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ACTIVIN INDUCES THE EXPRESSION OF THE *XENOPUS* HOMOLOGUE OF *SONIC HEDGEHOG* DURING MESODERM FORMATION IN *XENOPUS* EXPLANTS

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Summary: The *Xenopus* homologue of *sonic hedgehog* (*Xhh*) was detected in *Xenopus* embryos at stages 13 and 31 by RT-PCR, but it was not expressed in explants isolated from the animal hemisphere of *Xenopus* embryos at stage 8-9. Treatment of the animal cap with activin (1-100 ng/ml) induced the expression of *Xhh*. However, it was not induced by 100 ng/ml basic fibroblast growth factor (bFGF). Whole mount *in situ* hybridization confirmed the expression of *Xhh* in the animal cap treated with activin. The expression of *Xhh* induced by activin was not inhibited in the presence of cycloheximide, suggesting that *Xhh* is an early response gene induced by activin. © 1995 Academic Press, Inc.

The mesoderm induction that is the first step of pattern formation in *Xenopus* embryos, requires the cell to cell contact of the vegetal and animal hemisphere cells. During gastrulation, mesodermal cells of the organizer region give rise to the notochord followed by the induction of floor plate and can induce neural differentiation in competent ectoderm.

Recently, three vertebrate homologues of the *hedgehog* gene (*hh*), segment polarity gene in *Drosophila* (1-3), *Shh*, *Dhh* and *Ihh*, have been isolated from mice, chicks and zebrafishes (4-6). In mouse embryos, *Shh* is expressed in the notochord and highly expressed in the floor plate. Ectopic expression of *Shh* induces the differentiation of motor neurons in the neural tube (7), strongly suggesting that *Shh* mediates conserved signaling functions governing the patterning of the vertebrate central nervous system. In zebrafish, *Shh* is expressed in the early developmental stages, and its transcript is restricted to the inner cell layer of the embryonic shield, the equivalent to the Spemann organizer of *Xenopus* (6). Thus the expression of *Shh* seems to be closely related to mesoderm induction.

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bFGF and activin, which are both present in eggs during the time of the mesoderm induction, are capable of inducing mesoderm formation in ectoderm explants (animal cap) of *Xenopus* embryos (8-10). When applied to the isolated animal caps, bFGF can induce only ventral mesoderm such as coelomic epithelium, while activin can induce dorsal mesoderm such as the notochord. During the mesoderm induction of the animal cap, activin induces various homeobox genes such as *goosecoid, Xlim-1* and *pintallavis (XFKH1),* a member of the *HNF/fork head* family, whose expression is restricted to the organizer region and newly formed notochord *in vivo* (11-14).

In the present study, we examined the expression of *Xenopus* homologue of *Shh* (*Xhh*) during the mesoderm induction in animal cap by activin.

Materials and Methods

Mesoderm induction in animal cap by activin

The upper portion of the animal hemisphere (animal cap), presumptive ectoderm, was manually dissected at stage 8-9. The isolated animal cap was cultured in Steinberg's solution containing 0.1% bovine serum albumin with bFGF and activin A at various concentrations (1-100 ng/ml) for 30 min in the presence or absence of cycloheximide (10 μg/ml) and then further incubated for 9 hr without factors and reagents. Human recombinant activin A was prepared as previously described (15). Human recombinant bFGF was obtained from Mallinckrodt (Paris, France). RNA was prepared from these samples.

RT-PCR

RNA was prepared from the embryos at stages 13 and 31, and untreated or activin-treated animal caps (100 pieces) according to the guanidine thiocyanate method (16). The total RNA (1 μg) was subjected to the RT-PCR as described in the manual from Perkin- Elmer. The following primers were used for the detection of *Xbra*, *EF1 α* and *Xhh*; 5'-CACCGCTGGAAGTATGTGAA-3' *Xbra* (429-448) and 5'-TAGACCAGTTATCATGGGAC-3' *Xbra* (1098-1079) for *Xbra* (17), 5'-CAGATTGGTGCTGGATATGC-3'*EF1α* (1104-1123) and 5'-ACTGCCTTGATGACTCC TAG-3' *EF1 α* (1372-1353) for *EF1 α* (17), and 5'-CCAACTACAATCCCGACATC-3' *Xhh* (240-259) and 5'-TCGTAGTAGACCCAGTCGAA-3'*Xhh* (534-515) for *Xhh* (5, unpublished data).The annealing temperature was 60°C and 35 cycles were performed. The RT-PCR products were subjected to 3 % Nusieve gel (TaKaRa, Kyoto) and blotted to nylon filter (Amersham, Buckinghamshare, England). The filters were hybridized with [^{32}P]-labeled probes of *Xbra*, *EF1 α* and *Xhh*, which were prepared by PCR and whose DNA sequences were identified.

The RT-PCR product of *Xhh* obtained from the RNA of the embryos at stages 13 and 31 was cloned into the pCRII vector by a TA cloning kit (Invitrogen, San Diego, CA), and 20 clones were isolated and their DNA sequences were analyzed by a fully automatic DNA sequencer (Pharmacia, Milwaukee, WI).

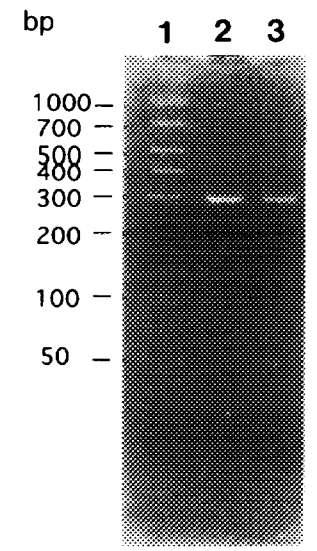
Whole mount *in situ* hybridization

Digoxigenin (DIG) -labeled RNA probes in sense and antisense orientations were prepared from *Xhh* using the DIG RNA Labeling Kit (Boehringer, Mannheim, DFG).Whole mount *in situ* hybridization was performed essentially as described elsewhere (18) .

Results

A fragment of the expected size (295 bp) of PCR product of *Xhh* was obtained from the RNA of *Xenopus* embryos at stages 13 and 31 (Figure 1A). Their DNA sequence was highly homologous, 82 % homology, to that of mouse *Shh* (Figure 1B).

The expression of *Xhh* was examined in the animal caps treated with activin or bFGF by RT-PCR and southern blot analysis, because of the limitation of the amount of sample available. *Xhh* was not expressed in untreated animal caps of stage 8-9. Activin (100 ng/ml) induced the



B

Xhh	CCAACTACAA	TCCCGACATC	ATCTTCAAGG	ATGAAGAGAA
Shh	CCAATTACAA	CCCCGACATC	ATATTTAAGG	ATGAGGAAAA
Xhh	CACAGGAGCT	GACCGACTGA	TGACTGAGCG	CTGCAAAGAC
Shh	CACGGGAGCA	GACCGGCTGA	TGACTCAGAG	GTGCAAAGAC
Xhh	AGAGTCAATG	CCTTGGCTAT	CTCTGTGATG	AACATGTGGC
Shh	AAGTTAAATG	CCTTGGCCAT	CTCTGTGATG	AACCAGTGGC
Xhh	CAGGCTTGAA	GCTGCGGGTG	ACCGAGGGCT	GGGATGAGGA
Shh	CTGGAGTGAG	GCTGCGAGTG	ACCGAGGGCT	GGGATGAGGA
Xhh	CGGCCAGCAC	GCTCAGGAGT	CTTTTCATTA	TGAAGGGCGA
Shh	CGGCCATCAT	TCAGAGGAGT	CTCTACACTA	TGAGGGTCGA
Xhh	GCGTTGGACA	TTACAACGTC	TGACCGAGAC	AGGAACAAGT
Shh	GCAGTGGACA	TCACCACGTC	CGACCGGGAC	CGCAGCAAGT
Xhh	ATGGCATGGT	GGCGCGTCTG	GCAGTGGAAG	CTGGCTTCGA
Shh	ACGGCATGCT	GGCTCGCCTG	GCTGTGGAAG	CAGGTTTCGA
Xhh	CTGGGTCTAC	TACGA		
Shh	CTGGGTCTAC	TATGA		

Figure 1.
Detection of *Xhh* in the *Xenopus* embryos at stages 13 and 31 by RT-PCR.
A) The RT-PCR products obtained from the RNA of *Xenopus* embryos at stages 13 and 31. Lane 1, molecular marker; lane 2, stage 13; and lane 3, stage 31. B) The DNA sequence of the RT-PCR products obtained. The forward and reverse primers used for the RT-PCR are underlined. Nucleotides of the *Xhh* that are not identical to those of the mouse *Shh* are indicated by squares. Twenty clones obtained from the embryos at stages 13 and 31 were identical. The same DNA sequence was also observed in six clones of the RT-PCR products obtained from the RNA of the animal caps treated with activin (100 ng/ml).

expression of *Xhh* in the animal cap, but bFGF (100 ng/ml) did not (Figure 2A). bFGF could increase the expression of *Xbra* in the animal caps under the conditions used. The DNA sequence of the PCR products obtained from the activin-treated animal caps was identical with that of *Xhh* (Figure 1B). When animal caps were treated with activin (1-100 ng/ml), most of the them became elongated in shape and the expression of *Xhh* was induced in them (Figure 2B). Expression of *Xhh* in the animal cap induced by activin (100 ng/ml) was not inhibited, rather stimulated, in the presence of cycloheximide (10 μg/ml) (Figure 2C).

The whole mount *in situ* hybridization confirmed the expression of *Xhh* in the animal cap treated with activin (Figure 3). *Xhh* was expressed in all of the animal caps treated with activin at more than 10 ng/ml, but weakly positive signals of *Xhh* were detected in the animal caps treated with activin at 1ng/ml (unpublished observation).

Discussion

Various genes are induced during the mesoderm induction in the animal cap of *Xenopus* embryos by activin or bFGF. *Xbra* and *Xwnt* are induced by both activin and bFGF (19,20),

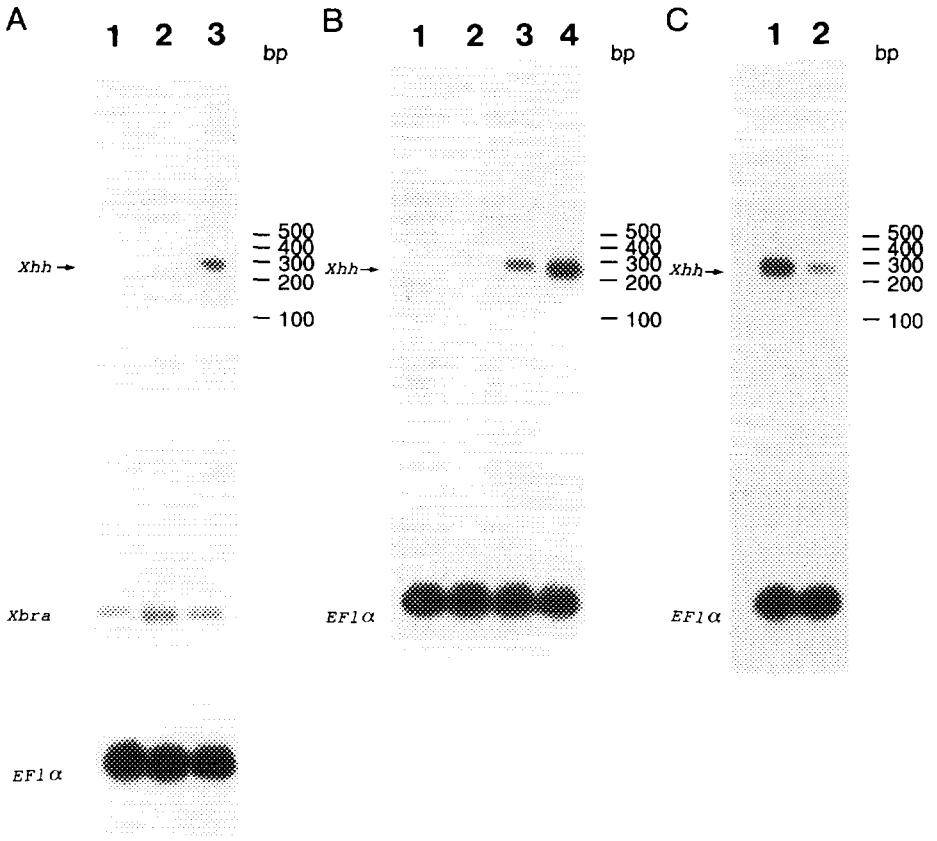


Figure 2.
Activin induced the expression of *Xhh* in the animal cap at stage 8-9.
A) *Xhh* was induced by activin (100 ng/ml), but not bFGF (100 ng/ml). Lane 1, untreated animal cap; lane 2, animal cap treated with bFGF (100 ng/ml); lane 3, animal cap treated with activin (100 ng/ml). B) Dose-dependency of the expression of *Xhh* induced by activin. Lane 1, without treatment; lane 2, activin (1 ng/ml); lane 3, activin (10 ng/ml); lane 4, activin (100 ng/ml). C) Activin induced the expression of *Xhh* in the animal cap in the presence or absence of cycloheximide (10 μg/ml). Lane 1, with cycloheximide; and lane 2, without cycloheximide.

while homeobox genes such as *Mix-1, goosecoid, Xlim-1* and *pintallavis (XFKH1)* are induced by only activin, but not bFGF (11-14). In the present study, we demonstrated that *Xhh* was also induced by activin (1-100 ng/ml) but not by bFGF (Figure 2 and 3). The notochord is appeared with high efficiency in the animal caps treated with activin (10-100 ng/ml), but not appeared in the animal caps treated with activin (1ng/ml) under the conditions used (21). Since the expression of *Xhh* was induced by activin even at the concentration of 1ng/ml and *Xhh* was expressed much earlier than the appearance of the notochord in the animal cap induced by activin, *Xhh* is not only the product of the notochord, but also may be involved in the dorsal mesoderm formation. The expression of *Shh* during the development of zebrafish also supports this; *Shh* is expressed in the organizer region before its expression in the notochord (6).

Hepatocyte nuclear factor (*HNF-3β*) encoding the *fork head* (*fkh*) domain, which is conserved in the *Drosophila* homeotic gene, shows several similarities in expression to *Shh* in

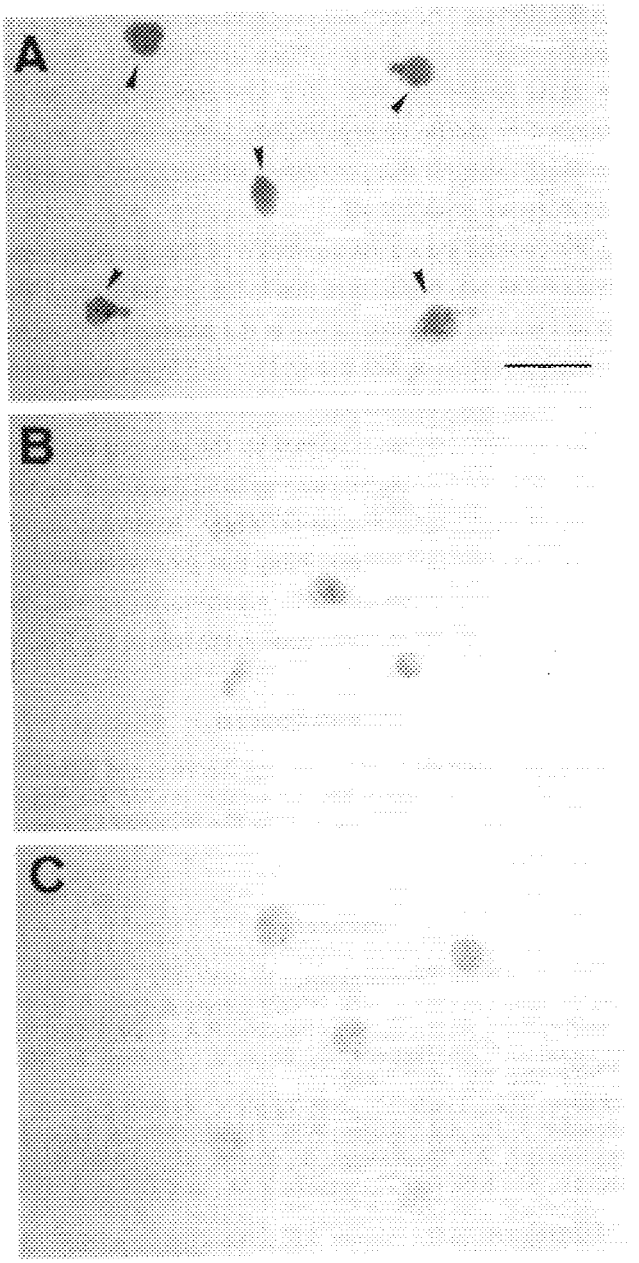


Figure 3.
Whole mount *in situ* hybridization of *Xhh* in the animal cap treated with activin.
Animal caps were incubated with or without activin (10 ng/ml) and then subjected to the whole mount *in situ* hybridization with antisense and sense probe of *Xhh*. A) Activin-treated animal cap hybridized with antisense probe. B) Activin-treated animal cap hybridized with sense probe. C) Untreated animal cap hybridized with antisense probe. Arrowheads indicate the positive signal of *Xhh*. Scale bar indicates 1 mm.

the mouse (4,22). The expression pattern of *HNF-3β* in the mouse is strikingly similar to that of *pintallavis (XFKH1),* a member of the *HNF/fork head* family in *Xenopus* embryos (13,14); *pintallavis (XFKH1)* is first detected in the late blastula (stage 9) and its expression peaks in

mid-gastrula (stage 12) embryos. The expression of *pintallavis (XFKH1)* is coincident with the organizer region after the onset of gastrulation. *Pintallavis (XFKH1)* is expressed at high levels in the notochord by stages 15 and 16. *HNF-3β* is also expressed in the notochord and floor plate during development of mouse embryo shortly before *Shh* is expressed (4,22). Taken together, these observations suggest that *pintallavis (XFKH1)* is a potential regulator for the expression of *Xhh* in *Xenopus* embryos (4).

However, activin can still induce the expression of *Xhh* in the presence of cycloheximide (Figure 2C). The level of *Xhh* was higher than that in the untreated caps. This overinduction is typical for many immediate early induced genes in the presence of cycloheximide (23). This result suggested that the expression of *Xhh* induced by activin is not dependent on new protein synthesis and that *Xhh* is a primary response gene induced by activin, like *pintallavis (XFKH1)* (13,14). The expression of *Xhh* may be regulated by the pre-existing transcriptional factors activated by the phosphorylation via a signal pathway from the activin receptor kinase (24, 25). However, it should be noted that *pintallavis (XFKH1)* is already expressed at stage 8-9 (13,14). Therefore, we can not exclude the possibility that gene product of *pintallavis (XFKH1)* is one of the candidates for the transcriptional factor regulating the expression of *Xhh*.

In conclusion, *Xhh* is the early response gene in the animal cap induced by activin.

Acknowledgments

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