A novel, activin-inducible, blastopore lip-specific gene of *Xenopus laevis* contains a *fork head* DNA-binding domain

Marie Luise Dirksen and Milan Jamrich¹

Laboratory of Molecular Pharmacology, Division of Biochemistry and Biophysics, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892 USA

The organizer region, or dorsal blastopore lip, plays a central role in the initiation of gastrulation and the formation of the body axis during *Xenopus* development. A similar process can also be induced in ectodermal explants by activin or by injection of activin mRNA into embryos. We have searched early embryo-specific cDNA libraries for genes containing the *fork head* box sequence that encodes a DNA-binding domain similar to that of the *Drosophila* homeotic gene *fork head* and rat hepatocyte nuclear factor *HFN3β*. These genes were subsequently tested for expression in the organizer region of blastula/gastrula-stage embryos as well as inducibility by activin. Our effort resulted in the isolation of a gene, *XFKH1*, that is primarily expressed in the dorsal blastopore lip of early gastrulae and is inducible by activin. At later stages it is expressed in the notochord and neural floor plate. Because of its spatial and temporal expression pattern, as well as its inducibility by activin, this gene is a good candidate to have a regulatory function in the initial processes of axis formation in *Xenopus laevis* embryos.

[Key Words: Activin; *fork head* domain; mesodermal induction; neural floor plate; neural induction; notochord]

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The molecular analysis of developmental biology, especially pattern formation, has experienced major growth during the last decade. This is primarily the result of the discovery that many of the genes controlling pattern formation are members of multigene families. They encode conserved DNA-binding domains such as the homeo domain (for review, see Scott et al. 1989; Wright et al. 1989), *paired* domain (Bopp et al. 1986; Walter et al. 1991), or POU domain (Herr et al. 1988; Kessel and Gruss 1990; Rosenfeld 1991). This fact has been utilized to isolate additional gene family members in a variety of species, including flies, frogs, worms, and man. In *Drosophila*, individual family members are expressed throughout the entire period of embryonic development in consecutive, spatially restricted waves that comprise a regulatory cascade. Consequently, genes expressed early in development are likely to be crucial in the initiation of the pattern-forming chain of events. In *Xenopus*, zygotic transcription starts 7 hr after fertilization following a period of transcriptional silence (Bachvarova and Davidson 1966; Newport and Kirschner 1982). This is also the time period during which cell type-specific transcrip-

tion begins (Jamrich et al. 1987). Therefore, if any zygotic gene has a role in the initiation of pattern formation it would have to be expressed at this stage of development. This period of embryogenesis (blastula/early gastrula) also coincides with the beginning of mesodermal and neural induction. A search for factors involved in mesodermal induction has led to the discovery that peptide growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF- β) can promote mesoderm formation in ectodermal explants (Kimelman and Kirschner 1987; Slack et al. 1987; Smith 1989; Roberts et al. 1990). It was found that activin, a growth factor related to TGF- β , is not only able to induce mesoderm formation but can also induce axis formation in uncommitted ectodermal explants (Asashima et al. 1990; Smith et al. 1990; Sokol et al. 1990). The resulting tissue frequently resembles a mini-embryo. Furthermore, microinjection of activin mRNA into embryos can also induce secondary axis formation (Thomsen et al. 1990), indicating that activin is somehow able to activate the correct pattern-forming chain of events. One would therefore expect that genes involved in the initiation of pattern formation would display a rapid activation response to mesoderm-inducing factors, especially activin. When morphologically visible pattern formation

¹Corresponding author.

begins, a region of the embryo termed the blastopore lip or the "organizer" plays a critical role (Nieuwkopp 1973). Although the steps leading to the determination of the dorsoventral axis begin at fertilization (Elinson and Kao 1989; Gerhart et al. 1989), the formation of the dorsal blastopore lip is the first morphological demonstration that the dorsoventral axis has been established. Mesodermal cells originating in this region migrate toward the animal pole of the embryo, inducing the overlying uncommitted ectodermal cells to form neural tissue. The dorsal blastopore lip has the extraordinary ability to induce a secondary axis when transplanted to another embryo (Spemann and Mangold 1924). One might therefore expect that a gene involved in the initial pattern-forming events would be expressed in this area of early gastrulae. For these reasons we have searched for genes that are expressed at the beginning of zygotic transcription in the dorsal marginal region and are at the same time inducible by activin. It has been found previously that some homeo box-containing genes respond to mesoderm inducers. The gene *Mix 1* shows a quick response to activin induction (Rosa 1989). It is, however, expressed in the entire endodermal region of the embryo, and it is not clear whether this gene is involved in axis formation. Two other homeo box-containing genes that respond to mesodermal inducers are thought to be involved in the later, posterior development of the embryo (Ruiz i Altaba and Melton 1989; Cho and De Robertis 1990; Cho et al. 1990).

Whereas several previous efforts have concentrated on homeo box-containing genes, we have screened early embryo-specific libraries for genes encoding the *fork head* (*fkh*) domain. This DNA-binding domain was originally described in the *Drosophila* homeotic gene *fkh* (Weigel et al. 1989; Weigel and Jäckle 1990) and in rat hepatocyte transcription factor *HNF3 α* (Lai et al. 1990, 1991). This 110-amino-acid domain is highly conserved between these two genes, suggesting that other species might also contain genes with this domain. In this paper we describe a *Xenopus* gene, *XFKH1*, which encodes a *fkh* domain protein. The *XFKH1* gene is activated at the onset of zygotic transcription in the dorsal marginal zone of the embryo and can be induced in ectodermal explants even in the absence of protein synthesis. Its expression pattern during early development, as well as its inducibility by activin, suggests that this gene might play a pivotal role in the initiation of pattern formation in *Xenopus* embryos.

Results

Isolation and characterization of the *XFKH1* gene

Using degenerate primers and polymerase chain reaction (PCR)-based technology (Saiki et al. 1988), we have isolated a cDNA clone containing a *Xenopus fkh* box. We have used degenerate primers corresponding to the conserved ITMAIQ and NMFENG sequences of the rat hepatocyte transcription factor *HNF3 α* and *Drosophila fkh* box. As a template we have used DNA prepared from a

gastrula-specific cDNA library (Sargent and Dawid 1983). After amplification, a fragment of the predicted size (240 bp) was cloned, sequenced, and used to screen stage 13 and stage 24 cDNA libraries for longer cDNA clones (Richter et al. 1988). We obtained a 1.8-kb-long cDNA clone that contains the entire coding sequence of the gene and additional 5'- and 3'-flanking sequences (Fig. 1A). We estimated from Northern blots that the entire *XFKH1* RNA is 2.5 kb long. Therefore, we miss ~700 bp, probably at the 5' end. The open reading frame encodes a 400-amino-acid-long protein. It has a conserved 110-amino-acid *fkh* domain that is 89% identical to the hepatocyte-enriched transcription factor HNF-3 β (Fig. 1B).

Spatial and temporal localization of *XFKH1* mRNA during embryogenesis

As can be seen in Figure 2A, *XFKH1* mRNA is not present in the oocyte. The gene is activated at, or shortly after, the midblastula transition. Considerable amounts of transcripts are present by stage 10. The peak of expression is during gastrulation between stages 10 and 12. After this stage, the RNA levels decline, although some transcripts are present until stage 40. Using Northern blot hybridization to RNA from eight different tissues (including brain) we did not observe any expression of this gene in adult animals. Northern blot analysis of microdissected tissues revealed that at stage 10, *FKH1* RNA is primarily localized in the dorsal, vegetal region of early gastrulae, within the quadrant of the embryo containing the dorsal blastopore lip (Fig. 2B). More detailed analysis of expression was performed using in situ hybridization to whole embryos with digoxigenin-labeled RNA probes (Figs. 3–5). We found that the expression of this gene can be visualized first in late blastulae (stage 9½). The gene is expressed in the dorsal marginal zone (Fig. 3A). The highest level of expression is in the middle of the dorsal marginal zone. This is the area where the future dorsal blastopore lip will form. During gastrulation, the highest concentration of *XFKH1* RNA is in the dorsal blastopore lip (Fig. 3B–D). In the process of gastrulation, this gene is first expressed in the invaginating mesoderm, followed closely by adjacent neural tissue (Fig. 4A,B). The expression within the mesodermal tissue is limited to the chordal mesoderm (see Fig. 5A–C). No expression can be seen in the presomitic mesoderm. In the neural plate, only the prospective floor plate shows expression. As neurulation proceeds, expression extends toward the anterior region of the embryo (Fig. 4C). At the same time, the gene starts to be progressively inactivated from the posterior end of the embryo. At the end of neurulation only the expression in the anterior neural region remains (Fig. 4D); at later stages the expression weakens. No expression can be seen in isolated tadpole brains (stage 45) using whole-mount in situ hybridization. The exact anterior limit of *XFKH1* hybridization is not known at present. From the whole-mount in situ hybridization with a combined probe of *XFKH1* and *engrailed 2* (Hemmati-Brivanlou and Harland 1989) we conclude

B

XFKH-1 TYRRNYSHAKPPYSYISLITMAIOQAPNKMMTLNEIYQWIVDLFPYYRQNOQRW
HNF-38 *****ST*****S*****L*****S*****M*****F*****
Fork head *****ST*****NN*TR*LS*****F*****F*****

XFKH-1 QNSIRHSLSFNDCFIKVPRSPKEKPGKGSYWTLPESGNMFENG CYLRRQKRFKCE
HNF-38 *****L*****A*****D*****F*****D*****
Fork head *****V*****I*****T*****D*****F*****D*****

Figure 1. The nucleotide sequence of *XFKH1* and its corresponding amino acid sequence. (A) Amino acids are in the single-letter IPAC code. The *fkh* domain is boxed in. The termination codon is indicated by an asterisk (*). (B) Comparison of the *fkh* domain of *XFKH1* to the similar domains in the rat transcription factor HNF-3 β [Lai et al. 1991] and *Drosophila* homeotic gene *fkh* [Weigel et al. 1989]. Asterisks indicate identities.

that the *XFKH1* expression domain extends into the midbrain. The *engrailed 2* probe hybridizes to the boundary between midbrain and hindbrain, and this double hybridization (Fig. 4E) clearly shows that *XFKH1* is expressed anterior to this boundary. At present, we have no

conclusive evidence that *XFKH1* is ever expressed in the forebrain.

The relative intensity of hybridization of the notochord and neural floor plate varies, depending on the stage of the embryo and plane of the section (Fig. 5).

Figure 2. Developmental expression of *XFKH1*. (A) Northern blot analysis of 10 μ g of total RNA from different stages of embryos. (Bottom) Ethidium bromide staining of the gel; (top) autoradiography. (Stage E) Egg; (stage 8) blastula; (stages 10,12) gastrula; (stages 15,20) neurula; (stages 30,40) tadpoles. The positions of 18S and 28S mRNAs are indicated by arrows. (B) Northern blot analysis of RNA from dissected halves of embryos. Equal amounts of RNA (10 μ g) were loaded per lane. (A) Animal; (V) vegetal; (D) dorsal; (Ve) ventral.

Whereas earlier, the notochord seems to be the major site of expression (Fig. 5A), later, especially in the anterior region, the neural floor plate hybridizes best (Fig. 5B). The hybridization within the notochord is not uniform. There is a slight dorsoventral gradient with more intense hybridization in the dorsal region (Fig. 5C).

The expression of *XFKH1* in the neural floor plate suggests that this gene is expressed in this tissue as a result of induction from underlying notochord. Alternatively, the signal could be generated in the organizer region and travel within the plane of the ectoderm. In this case, the contact of ectodermal cells to the underlying mesodermal cells would not be essential for the expression of this gene. To address this question we have treated embryos with high salt buffer. This treatment induces formation of exogastrulae. In these embryos the normal contact of

chordal mesoderm and ectoderm is eliminated. Whole-mount in situ hybridization of complete exogastrulae shows that *XFKH1* is expressed in the notochord but not in the ectoderm (Fig. 4F). This result suggests that a contact between mesoderm and ectoderm is required for the normal level of expression of this gene in the neural plate. No such contact is required for the expression of this gene in the notochord.

Induction of *XFKH1* by activin

Because it has been shown that activin can induce axis formation in animal caps, we have examined the *XFKH1* gene for inducibility by growth factors. As seen in Figure 6, this gene is specifically induced by activin. Animal caps cultured in saline did not express this gene, and FGF alone was not sufficient to induce gene activity. This result agrees well with general expectations as it is known that TGF- β -like growth factors induce preferentially dorsal structures, whereas FGF-like growth factors induce mostly ventral mesoderm. In comparison, gene 1A11 (Rosa and Dawid 1990), which was used in this experiment as a control, is induced by activin and FGF. Because the *XFKH1* RNA appeared rapidly after activin induction, we tested whether the *XFKH1* gene requires protein synthesis for its induction. As seen in Figure 7, *XFKH1* gene expression can be induced in animal caps even if the animal caps are preincubated in cyclohexamide, a potent inhibitor of protein synthesis. This treatment reduces the protein synthesis by 75–95% (Rosa 1989; Cho et al. 1991b). The amount of RNA is reduced but not eliminated. The partial reduction of RNA synthesis is probably due to the fact that cyclohexamide inhibits DNA replication (Cascio and Gurdon 1987) and, therefore, fewer gene templates are available for transcription. After correction for the template copies, the level of *XFKH1* is higher than in uninduced caps. This overinduction is typical for many immediate early induced genes in the presence of protein synthesis inhibitor (Ringold 1979). This result indicates that activation of *XFKH1* is an immediate early response to activin induction.

Discussion

Although it has been known since the beginning of the century that a transplanted blastopore lip can organize a secondary body axis in amphibian embryos (Spemann and Mangold 1924), this process is poorly understood at the molecular level. To gain a better understanding of this process, we have searched for potential regulatory genes expressed in the organizer region of *Xenopus* blastula/gastrula-stage embryos.

In our search, we have concentrated on a new family of DNA-binding proteins—those containing the *fkh* DNA-binding domain. This domain was originally found in the *Drosophila* homeotic gene *fkh*. Later, a similar domain was found in rat hepatocyte-enriched transcription factor *HNF3 α* . The *fkh* gene of *Drosophila* encodes a nuclear protein that acts in the pattern-forming pathway by

Figure 3. Whole-mount in situ hybridization analysis of *XFKH1* expression in *Xenopus* blastula and gastrula. (A) In situ hybridization of *XFKH1* to stage 9½ *Xenopus* blastula. This view from the vegetal pole of the embryo shows that the *XFKH1* RNA is expressed in the dorsal marginal zone of the embryo. (d) Dorsal; (v) ventral. (B) In situ hybridization of *XFKH1* to stage 11 *Xenopus* gastrula. Notice intense hybridization in the dorsal marginal zone in this vegetal view of the embryo. (d) Dorsal; (v) ventral. (C) Lateral view of a stage 10½ embryo hybridized with *XFKH1* cRNA. Most of the signal is in the organizer region (arrow). (D) Lateral view of stage 11½ embryo. Hybridization is primarily in the invaginating marginal zone of the dorsal blastopore lip (arrow).

influencing the development of terminal regions of the embryos. It is genetically distinct from the homeotic selector gene network that is regulated by the *Polycomb* group of genes. The genetic differences are paralleled by the different molecular structure of the gene (Weigel et al. 1989). The *fkh* gene does not have a homeo box, paired box, or POU box. Instead it encodes a distinct 110-amino-acid DNA-binding domain, the *fkh* domain.

Rat *HNF3α* encodes a sequence-specific DNA-binding protein that is involved in the transcriptional regulation of liver-specific genes. Two more *HNF3α*-like genes (*HNF3β*, *HNF3γ*) have been described recently (Lai et al. 1991). They all contain the highly conserved *fkh* DNA-binding domain. We have isolated a *Xenopus* gene that contains a *fkh* DNA domain. It is most similar to rat

hepatocyte-enriched transcription factor *HNF3β*. By analogy, we believe that this gene is also a transcriptional regulator. Although the two genes have similarities outside the *fkh* domain, the patterns of expression are sufficiently different, making it unlikely that these two genes are true homologs. Indeed, we have evidence that there is a family of genes in *Xenopus* that have similar DNA-binding motifs but distinct expression patterns.

Developmental expression of *XFKH1*

XFKH1 mRNA can first be detected in late blastula-stage embryos, suggesting that this gene is not transcribed in the oocyte. Using total RNA Northern blots, we can de-

Figure 4. Whole-mount in situ hybridization of *XFKH1* to *Xenopus* neurulae. (A) Dorsal view of stage 12 embryo showing the extent of hybridization of *XFKH1* in the dorsal region of the embryo. Anterior is left; posterior is right. (B) Posterior view of stage 12½ embryo hybridized with *XFKH1* showing the dorsal labeling of *XFKH1* RNA. Hybridization is to a narrow strip of chordal mesoderm and overlying neural tissue. (C) Dorsal view of stage 18 embryo showing the *XFKH1*-labeling pattern. At this point of development, labeling extends into the brain area. (D) Lateral view of stage 20 embryo showing the inactivation of *XFKH1* gene in the posterior region of the embryo. Forebrain area appears to be negative as well (arrow). (E) In situ hybridization of *XFKH1* and *engrailed 2* to a stage 18 embryo. Arrow indicates the transverse band of *engrailed 2* hybridization. The hybridization resulting from the *XFKH1* probe extends anteriorly beyond that of *engrailed 2*, which marks the boundary between midbrain and hindbrain. (F) Whole-mount in situ hybridization of *XFKH1* to *Xenopus* exogastrula. Lateral view of a *Xenopus* exogastrula. Hybridization signal is visible in the notochord. No signal appears in the ectoderm (arrow). The ectodermal part broke off during the examination of the embryo.

Figure 5. Sections of embryos hybridized with *XFKH1*. (A) Transverse section through the trunk region of a stage 15 embryo. Hybridization of *XFKH1* is to the notochord and putative neural floor plate. (B) In situ hybridization of *XFKH1* to a stage 16 embryo. Section is through the hind-brain region. The upper arrow shows the hybridization in the neural floor plate; the lower arrow shows the hybridization to the notochord. This transverse section is slightly oblique. The dorsal area is more anterior than ventral. (C) Higher magnification of the *XFKH1* hybridization to the stage 16 embryo. Hybridization is to the notochord (n) and neural floor plate (arrow). Somites(s) and other areas of the embryo are negative.

tect *XFKH1* RNA as early as stage 9 (data not shown). It is likely that by using more sensitive methods such as RNase protection or quantitative PCR, we would detect transcripts earlier. In late blastula, transcripts are localized in the dorsal marginal zone of the embryo, appearing

Figure 6. Effects of growth factors on the expression of *XFKH1*. Northern blot analysis of RNA (10 µg) from induced and uninduced animal caps. (Left) Ethidium bromide staining; (upper right) control hybridization with clone 1A11 that is known to be induced by activin and FGF; (lower right) same filter hybridized by *XFKH1* (induction by activin only). (Lane 1) FGF; (lane 2) control; (lane 3) activin.

before the dorsal lip is formed. Initially this gene is expressed in an arc encompassing the dorsal half of the embryo. At later stages, as the expression becomes more intense, it becomes progressively more restricted to the organizer region of the embryo. During the process of gastrulation, expression can be observed in the invaginating marginal zone. However, these transcripts are limited to chordal mesoderm. Shortly after the onset of expression in the chordal mesoderm, expression can be observed in the overlying neural plate. Expression in the neural plate is limited to what appears to be the prospective floor plate. In the process of neurulation, the expression of the *XFKH1* gene extends into the brain area. At this point, we are not sure exactly how far anterior this expression extends. From the double-labeling experiments using *EN2* and *XFKH1* probes for in situ hybridization, it is clear that the expression extends at least into the midbrain region. We do not know whether any expression is taking place in the forebrain. At later stages, the forebrain area is largely negative; however, this might be the result of the increase in volume in the forebrain area. The pattern of *XFKH1* expression, which initiates in the mesoderm followed immediately by expression in the overlying neuroectoderm, is reminiscent of *Hox-2.9* gene expression in the different germ layers of early mouse embryos (Frohman et al. 1990) and suggests that the expression in the neuroectoderm is specified by

Figure 7. Northern analysis of *XFKH1* induction in the presence and absence of protein synthesis. Equal amounts of RNA (10 μ g) from induced and uninduced animal caps were loaded per lane. (Lane 1) Control; (lane 2) activin (no cycloheximide); (lane 3) activin and cycloheximide.

the expression in the adjacent notochord. This idea of homeogenetic induction across germ layer boundaries (DeRobertis et al. 1989), in which the expression of a transcription factor in one tissue induces the expression of the same factor in the other tissue, is supported further by the experiments with exogastrulae. In these embryos, where the contact between notochord and ectoderm is experimentally eliminated, the expression of *XFKH1* in the notochord is unaltered but the expression corresponding to the neural floor plate is eliminated. This experiment suggests that the notochord is necessary for the formation of the neural floor plate. The lack of expression in the ectoderm of exogastrulae might be surprising in view of results of Kintner and Melton (1987) and Ruiz i Altaba (1990), who showed that neural-specific markers *N-CAM* and *Xhox3* are expressed in the exogastrula. The likely explanation for this discrepancy is that some neural-specific genes require normal contact between mesoderm and ectoderm and others do not.

XFKH1 is an immediate early response to activin induction

Peptide growth factors related to TGF- β and bFGF can induce mesoderm formation in ectodermal animal caps. In addition, activin A, a growth factor related to TGF- β , can also induce axis formation in these explants, mimicking the function of the organizer region of amphibian gastrula. Although it is not known whether activin is the natural inducer in vivo, activin-mediated induction can be used as a test for molecules involved in the initial

steps of axis formation. We have tested *XFKH1* for inducibility by activin in this animal cap assay. The data obtained show that the *XFKH1* gene is induced by activin even in the absence of protein synthesis. This indicates that *XFKH1* is a primary response gene induced by activin-like growth factors and suggests that processes other than translation (e.g., phosphorylation) are needed for transcriptional activation of this gene. This is not entirely surprising, as it is known that the activin receptor has a serine kinase activity (Mathews and Vale 1991).

In summary, we have isolated a gene that is a good candidate to play a regulatory role in the initial steps of pattern formation in *Xenopus* embryos. In its initial expression pattern at blastula and gastrula, this gene is similar to two previously isolated homeo box genes, *gooseoid* (Blumberg et al. 1991; Cho et al. 1991a,b) and *Xlim1* (Taira et al. 1992). Both of these genes are expressed in the organizer region and are inducible by activin. At present, only *gooseoid* has been shown to induce a secondary axis after injection of its mRNA. Alternatively, the *XFKH1* gene could be involved in the formation of the dorsal midline and is similar in its function to that of *single-minded* gene of *Drosophila* (Nambu et al. 1991), which acts as a marker regulator of CNS midline development and the cyclop mutation of zebrafish (Hatta et al. 1991) which, in turn, blocks specification of the floor plate. At present, we cannot distinguish between these two possibilities. Unfortunately, we cannot learn much about its function from comparison to *Drosophila fkh* expression. *Drosophila fkh* is expressed in a very different way, primarily in the most anterior and most posterior regions of the embryo. The comparison is made even more difficult by the fact that *Xenopus* might have as many as 10 different genes containing the *fkh* domain (M. Dirksen and M. Jamrich, unpubl.). These genes seem to have vastly different expression patterns; therefore, any attempt to make a detailed comparison of *XFKH1* and *Drosophila fkh* would only be misleading. Although a specific comparison might not be useful at present, in general, it is likely that in *Xenopus*, as in *Drosophila*, some of the early functions in embryonic development are performed by homeotic selector genes such as homeo box-containing genes; others are performed by region-specific homeotic genes like *fkh* or *spalt* (Frei et al. 1988; Jürgens 1988; Jürgens and Weigel 1988).

Materials and methods

PCR reactions

PCR reactions (Saiki et al. 1988) with degenerate primers to ITMAIQ and NMFENG sequences were performed using the Perkin-Elmer Cetus PCR reaction kit. The reaction cycles and conditions were performed as described in Mackem and Mahon (1991).

Embryos

Developmental stages of embryos were determined according to Nieuwkoop and Faber (1967). For exogastrulation experiments,

embryos were placed into $1\times$ nuclear magnetic resonance at two-cell stage, and the vitelline membrane was manually removed. Exogastrulae were monitored carefully during formation, and only complete exogastrulae were selected for further analysis.

Preparation of RNA

Total RNA from embryos and tissues was prepared according to Sargent et al. (1986). Ten micrograms of RNA was loaded per lane on 1.2% agarose gels containing 5 mM methylmercury hydroxide (Bailey and Davidson 1976). Gels were blotted to Nytran filters using vacuum transfer. DNA probes were made using the BRL random primer labeling system with [α - P^{32}]dCTP. Hybridization was performed as described by Church and Gilbert (1984).

Induction experiments

Animal caps were isolated from stage 8 blastulae. They were treated with FGF, activin, or cycloheximide according to Rosa et al. (1988) and Rosa (1989). We have used the following factor concentrations: 50 pM activin; 100 ng/ml of bFGF.

Whole-mount in situ hybridization

In situ hybridization was performed following the method of Hemmati-Brivanlou et al. (1990) and Harland (1991). Antisense RNA probes were prepared using the Boehringer RNA labeling kit with digoxigenin-UTP. A clone containing the entire *XFKH1* cDNA was used as the template for transcription.

Embryo sections

Embryos that gave a positive signal with whole-mount in situ hybridization were incubated 2 hr in 100% methanol, 2 hr in xylene, 1 hr in 1:1 xylene-paraffin (Paraplast-Plus) and, finally, 6 hr in paraplast. Incubations in paraffin were performed at 60°C. The paraffin was solidified by transferring it to room temperature, and 30- μ m sections were cut and dried down on polylysine-coated slides. Paraffin was dissolved in xylene, and xylene was washed out in 100% ethyl alcohol. Sections were viewed and photographed under a coverslip in 100% alcohol.

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M L Dirksen and M Jamrich

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