Essential role of the transcription factor Ets-2 in *Xenopus* early development

Kaoru Kawachi, Norihisa Masuyama, and Eisuke Nishida

From the Department of Cell and Developmental Biology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Address correspondence to: Eisuke Nishida
Department of Cell and Developmental Biology
Graduate School of Biostudies
Kyoto University
Sakyo-ku, Kyoto 606-8502
Japan
TEL: +81-75-753-4230
FAX: +81-75-753-4235
E-mail: L50174@sakura.kudpc.kyoto-u.ac.jp

Running Title: Essential role of Ets-2 in Xenopus early development
SUMMARY

The FGF/MAPK pathway plays an important role in early *Xenopus* developmental processes, including mesoderm patterning. The activation of the MAPK pathway leads to induction of *Xenopus* Brachyury (Xbra) that regulates the transcription of downstream mesoderm-specific genes in mesoderm patterning. However, the link between the FGF/MAPK pathway and the induction of Xbra has not been fully understood. Here we present evidence suggesting that Ets-2 is involved in the induction of Xbra and thus in the development of posterior mesoderm during early embryonic development. Overexpression of Ets-2 caused posteriorized embryos and led to the induction of mesoderm in ectodermal explants. Expression of a dominant negative form of Ets-2 or injection of antisense morpholino oligos against Ets-2 inhibited the formation of the trunk and tail structures. Overexpression of Ets-2 resulted in the induction of Xbra, and expression of the dominant-negative Ets-2 inhibited FGF- or constitutively active MEK-induced Xbra expression. Moreover, overexpression of Ets-2 upregulated the transcription from Xbra promoter reporter gene constructs. Ets-2 bound to the Xbra promoter region *in vitro*. These results taken together indicate that Xenopus Ets-2 plays an essential role in mesoderm patterning, lying between the FGF/MAPK pathway and the Xbra transcription.
INTRODUCTION

During Xenopus early embryonic development, mesoderm arises from ectoderm by induction that requires signals from the vegetal hemisphere of the embryo (1, 2). The FGF/MAP kinase (MAPK) pathway has been shown to be involved in early mesodermal patterning (3-8). One of the genes that are thought to be regulated directly by FGF via the MAPK signal transduction pathway is Xenopus Brachyury (Xbra). Brachyury is an important regulatory gene in early vertebrate development (9-14). Loss of Brachyury function in mouse, zebrafish, and Xenopus embryos causes defects in posterior mesoderm and notochord differentiation (9,15-17). However, the link between the FGF/MAPK pathway and the induction of Xbra expression has not been fully defined.

The ETS family of transcription factors, comprising more than 30 different members, has been found to play a crucial role in controlling transcription of a variety of genes involved in important cellular processes, such as proliferation and differentiation (19,20). They share a unique DNA binding domain, the ETS domain, which interacts specifically with GGA(A/T)-based recognition sites (18). As targets of the Ras-MAPK signaling pathway, Ets transcription factors are phosphorylated by MAPK and function as critical nuclear integrators of ubiquitous signaling cascades. The ETS family is divided into subfamilies by sequence similarity based on the ETS-domain or additional sequence motifs. Ets-2 is a member of the ETS subfamily which consists of three members: Ets-1, Ets-2 and Drosophila Pointed (21, 22). Studies with mammalian cultured cells have shown that Ets-2 is activated by MAPK-dependent phosphorylation of threonine 72 in an N-terminal regulatory domain (the Pointed domain) (21,23). Xenopus Ets-2 is maternally expressed in both the animal pole and the intermediate zone (24,25). On the basis of these results, we hypothesized that Ets-2 relays the FGF/MAPK signaling and induces mesoderm, by inducing Xbra gene transcription. In this study, we have presented several lines of evidence indicating that Ets-2 plays an essential role in mesodermal patterning, lying between the FGF/MAPK pathway and the Xbra transcription.
**EXPERIMENTAL PROCEDURES**

*Plasmid construction*—Xenopus Ets-2 cDNA was obtained by screening a λZAP II cDNA library made from stage 10.5 embryos. To construct CS2-En-N', the 888 bp fragment coding for amino acids 1–294 of *Drosophila* engrailed protein was inserted into the StuI site of CS2+. To generate EtsΔN En-R', the DNA binding domain of Ets-2 (amino acid residues 310–468) was subcloned into CS2-En-N. *In vitro* synthesis of capped mRNA was performed using mMESSAGE mMACHINE kit (Ambion) according to the manufacturer’s instruction.

*Embryo manipulation and animal cap assay*—Embryos were *in vitro* fertilized, dejellied and cultured in 0.1 × MBS [1.5 mM HEPES pH 7.4, 8.8 mM NaCl, 0.1 mM KCl, 0.24 mM NaHCO₃, 0.082 mM MgSO₄, 0.03 mM Ca(NO₃)₂ and 0.041 mM CaCl₂]. Embryos were staged according to Nieuwkoop and Faber (26). Embryos at 4-cell stage were injected with mRNA as described in the text and figure legends. Animal caps were dissected from the injected embryos at stage 8-8.5 and cultured in 1 × Steinberg solution containing 0.1% BSA to various stages for further analysis as described in the figure legends. RT-PCR experiments were performed according to standard protocol. The primer pairs used here for RT-PCR have been described elsewhere (27, 28). For morpholino oligo injections, an Ets-2 antisense oligo with the sequence 5'-AGCTGAGGGAGGGTATGTCCTTCC-3' was obtained from Gene Tools, LLC. Oligos were resuspended in sterile, filtered water and injected into 4-cell stage embryos with indicated amounts (29).

*Luciferase assay*—Embryos were injected with 200 pg of Xbra-pOLUC (provided by Dr. K. W. Y. Cho) and 100pg of pCMV-β-galactosidase together with 1ng of Ets-2 mRNA into animal poles at 2-cell stage. Dissected animal caps were assayed for luciferase and β-galactosidase activities at stage 11 (13, 14). The ratio of luciferase to β-galactosidase activity provides a normalized measure of luciferase expression. In all cases, fold activation was calculated using the results from only Xbra pOLUC and CMV-β-galactosidase injected embryos as background. Each experiment was performed
three times to ensure reproducibility of results.

_Gel mobility shift assay_—To obtain the recombinant GST-Ets-2 protein, the entire coding region of Ets-2 was subcloned into pGEX-6P. Production of GST-Ets-2 and GST protein was performed as described (30). A DNA sequence for oligonucleotides of the Ets binding site was 5′-CAGGTGTCAGTTACTGGATGTAAGTTTATTGAAGGCA-3′. Gel mobility shift assay was carried out as described (31). For competition assay, the Ets binding site was mutated using Site-Directed Mutagenesis Kit (Stratagene). The Ets binding site was mutated using forward primer 5′-CAGGTGTCAGTTACTGGCCGTAAGTTTATTGAAGGC-3′ and reverse primer 5′-TGCCCTCAATAAAACTTACGGCCAGTAAGAACTGACACCTG-3′.

**RESULTS**

_Ets-2 is required for mesoderm patterning in FGF signaling_—We first examined the effect of overexpression of wild type _Xenopus_ Ets-2 mRNA on early embryonic development. Ets-2 mRNA was injected into the dorsal or ventral marginal zones of 4-cell stage _Xenopus_ embryos. Compared with normal uninjected control embryos at tadpole stage 35, embryos with dorsal injections of Ets-2 showed anterior truncation and shorten body axis. The embryos injected with relatively high doses of Ets-2 mRNA (1 to 2 ng) lacked eyes and cement glands (Fig. 1A). About 70 % of embryos injected with 2 ng of Ets-2 mRNA showed the severe anterior defects (n=34). A typical image is shown in Fig. 1A. About 40 % of embryos injected with 1 ng of Ets-2 mRNA showed the similar severe defects (n=45). A typical phenotype is shown (Fig. 1A, Ets-2 1 ng DMZ). Moreover, injections with high doses of mRNA sometimes resulted in embryos with tail like protrusions (data not shown). The ventral injections had little or no effect on the development of the embryos (Fig. 1A, Ets-2 1 ng VMZ). To investigate these embryos in more detail, we examined the wide range of molecular markers in Ets-2 mRNA injected
embryos. The results of the RT-PCR analysis carried out on stage 26 and 37 whole embryos injected with Ets-2 mRNA of 1 ng are shown (Fig. 1B). Sibling embryos were used as control. Overexpression of Ets-2 mRNA resulted in remarkable reduction of expression of a forebrain marker Otx2 and a cement gland marker XAG. Moreover, expression of a pan-neural marker NCAM was also reduced by injection of Ets-2 mRNA. Thus, Ets-2 overexpression suppressed expression of anterior markers. Moreover, Ets-2 overexpression increased expression of the posterior markers such as Xbra and Xcad3 (Fig. 1B). A transverse section through the anterior of the posteriorized embryo showed no development of brain ventricles (data not shown). These data suggest that overexpression of Ets-2 causes anterior truncation and induction of posterior mesoderm. Next, to examine whether Ets-2 functions downstream of FGF signaling, we tested whether expression of Ets-2 could rescue the defects caused by inhibition of FGF signal in Xenopus embryos. Dominant negative FGF receptor (XFD) mRNA with or without Ets-2 mRNA was injected into dorsal marginal zones of 4-cell stage. Overexpression of XFD caused a severe posterior defect (18 out of 18, Fig. 2). This defect is thought to result from inhibition of both gastrulation movement and posterior mesoderm formation (32). Co-injection of Ets-2 rescued this morphological defect, and the injected embryos developed almost normally (22 out of 47) (Fig. 2). This rescue of the XFD-induced phenotype by Ets-2 suggests that Ets-2 functions in mesoderm patterning downstream of FGFs.

To confirm the effects of Ets-2, we analyzed various markers. Animal caps were dissected from Ets-2 mRNA injected embryos and cultured to stage 10.5 or stage 22. In stage 10.5 animal cap explants, Ets-2 induced the expression of the pan-mesodermal marker Xbra in a dose-dependent manner (Fig. 3A). The dorsal mesoderm marker Goosecoid and the ventral mesoderm marker Xwnt8 were not induced by Ets-2 overexpression (data not shown). In stage 22 animal caps, Ets-2 strongly induced the expression of the posterior markers Xcad3 and Xhox3 (Fig. 3B). The expression of Xlhbox6 (HoxB9), which is expressed in lateral mesoderm and spinal chord, was a little induced by the injection of Ets-2 mRNA.
Inhibition of Ets-2 causes posterior mesoderm defects in embryos—To examine whether Ets-2 is necessary for mesoderm patterning, we generated a dominant negative form of Ets-2. The schematic diagram of the Ets-2 based construct used here was shown (Fig. 4A). Ets-2 possesses a highly conserved N-terminal regulatory domain (the Pointed domain) and a C-terminal motif that comprises the DNA binding domain (the ETS domain). EtsΔN En-R was made consisting of the C-terminal region of Ets-2, which contains the DNA binding domain but lacks the activation function, fused to the transcription repressor domain of the Drosophila Engrailed (En-R). We examined the effect of overexpression of EtsΔN En-R (Fig. 4B). EtsΔN En-R mRNA was injected into dorsal marginal zones of 4-cell stage embryos and these embryos were cultured until stage 34. A typical image is shown in Fig. 4B. EtsΔN En-R injected embryos showed the severe posterior defect (47 out of 52). Body axis truncation and dorsal bending of embryos were also observed. These features of the injected embryos were similar to those of the XFD injected embryos (see Fig. 2). Embryos injected ventrally developed almost normally (Fig. 4B).

We then examined whether EtsΔN En-R is able to block FGF- or constitutively active MAPKK (MAPKK SESE)-induced expression of Xbra (Fig. 4C). MAPKK (MEK) is a specific activator of classical MAPK (ERK MAPK) (33-36). While uninjected animal caps with FGF treatment expressed a high level of Xbra, EtsΔN En-R injected animal caps with FGF treatment did not induce Xbra expression. While injection of active MAPKK alone induced Xbra expression strongly, co-injection of EtsΔN En-R resulted in great reduction of Xbra expression. These results suggest that the FGF/MAPK pathway induces Xbra expression via Ets-2.

To test the specificity of the EtsΔN En-R construct, EtsΔN En-R mRNA was injected with wild type Ets-2 mRNA and expression of Xbra was analyzed by RT-PCR with whole embryos (Fig. 4D). EtsΔN En-R injected whole embryos showed greatly reduced expression of Xbra compared to uninjected whole embryos. The wild type Ets-2 co-injected embryos showed recovered expression of Xbra, indicating that wild type Ets-2 rescued the effect brought by EtsΔN En-R.
Next, we tested the effect of expression of antisense morpholino oligos (MO) against Ets-2. We injected the Ets-2 MO into dorsal marginal zones in 4-cell stage embryos. About 80% of embryos injected with the Ets-2 MO in dorsal sides showed a severe posterior defect (n=42). A typical phenotype is shown in Fig. 5. Expression of control MO in dorsal sides of embryos had no effect (Fig. 5). Embryos injected with the Ets-2 MO in ventral sides were almost normal (data not shown). To confirm that the Ets-2 MO-induced phenotype is specifically caused by blocking the Ets-2 function, we co-injected Ets-2 mRNA with Ets-2 MO. In about 60% of embryos co-injected with Ets-2 mRNA were rescued the defects in posterior structures induced by Ets-2 MO (n=28). A typical image is shown (Fig. 5, lower panel). Taken together these results suggest that Ets-2 is necessary for patterning of posterior mesoderm.

Xbra transcription is regulated by Ets-2—Our RT-PCR analysis showed that expression of Ets-2 is able to induce expression of Xbra. We supposed that Ets-2 regulates the Xbra transcription. To test the role of Ets-2 in regulation of Xbra transcription, we performed a reporter assay using the luciferase reporter plasmid containing the 1562-bp fragment of the Xbra promoter region (13, 14). The Xbra reporter construct was co-injected with Ets-2 mRNA and the internal control β-galactosidase into animal poles of 2-cell stage embryos. Animal caps were dissected at stage 8 and assayed for luciferase and β-galactosidase activities at stage 11. The ratio of luciferase to β-galactosidase activity provides a normalized measure of luciferase expression. Ets-2 activated luciferase expression about 4-fold relative to control (Fig. 6A). With FGF treatment, the luciferase activity by Ets-2 was slightly increased. On the contrary, EtsΔN En-R repressed the luciferase activity markedly. Similarly, EtsΔN En-R strongly inhibited the FGF-induced luciferase activity (Fig.6A). These results are in good accordance with our hypothesis that Ets-2 regulates the transcription of Xbra. Then, we investigated whether Ets-2 protein binds to the promoter region of Xbra. A recent study has shown that a restricted upstream region of the Xbra promoter is necessary for its expression (37). We searched for the Ets binding sites within this region of Xbra promoter. We found two Ets binding motifs in the Xbra promoter and
tested their ability to bind to Ets-2 in the gel mobility shift assay. The gel mobility shift assay was performed on −310 to −271 and −259 to −219 fragments of the Xbra promoter using the bacterially expressed glutathione-S-transferase (GST)-Ets-2 fusion protein or GST alone. The −259/−219 fragment bound only very weakly to GST-Ets-2 (data not shown), whereas the −310/−271 region of the Xbra promoter bound to Ets-2 especially (Fig. 6C). Oligonucleotide probes corresponding to the wild type and the mutated −310/−271 region were designed for the gel mobility shift assay (Fig. 6B). The GST-Ets-2 fusion protein bound to the wild-type oligonucleotide probe strongly while GST alone did not bind to the probe (Fig. 6C). The binding was inhibited by preincubation with an excess of unlabeled oligonucleotide (Fig. 6C, competitor). However, the binding persisted with an excess of unlabeled mutant oligonucleotide (Fig. 6C, mutant competitor). Thus, Ets-2 is functional in forming a specific DNA-protein complex with a Xbra promoter region.

**DISCUSSION**

It is known that FGFs can induce mesoderm via the Ras/MAPK pathway in early *Xenopus* development (3-8). Previous studies have shown that members of at least six subfamilies of ETS proteins (ETS, YAN, ELG, PEA3, ERF, TCF) are nuclear targets of the Ras/MAPK pathway. Elk-1 is a member of the TCF subfamily, and it is well known that phosphorylation of Elk-1 by MAPK enhances activities of Elk-1. However, the FGF-induced Xbra expression was not reduced by overexpression of dominant negative Elk-1 in animal cap assay (38). The other member of ETS family, ER81, which belongs to the PEA3 subfamily, was identified in *Xenopus*. Although XER81 has been reported to be a target of FGF signaling, XER81 alone did not induce Xbra expression in animal cap explants (39). Moreover, overexpression of XER81 did not change the expression pattern of Xbra transcript (40). Our results here have demonstrated that Ets-2 induces
Xbra expression and plays an essential role in mesoderm patterning downstream of the FGF/MAPK pathway. Furthermore, overexpression of Ets-2 caused posteriorized embryos. Similar phenotypes were reported to be induced by expressing Xcad3 that is required for posterior development downstream of FGF signaling (41). Both Ets-2 and Xcad3 appear to posteriorize anterior neural tissue. How Ets-2 cooperates with Xcad3 and the Hox gene pathway in posterior development remains to be studied (42).

In Xenopus mesoderm patterning, no transcription factor that would relay the FGF signal at the Xbra promoter has been identified (43). Our results suggest that Ets-2 regulates the transcription of Xbra downstream of the FGF/MAPK pathway. Mullick et al. have recently shown that Ets-2 protein binds to a “weak” Ets-like site of the Cytochrome P-450c27 promoter. They provide new insights on the role of putative weak consensus Ets sites in transcription activation, possibly through synergistic interaction with other gene-specific transcription activators (44). Because the putative weak Ets consensus sites are widely distributed on the Xbra promoter, it is possible that interactions with several Ets-like sites synergistically regulate Xbra transcription. Furthermore, other transcription factors, such as AP-1, potentially participate in transcriptional regulation of Xbra (45). It is likely that other transcription factors in coordination with Ets-2 regulate Xbra transcription. Elucidation of the precise relationship of Ets-2 with other transcription factors will be a necessary step toward understanding the regulation of Xbra.

Our results reported here strongly suggest that Ets-2 plays an essential role in mesoderm patterning. Previously, HpEts has been identified as a sea urchin homologue of Ets-2. Overexpression of HpEts in sea urchin embryos caused primary mesenchyme cells to extinguish cellular adhesion and to migrate (46, 47). In Xenopus, it is likely that Ets-2 is also involved in cellular adhesion and migratory cell guidance. We are currently investigating other roles of Ets-2 in Xenopus developmental processes.
Acknowledgments—We thank Dr. Ken W. Y. Cho for the Xbra-Luc plasmid. We also thank H. Hanafusa and M. Kusakabe in our laboratory for their technical advice and support.
REFERENCES

1. Sive, H. L. (1993) Genes Dev. 1, 1-12
2. Slack, J. M. (1994) Curr. Biol. 4, 116-126
3. Whitman, M., and Melton, D. A. (1992) Nature 357, 252-254
4. MacNicol, A. M., Muslin, A. J., and Williams, L. T. (1993) Cell 73, 571-583
5. Gotoh, Y., Masuyama, N., Suzuki, A., Ueno, N., and Nishida, E. (1995) EMBO J. 14, 2491-2498
6. LaBonne, C., Burke, B., and Whitman, M. (1995) Development 121, 1475-1486
7. Umbhauer, M., Marshall, C. J., Mason, C. S., Old, R. W., and Smith, J. C. (1995) Nature 376, 58-62
8. Kusakabe, M., Masuyama, N., Hanafusa, H., and Nishida, E. (2001) EMBO Rep. 2, 727-735
9. Herrmann, B. G., Labeit, S., Poustka, A., King, T. R., and Lehrach, H. (1990) Nature 343, 617-622
10. Smith, J. C., Price, B. M., Green, J. B., Weigel, D., and Herrmann, B. G. (1991) Cell 67, 79-87
11. Schulte-Merker, S., Ho, R. K., Herrmann, B. G., and Nusslein-Volhard, C. (1992) Development 116, 1021-1032
12. Kispert, A., Ortner, H., Cooke, J., and Herrmann, B. G. (1995) Dev Biol. 168, 406-15
13. Latinkic, B. V., Umbhauer, M., Neal, K. A., Lerchner, W., Smith, J. C., and Cunliffe, V. (1997) Genes Dev. 23, 3265-3276
14. Artinger, M., Blitz, I., Inoue, K., Tran, U., and Cho, K. W. (1997) Mech. Dev. 65, 187-196
15. Halpern, M. E., Ho, R. K., Walker, C., and Kimmel, C. B. (1993) Cell 75, 99-111
16. Schulte-Merker, S., van Eeden, F. J., Halpern, M. E., Kimmel, C. B., and Nusslein-Volhard, C. (1994) Development 120, 1009-1015
17. Conlon, F. L., Sedgwick, S. G., Weston, K. M., and Smith, J. C. (1996) Development 122, 2427-2435
18. Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998) *Trends Biochem. Sci.* **23**, 213-216

19. Yordy, J. S., and Muise-Helmericks, R. C. (2000) *Oncogene* **55**, 6503-6513

20. Sharrocks, A. D. (2001) *Nat. Rev. Mol. Cell. Biol.* **11**, 827-837

21. Yang, B. S., Hauser, C. A., Henkel, G., Colman, M. S., Van Beveren, C., Stacey, K. J., Hume, D. A., Maki, R. A., and Ostrowski, M. C. (1996) *Mol. Cell. Biol.* **16**, 538-547

22. Wasylyk, C., Bradford, A. P., Gutierrez-Hartmann, A. and Wasylyk, B. (1997) *Oncogene* **14**, 899-913

23. McCarthy, S. A., Chen, D., Yang, B. S., Garcia Ramire, J. J., Cherwinski, H., Chen, X. R., Klagsbrun, M., Hauser, C. A., Ostrowski, M. C., and McMahon, M. (1997) *Mol. Cell. Biol.* **17**, 2401-2412

24. Wolff, C. M., Stiegler, P., Baltzinger, M., Meyer, D., Ghysdael, J., Stehelin, D., Befort, N., and Remy, P. (1991) *Cell. Growth. Differ.* **9**, 447-456

25. Meyer, D., Durliat, M., Senan, F., Wolff, M., Andre, M., Hourdry, J., and Remy, P. (1997) *Int. J. Dev. Biol.* **41**, 607-620

26. Nieuwkoop, P. D., and Faber, J. (1967) *Normal Table of Xenopus laevis (Daudin)*, North Holland Publishing Co., Amsterdam

27. Masuyama, N., Hanafusa, H., Kusakabe, M., Shibuya, H., and Nishida, E. (1999) *J. Biol. Chem.* **274**, 12163-12170

28. Hanafusa, H., Masuyama, N., Kusakabe, M., Shibuya, H., and Nishida, E. (2000) *EMBO Rep.* **1**, 32-39

29. Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S., and Nishida, E. (2002) *EMBO Rep.* **3**, 69-75

30. Basuyaux, J. P., Ferreira, E., Stehelin, D., and Buttice, G. (1997) *J. Biol. Chem.* **272**, 26188-26195

31. Ma, X., Neurath, M., Gri, G., and Trinchieri, G. (1997) *J. Biol. Chem.* **272**, 10389-10395

32. Amaya, E., Musci, T. J., and Kirschner, M. W. (1991) *Cell* **66**, 257-270
33. Sturgill, T. W., and Wu, J. (1991) *Biochim. Biophys. Acta* **1092**, 350-357
34. Nishida, E., and Gotoh, Y. (1993) *Trends Biochem. Sci.* **18**, 128-131
35. Kyriakis, J. M., and Avruch, J. (2001) *Physiol. Rev.* **81**, 807-869
36. Pouyssegur, J., Volmat, V., and Lenormand, P. (2002) *Biochem. Pharmacol.* **64**, 755-763
37. Lerchner, W., Latinkic, B. V., Remacle, J. E., Huylebroeck, D., and Smith, J. C. (2000) *Development* **127**, 2729-2739
38. Panitz, F., Krain, B., Hollemann, T., Nordheim, A., and Pieler, T. (1998) *EMBO J.* **17**, 4414-4425
39. Chen, Y., Hollemann, T., Grunz, H., Pieler, T., (1999) *Mech. Dev.* **80**, 67-76
40. Münchberg, S. R., Steinbeisser, H., (1999) *Mech. Dev.* **80**, 53-65
41. Isaacs, H. V., Pownall, M. E., and Slack, J. M. (1998) *EMBO J.* **17**, 3413-3427
42. Pownall, M. E., Tucker, A. S., Slack, J. M., and Isaacs, H. V. (1996) *Development* **122**, 3881-3892
43. Tada, M., O'Reilly, M. A., and Smith, J. C. (1997) *Development* **124**, 2225-2234
44. Mullick, J., Anandatheerhavarada, H. K., Amuthan, G., Bhagwat, S. V., Biswas, G., Camasamudram, V., Bhat, N. K., Reddy, S. E. P., Rao, V., and Avadhani, N. G. (2001) *J. Biol. Chem.* **276**, 18007-18017
45. Kim, J., Lin, J. J., Xu, R. H., and Kung, H. F. (1998) *J. Biol. Chem.* **273**, 1542-1550
46. Kurokawa, D., Kitajima, T., Mitsunaga-Nakatsubo, K., Amemiya, S., Shimada, H., and Akasaka, K. (1999) *Mech. Dev.* **80**, 41-52
47. Kurokawa, D., Kitajima, T., Mitsunaga-Nakatsubo, K., Amemiya, S., Shimada, H., and Akasaka, K. (2000) *Zygote* **8**, S33-S34
*This work was supported by grants from the Ministry of Education, Science and Culture of Japan (to E.N.).

¹The abbreviations used are: FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; Xbra, Xenopus brachyury; En-R, repressor domain of Drosophila Engrailed; XFD, Xenopus dominant negative form of FGF receptor; DMZ, dorsal marginal zone; VMZ, ventral marginal zone; RT-PCR, reverse transcription-coupled polymerase chain reaction; BSA, bovine serum albumin; GST, glutathione S-transferase.
FIGURE LEGENDS

FIG. 1 Overexpression of Ets-2 induces posteriorized embryos. A, tadpole stage (stage 35) embryos and sibling embryos that have been injected with Ets-2 mRNA into dorsal marginal zones (DMZ) or ventral marginal zones (VMZ) at 4-cell stage at indicated doses. B, expression of marker genes in whole embryos that were injected with 1 ng of Ets-2 mRNA into DMZ. Injected embryos were cultured until sibling embryos reached stage 26 or stage 37. Expression of indicated marker genes was analyzed by RT-PCR. EF1-α served as a loading control. RNA from whole embryo (indicated as embryo) provides a positive control. No signal was observed in the absence of reverse transcription (-RT).

FIG. 2 Ets-2 rescues the defects by XFD. Embryos were injected into DMZ at 4-cell stage and cultured until stage 35. 0.5 ng of XFD mRNA was injected with or without 1.5 ng of Ets-2 mRNA.

FIG. 3 Ets-2 induces expressions of mesodermal markers in animal cap explants. A, Ets-2 mRNA was injected into animal poles of 2-cell stage embryos at the indicated doses. Animal caps were dissected at blastula stage and cultured until sibling embryos reached stage 11. Expression of indicated marker genes was analyzed by RT-PCR. B, Ets-2 mRNA was injected as in A. Animal caps were dissected at blastula stage and cultured until sibling embryos reached stage 26. Indicated markers were analyzed by RT-PCR.

FIG. 4 Inhibition of Ets-2 causes defects in mesodermal patterning. A, A schematic diagram of Ets-2 construct fused with an Engrailed repressor domain (En-R). B, tadpole stage (stage 35) embryos that have been injected with 1 ng mRNA encoding EtsΔN En-R into dorsal or ventral marginal zones at 4-cell stage. C, An inhibitory effect of EtsΔN En-R on FGF- or MAPK SESE-induced Xbra expression in isolated animal
caps. Animal caps were dissected at blastula stage from embryos that had been injected with Ets\(\Delta N\) En-R mRNA (1 ng) together with MAPKK SESE mRNA (0.1 ng) at 2-cell stage and were cultured until sibling embryos reached stage 11. On FGF treatment, animal caps were cultured in the medium including 50 ng/ml bFGF. Expression of Xbra was analyzed by RT-PCR. D, wild type Ets-2 rescued the reduction of Xbra expression caused by Ets\(\Delta N\) En-R. Ets\(\Delta N\) En-R mRNA (50 pg) was injected together with wild type Ets-2 mRNA (1 ng) into marginal zones of 2-cell embryo. Injected embryos were cultured until sibling embryos reached stage 11. Expression of Xbra was analyzed by RT-PCR with injected whole embryos.

FIG. 5 **Ets-2 is necessary for patterning of posterior mesoderm.** Embryos were injected with Ets-2 MO (50 ng) or control MO (50 ng) into dorsal marginal zones at 2-cell stage and cultured until stage 33. For rescue of Ets-2 depletion, Ets-2 mRNA (1.5 ng) was co-injected with Ets-2 MO.

FIG. 6 **Ets-2 is essential for Xbra transcription.** A, Ets-2 regulated expression of reporter gene construct containing 1.5 kb fragment of the Xbra promoter region. The Xbra promoter construct was co-injected with Ets-2 mRNA or Ets\(\Delta N\) En-R mRNA into animal poles of 2-cell stage embryos. Animal caps were dissected at stage 8 and cultured with or without FGF (50 ng/ml). Cultured animal caps were assayed for luciferase activity at stage 11. B, the sequence of the wild type and the mutant Ets binding sites. C, Ets-2 bound to the –310/–271 region of the Xbra promoter. The gel mobility shift assay was performed using radiolabeled double-strand oligonucleotide probe of Ets-binding site. After incubation of the radiolabeled probe with protein extracts, DNA-protein complex were analyzed by autoradiography following electrophoresis of binding reactions on 4% polyacrylamide gels. Upper arrow indicates the position of DNA-protein complex. The recombinant GST or GST-Ets-2 fusion protein was incubated with radiolabeled oligonucleotide probe containing Ets-binding site. For competition assay, binding reactions were preincubated with a 200-fold molar
excess of unlabeled oligonucleotide probe as competitor, then binding reactions with GST·Ets·2 and labeled probe were incubated as described above. The same competition assay was performed with unlabeled mutant oligonucleotide.
FIG. 2

uninjected  XFD 0.5 ng  XFD 0.5 ng + Ets-2 1.5 ng
FIG. 6

A

Fold activation

control  FGF  Ets-2  FGF+Ets-2  Ets+EN-RE  FGF+Ets+EN-R

B

-310
CAGGTGTCAGTTCTTACTGGTGAAGTTTATTGAAGGCA  wild type

-271

CC

mutant

C

probe  GST  Ets-2  competitor

mutant competitor  free probe
