished when the HNF4-binding site is mutated (Fig. 5G). From these data, we conclude that the HNF4-binding site plays the major role in the activation of the *HNF1α* gene by activin A together with retinoic acid.

HNF1α is induced by activin A but not by retinoic acid

To investigate whether the combined action of activin A and retinoic acid is essential to stimulate injected HNF1α promoter constructs, we analyzed the activation of the HNF1α promoter construct −594/−207 by either activin A or retinoic acid. When explants of the injected embryos are incubated with retinoic acid alone, the promoter is not activated whereas significant stimulation is observed after treatment with activin A (Fig. 6A). To analyze whether the activation of the injected promoter construct reflects the induction of the endogenous gene, we performed Western blots from extracts of animal caps incubated with either retinoic acid or activin A. In explants treated with retinoic acid no induction of HNF1α is observed (Fig. 6B, lane 3), whereas in explants treated with activin A the HNF1α protein can clearly be detected (lane 4). Thus, luciferase activity driven by the HNF1α promoter reflects perfectly the induction of the endogenous gene in explants treated with activin A or retinoic acid. However, activin A and retinoic acid act synergistically in activation of the endogenous HNF1α gene, as in the presence of both factors the amount of protein is about 6 fold increased compared to the activation triggered by activin A alone (Fig. 6B, lane 4 and 5). In contrast to that, no significant difference between the stimulation of the injected promoter construct by activin A alone and the one induced by activin A together with retinoic acid is detected as based on the Wilcoxon statistic test (Fig. 6A).

The HNF4-binding site functions as an activin A responsive element in the HNF1α promoter

As activin A alone is sufficient to induce HNF1α, we wondered whether the activaton is mediated via the same promoter element responsible for stimulation by activin A and retinoic acid. Therefore, the promoter constructs shown in Fig. 5 were injected and explants of the injected blastulae were incubated without inducing factors or with activin A. As Fig. 7 illustrates, deletion of the OZ-element (compare Fig. 7A and B) and mutation of the HNF1-binding sites (compare Fig. 7C and D) do not reduce the stimulation of the promoter but significant activation is lost when the HNF4-binding site is mutated (Fig. 7E). Furthermore, the 69 bp promoter fragment containing the HNF4-binding site and the transcription start site is sufficient to activate reporter gene expression in explants treated with activin A (Fig. 7F) and this activation is completely abolished when the HNF4-binding site is mutated (Fig.

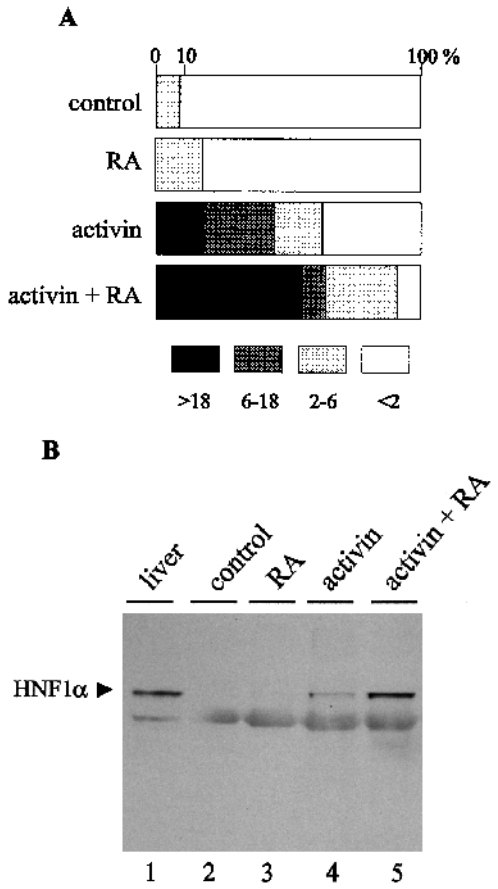

Fig. 6. Activation of the *HNF1α* gene by either activin A or retinoic acid. (A) Stimulation of the HNF1α promoter construct –594/–207 (Fig. 5C) in animal cap explants incubated in the absence of inducers (control), with 10^{-5} M retinoic acid (RA), 10 ng/ml activin A (activin) or combination of both. The percentage of animal caps scored into groups as defined in Fig. 4A is shown. Each bar corresponds to 11 explants analyzed. (B) Animal caps were incubated with inducing factors as described in A and cultured 3 days. Extracts of 21 animal cap equivalents from each pool were analyzed in a western blot using the monoclonal antibody XAD5 specific for HNF1α (Bartkowski et al., 1993). In lane 1 the comigration of HNF1α from adult *Xenopus* liver is shown.

7G). Based on these results, we deduce that the HNF4-binding site functions as an activin-A responsive element in the HNF1α promoter.

DISCUSSION

HNF1α gene expression induced in ectoderm explants

Our data demonstrate that activin A together with retinoic acid induces HNF1α in isolated animal caps, where the protein is localized in pronephric tubules as well as in gut cells (Fig. 2). This induction of HNF1α in mesodermal as well as in endodermal cell types reflects the expression pattern of HNF1α in the larvae (Fig. 1), suggesting that the activation of the *HNF1α* gene in animal caps mimicks the events occurring during normal development. The expression of the *HNF1α* gene in tissues that derive from both germ layers confirms the hypothesis that mesoderm and endoderm formation are part of a single process. It has already been shown that differentiation of tissues originating from both germ layers can be induced in animal caps by the same molecules. For instance, purified vegetalizing factor (identified as activin A) has been shown to induce all kinds of mesoderm as well as intestine and differentiated yolk-rich (endodermal) tissue in *Triturus* ectoderm (Asashima et al., 1991). Furthermore, gut cells defined by expression of the 4G6 gut marker are induced by purified XTC-MIF (*Xenopus* activin A) as well as recombinant bFGF in *Xenopus* animal cap explants, and both these factors also induce mesodermal tissues (Jones et al., 1993). The data presented in this paper demonstrate that activin A triggers the activation of the same transcriptional regulator in mesodermal as well as endodermal cells, suggesting that similar mechanisms are involved in differentiation of both cell types. This assumption is supported by the fact that, in addition to HNF1α, there are other transcription factors known that are expressed at the appropriate time point in the presumptive endoderm and mesoderm of the embryo and are induced in the mesoderm induction assay by activin A. Such factors are Mix.1, a homeodomain-containing protein that is transiently expressed during the blastula and gastrula stages (Rosa, 1989) and XFKH2, the product of a *forkhead* related gene, most similar to the rat liver transcription factor HNF3-α (Bolce et al., 1993).

The HNF4-binding site acts as an activin-A-responsive element in the mesoderm induction assay

The mesoderm induction assay has been widely used to analyze the mesoderm-inducing capacity of soluble molecules as well as to look for the genes that are activated in response to these factors. Although activin A is one of the most competent inducers in this assay, leading to the differentiation of a wide range of tissues, the signal transduction pathway triggered by activin A is largely unknown (reviewed in Smith, 1995). Injecting promoter reporter constructs into fertilized eggs and analyzing their activation in isolated animal caps upon treatment with activin A, we identified the activin-A-responsive element in the promoter of the *HNF1α* gene. A similar approach was used by Krieg et al. (1993) to demonstrate that the NCAM promoter is activated in response to neural-inducing factors. However, as the molecules responsible for neural induction have not been identified, they used vegetal pole cells as the source of inducing signals. Mayor et al. (1993) analyzed the activation of the injected *X*sna promoter in animal cap explants treated with activin A or bFGF. Although the amount of *X*sna mRNA is clearly increased in explants cultured with these inducers (Sargent and Bennet, 1990), reporter gene expression driven by a 2 kb promoter construct is not, suggesting that the mesoderm-inducing factors used affect the stability of the *X*sna mRNA rather than the transcription of the gene itself. In contrast, our data establish that reporter gene expression driven by the HNF1α promoter is clearly increased in response to activin A. The activation of the injected HNF1α promoter reflects the induction of the endogenous gene (Fig. 6) and is a specific effect, as expression of unrelated promoter reporter constructs is not stimulated upon treatment with the inducers (Fig. 4). As mutation of the HNF4-binding site abolishes promoter activa-

tion (Fig. 7E), we conclude that the HNF4-binding site is essential for the transcriptional activation of the *HNF1α* gene in response to activin A. This conclusion is further supported by our finding that the promoter construct of 67 bp, which includes the HNF4-binding site and the transcriptional start site, is also activated by activin A. More importantly, mutation of the HNF4-binding site in this minimal promoter abolishes activin A response and thus excludes the posibility that there is an activin-responsive element between the HNF4-binding site and the transcription start site. Therefore, the HNF4-binding site is the first activin-A-responsive element so far identified mediating transcriptional activation during development.

Activation of the *HNF1α* gene during embryogenesis

The major role of the HNF4-binding site for gene activation in the mesoderm induction assay is consistent with our previous results demonstrating that the HNF4-binding site is also essential to drive appropriate reporter gene expression in the embryo (Holewa et al., 1996). More importantly, we demonstrated that injection of synthetic HNF4 mRNA into fertilized eggs causes ectopic expression of the endogenous HNF1α strongly suggesting that HNF4 is the factor actually binding to the HNF4 consensus site. Although heterodimers of the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), which are both also members of the steroid hormone receptor superfamily, interact with the HNF4-binding site in vitro, they do not stimulate the HNF1α promoter in transient transfection experiments (unpublished data). Therefore, it seems unlikely that RAR/RXR heterodimers transactivate the HNF1α promoter via the HNF4-binding site. Consistent with this assumption, we observed that retinoic acid is not able to activate the HNF1α promoter in animal caps (see Fig. 6). Based on this finding, we conclude that retinoic acid only modulates the activin response and this effect seems to involve a more efficient accumulation of the HNF1α protein (Fig. 6B). Taking these data together, we propose that an activin-A-dependent signal transduction pathway initiates the transcriptional activation of the *HNF1α* gene in the early gastrula stage (Bartkowski et al., 1993) by activating HNF4.

However, the stimulation of injected HNF1α promoter constructs in the mesoderm induction assay differs in one important respect from the stimulation in the larvae. In the larvae, deletion of the OZ-element (Fig. 7, construct B) totally abolishes reporter gene expression (Zapp et al., 1993), whereas this element is not required for promoter activation by activin A in ectoderm explants (Fig. 7B). These results suggest that, in the marginal zone and the vegetal region of the embryo, from where the HNF1α-expressing tissues naturally originate, the OZ-element is required in addition to the HNF4-binding site to activate *HNF1α* transcriptionally.

Possible mechanisms for activation of the *HNF1α* gene

Activin A is a secreted morphogen binding to transmembrane receptors characterized by an intrinsic serine/threonine kinase activity. In fact, an activin A homologue is present in the unfertilized *Xenopus* egg as well as in blastula-stage embryos (Fukui et al., 1994). But the natural mesoderm inducer might also be another member of the TGFβ superfamily acting through the same transmembrane receptors. A good candidate that shows the expected localization during early embryogenesis is Vg-1, a factor whose mRNA is present as a maternal transcript in the vegetal hemispere (reviewed in Dawid, 1994; Smith, 1995).

The second important component for *HNF1α* gene activation is HNF4. This transcription factor is present as a maternal protein in the egg and the amount of protein remains constant during development (Holewa et al., 1996). Consistent with this, we showed that the amount of HNF4 protein is not increased in animal caps treated with activin A or activin A together with retinoic acid compared to the uninduced controls (western blots not shown). One possibility for activation of HNF4 might be its post-transcriptional modification. Using a pituitary somatotrope cell line, it has recently been shown that pit-1, a transcription factor of the POU-homeobox family, is

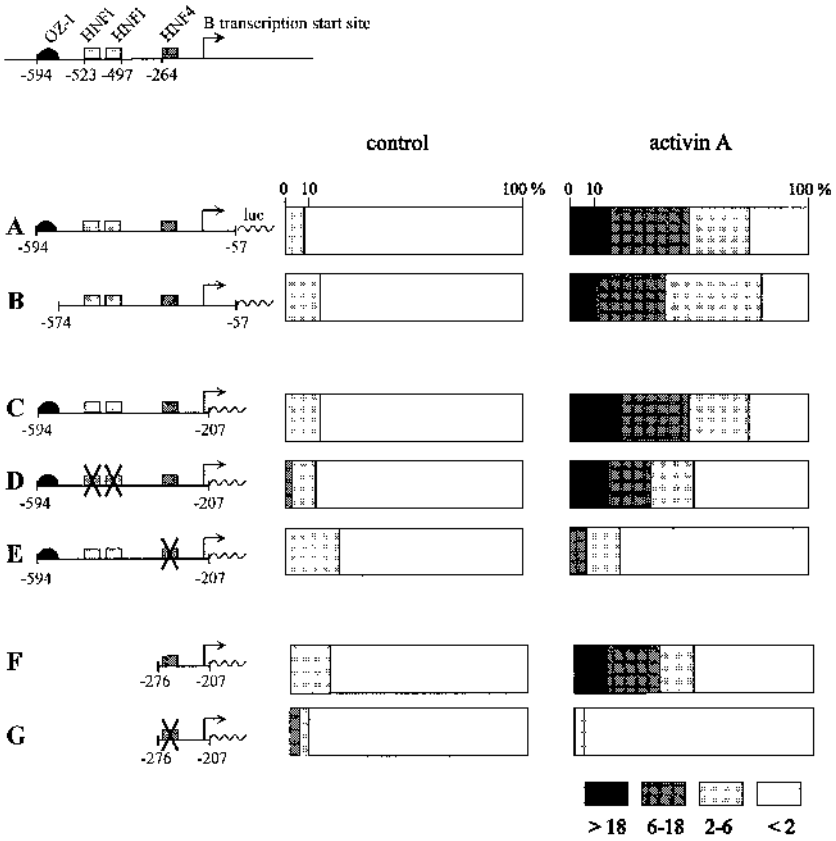

Fig. 7. Activation of the *luciferase* gene driven by HNF1α promoter mutants in ectoderm explants treated with activin A. The injected promoter mutants (A-G) correspond to the schemes represented in Fig. 5. Each bar corresponds to the data derived from 30-40 explants.

phosphorylated in response to the activin A signal. In contrast to the HNF1α promoter, which is activated in response to activin A, phosphorylation of pit-1 inhibits its binding to the growth hormone promoter thereby reducing growth hormone synthesis (Gaddy-Kurten et al., 1995). The activation of HNF4 by phosphorylation is a striking hypothesis as tyrosine phosphorylation of HNF4 is required for DNA binding and consequently for transactivation by HNF4 in cell-free systems as well as in cultured mammalian cells (Ktistaki et al., 1995). Furthermore, these authors have demonstrated that, irrespective of the phosphorylation state, HNF4 is localized in the nucleus. We would expect the same for regulation of HNF4 in the mesoderm induction assay, since, in the blastula stage, HNF4 is already present in the nuclei of the animal caps (Holewa et al. 1996). An alternative possibility for activation of HNF4 is the elimination of a potential inhibitor. There are two proteins known, calreticulin (Burns et al., 1994; Dedhar et al., 1994) and TRUP (Burris et al., 1995), which specifically interact with a subclass of steroid hormone receptors thereby inhibiting the binding to DNA-response elements. Corresponding inhibitory factors might exist in the embryo that antagonize the transactivation by HNF4 and whose functions are repressed by activin A.

We thank Y. Eto for the generous gift of recombinant human activin A, I. Spratte for culturing hybridomas for monoclonal antibody production, T. Drewes for helpful discussions on the statistical evaluation of the luciferase activities and E. Pogge v. Strandmann for critically reading this manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (Ry 5/3-2).

REFERENCES

Asashima, M., Uchiyama, H., Nakano, H., Eto, Y., Ejima, D., Sugino, H., Davids, M., Plessow, S., Born, J., Hoppe, P., Tiedemann, H. and Tiedemann, H. (1991). The vegetalizing factor from chicken embryos: its EDF (activin A)-like activity. *Mech. Dev.* **34**, 135-141.

Asashima, M. (1994) Mesoderm induction during early amphibian development. *Develop. Growth and Differ.* **36,** 343-355.

Bartkowski, S., Zapp, D., Weber, H., Eberle, G., Zoidl, C., Senkel, S., Klein-Hitpass, L. and Ryffel, G. U. (1993). Developmental regulation and tissue distribution of the liver transcription factor LFB1 (HNF1) in *Xenopus* laevis. *Mol. Cell. Biol.* **13,** 421-431.

Blumenfeld, M., Maury, M., Chouard, T., Yaniv, M. and Condamine, H. (1991). Hepatic nuclear factor 1 (HNF1) shows a wider distribution than products of its known target genes in developing mouse. *Development* **113,** 589-599.

Bolce, M. E., Hemmati-Brivanlou, A. and Harland, R. M. (1993). XFKH2, a Xenopus HNF-3α homologue, exhibits both activin-inducible and autonomous phases of expression in early embryos. *Dev. Biol.* **160,** 413-423.

Burns, K., Duggan, B., Atkinson, E. A., Famulski, K. S., Nemer, M., Bleackley, R. C. and Michalak, M. (1994). Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* **367,** 476-480.

Burris, T. P., Nawaz, Z., Tsai, M. J. and O´Malley, B. W. (1995). A nuclear hormone receptor associated protein that inhibits transactivation by the thyroid hormone and retinoic acid receptors. *Proc. Natl. Acad. Sci.* **92,** 9525-9529.

Dawid, I. B. (1994). Intercellular signaling and gene regulation during early embryogenesis of *Xenopus* laevis. *J. Biol. Chem.* **269,** 6259-6262.

Dedhar, S., Rennie, P. S., Shago, M., Hagesteijn, C.-Y. L., Yang, H., Filmus, J., Hawley, R. G., Bruchowsky, N., Cheng, H., Matusik, R. J. and Giguère, V. (1994). Inhibition of nuclear hormone receptor activity by calreticulin. *Nature* **367,** 480-483.

Demartis, A., Maffei, M., Vignali, R., Barsacchi, G. and De Simone, V. (1994). Cloning and developmental expression of LFB3/HNF1ß transcription factor in *Xenopus* laevis. *Mech. Dev.* **47,** 19-28.

De Simone, V., De Magistris, L., Lazzaro, D., Gerstner, J., Monaci, P., Nicosia, A. and Cortese, R. (1991). LFB3, a heterodimer-forming homeoprotein of the LFB1 family, is expressed in specialized epithelia. *EMBO J.* **10,** 1435-1443.

Drewes, T., Clairmont, A., Klein-Hitpass L. and Ryffel, G. U. (1994). Estrogen-inducible derivatives of hepatocyte nuclear factor-4, hepatocyte nuclear factor-3 and liver factor B1 are differentially affected by pure and partial antiestrogens. *Eur. J. Biochem.* **225,** 441-448.

Fukui, A. and Ashashima, M. (1994). Control of cell differentiation and morphogenesis in amphibian development. *Int. J. Dev. Biol.* **38,** 257-266.

Fukui, A., Nakamura, T., Uchiyama, H., Sugino, K., Sugino, H. and Asashima, M. (1994). Identification of activin A, AB and B and follistatin proteins in Xenopus embryos. *Dev. Biol.* **163,** 279-281.

Gaddy-Kurten, D., Tsuchida, K. and Vale, W. (1995). Activins and the receptor serine kinase superfamily. *Rec. Progr. Horm. Res.* **50,** 109-129.

Gotoh, Y., Masuyama, N., Suzuki, A., Ueno, N. and Nishida, E. (1995). Involvement of the MAP kinase cascade in *Xenopus* mesoderm induction. *EMBO J.* **14,** 2491-2498.

Heldin, C.-H. (1995). Dimerization of cell surface receptors in signal transduction. *Cell* **80,** 213-223.

Hogan, B. L. M., Blessing, M., Winnier, G. E., Suzuki, N. and Jones, C. M. (1994). Growth factors in development: the role of TGF-ß related polypeptide signalling molecules in embryogenesis. *Development* **1994 Supplement,** 53-60.

Holewa, B., Pogge v. Strandmann, E., Zapp, D., Lorenz, P. and Ryffel, G. U. (1996): Transcriptional hierarchy in *Xenopus* embryogenesis: HNF4 a maternal factor involved in the developmental activation of the gene encoding the tissue specific transcription factor HNF1α (LFB1). *Mech. Dev.* **54,** 45-57.

Johnson, G. D. and Nogueira Aranjo, G. M. (1981) A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods* **43:** 349-350.

Jones, E. A., Abel, M. H. and Woodland, H. R. (1993). The possible role of mesoderm growth factors in the formation of endoderm in Xenopus laevis. *Roux´s Arch. Dev. Biol.* **202,** 233-239.

Ktistaki, E., Ktistakis, N. T., Papadogeorgaki, E. and Taliandis, I. (1995). Recruitment of hepatocyte nuclear factor 4 into specific intranuclear compartments depends on tyrosine phosphorylation that affects its DNA-binding and transactivation potential. *Proc. Natl. Acad. Sci. USA* **92,** 9876-9880.

Krieg, P. A., Zorn, A., Ovsenek, N. and Johnson A. (1993). Gene activation in response to neural induction. *Cell. Mol. Biol. Res*. **39,** 377-383.

LaBonne, C. and Whitman, M. (1994). Mesoderm induction by activin requires FGF-mediated intracellular signals. *Development* **120,** 463-472.

LaBonne, C., Burke, B. and Whitman, M. (1995). Role of MAP kinase in mesoderm induction and axial patterning during *Xenopus* development. *Development* **121,** 1475-1486.

MacNicol, A. M., Muslin, A. J. and Williams, L. T. (1993). Raf-1 kinase is essential for early *Xenopus* development and mediates the induction of mesoderm by FGF. *Cell* **73,** 571-583.

Mayor, R., Essex, L. J. Bennett, M. F. and Sargent, M. G. (1993). Distinct elements of the Xsna promoter are required for mesodermal and ectodermal expression. *Development* **119,** 661-671.

McLean, I. W and Nakane, P. K. (1974). Perjodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem*. **22,** 1077-1083.

Mendel, D. B., Hansen, L. P., Graves, M. K., Conley, P. B. and Crabtree, G. R. (1991). HNF-1α and HNF-1ß (vHNF-1) share dimerization and homeodomains, but not activation domains, and form heterodimers in vitro. *Genes Dev*. **5,** 1042-1056.

Moriya, N., Uchiyama, H. and Asashima, M. (1993). Induction of pronephric tubules by activin and retinoic acid in presumptive ectoderm of *Xenopus laevis. Develop. Growth and Differ.* **35,** 123-128.

Nieuwkoop, P. D. (1973). The 'organization center' of the amphibian embryo; its origin, spatial organization and morphogenetic action. *Adv. Morphog*. **10,** 2-39.

Nieuwkoop, P. D. and Faber, J. (1975). *Normal Table of Xenopus laevis.* (Daudin), 2nd. ed. Amsterdam: Elsevier/North-Holland Publishing Co.

Nordeen, S. K. (1988). Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques* **6,** 454-457.

Ovsenek, N., Zorn, A. M. and Krieg, P. A. (1992). A maternal factor, OZ-1, activates embryonic transcription of the *Xenopus* laevis GS17 gene. *Development*. **115,** 649-655.

Peng, H. B. (1991). Appendix A: Solutions and Protocols. *Meth. Cell Biol*. **36,** 657-662.

Pogge v. Strandmann, E. and Ryffel, **G. U.** 1995. Developmental expression of the maternal protein XDCoH, the dimerization cofactor of the homeoprotein LFB1 (HNF1). *Development* **121,** 1217-1226.

Rosa, F. M. (1989). Mix. 1, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. *Cell* **57,** 965-974.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning: a laboratory manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sargent, M. G. and Bennett, M. F. (1990). Identification in *Xenopus* of a structural homologue of the *Drosophila* gene *Snail*. *Development* **109,** 967-973.

Slack, J. M. W. (1994). Inducing factors in *Xenopus* early embryos. *Curr. Biol.* **4,** 116-126

Sladek F. M. (1994). Hepatocyte nuclear factor 4 (HNF-4). In *Liver Specific Gene Expression* (eds. F. Tronche and M. Yaniv), pp. 207-230. Austin, Texus: R. G. Landes Co.

Smith, J. C. (1993). Mesoderm inducing factors in early vertebrate development. *EMBO J.* **12,** 4463-4470.

Smith, J. C. (1995). Mesoderm inducing factors and mesodermal patterning. *Curr. Biol.* **7,** 856-861.

Tronche, F. and Yaniv, M. (1992). HNF1, a homeoprotein member of the hepatic transcription regulatory network. *BioEssays* **14,** 579-587.

Vize, P. D., Jones, E. A. and Pfister, R. (1995). Development of the *Xenopus* pronephric system. *Dev. Biol.* **171,** 531-540.

Weber, H., Pogge v. Strandmann, E., Holewa, B., Bartkowski, S., Zapp, D., Zoidl, C. and Ryffel, G. U. (1996). Regulation and function of the tissue-specific transcription factor HNF1α (LFB1) during *Xenopus* development. *Int. J. Dev. Biol.* (special issue), in press.

Whitman, M. and Melton, D. A. (1992). Involvement of p21ras in *Xenopus* mesoderm induction. *Nature* **357,** 252-254.

Zapp, D., Bartkowski, S., Holewa, B., Zoidl, C., Klein-Hitpass, L. and Ryffel, G. U. (1993). Elements and factors involved in tissue specific and embryonic expression of the liver transcription factor LFB1 in *Xenopus* laevis. *Mol. Cell. Biol.* **13,** 6416-6426.

(Accepted 15 March 1996)