Vol. 216, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 2, 1995 Pages 26-33

TRUNCATED TYPE II RECEPTOR FOR BMP-4 INDUCES SECONDARY AXIAL STRUCTURES IN *XENOPUS* EMBRYOS

Tetsuya Ishikawa,[1] Hidefumi Yoshioka,[2] Hideyo Ohuchi,[2] Sumihare Noji,[2] and Tsutomu Nohno[3]*

[1]*Department of Biochemistry and* [3]*Department of Pharmacology, Kawasaki Medical School, Kurashiki 701-01, Japan*

[2]*Department of Biological Science and Technology, Faculty of Engineering, University of Tokushima, Tokushima 770, Japan*

Received September 4, 1995

Summary: BRK-3 is a vertebrate type II receptor for BMP-4 distantly related to invertebrate type II receptors for BMP-2/BMP-4/dpp, such as daf-4 and punt. BRK-3 has a long carboxy-terminal sequence following intracellular kinase domain and is capable of forming a high-affinity complex with a type I receptor, BRK-2. To examine the role of BRK-2 + BRK-3 receptor complex in BMP signaling during early embryogenesis, the dominant-negative form of BRK-3 was ectopically expressed in the *Xenopus* embryos. A secondary body axis expressing the *Sonic hedgehog* and N-CAM genes is induced by injecting mRNA encoding truncated form of BRK-3 into ventral marginal region, implicating the BMP signaling in axial mesoderm induction. Formation of the secondary axis depends on whether the deletion extends into the kinase domain, not into the carboxy-terminal tail, suggesting that the kinase domain, but not the tail region, is essential for BMP signaling.

Understanding body plan determination is fundamental for embryology and much of our knowledge comes from studies in amphibia. One of the earliest developmental decisions in *Xenopus* embryos involves induction of the axial mesoderm that organizes the body axis. The ground state for the mesoderm is ventral, and signals from the Nieuwkoop center are known to induce dorsal mesoderm. It is becoming clearer from the recent studies that various growth factors contribute to determination of the dorsoventral pattern (1). In addition to Wnt-11 and noggin, activin of the transforming growth factor-β (TGF-β) superfamily has a potent mesoderm-inducing activity (1,2), and was presumed to be an inducing factor for dorsal mesoderm albeit controversy remains.

Bone morphogenetic proteins (BMPs) that induce cartilage and endochondral bone formation belong to the DVR subgroup of the TGF-β superfamily and play important roles in early embryogenesis (3,4). BMP-4 is present maternally and induces ventral mesoderm from

*To whom correspondence should be addressed (FAX: 81-86-462-1199).

0006-291X/95 $12.00

prospective epidermis, resulting in formation of most ventral mesenchyme (5-8). When overexpressed in the early embryo, BMP-4 ventralizes the embryo resulting in complete loss of dorsal structure (6,7). On the contrary, overexpression of dominant-negative form of the BMP type I receptor that blocks the normal BMP signal produces more dorsal fate to become in the ventral mesoderm (9,10), and microinjection of the truncated type I receptor mRNA into ventral side resulted in twinned embryos, though the secondary body axis was incomplete (10). These experiments show that active BMP signal is required to form most ventral tissues.

To determine whether BRK-3, a human type II receptor for BMP-4, comprises a functional receptor system for BMP-4, we utilize the *Xenopus* embryos. We show here that the truncated form of BRK-3 lacking the kinase domain inhibits the BMP signaling pathway during *Xenopus* embryogenesis, resulting in induction of a secondary body axis. Endogenous BMP-4 is presumed to be involved in the dorsoventral specification in *Xenopus* embryo, and long carboxy terminal sequence of BRK-3 may not be essential for signaling activity.

MATERIALS AND METHODS

Construction of truncated type II BMP receptors: The BRK-3 cDNA encoding type II BMP receptor was isolated from human skin fibroblast cDNA libraries (Fig. 1A) (11). Dominant negative form of the BRK-3 gene was constructed by removing sequence downstream of the transmembrane region at Asp201. The truncated BRK-3 gene was finally ligated into expression vector pSP64TL, a derivative of pSP64T (12) containing polylinker sequence at the *Bgl*II site. The resultant tBRK-3K gene encodes truncated type II receptor containing the first 200 amino acids of BRK-3 and additional 17 amino acids (ERPQAYRPITSGRLAHR) derived from vector sequence. A partial deletion was also constructed that extends only in the carboxy-terminal sequence. The resultant tBRK-3T gene encodes type II receptor containing the first 657 amino acids of BRK-3 and additional 9 amino acids (ITSGRLGHR) derived from the vector sequence. RNA was synthesized using SP6 polymerase after digesting the plasmids by restriction digestion.

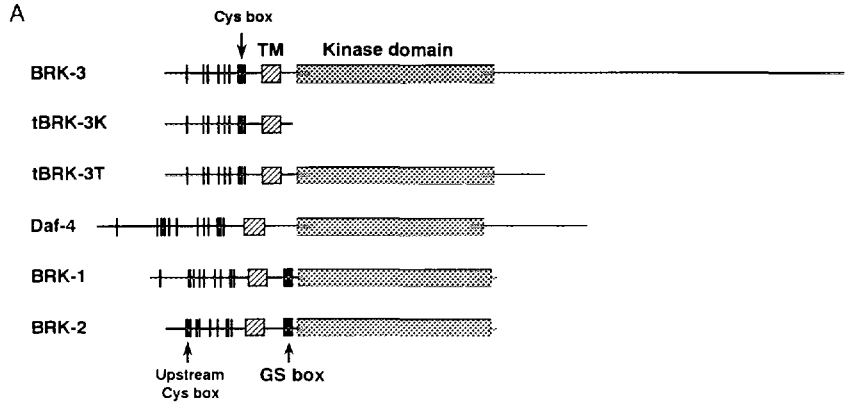
Ectopic expression of the BRK-3 gene in Xenopus embryos: Embryos were staged according to Nieuwkoop and Faber (13). Transcribed RNAs were injected into the ventral equatorial region of *Xenopus laevis* embryos at four-cell stage, and allowed to develop until at stages 15-30 to observe the phenotype. Expression of the neural marker gene neural cell adhesion molecule (N-CAM, 14) was detected by whole mount *in situ* hybridization (15). The *Xenopus Sonic hedgehog* gene was isolated from the cDNA library (Tashiro et al., manuscript in preparation), and used as a notochord and floor plate marker gene (16).

RESULTS

BRK-3 encodes type II receptor for BMP-4

We have identified a human type II receptor for BMP-4, called BRK-3, and the binding characteristics were already described (11). In brief, cotransfection of the BRK-3 type II receptor with BRK-2 type I receptor greatly enhanced affinity labeling of BMP-4 to BRK-2. The BRK-2 and BRK-3 heteromer represents a functional receptor complex for BMP-4. The nucleotide sequence of the BRK-3 cDNA is identical to the T-ALK receptor isolated using yeast two-hybrid system as a novel type II receptor interacting with type I receptor for TGF-β1 (17).

The BRK-3 protein contains a long carboxy terminal tail rich in Ser, Thr and Pro residues following the intracellular Ser/Thr kinase domain (Fig. 1A). No significant similarity is detectable in the extracellular domain among the other type II receptors for TGF-β superfamily, although the downstream Cys box and several Cys residues are conserved (Fig. 1B). Several charged and



B

```
          *                    *           *
BRK-3   (34) CAFKDPYQQDLGIGESRISHENGTIL-----C-----SKGST-CYGLWEKSKGDINLVKQ----------
Daf-4   (90) CVYYDEMECEKSGDCEITKKT----------CYSEAHLKAVG-CLAVFGLPTQEINSTEPYLKVDKPQYKSL
Punt    (36) CEHFDEKMCNTTQQCETRIEH----------C-KMEADKFPS-CYVLWSVNETTGILRIKMK----------
MISRII  (24) CVFFEAPGVRGSTKTLGEVVDAGPGPPKGIRC-----LYSHC-CFGIWNLTHGRAQVEWQ----------
ActRII  (30) CLFFNANWERDRTNQTGVEP----------C-YGDKDKRRH-CFATWKNISGSIEIVKQ----------
ActRIIB (29) CIYYNANWELERTNQSGLER----------C-EGEQDKRLH-CYASWRNSSGTIELVKK----------
TGFbRII (51) CKFCDVTLSTCDNQKSCMSNCSVTSI-----C-----EKPQEVCVAVWRKNDKNITLET----------

                *           *           * *   *
BRK-3       GCWSHIGDPQE-CHYEE----CVVTTTPPSIQNGTYRFCCCSTDLCN (124)
Daf-4       GCMPYQHADSMNCENESS---CRQGRSFRGGIG----MCCCSTNNCN (190)
Punt        GCFTDMHE----CNQTE----CVTSAEPRQGNIH---FCCCKGSRCN (121)
MISRII      GCLDSDEPG---CESLH----CDPVPRAHPSPSSTLFTCSCGTDFCN (117)
ActRIIA     GCWLDDIN----CYDRTD---CIEKKDSPEVY-----FCCCRGNMCN (111)
ActRIIB     GCWLDDFN----CYDRQE---CVATEENPQVY-----FCCCEGNFCN (110)
TGFbRII     VCHDPKFTYHGFTLEDATSPTCVMKEKKRAGETFF--MCSCNTEECN (144)
```

Fig. 1. Structure of BRK-3 and other BMP receptors.

A: Schematic presentation of BRK-3, tBRK-3K, tBRK-3T, Daf-4, BRK-1, and BRK-2. Cys residues in the extracellular domain are shown by vertical bars. **B**: Alignment of the extracellular ligand binding domain of the type II receptors. Conserved Cys residues are shown by asterisks, and several charged residues are highlighted. **C**: Dendrogram of alignment for the kinase domain of receptor Ser/Thr protein kinase. ActRI, mouse activin type I receptor (26); ActRIB, human activin type IB receptor (27); ActRII, mouse activin type II receptor (28); ActRIIB, mouse activin type IIB receptor (29); ALK-1, human activin receptor-like kinase-1 (27); Atr-I, *Drosophila* activin type I receptor (30); Atr-II, *Drosophila* activin type II receptor (20); BRK-1, mouse BMP type I receptor (24); BRK-2, mouse BMP type I receptor (31); BRK-3, human BMP type II receptor (this study); Daf-1, *Caenorhabditis elegance* daf-1 (32); Daf-4, *C. elegance* BMP type II receptor (18); MISRII, human Müllerian-inhibiting substance type II receptor (21); Sax, *Drosophila* saxophone (33); Tkv, *Drosophila* thick veins (34); TGFbRI, human TGF-β type I receptor (27); TGFbRII, human TGF-β type II receptor (35).

neutral residues are present between these Cys residues in the type II BMP receptors, such as BRK-3, daf-4 (18) and punt/AtrII (19,20), and may contribute to the ligand binding with the BMP-2/BMP-4/dpp subgroup. To evaluate phylogenetic relationship of BRK-3 with other receptor Ser/Thr kinases, a dendrogram is calculated within the kinase domain (Fig. 1C). BRK-3 and MISRII (C14) (21) are most distantly related to other type II receptors. The kinase domains of the type II receptor subgroup are divergent as compared to the type I receptor subgroup.

Truncated BRK-3 inhibits the endogenous BMP signaling

To examine the biological function of BRK-3 in mediating BMP signaling during *Xenopus* development, we construct two forms of truncated BRK-3 both of which have an intact extracellular domain. The truncated receptor mRNA was injected into the ventral equatorial region

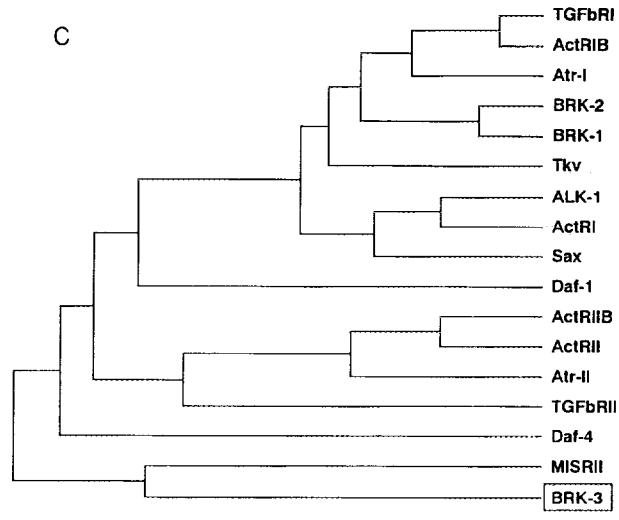


Figure 1 -- *Continued*

of the blastmeres of *Xenopus* embryos at stage 3, and the embryos were developed until at stages 15 to 30. When the deletion extends into the kinase domain, microinjection of tBRK-3K resulted in bifurcation of the neural tissue (Fig. 2A) and eventually formed a secondary body axis (Fig. 2B). Formation of the secondary axis was dose-dependent, since the frequency increased from 50% to 74% by doubling the amount of tBRK-3K mRNA (Table 1). The induced secondary axes usually lack anterior structure, and almost all of the injected embryo revealed an identical phenotype. However, the secondary axis was not induced when tBRK-3K mRNA was injected into the dorsal equatorial region (Table 1). Injection of 1 ng or more RNA resulted in the increased lethality, while less than 20 pg RNA had no effect on the phenotype (data not shown).

We examined expression pattern of the *Sonic hedgehog* gene and N-CAM gene by whole-mount *in situ* hybridization to determine the cell type in the secondary axis. In the normal embryo, the *Sonic hedgehog* gene was expressed in the notochord, floor plate, oral cavity, and gut primordium (Fig. 2C). Expression in the gut primordium was also detectable in the secondary axis (Figs. 2D, 2G). The N-CAM gene was expressed in the neural tube of the primary axis (Figs. 2E, 2F, 2H) as well as in the neural tissue of the secondary axis (Figs. 2F, 2H). These results indicate that the secondary axis induced by dominant negative form of BRK-3 contains neural and endodermal tissues, thus suggesting temporal correlation of *Sonic hedgehog* expression and N-CAM expression during secondary axis formation.

The secondary axis was not induced by injecting tBRK-3T mRNA that encodes truncated BRK-3 lacking only the most part of carboxy terminal tail (Table 1). About one-tenth of the embryos have defects in the posterior structure as observed in the medium-injected control. These findings imply that BRK-3 ligands are required for dorsoventral axis determination as an endogenous ventralizing factor, and that kinase domain, but not the tail region, is essential for BMP signaling.

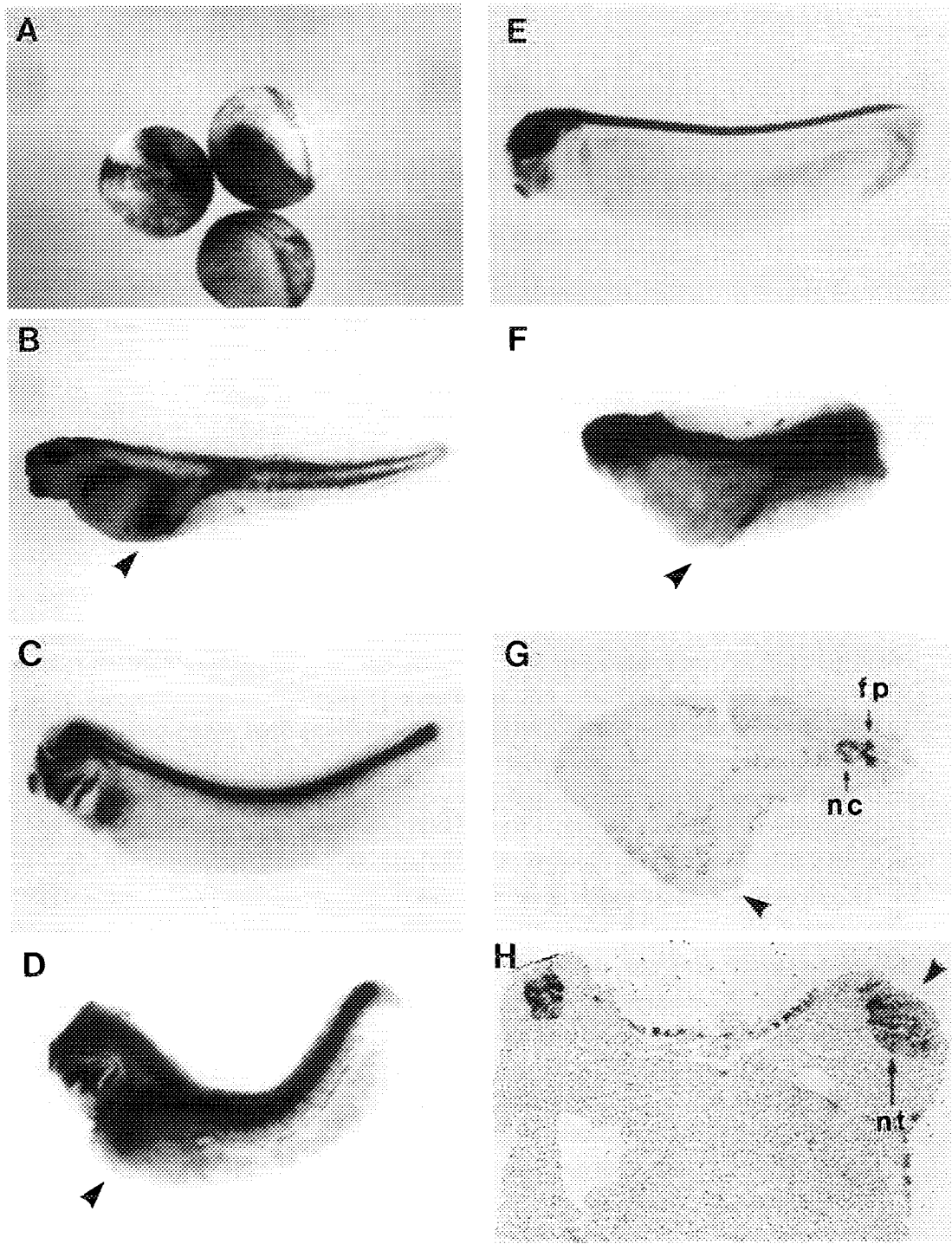


Fig. 2. Inhibition of endogenous BMP signaling pathway by tBRK-3K in the developing *Xenopus* embryo.

A: Neurula embryo after tBRK-3K injection into ventral marginal zone. **B**, **D**, and **F**: Embryos at stage 30 showing induction of the secondary axial structures (arrowheads) by tBRK-3K. **C** and **E**: Normal embryos at stage 30. **C** and **D**: Expression pattern of the *Sonic hedgehog* gene. **E** and **F**: N-CAM expression determined by whole-mount *in situ* hybridization. **G** and **H**:

Table 1. Phenotype of embryos injected with truncated BRK-3 mRNAs

RNA injected (pg)		Injection	Number*	Phenotype (%)		
tBRK-3K	tBRK-3T	site		Normal	Secondary axis	Defects†
0‡		Ventral	36	89	0	11
250		Ventral	52	44	50	6
500		Ventral	73	19	74	7
	250	Ventral	23	87	0	13
	500	Ventral	31	80	0	20
250		Dorsal	62	94	0	6
500		Dorsal	53	91	0	9

The table summarizes the results of three independent experiments performed under similar conditions. *The total number of embryos at stage 30. †Defects include incomplete invagination and posterior truncation. ‡Water was injected in the ventral region.

DISCUSSION

Experiments using dominant-negative form of growth factor receptors suggest involvement of the endogenous ligands in normal development of *Xenopus* embryos. Several TGF-β related proteins, such as Vg-1, activin and BMP-4, have been implicated in the process of mesoderm formation and dorsoventral patterning in the *Xenopus* embryo (1,2). The TGF-β superfamily generally requires both the type I and the type II receptors for signal recognition, thereby forming a high-affinity heteromeric complex that transduces subsequent intracellular signaling (3,22). Coexpression of the type I and type II receptors is therefore a prerequisite for specific recognition of the ligand, and the type II receptor participates in preferential ligand binding while the type I receptor determines subsequent intracellular signaling.

Several type I receptors for BMPs have been identified: BRK-1/BMPR-IA/TFR11/ALK-3 binds BMP-2 and BMP-4 more preferentially than BMP-7 (23,24); BRK-2/RPK-1/BMPR-IB/ALK-6 binds both BMP-4 and BMP-7 efficiently (11,23); and ActRI/SKR1/ALK-2 binds both activin and BMP-7 but not BMP-4 (23,25). BRK-3, the type II receptor for BMP-4 from the vertebrate, can form high affinity complex with BRK-2, but not with BRK-1 (11), implicating that both BRK-2 and BRK-3 are required to transduce BMP-4 at physiological concentrations. Because BMP-4 has been suggested to act as a ventralizing signal in the *Xenopus* embryo (4), the dominant-negative form of BRK-3 takes great advantage to examine the physiological role of BMP-4 signaling. Therefore, we used BRK-3 rather than other type I receptors to block the BMP-4 signal specifically, and evaluated dominant negative effect during *Xenopus* embryogenesis. Our results were consistent to those obtained with dominant negative BRK-1 in the *Xenopus* embryo (9,10), thus confirming that BRK-3 is participate in the formation of

Transverse sections of the whole-mount hybridized embryos for the *Sonic hedgehog* gene and the N-CAM gene, respectively. The secondary axis is indicated by an arrowhead. Abbreviations: nc, notochord; fp, floor plate of the spinal cord; nt, neural tissue.

receptor complex for BMP-4. Induction of the secondary axis by tBRK-3K strongly suggests that an endogenous BRK-3 ligand is involved in the dorsoventral specification. Since BMP-4 binding to BRK-2 is greatly enhanced by coexpressing the BRK-3 gene in COS cells (11), BMP-4 is presumed to be the endogenous ligand.

On the other hand, tBRK-3T, which encodes the intact extracellular domain, the transmembrane region and the kinase domain without long carboxy terminal sequence, has no effect on the phenotype of *Xenopus* embryos. The results suggest that the long carboxy terminal sequence following the kinase domain may not be essential for the downstream signaling pathway, at least, in the early embryogenesis.

Inability to form most anterior structure by ectopic tBRK-3K expression suggests that BMP-4 is involved in the formation of posterior structure. Although the N-CAM gene was intensely expressed in the neural tissue, expression of the *Sonic hedgehog* gene was weak in the secondary axis and confined presumably to the gut primordium, resulting in incomplete formation of the neural tube in the secondary axis. The BRK-3 ligand may be involved in patterning of the neural tube.

Acknowledgments: We thank Drs. A. Suzuki, N. Ueno, and M. Yuge for their helpful advice in microinjecting mRNA into *Xenopus* embryos, and Dr. K. Tashiro for sharing the *Xenopus Sonic hedgehog* cDNA. This work was supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture, and by a Research Project Grant from Kawasaki Medical School.

REFERENCES

1. Slack, J.M. (1994) Curr. Biol. 4, 116-126.
2. Klein, P.S.and Melton, D.A. (1994) Endocr. Rev. 15, 326-339.
3. Kingsley, D.M. (1994) Genes Dev. 8, 133-146.
4. Harland, R.M. (1994) Proc. Natl. Acad. Sci., USA 91, 10243-10246.
5. Koster, M., Plessow, S., Clement, J.H., Lorenz, A., Tiedemann, H., and Knochel, W. (1991) Mech. Dev. 33, 191-199.
6. Jones, C.M., Lyons, K.M., Lapan, P.M., Wright, C.V., and Hogan, B.L. (1992) Development 115, 639-647.
7. Dale, L., Howes, G., Price, B.M., and Smith, J.C. (1992) Development 115, 573-585.
8. Nishimatsu, S., Suzuki, A., Shoda, A., Murakami, K., and Ueno, N. (1992) Biochem. Biophys. Res. Commun. 186, 1487-1495.
9. Graff, J.M., Thies, R.S., Song, J.J., Celeste, A.J., and Melton, D.A. (1994) Cell 79, 169-179.
10. Suzuki, A., Thies, R.S., Yamaji, N., Song, J.J., Wozney, J.M., Murakami, K., and Ueno, N. (1994) Proc. Natl. Acad. Sci., USA 91, 10255-10259.
11. Nohno, T., Ishikawa, T., Saito, T., Hosokawa, K., Noji, S., Wolsing, D.H., and Rosenbaum, J.S. (1995) J. Biol. Chem., in press.
12. Krieg, P.A. and Melton, D.A. (1984) Nucleic Acids Res. 12, 7057-7070.
13. Nieuwkoop, P.D. and Faber, J. (1967) Normal Tables of *Xenopus laevis*, North-Holland, Amsterdam.
14. Kintner, C.R. and Melton, D.A. (1987) Development 99, 311-325.
15. Harland, R.M. (1991) Methods Cell Biol. 36, 685-695.
16. Echelard, Y., Epstein, D.J., St Jacques, B., Shen, L., Mohler, J., McMahon, J.A., and McMahon, A.P. (1993) Cell 75, 1417-1430.
17. Kawabata, M., Chytil, A., and Moses, H.L. (1995) J. Biol. Chem. 270, 5625-5630.

18. Estevez, M., Attisano, L., Wrana, J.L., Albert, P.S., Massague, J., and Riddle, D.L. (1993) Nature 365, 644-649.
19. Letsou, A., Arora, K., Wrana, J.L., Simin, K., Twombly, V., Jamal, J., Stachling-Hampton, K., Hoffmann, F.M., Gelbart, W.M., Massague, J., and O'Connor, M.B. (1995) Cell 80, 899-908.
20. Childs, S.R., Wrana, J.L., Arora, K., Attisano, L., O'Connor, M.B., and Massague, J. (1993) Proc. Natl. Acad. Sci., USA 90, 9475-9479.
21. Baarends, W.M., van Helmond, M.J.L., Post, M., van der Schoot, P.J.C.M., Hoogerbrugge, J.W., de Winter, J.P., Uilenbroek, J.T.J., Karels, B., Wilming, L.G., Meijers, J.H.C., Themmen, A.P.N., and Grootegoed, J.A. (1994) Development 120, 189-197.
22. Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) Nature 370, 341-347.
23. ten Dijke, P., Yamashita, H., Sampath, T.K., Reddi, A.H., Estevez, M., Riddle, D.L., Ichijo, H., Heldin, C.H., and Miyazono, K. (1994) J. Biol. Chem. 269, 16985-16988.
24. Koenig, B.B., Cook, J.S., Wolsing, D.H., Ting, J., Tiesman, J.P., Correa, P.E., Olson, C.A., Pecquet, A.L., Ventura, F., Grant, R.A., Chen, G.X., Wrana, J.L., Massague, J., and Rosenbaum, J.S. (1994) Mol. Cell. Biol. 14, 5961-5974.
25. Matsuzaki, K., Xu, J., Wang, F., McKeehan, W.L., Krummen, L., and Kan, M. (1993) J. Biol. Chem. 268, 12719-12723.
26. Ebner, R., Chen, R.H., Shum, L., Lawler, S., Zioncheck, T.F., Lee, A., Lopez, A.R., and Derynck, R. (1993) Science 260, 1344-1348.
27. ten Dijke, P., Ichijo, H., Franzen, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C.H., and Miyazono, K. (1993) Oncogene 8, 2879-2887.
28. Mathews, L.S. and Vale, W.W. (1991) Cell 65, 973-982.
29. Attisano, L., Wrana, J.L., Cheifetz, S., and Massague, J. (1992) Cell 68, 97-108.
30. Wrana, J.L., Tran, H., Attisano, L., Arora, K., Childs, S.R., Massague, J., and O'Connor, M.B. (1994) Mol. Cell. Biol. 14, 944-950.
31. ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M., Miyazono, K., and Heldin, C.H. (1994) Science 264, 101-104.
32. Georgi, L.L, Albert, P.S., and Riddle, D.L. (1990) Cell 61, 635-645.
33. Xie, T., Finelli, A.L., and Padgett, R.W. (1994) Science 263, 1756-1759.
34. Brummel, T.J., Twombly, V., Marques, G., Wrana, J.L., Newfeld, S.J., Attisano, L., Massague, J., O'Connor, M.B., and Gelbart, W.M. (1994) Cell 78, 251-261.
35. Lin, H.Y., Wang, X.F., Ng-Eaton, E., Weinberg, R.A., and Lodish, H.F. (1992) Cell 68, 775-785.