CLONING AND CHARACTERIZATION OF A cDNA ENCODING er1, A NOVEL DEVELOPMENTALLY REGULATED FGF RESPONSE GENE

CENTRE FOR NEWFOUNDLAND STUDIES

TOTAL OF 10 PAGES ONLY
MAY BE XEROXED

(Without Author's Permission)

YU LI

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-47427-5

Canada

Cloning and Characterization of a cDNA encoding *er1*, a novel developmentally regulated FGF response gene

by
Yu Li B. Sc.

A thesis submitted to the
School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Master of Science

Terry Fox Laboratories, Faculty of Medicine
Memorial University of Newfoundland

© 1998

St. John's Newfoundland

to my parents

Abstract

In order to investigate the molecular mechanism of mesoderm induction by FGF in *Xenopus* Laevis, I have utilized the polymerase chain reaction (PCR)-based differential display methodology (Liang and Pardee, 1992) to identity a novel transcript whose expression level increased in *Xenopus* embryo explants during mesoderm induction by fibroblast growth factor (FGF). The PCR product was used to clone a 2.3-kb cDNA representing this transcript, which I have named *er1*. The *er1* cDNA contains a single open reading frame (ORF) predicted to encode a protein of 493 amino acid residues. Northern blot analysis revealed a single 2.8-kb mRNA that was observed predominantly during the initial cleavage and blastula stages of *Xenopus* development, with little or no detected mRNA during subsequent development. *In vitro* translation of *er1* using a rabbit reticulocyte lysate system produced a protein with an apparent molecular mass of 74kDa. A database homology search revealed that the predicted *er1* amino acid sequence contains three regions of similarity to the rat metastasis-associated gene *mta1*. FGF is known to play an important role in both mesoderm induction and gastrulation movement during amphibian development, elucidation of the function of this *mta1*-related FGF response gene may lead to a better understanding of the early development of *Xenopus* Laevis.

Acknowledgements

I would like to express my appreciation to my supervisor, Dr. Laura Gillespie, much of my work done in this laboratory is attributed to her logical design of our project. Her patience and encouragement helped me to overcome many difficulties I encountered during my program. I would also like to extend my gratitude to Gang Chen, who introduced me to this laboratory and helped me in many ways. Special thanks are given to Dr. Gary Paterno, Dr. Ken Kao and Dr. Jon Church for their guidance and enlightening discussion, to Paula Ryan for her research assistance. Finally, I sincerely thank my colleagues at Terry Fox laboratory.

Contents

List of Tables

List of Figures

List of Abbreviations and Symbols Used

1. **aFGF** acidic FGF
2. **AIGF** Androgen-induced growth factor
3. **bFGF** basic FGF
4. **BMP-4** bone morphogenesis protein 4
5. **bp** base pair
6. **cDNA** complementary DNA
7. **CHX** cycloheximide
8. **DAG** diaylglycerol
9. **DEPC** diethylpyrocarbonate
10. **DTT** dithiothreitol
11. **EDTA** ethylene diaminetetraacetic acid
12. **FGF** fibroblast growth factor
13. **FGFR** FGF receptor
14. **GAF** Glia-activating factor
15. **HSPG** heparin sulfate proteoglycan

16. **IP_3** Inositol 1, 4, 5-triphosphate

17. **k-FGF** Kaposi's Sarcoma-FGF

18. **kb** kilobase

19. **KGF** keratinocyte growth factor

20. **kD** kilodalton

21. **MIFs** mesoderm inducing factors

22. **MMLV** Moloney Murine Leukemia virus

23. **mRNA** messanger RNA

24. **PCR** polymerase chain reaction

25. **PKC** protein kinase C

26. **PLC-γ** phospholipase C-γ

27. **RA** retinoic acid

28. **RT** reverse transcription

29. **SH_2** Src-homology domain 2

30. **SH_3** Src-homology domain 3

31. **Ser** Serine

32. **ssDNA** Samon sperm DNA

33. **SDS** sodium dodecyl sulfate

34. **TGF-β** transforming growth factor β

35. **Thr** Threonine

36. **TPA** phorbol ester

37. **UV** ultra-violet

38. **Wnt** wingless

39. **XbFGF** *Xenopus* basic FGF

40. **XeFGF** *Xenopus* embryo FGF

41. **XWnt** *Xenopus*-wingless

Chapter 1

Introduction

1.1 The *Xenopus laevis* System

Xenopus embryos are popular material for developmental biological studies because of their technical advantages:

1. *Xenopus* eggs are large, making micro-operative procedures such as microinjection and microdissection relatively easy.

2. *Xenopus* embryos may be obtained in large numbers which, together with their large size, enable enough material to be extracted for biochemical analysis.

3. Their fertilization can be carried out *in vitro*; thus, large numbers of synchronously developing embryos can be obtained, which are available for experimentation at all development stages, from egg to tadpole.

4. Their development is rapid—the body plan is established and tissue-specific gene activation occurs within twenty-four hours.

5. Explants cut from early embryos can continue to develop if incubated in simple salt solutions. Thus, it is possible to test certain molecules for their effects on differentiation by adding them into the culture medium.

6. The *Xenopus* oocyte and embryo have been shown to be a good *in vivo* translation system which can translate injected mRNAs faithfully. Thus, by injecting mRNAs, we can investigate the function of some genes whose products may play an important role in regulating developmental events.

7. *Xenopus* embryos do not grow during development, which means that inert cell lineage labels introduced by the experimenter do not become diluted during development.

1.2 Early Development in *Xenopus laevis*

Xenopus oocytes are formed from a dividing stem cell population called oogonia (Wylie *et al.*, 1985). After oogenesis and maturation, they become fertilizable eggs. These eggs are highly polarized with a strongly pigmented animal half and nearly unpigmented vegetal half. After fertilization and before first cleavage, *Xenopus* embryos undergo cortical rotation, which initiates the dorsal-ventral axis. During cleavage stages, the embryos undergo a series of rapid and synchronous cleavage division, which leads the embryos to develop from one large zygotic cell to a hollow ball . After the twelfth division, the cell division rate slows down, and zygotic transcription

starts. This stage is referred to as the mid-blastula transition or MBT (Newport and Kirschner, 1982). The next phase of development is an extensive cellular movement and cellular interaction process called gastrulation. During gastrulation, the embryo is reorganized from a hollow ball to a sphere with three distinct germ layers – ectoderm, mesoderm, and endoderm – each of which forms different tissues and organs at later stages.

1.3 Mesoderm Induction and Mesoderm Inducing Factors (MIF)

Before the 64-cell stage, the *Xenopus* embryo can be considered to consist of only two cell types—prospective ectoderm in the animal hemisphere and prospective endoderm in the vegetal hemisphere (Jones and Woodland, 1986). At the 128-cell stage, the blastocoel begins to appear and separates animal pole cells (also called animal cap) from vegetal hemisphere cells. When the animal cap is cut from a mid-blastula stage embryo and is cultured in isolation *in vitro*, it will develop only into epidermis. However, when cultured together with vegetal tissue, it will develop into a variety of mesodermal tissues, as well as epidermis (Nieuwkoop, 1969) (Figure 1.1 (B)). One interpretation of this result is that mesoderm formation in *Xenopus* results from an inductive interaction, which is referred to as mesoderm induction—vegetal cells emit signals and induce overlying animal cells to develop into mesoderm. These signals cannot penetrate the blastocoel and reach the "animal cap" region. Thus, although both "animal cap" cells and equatorial region cells are capable of responding to the

signals, only the latter can receive the signals *in vivo*, and as a result, they will give rise to mesoderm; the "animal cap" cells do not receive the signals *in vivo*, and therefore give rise to epidermis (Smith, 1989 & 1993; Dawid *et al.*, 1992a) (Figure 1.1 (A)).

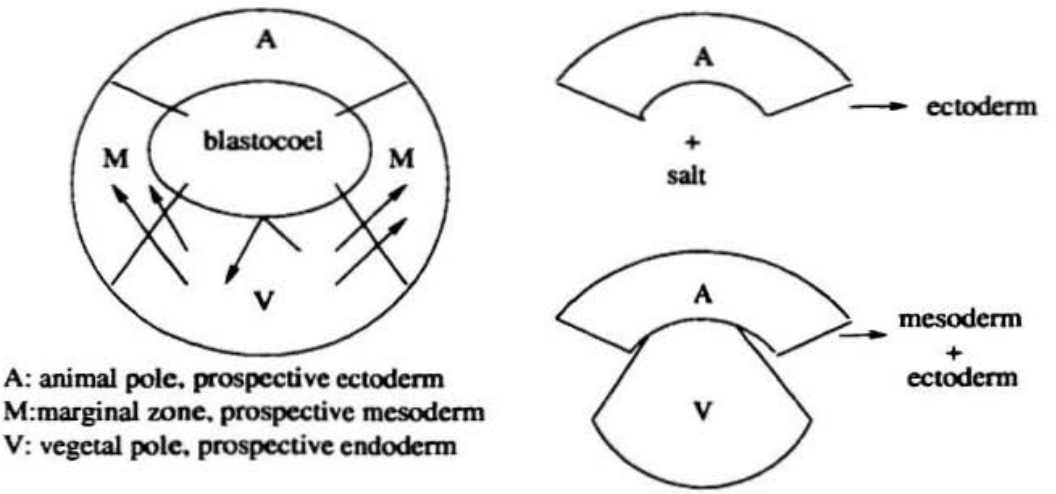
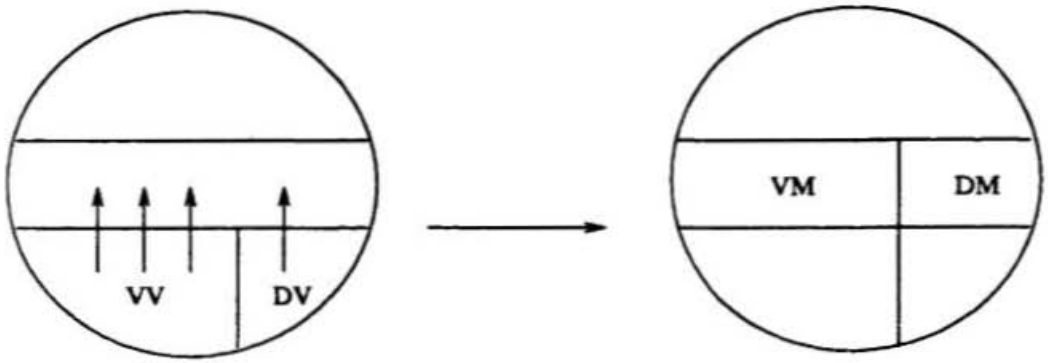


Figure 1.1: **Mesoderm Induction *in vivo* (A) and *in vitro* (B).** (A): Signals released from the vegetal pole induce the overlying marginal zone cells to develop into mesoderm; the signals can not reach the animal cap cells, therefore, the animal pole cells will develop into ectoderm; the vegetal pole cells will develop into endoderm. The arrows represent the inducing signals. (B): When the animal cap is cut from the embryo and cultured in a salt solution *in vitro*, it will develop into ectoderm only, however, if it is cultured together with the vegetal pole, it will develop into mesoderm as well as ectoderm.



VV: ventral vegetal
DV: dorsal vegetal

VM: ventral mesoderm
DM: dorsal mesoderm

Figure 1.2: **Two types of mesoderm are induced by vegetal tissues** Dorsal vegetal cells induce a small equatorial region to form dorsal mesoderm, and the ventral vegetal cells induce a large equatorial region to form ventral mesoderm. The arrows represent the signals released from the vegetal cells.

Two types of mesoderm are induced in the equatorial region during blastula stage – dorsal mesoderm and ventral mesoderm (Dale and Slack, 1987a) (Figure 1.2). The "dorsal vegetal center", also called "Nieuwkoop center", in the dorsal vegetal quadrant, induces a small equatorial region ($60-90°$) to form "dorsal mesoderm" characterized by notochord and muscle. This "dorsal mesoderm" is also called the "organizer". The rest of the vegetal cells (also referred to as "ventral vegetal cells") induce a large equatorial region ($270-300°$) to form "ventral mesoderm" characterized by mesenchyme, mesothelium and blood.

Those signals which are emitted from the vegetal cells and have the mesoderm induction capability are termed mesoderm induction factors (MIFs). During the past

ten years, research has been concentrated on identifying the nature of the signals emitted from vegetal tissues. One important discovery is that some growth factors can mimic the vegetal tissue's function to induce mesoderm. These growth factors belong to either FGF family (Slack *et al.*, 1987; Paterno *et al.*, 1989), or TGF-β superfamily (Smith, 1987; Van den Eijnden-Van Raai j, A.J.M. *et al.*, 1990).

Within the TGF-β family, activin is the most potent mesoderm inducing agent. Activin induces both dorsal and ventral mesoderm, the former at a higher concentration, and the latter at a lower concentration. (Cooke, 1989; Green *et al.*, 1992). FGFs induce different types of mesoderm in a concentration dependent manner too, but the resulting mesoderm types are different from those induced by activin. At low concentration, bFGF induces "ventral mesoderm"; as concentration increases, it induces more and more muscle, but rarely notochord or organizer (Paterno *et al.*, 1989).

Ample evidence suggests that MIFs can induce mesoderm both individually and synergistically. bFGF's capacity to induce muscle actin expression can be increased by TGF-β_1 (Kimelman and Kirchner, 1987). On the other hand, low concentrations of bFGF can lower the concentration of activin required for muscle induction (Green *et al.*, 1992). BMP-4 is a member of TGF-β family. Unlike activin, BMP-4 is not capable of inducing dorsal mesoderm and it is a weak ventral mesoderm inducer (Smith, 1993). Animal caps treated with a combination of BMP-4 and activin result in the formation of ventral mesoderm (Dale *et al*, 1992; Jones *et al*, 1992). Overexpression of BMP-4 in the embryo enhances the formation of ventral mesoderm, and overexpression of a dominant-negative BMP-4 receptor that inhibits BMP-4 ac-

tivity in ventral blastomeres of *Xenopus* embryos leads to the formation of dorsal mesoderm on the prospective ventral side (Suzuki *et al.*, 1994).

1.4 MIFs and Gastrulation

Besides mesoderm induction, some MIFs have other functions in normal development. If animal caps are exposed to some MIFs, they will change their shape, a process involving elongation and constriction. In addition, dispersed animal cap cells treated with some MIFs are capable of spreading and migrating on fibronectin, whereas uninduced cells are not (Howard and Smith, 1993). These movements resemble the events of gastrulation (Keller and Danilchik, 1988). Hence, some MIFs may play a role in gastrulation.

1.5 MIFs and Antero-posterior Axis Specification

Several studies have implicated FGFs and activins in antero-posterior specification. If animal caps are treated with FGF or activin and subsequently implanted into early blastulae, activin treated caps tend to induce heads, whereas FGF treated caps tend to induce tails (Ruiz i Altaba and Melton, 1989a). Xhox-3 is an homeobox gene expressed in the posterior mesoderm. It can be induced by both FGF and activin, but induced by FGF to a higher degree (Ruiz i Albata and Melton , 1989b). XIHbox1, a homeobox gene normally expressed in the anterior trunk region, is preferentially activated by activin (Cho and De Robertis, 1990). On the other hand,

XIHbox6, a homeobox gene normally expressed in the mid and hind-trunk region, is preferentially activated by FGF (Cho and De Robertis, 1990). These data suggested that FGF is the ventral-posteror mesoderm inducer and activin is the dorsal-anterior inducer. Thus, these two types of MIFs have distinct functions in specification of mesoderm.

1.6 The Fibroblast Growth Factor Family and their Function in *Xenopus* Embryos

Up until now, we have sufficient evidence to prove that members of FGF family and TGF-β superfamily can induce mesoderm in explants. However, whether they are involved in mesoderm induction during normal development is not yet clear. Though mesoderm tissues are derived from the equatorial region, it is very difficult to use cells from this region to study mesoderm induction, since they may already been induced before we cut them from the embryos. Thus, we use animal caps, which do not form mesoderm during normal development, to study mesoderm induction, keeping in mind that cells in the animal caps may not be exactly the same as the cells at the equatorial region. Furthermore, though these MIFs can mimic vegetal tissue's function to induce animal caps to form mesoderm, they may not be the signals produced by the vegetal tissue. Instead, MIFs may be the downstream genes to those vegetal signals (Isaacs *et al.*, 1994). One approach to address these questions is to study the molecular mechanisms of mesoderm induction. My specific interest is to study the molecular mechanism of mesoderm induction by FGF.

1.6.1 The FGF Family Members

Fibroblast growth factor (FGF) was originally identified from extracts of pituitary and brain by its ability to stimulate the growth of BALB/C 3T3 fibroblasts (Armelin, 1973; Gospodarowicz, 1974a). To date, nine FGF family members have been identified. The proteins are related by their ability to bind to heparin with high affinity, and share 33-65% homology at the amino acid level.

bFGF (*FGF-1*) was first identified by its ability to cause the proliferation (Gospodarowicz, 1974a) and phenotypic transformation of 3T3 cells (Gospodarowicz and Moran, 1974b). *aFGF* (*FGF-2*) was first identified by its ability to cause proliferation and delayed differentiation of myoblasts as well as to stimulate endothelial cell proliferation (Gospodarowicz *et al.*, 1975; Lemmon *et al.*, 1982; Maciag et al., 1979). The two share 55% homology and interact with the same receptor (Gospodarowicz *et al.*, 1986) . *Int-2* (*FGF-3*) is a proto-oncogene (Dickson and Peters, 1987) which was originally identified by virtue of its frequent proximity to integrated proviral DNA in carcinomas induced by mouse mammary tumor virus (MMTV) (Moore *et al.*, 1986). *kFGF* (*hst/ks3*; *FGF-4*) is a human oncogene that was isolated from Kaposi's Sarcoma by its ability to transform NIH 3T3 cells (Delli Bovi and Basilico, 1987a). Its protein is capable of stimulating cell proliferation (Delli Bovi *et al.*, 1987b). *FGF-5* is a human oncogene with transforming potential. It was detected by the transfer of DNAs from human tumour cell lines into NIH 3T3 cells (Zhan *et al.*, 1987). *FGF-6* was isolated by screening a mouse Cosmid library under low stringency conditions with a human *kFGF* probe (Marics *et al.*, 1989). *FGF-6* appears to be more closely related to *kFGF* than to any other FGF family members.

Keratinocyte growth factor (*KGF* or *FGF-7*) is a human mitogen that is specific for epithelial cells. It is important in the normal mesenchymal stimulation of epithelial cell growth (Finch *et al.*, 1989). Androgen – induced growth factor (*AIGF*, *FGF-8*) was isolated from an androgen dependent mouse mammary carcinoma cell line (SC-3). It is believed that androgen-dependent growth of SC-3 cells are mediated by *AIGF* through an autocrine mechanism (Tanaka *et al.*, 1992). Glia-activating factor (*GAF*, *FGF-9*) was purified from the culture supernatant of a human glioma cell line. It is one of the three members of the FGF family which lack typical signal sequence at the N-terminus. However, unlike *bFGF* and *aFGF*, which are not secreted from the cell in a conventional manner, *GAF* is secreted from the cell through a unique secretion mechanism (Miyamoto *et al.*, 1993). *XeFGF* is a newly identified FGF in *Xenopus* which shares 70% homology to mammalian *kFGF* and *FGF-6* (Isaacs *et al.*, 1992). In the FGF family, *aFGF*, *bFGF*, *int-2*, *kFGF* and *XeFGF* are capable of inducing animal caps to form mesoderm *in vitro* (Slack *et al.*, 1987; Paterno *et al.*, 1989; Isaacs *et al.*, 1992).

1.6.2 Temporal and Spatial Expression of FGFs in *Xenopus laevis*

Endogenous MIFs have to be expressed maternally since ample evidence suggests that mesoderm induction occurs before the zygotic transcription begins. In addition, MIFs should be preferentially located in the vegetal region. It has been shown that bFGF protein is present in both unfertilized eggs and blastula- stage embryos at a concentration about 7ng/ml, which is sufficient to induce mesoderm

(Slack and Isaacs, 1989). bFGF mRNA is also found to be present in both oocyte and later stage embryos (Kimelman *et al.*, 1988). In addition, results from immunocytochemical staining shows that bFGF is predominantly located in the marginal and vegetal regions during cleavage and blastula stages (Shiurba *et al.*, 1991). This evidence combined with bFGF's activity in mesoderm induction *in vitro* implicates bFGF as an endogenous mesoderm inducer. However, antibodies against *Xenopus* bFGF could not inhibit mesoderm induction in transfilter experiments (Slack, 1991). (Transfilter experiments are similar to "combination" experiments except that the animal cap is separated from vegetal tissue with a 0.1 μm nucleopore filter. MIFs can pass through the filter and induce mesoderm *in vitro*.) Furthermore, overexpression of bFGF in *Xenopus* embryos shows little mesoderm inducing activity (Thompson and Slack, 1992). These results together with the fact that bFGF lacks a signal sequence makes it unclear whether bFGF acts as an endogenous mesoderm inducer or not.

Like bFGF, XeFGF mRNA is also expressed both maternally and zygotically, but it contains a signal sequence. However, its maternal expression does not show a preferential vegetal location. On the other hand, its zygotic expression is located near the dorsal lip in the early gastrula and then predominantly located in the posterior region during the formation of the antero-posterior axis (Isaacs *et al.*, 1992).

1.6.3 Inhibitors of FGFs Inhibit Mesoderm Formation *in vivo*

Overexpression of inhibitors of endogenous MIFs should be able to inhibit mesoderm formation in *Xenopus* embryos effectively. Therefore, to examine whether FGFs' inhibitors such as heparin and suramin inhibit mesoderm formation may indirectly elucidate FGFs' role in mesoderm induction *in vivo*.

Addition of heparin to bFGF inhibits bFGF's mesoderm inducing activity *in vitro* (Slack *et al.*, 1987) and the presence of either heparin or suramin inhibits mesoderm formation in transfilter experiments (Slack, 1989). In addition, microinjection of either suramin or heparin into the blastocoel cavity of *Xenopus* embryos affects gastrulation (Gerhart *et al.*, 1989 , 1991; Mitrani, 1989) and mesoderm formation (Cardellini *et al.*, 1994).

1.6.4 FGF Mediated Signal Transduction and Mesoderm Induction

FGF mediated signal transduction is triggered by the binding of FGFs to the extracellular domain of their specific transmembrane FGF receptors (FGFR). This activates the FGFRs through dimerization followed by autophosphorylation of the tyrosine residues of the FGFRs (see review in Heldin, 1995). The phosphorylated FGFR forms a signalling complex by binding a number of intracellular substrates which results in activation of several signalling pathways. Ultimately, the FGF signal will be transmitted to the nucleus and lead to activation of response genes (see review

in Pawson, 1995).

Therefore, FGF mediated signal transduction can be controlled by modifying 1) the expression or function of FGFRs in the cell surface (cell membrane), 2) intracellular signalling (cell cytoplasm), 3) the transcription of the response genes (cell nucleus).

FGFR and Mesoderm Induction

There exists two distinct FGFR families—high-affinity FGF receptors with tyrosine kinase activity, and low-affinity FGF receptors which have been identified as heparin sulfate proteoglycan (HSPG). It is believed that the binding of FGFs to their high-affinity tyrosine kinase receptors requires the function of low-affinity FGF receptors (see review in Robinson, 1991).

The high-affinity FGF receptor family contains four structurally related FGF receptors—FGFR-1, FGFR-2, FGFR-3 and FGFR-4. They all have an extracellular region containing three immunoglobin (Ig)-like domains, one transmembrane region, one intracellular region containing highly conserved tyrosine kinase domains that are split by a 14 amino acid insertion, and a C-terminal tail.

Isoforms of FGFR-1, FGFR-2 and FGFR-3 are generated by the alternative splicing of their mRNA transcripts. The significance of this alternative splicing is not completely known. However, there is evidence that alternative splicing is important in regulating both the receptor-ligand-binding affinity and FGFR intracellular signalling (see review in Friesel and Maciag, 1995).

FGF receptors are present in *Xenopus* embryos during mesoderm induction stages. They are present in the animal cap region, marginal zone, and vegetal region. Their density is the highest in the marginal zone, which is destined to become mesoderm (Gillespie *et al.*, 1989). *Xenopus* FGFR-1 mRNA is expressed throughout early development and its expression can be regulated by FGF or activin (Musci *et al.*, 1990; Friesel and Dawid, 1991). An isoform of FGFR-1, which results from alternative splicing and lacks the protein kinase C phosphorylation site, has also been identified (Gillespie *et al.*, 1995).

When animal caps from blastula stage embryos are treated with FGF *in vitro*, FGFRs on the animal cap cell surfaces are phosphorylated at their tyrosine residues. Thus, mesoderm induction by FGF is initiated by the activation of FGFRs through autophosphorylation at their tyrosine residues (Gillespie *et al* ., 1992).

The presence of FGFRs in *Xenopus* embryos during mesoderm induction and the activation of FGFR during FGF-induced mesoderm differentiation suggest that FGFRs are involved in mesoderm differentiation *in vivo*. In addition, direct evidence for FGFR involvement in mesoderm induction comes from the expression of a dominant negative mutant of the FGFR in *Xenopus* embryos. This dominant negative mutant of FGFR, which contains both extracellular and transmembrane domains but lacks the intracellular tyrosine kinase domain, can effectively inhibit endogenous FGFR function by forming non-functional heterodimers with endogenous FGFRs (Amaya *et al.*, 1991). Embryos expressing this dominant negative mutant of the FGFR show specific defects in gastrulation and in posterior mesoderm development;

on the other hand, animal caps from these embryos cannot be induced to form mesoderm by FGF (Amaya *et al.*, 1991). In addition, microinjection of dominant negative mutant FGFR mRNA into embryos not only inhibits muscle differentiation, which can be induced by FGF *in vitro*, but also notochord differentiation, which is not normally induced by FGF *in vitro*. This suggests that FGF signalling are involved in both ventral and dorsal mesoderm induction (Amaya *et al.*, 1993).

FGF Intracellular Signalling and Mesoderm Induction

Autophosphorylation of FGFRs on their tyrosine residues results in the binding of Src-Homology domain 2 (SH_2)-containing proteins to FGFRs and the formation of "FGFR signalling complexes". Some of these SH_2-containing proteins are subsequently phosphorylated on tyrosine residues which serves to modify their activity. Alternatively, binding of SH_2-containing proteins to phosphorylated FGFRs serves to recruit these proteins to the plasma membrane. One signalling pathway involves recruitment of Grb-2 and SOS complex to the FGFR complex (Figure 1.3). Grb-2 is an adaptor molecule and it is complexed with the guanine nucleotide exchange factor SOS in the cytoplasm via its SH_3 domain. SOS subsequently activates Ras by catalyzing the exchange of bound GDP for GTP. Once Ras is activated, it in turn activates the Ser-Thr kinase Raf-1, which activates MAPK kinase (MEK). MEK subsequently activates MAP kinase (MAPK), which activates transcription factors in the nucleus and these factors then directly activate the expression of genes in the nucleus.

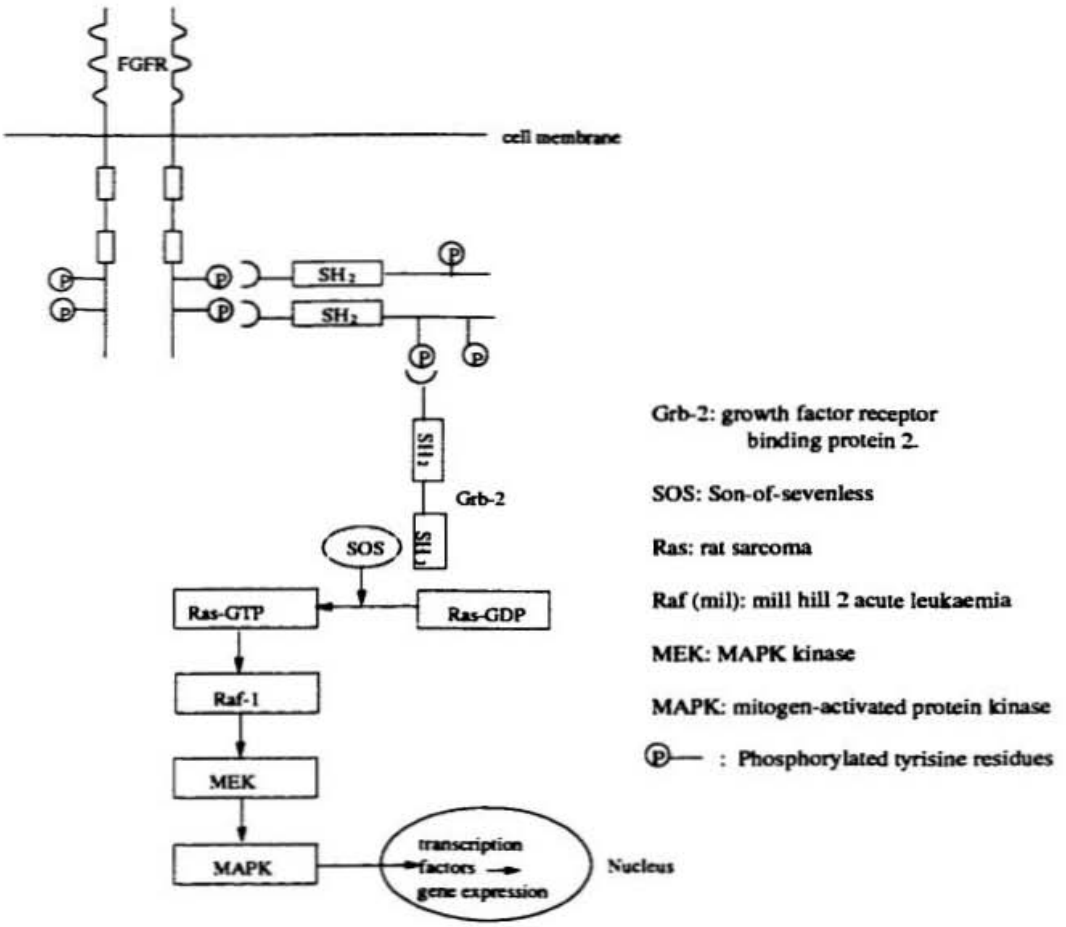


Figure 1.3: **General intracellular signalling cascade** FGFRs are activated through dimerization and autophosphorylation on tyrosine residues. These phosphorylated tyrosine residues subsequently bind to the SH_2 domains of various proteins to form FGFR complexes. The SH_2 proteins become phosphorylated on their tyrosine residues and recruit Grb-2 and SOS to the complexes. SOS subsequently activates RAS by catalyzing the exchange of bound GDP for GTP. activated RAS activates RAf-1, which in turn activates MEK. Activated MEK activates MAPK, which activates transcription factors in the nucleus and these factors activate the gene expression. arrows in the figure indicate the direction of activation.

It has been found that phospholipase C-γ (PLC-γ) (Burgess *et al.*, 1990) and a non-receptor tyrosine kinase oncogene Src (Zhan *et al.*, 1994) directly associate with FGFR-1 and are phosphorylated upon association with FGFR-1. Phosphorylation of PLC-γ leads to the phosphatidyl inositol bisphosphate hydrolysis and generation of inositol 1, 4, 5-trisphosphate (IP_3) and diaylycerol (DAG) which in turn activates protein kinase C (PKC). An adaptor protein, Shc, is also phosphorylated in response to activation of FGFR-1. It may directly associate with FGFR-1 (Klint *et al.*, 1995) or indirectly associate with FGFR-1 through Src (Vainikka *et al.*, 1994; Wang *et al.*, 1994). Grb-2 does not bind to FGFR-1 directly, but does indirectly through Shc, or through a 89-kDa component (Klint *et al.*, 1995).

It has been shown that during mesoderm induction in *Xenopus* embryos, PLC-γ is associated with FGFR-1. Other intracellular SH_2-containing substrates such as nck, GAP, Grb-2 are also involved in the FGFR signalling complexes (Ryan and Gillespie, 1994).

Experimental results from several groups demonstrated that some of the intracellular substrates, which are coupled to the FGFR signalling complex, play a crucial role in mesoderm induction *in vivo*. Microinjection of a dominant inhibitory mutant Ras into *Xenopus* embryos results in the blockage of the capacity of animal caps to respond to FGF, activin, and endogenous inducing signals from vegetal tissues (Whiteman and Melton, 1992). Microinjection of a dominant negative Raf-1 mutant into *Xenopus* embryos blocks animal caps' ability to respond to FGF, but does not block the response to activin. Furthermore, whole embryos injected with this

dominant negative Raf-1 mutant exhibits severe posterior mesodermal deficiencies (MacNicol *et al.*, 1993). It has also been found that overexpression of MAPK kinase (MEK) or MAP kinase (MAPK) induces ventral mesoderm, and overexpression of MAP kinase phosphatase (MKP-1), which is capable of inactivating endogenous MAP kinase, blocks mesoderm induction by either FGF or activin, and causes posterior mesodermal deficiencies in intact embryos (Umbhauer *et al.*, 1995; LaBonne *et al.*, 1995).

Although PLC-γ associates with FGFR-1, and is tyrosine phosphorylated by FGFR-1 in response to FGF(Ryan and Gillespie,1994) ,tyrosine phosphorylation of PLC-γ is not required for mesoderm induction in *Xenopus* (Muslin *et al.*, 1994). Since tyrosine phosphorylation of PLC-γ is thought to enhance its enzymatic activity (Kim *et al.*, 1991), and therefore lead to an increase in PKC activation, it is likely that PKC is not required for mesoderm induction. This idea is consistent with results from experiments with the phorbol ester TPA, an activator of PKC, which showed that TPA does not induce mesoderm in animal caps but in fact, inhibits mesoderm induction by FGF, suggesting that PKC may play a negative feedback regulatory role in mesoderm induction (Gillespie *et al.*, 1992).

FGF-induced Early Response Genes and Mesoderm Induction

When the signal is finally transmitted into the nucleus, some genes are activated immediately and these genes are referred to as "early response genes". After the early response genes are transcribed, their mRNAs are transported to the cytoplasm from the nucleus and their proteins are subsequently synthesized in the cytoplasm.

Some of these proteins are translocated into the nucleus and function as transcription factors to activate the expression of other genes; the latter are referred to as "delayed response genes". Hence, induction of "early response genes" is not dependent upon protein synthesis, whereas induction of the "delayed response genes" is.

During the past few years, several early mesodermal response genes have been identified. Some of them can be induced both by FGF and activin, and others can be only induced by activin. Mix.1 was the first early response gene to be identified. It can be induced by activin, even in the presence of protein synthesis inhibitor CHX, but cannot be induced by either FGF or TFG-β_2. It can be induced, however, by a combination of TGF-β_2 and FGF.

Mix.1 mRNA encodes a homeodomain-containing protein. Since homeobox proteins in general act as transcription factors, Mix.1 may play an important regulatory role in mesoderm formation (Rosa, 1989). Goosecoid is another homeobox gene which can be induced by activin, but not by FGF (Cho *et al.*, 1991). Goosecoid is expressed on the dorsal side of the embryo before the dorsal lip is formed, and microinjection of goosecoid mRNA into the ventral side of the embryos mimics the properties of Spemann's organizer and produces secondary axes (De Robertis *et al.*, 1992). Induction of goosecoid by activin is not inhibited by CHX either, but is stimulated by CHX. It is believed that this is because the addition of CHX inhibits the synthesis of some proteins which may inhibit the expression of goosecoid. Xbra is a mesodermal early response gene which can be induced both by activin, FGF, and vegetal tissue cells. It is expressed throughout the marginal zone of the embryo,

which is destined to become mesoderm (Smith *et al.*, 1991). Surprisingly, it has been found that during blastula stages, not only can XeFGF induce Xbra, but Xbra can induce the expression of XeFGF and XeFGF is required to maintain the expression of Xbra during gastrula stages (Isaacs *et al.*, 1994). This suggests that FGF is not only involved in mesoderm induction, but may also be important for gastrulation. Xnot is an homeobox gene which is expressed predominantly in the organizing region during gastrula stages. It can be induced by both activin and FGF, and induction is not inhibited by the protein synthesis inhibitor, CHX. Furthermore, expression of a dominant negative FGF receptor mutant eliminates Xnot expression. This suggests that FGF is not only involved in ventral mesoderm formation, but also involved in dorsal mesoderm formation (Von Dassow *et al.*, 1993).

Recently, results from several research groups have shown that mesoderm induction by activin requires FGF (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). Expression of dominant negative FGF mutant inhibits the capacity of activin to induce Xbra, Xnot, Mix.1, but does not affect activin's capacity to induce goosecoid (LaBonne and Whitman, 1994).

In summary, FGFs have been implicated in a number of developmental processes, including mesoderm induction, gastrulation and antero-posterior axis formation in *Xenopus laevis* and my interest is to investigate the molecular basis of FGF action. FGF mediated intracellular signal transduction leads to transcription of early response genes. The product of such genes in turn regulate the expression of delayed response genes and ultimately the cell's fate. Hence, the identification of genes which

are induced immediately by FGFs is an important step in the elucidation of FGF action. During the past few years, only a few FGF early response genes have been identified and these are not sufficient to explain the molecular mechanism of FGF's function. Therefore, it is important to identify additional FGF response genes in *Xenopus* embyos.

1.7 Differential Display RT-PCR

To isolate genes which are induced by FGF, two kinds of cloning strategies may be selected. The first is an expression cloning strategy which relies on a functional assay. The advantage of this method is that the isolated genes usually have interesting functions. For instance, two *Xenopus* genes-noggin and siamois, which can induce the second axis formation, were isolated this way (Smith and Harland, 1992; Lamaire *et al*, 1995). If this method is used to isolate FGF response genes, a plasmid cDNA library from mRNAs of FGF-treated animal caps has to be constructed. The library is partitioned and mRNAs from each fraction are synthesized *in vitro*, followed by microinjection and animal caps assay. Next, the active fractions are partitioned further followed by further microinjection and animal activity assay. This process will be repeated until the single active clone is isolated. (For detailed procedure, see Smith and Harland, 1991). The disadvantage of this method for the FGF-response gene cloning is that one needs a large amount of mRNA from the manually obtained animal caps.

A second kind of cloning strategy is differential screening based on the distinction of mRNAs from different sources. For instance, FGF can divert the development of animal caps from ectoderm to mesoderm. Therefore, the mRNAs from untreated animal caps and FGF-treated animal caps can be classified into three groups: the first group of mRNAs are expressed at the same level in the FGF-treated animal caps and in the untreated animal caps; the second group of mRNAs are expressed at a higher level in the FGF-treated animal caps than in the untreated animal caps, so these genes are induced by FGF; the third group of mRNAs are expressed at a lower level in the FGF-treated animal caps than in the untreated animal caps, so these genes are repressed by FGF. The principle of differential screening is to try to isolate the latter two groups of mRNAs from the first group of mRNAs.

One of the most popular differential screening method is the substractive hybridization technique. For instance, Mix.1, which can be induced by activin, was isolated this way. To use this method to isolate FGF-response genes requires construction of two cDNA libraries from FGF-induced and untreated animal caps, respectively. Briefly this method involves isolation of mRNAs from FGF-treated animal caps and untreated animal caps, followed by cDNA synthesis and hybridization. (For detail procedure, see Maniatis *et al.*, 1982). Unhybridized cDNAs are subsequently labeled and used to probe the above two cDNA libraries. The advantage of this method is that large or full length cDNAs can be cloned, however, I didn't choose to use this method because it requires a large amount of mRNA(about 50ug) from animal caps, which have to be obtained manually.

Differential display is a more recent technique developed by Liang and Pardee as a method to identify differentially expressed genes using the advantages of the polymerase chain reaction (PCR) (Liang and Pardee, 1992). This method is actually a modified RT-PCR method. Instead of using oligodT or random primers as is normal for RT-PCR, it uses an anchored oligo-dT primer, 5'-$T_{11}MN$-3' to carry out the reverse transcription. "M" could be either A, C, G; "N" could be either A, C, G, T. Thus, mRNA are divided into twelve groups which can be reverse transcribed to cDNAs by primer 5'-$T-11AA$, 5'-$T-11AC$, 5'-$T-11AG$, 5'-$T_{11}AT$, 5'-$T_{11}CA$, 5'-$T_{11}CC$, 5,-$T_{11}CG$, 5'-$T_{11}CT$, 5'-$T_{11}GA$, 5'-$T_{11}GC$, 5'-$T_{11}GG$, 5'$T_{11}GT$, respectively. Reverse transcription is followed by ^{35}S-dATP labeled PCR using the same 5'-$T_{11}MN$ primer and a 10-mer primer which is randomly selected (Liang and Pardee, 1992). The amplified PCR fragments are then separated by electrophoresis on a DNA sequencing gel. Differentially expressed mRNAs can be identified by comparing cDNA bands from different mRNA sources (Figure 1.4). Then the differentially expressed bands can be cut from the gel, and the cDNAs can be eluted and cloned directly.

The differential display method has several advantages over the subtractive hybridization method: 1). Small amounts of RNA (about 2μg total RNA per reaction) are required, which is particularly beneficial for our study, where RNAs are to be extracted from manually dissected animal caps. 2).It is very easy. It is based on two of the most widely used molecular biological techniques: RT-PCR and DNA sequencing gel electrophoresis. 3). It takes only 2–3 days to obtain the differentially expressed bands and another 2–3 days to clone these bands. Differential display has

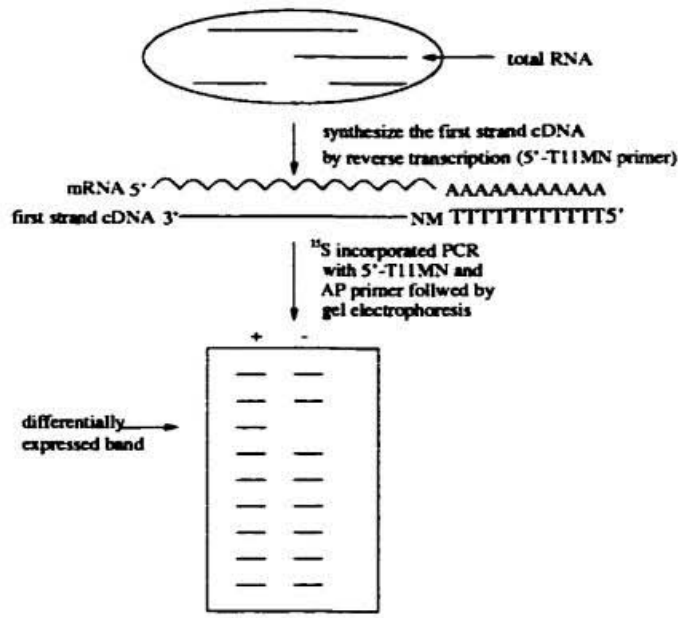


Figure 1.4: **Principle of the differential display method** mRNAs are reverse transcribed with anchored primer $T_{11}MN$, M, N could be A,C, G, or T. cDNA fragments are amplified by PCR using primers $T_{11}MN$, which hybridizes to the polyA tail of the mRNAs and either AP_1 or AP_2 primer, which randomly hybridizes to the first strand cDNAs. The ^{35}S-labelled PCR fragments are separated on a sequencing gel, and the differentially expressed mRNAs can be identified by comparing cDNA bands from different mRNA sources.

a few disadvantages, for example, using this method, shorter cDNAs(usually 200-300 bp) are cloned with higher risk of error caused by poor proof-reading ability of thermostable DNA polymerase during PCR.

Given these advantages and disadvantages of the different cloning strategies, I chose the differential display method to identify FGF-response genes because of technical efficiency. This thesis describes cloning and characterization of one such FGF-response gene.

Chapter 2

Materials and Methods

2.1 Materials

Xenopus Laevis were purchased from Nasco (Wisconson, USA). XbFGF used for induction was expressed and purified from recombinant cDNA according to Kimelman *et al*, 1988, then stored in −20°C. The stage 8 *Xenopus* (lambda ZAPII) cDNA library used for cloning the cDNA was constructed in this lab by Gang Chen (Gillespie *et al.*, 1995). Primers used for cloning and PCR were synthesized by either *Oligos Etc. Inc.* or *GSD general synthesis and diagnostics*, and their sequences are listed in Table 2.1. Culture mediums used for raise the embyos and explants are listed in Table 2.2.

Table 2.1: Sequences of oligonucleotide primers used in cloning, sequencing and RT-PCR

AP_1	primer:	5'- CTG ATC CAT G -3'
AP_2	primer:	5'- CTG CTC TCA G -3'
DOP	primer:	5'- CCG ACT CGA GNN NNN NAT GTG G -3'
mta_1	primer:	5'- TCC GTT ACA CCA GGA TGT AG -3'
mta_2	primer:	5'- GGC TGA AAT TCC AGT TGG TA -3'
MT_1	primer:	5' -CTA CAT CCT GGT GTA ACG GA -3'
MT_2	primer:	5' -TAC CAA CTG GAA TTT CAG CC -3'
MTU	primer:	5'- GAT GTA CGA GAA GTA ATC CG -3'
CH-1	primer:	5'- GGC CCA TGT GCA ATA ACT GC -3'
CH-2	primer:	5'- CAC TTT CTC TTT CAA GGT GC -3'
CH-3	primer:	5'- CTT TCA AAG GCT ACA AAG AG -3'
EL-1	primer:	5'- CTT CAG GGT TTA TTA AAT TA -3'
EL-2	primer:	5'- AAA TGT CAC TAT CAA CTA -3'
EL-3	primer:	5'- CGG CAC GAG AGC TGA CAT GC -3'
mta_9	primer:	5'- GCA TCA GCT GCA GAT CAA GG -3'
mta_{10}	primer:	5'- GTT TAA GAA AGG GCA GTT CG -3'
mta_{12}	primer:	5'- TAT GGA AGG AGT TGT TTG AGA -3'
PO_1	primer:	5'- GAG AAT TCG TCG ACA TCG ATT TTT TTT TTT TTT TT -3'
PO_2	primer:	5'- GAG AAT TCG TCG ACA TCG AT -3'
EF-1α	primer pairs:	Up: 5'- CAG ATT GGT GCT GGA TAT GC -3'
		Down: 5'- ACT GCC TTG ATG ACT CCT AG -3'
T_3	primer:	5'- ATT AAC CCT CAC TAA AG -3'
T_7	primer:	5'- AAT ACG ACT CAC TAT AG -3'
SP_6	primer:	5'- AT TTA GGT GAC ACT ATA -3'

Table 2.2: Culture Mediums for Embryos and Explants

100ml culture medium	10× NAM salt	Gentamycin (10mg/ml)	0.1M NaBiCarb	BSA
NAM/20	0.5ml	0.25ml		
NAM/2	5ml	0.25ml	1ml	1mg/ml
NAM	10ml	0.25ml	1ml	

Per 1L 10×NAM salt contains 65g $NaCl$, 1.5g KCl, 2.4g $Ca(NO_3)_2 \cdot 4H_2O$, 2.5g $MgSO_4 \cdot 7H_2O$, 2ml 0.5M EDTA, pH8.0 and 100ml 1M Hepes, pH7.5.

2.2 Methods

2.2.1 Embryos, Animal Caps and XbFGF Induction

Eggs were obtained from female *Xenopus laevis* injected 14 hours previously with 750 I.U. human chorionic gonadotrophin. Embryos were obtained by artificially fertilizing these eggs, using testes of a sacrificed male *Xenopus laevis*. Fertilized embryos were chemically dejellied using 2.5% L-cysteine hydrochloride (pH7.8–8.1), washed and transferred to petri dishes containing NAM/20 culture medium. They were raised at room temperature (22°C). Embryos were staged according to Nieuwkoop staging tables (Nieuwkoop and Faber, 1967). *Xenopus* animal caps were obtained by microsurgery. When the embryos reached stage 8 (about 5 hours after fertilization at room temperature), they were transferred to petri dishes coated with 1.5% agar and containing NAM culture medium. The vitelline membrane was removed using forceps and animal caps were cut from the embryos with a tungsten needle. They were transferred to petri dishes containing either NAM/2 culture medium, or XbFGF medium[1]. Animal caps were cultured at room temperature for

[1] 100ng/ml XbFGF in NAM/2 culture medium.

half an hour prior to RNA extraction.

2.2.2 Extraction of Total RNA From Whole Embryos and Animal Caps

Extraction of Total RNA From Whole Embryos

Total RNA from the whole embryos was extracted as described (Sambrook *et al.*, 1989). 12 *Xenopus* embryos at the same developmental stage were transferred to a 1.5ml eppendorf tube containing 300μl extraction buffer (3M LiCl, 6M Urea, 10mM NaOAc, pH7.5, 0.1% SDS and 0.5% 2-mercaptoethanol), These embryos were homogenized on ice by pipetting up and down at least 15 times, then the tube was covered and left on ice in cold room (4°C) overnight. The next day, the tube was centrifuged at 4°C, 12,000rpm for 30 minutes. After centrifugation, a thick lipid layer was removed and the supernatant was discarded carefully. Then 240μl DEPC water solution containing 0.3M NaOAc, pH7.5 and 0.5% SDS was added to the eppendorf tube and the pellet was loosened by pipetting up and down. This was followed by addition of an equal volume of Phenol/Chloroform/IAA. The aqueous phase was transferred to a new 1.5ml eppendorf tube and kept on ice, and the interphase and organic layers were re-extracted with an equal volume of DEPC water solution containing 0.3M NaOAc, pH7.5 and 0.5% SDS. The aqueous phases were pooled, and re-extracted with an equal volume of Phenol/Chloroform. Total RNA was precipitated with 2.5 times the volume of ethanol at -20°C overnight, collected by centrifugation, washed with cold 70% ethanol, and dried under vacuum. The entire RNA sample was then used for Northern analysis.

Extraction of Total RNA From Animal Caps

Total RNA from animal caps were extracted as described in (Reynolds *et al.*, 1996). Five animal caps cultured either in control medium or XbFGF medium were transferred to a 1.5ml eppendorf tube containing 200μl NETS solution (0.1M NaCl, 100mM EDTA, pH8.0, 10mM Tris, pH7.5 and 0.2% SDS). The embryos were homogenized by pipetting up and down, followed by extraction with an equal volume of 25:24:1 Phenol/Chloroform/iso-amylalcohol. The aqueous phase was removed to a new 1.5ml eppendorf tube and the interphase and organic phases were re-extracted with equal volume of NETS solution. The two aqueous phases were then pooled to the same tube and precipitated with 2.5 times the volume of ethanol and $\frac{1}{10}$ volume of 3M NaOAc, pH5.2 at $-20°C$ overnight. The next day, the pellet was spun down, washed with cold 70% ethanol and dried under vacuum. The pellet was then dissolved in 50μl DNase buffer (40mM Tris, pH8.0, 10mM NaCl, 6mM $MgCl_2$ and 10mM $CaCl_2$.), and digested with 3μl RQ RNase-free DNase at 37°C for 20 minutes. Then 250μl DEPC H_2O was added and followed by extraction with an equal volume of 25:24:1 Phenol/Chloroform/iso-amylalcohol. The RNA was precipitated with 2.5 times the volume of ethanol and $\frac{1}{10}$ volume of 3M NaOAc, pH5.2 at $-20°C$ overnight. The next day, the total RNA was spun down and washed with cold 70% ethanol and dried under vacuum. The entire sample was used for synthesis of first strand cDNA.

2.2.3 Identification of FGF Early Response Genes by the Differential Display Method

Differential display RT-PCR was carried out according to Liang and Pardee (1992).

Reverse Transcription of mRNA from FGF-treated or Control Animal Caps

Vacuum dried total RNA from 5 FGF-treated or 5 control animal caps was dissolved in 17μl DEPC-H_2O, followed by addition of 2μl 5'-$T_{11}AC$ (100ng/μl) primer. This RNA-primer mixture was then heated at 70°C for 10 minutes and quickly chilled on ice for about 5 minutes. This was followed by addition of 8μl 5×first strand buffer, 2μl 10mM each dNTPs, 4μl 100mM DTT, 1μl RNAguard, 6μl DEPC-H_2O and 2μl MMLV-reverse transcriptase. The reverse transcription reaction was then carried out at 37°C for 1 hour. This reverse transcription product mixture (RT-mix) was used directly for the PCR reaction.

Amplification of the DNA Fragments By PCR Method

PCR reactions were carried out as 40 cycles of 94°C for 30 seconds, 40°C for 2 minutes, and 72°C for 30 seconds; 1 cycle of 72°C for 5 minutes. Each reaction contained 2μl 10×PCR buffer, 1.6μl 2.5μM each dNTPs, 1.2μl 25mM $MgCl_2$, 2μl 2μM AP_1 primer or AP_2 primer, 2μl 10μM $T_{11}AC$ primer, 2μl RT-mix, 8μl dH_2O, 1μl α-^{35}S-dATP and 0.2μl Ampli Taq. The PCR reaction products were separated by electrophoresis on a DNA sequencing gel.

Electrophoresis of The Amplified cDNA Fragments on a DNA sequencing Gel

3.5μl of each PCR reaction product and 2μl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in dH_2O) were mixed, then denatured at 80°C for 2 minutes, followed by loading onto a 6% DNA sequencing gel (in a 100ml final volume containing 48g Urea, 6% 19:1 acrylamide/bis acrylamide and 1×TBE. 100ml sequencing gel was polymerized with 40μl TEMED and 500μl 10% ammonium persulfate.). 100bp ladder was used as marker.

Electrophoresis was carried out for about 4 hours at 55W constant power until the xylene dye migrated to the bottom. The gel was then transferred to 3MM paper, dried at 80°C for 2 hours, and exposed overnight to a X-ray film (Kodak X-AR).

Reamplification of cDNA Probes

Differentially expressed cDNA bands were selected, numbered, and cut from the gel. Each gel slice along with 3MM paper was put in a screw capped tube containing 100μl dH_2O, and soaked for about 30 minutes at room temperature. The tube was then boiled for 20 minutes, and spun for 2 minutes to pellet the gel and paper debris. The supernatant was then transferred to a new 1.5μl eppendorf tube. The cDNA fragment was precipitated at -20°C overnight by adding 10μl 3M NaOAc, pH5.2, 2.5μl 20mg/ml glycogen and 450μl 95% ethanol. The next day, the DNA pellet was centrifuged at 4°C, 12,000rpm for 15 minutes, washed with cold 85% ethanol and dried under vacuum. The pellet was then dissolved with 10μl dH_2O, and 4μl of this sample was used for reamplification.

Reamplification was carried out by PCR using the same conditions as described above except that ①it used 250μM dNTPs instead of 25μM dNTPs; ②no isotope was used; ③the last extension at 72°C was for 12 minutes instead of 5 minutes. The size and concentration of PCR products were checked by agarose-gel electrophoresis (agarose-gel contains 1.2% agarose and 1×TBE.). 1kb DNA ladder was used as a marker and electrophoresis was carried out for about 30 minutes in 1×TBE at 100V. The PCR product was then used for cloning the cDNA fragment.

2.2.4 Cloning of cDNA Fragments

One of the differentially expressed fragment numbered as IVB^+ was cloned by using a TA cloning kit (Invitrogen).

I. Ligation

The first step of cloning was to ligate the IVB^+ cDNA fragment into the specifically designed pCR™II vector (Figure 3.3). The ligation mixture contained 1μl 10×ligation buffer, 2μl 25ng/μl pCR™II vector, 5μl (about 10ng) freshly reamplified PCR product and 2μl T_4 ligase. The ligation reaction was carried out at 14°C for about 24 hours.

II. Transformation

The second step of cloning was to transform the vector-fragment ligation into competent E.Coli. Transformation was carried out according to the instructions included with the TA cloning kit. In short, the ligation reaction was spun down and

put on ice. One vial containing 50μl of frozen one shot™ competent cells was thawed on ice, followed by adding 2μl 0.5M β-mercaptoethanol and 10μl of the ligation reaction to the vial. This vial was then incubated on ice for 30 minutes, followed by heat shock for 45 seconds in a 42°C water bath. The vial was incubated on ice for 2 minutes and 450μl 37°C SOC medium was added to the vial, followed by shaking at 37°C, 225rpm for 1 hour. 300μl from this vial was plated on a X-Gal LB plate containing 90μg/ml ampicillin. The plate was then incubated at 37°C for about 12 hours to allow for growth of the transformants.

III. Analysis of Transformants

10 white transformants were numbered and analyzed by PCR using T_7 and SP_6 primers. The PCR reaction was carried out using 1 cycle of 94°C for 4 minutes and 30 cycles of 94°C for 50 seconds, 57°C for 50 seconds, 72°C for 50 seconds. Each reaction mixture contained 0.6μl 10mM dNTPs, 3μl 10×PCR buffer, 0.6μl 100ng/μl T_7 primer, 0.6μl 100ng/μl SP_6 primer, 1.8μl 25mM $MgCl_2$, 0.4μl AmpliTaq, 21μl dH_2O and a small amount of transformant. The concentration and size of the PCR products were then analyzed by agarose-gel electrophoresis as described on page 32.

2.2.5 Confirmation of Differential Expression by DOP-PCR-Southern Blotting

I. DOP-PCR

Degenerate Oligonucleotide Primers (DOP)-PCR was performed according to the manufacturer's instructions (Boehringer). Total RNA from 5 FGF-treated or

control animal caps were extracted . The first strand cDNAs were synthesized using random primers or oligodT primers. The DOP PCR reaction was carried out as 1 cycle of 95°C for 5 minutes; 5 cycles of 94°C for 1 minute, 30°C for 1.5 minutes, 72°C for 3 minutes, ramp 4.3; 35 cycles of 94°C for 1 minute, 62°C for 1 minute, 72°C for 2 minutes, time inc 14 seconds; 1 cycle of 72oC for 7 minutes. Each reaction mixture contains 4μl 10×PCR buffer, 2.4μl 25mM $MgCl_2$, 3.2μl 250μM dNTPs, 4μl 100ng/μl DOP-primers, 4μl RT-mix, 0.4μl AmpliTaq and 22μl dH_2O.

II. Electrophoresis

30μl of each DOP-PCR product was mixed with 3μl 10×loading buffer and loaded onto a 15 × 15cm 1.2% agarose gel.

Electrophoresis was carried out in 1×TBE buffer for about 2 hours at 120V.

III. Denaturation

After electrophoresis, the whole gel was carefully transferred to a plastic box containing 500ml 0.25M HCl solution. The plastic box was then shaken gently for about 15 minutes at room temperature. The 0.25M HCl solution was decanted, 250ml 0.5M NaOH plus 1.5M NaCl was added and the gel was shaken gently for 45 minutes at room temperature. The NaOH-NaCl solution was removed and substituted with 500ml 1M Tris, pH7.6 plus 1.5M NaCl and the gel was shaken gently for 45 minutes at room temperature.

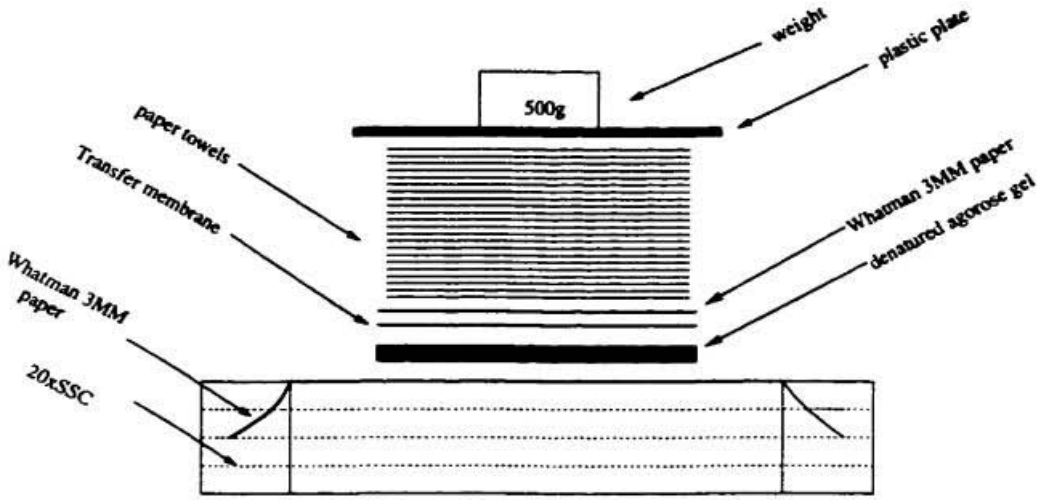


Figure 2.1: Transfer of DNAs or RNAs to a Nylon Membrane

IV. Transfer of DNA Fragments to a Nylon Membrane

Southern transfer was carried out as shown in Figure 2.1 for about 16 hours. 20×SSC was used as the transfer buffer. DNA fragments were transferred to Hybond™-N transfer membrane (Amersham). After Southern transfer, the blot was quickly rinsed with 6×SSC solution and air dried at room temperature for about 15 minutes. Finally, the blot is put between two pieces of 3MM paper and baked at 80°C for 2 hours in a vacuum oven.

V. Prehybridization

Before prehybridization, the blot was put into a plastic box containing 6×SSC solution. After the blot was completely wet, it was put into a plastic bag with 12ml hybridization solution (100ml hybridization solution contains 1g milk powder,

200μl 0.5M EDTA, 6.9g NaH_2PO_4 and 7g SDS. The final pH is 7.2.). The bubbles were removed and the plastic bag was sealed and put into a 65°C water bath to prehybridize for about 4 hours with constant shaking.

VI. Making the Hybridization Probe

100ng of the PCR product of clone IVB^+ from step 2.2.4(III) was used as a probe template. ^{32}P-labeled probe was made by incorporation of ^{32}P-dATP according to the instructions included with the Random Labeling Kit (GIBCO). Briefly, 5μl of the PCR product of clone IVB^+ (about 100ng) was transferred to a 1.5ml screw cap tube containing 15μl dH_2O, followed by boiling for 5 minutes, then the tube was immediately put on ice for 5 minutes. Next, 2μl 100mM dCTP, 2μl 100mM dGTP, 2μl 100mM dTTP, 15μl random primer mix, 5μl α-^{32}P-dATP, 2μl dH_2O and 2μl Klenow fragment were added. The reaction was then carried out at room temperature for about 2 hours. After the reaction was complete, the ^{32}P-dATP incorporated probe was denatured by boiling for 5 minutes, just before use. Histone H_4 probe was also prepared using the same method.

VII. Hybridization

After 4 hours prehybridization, the hybridization buffer was removed and substituted with another 12ml fresh hybridization buffer. The denatured probe was then added to the plastic bag and the bubbles were removed, the plastic bag was sealed and put back into the 65°C water bath for hybridization overnight.

VIII. Washing

Washing was carried out at low stringency (2×SSC, 1% SDS for 30 minutes at room temperature.) and at high stringency (0.1×SSC, 0.1% SDS 65°C for 40 minutes.). Then the blot was exposed overnight to a X-ray film (Kodak X-AR) at −70°C with an intensifying screen.

2.2.6 Large Scale Plasmid Extraction

A small amount of clone IVB^+ was transferred into a one litre sterile flask containing 500ml LB medium (1L LB medium contains 10g bacto-tryptone, 5g bacto-yeast extract and 10g NaCl, the final pH is 7.0.) with 70μg/ml ampicillin. The bacteria were grown by shaking the flask at 37°C, 225rpm for about 20 hours. The bacteria were then transferred into two 250ml plastic bottles to spin down the bacteria at 4°C, 500rpm for 10 minutes. The supernatant was discarded and the pellet was homogenized with 9ml/bottle of Solution I (50mM glucose, 25mM Tris·Cl, pH8.0, and 10mM EDTA, pH 8.0.), followed by addition of 17.5ml/bottle freshly prepared Solution II (0.2M NaOH and 1% SDS.). The bottles were left at room temperature for 10 minutes, 12.75ml cold Solution III (per 100ml Solution III contains 60ml 5M Potassium acetate, 11.5ml glacial acetic acid, and 28.5ml dH_2O, final pH4.8.) was added into each bottle and shaken by hand then put on ice for 10 minutes. Next, the bottles were centrifuged at 4°C, 7500rpm for 15 minutes. The supernatants were filtered into one fresh 250ml plastic bottle through four layers of cheese cloth. An equal volume of isopropanol was added to the filtrate and left at room temperature for about 2 hours to precipitate the DNA. After centrifugation at room temperature,

10,000rpm for about 20 minutes, the pellet was washed with 70% ethanol, transferred to a corex glass tube, dried under vacuum, and resuspended in 4ml TE buffer (100mM Tris, 1mM EDTA.), pH 8.0. Then, 4ml cold 5M LiCl was added to the corex tube and the tube was put on ice for 5 minutes, followed by centrifugation at 4°C, 10,000rpm for 10 minutes. The supernatant was subsequently transferred to a fresh corex tube and 8ml isopropanol was added to the same tube. After precipitation at room temperature for about 30 minutes, the isopropanol mixture was centrifuged at room temperature, 10,000rpm for 10 minutes. The supernatant was discarded and the pellet washed with 70% ethanol, dried under vacuum, then transferred to a 1.5ml eppendorf tube, and resuspended with 500μl TE, pH8.0 buffer. After incubating at room temperature for 30 minutes, 500μl 1.6M NaCl plus 13% PEG was added. The precipitated plasmid was centrifuged at 4°C, 12,000rpm for 15 minutes. The plasmid pellet was resuspended with 400μl TE, pH8.0 buffer and extracted with an equal volume 23:24:1 Phenol:Chloroform:iso-amylalcohol, twice. The plasmid DNA was precipitated at room temperature for 10 minutes by adding 2.5 times the volume of ethanol and $\frac{1}{10}$ volume of 3M NaOAc, pH5.2. The pellet was collected by centrifugation at 4°C, 12,000rpm, washed with 70% ethanol and vacuum dried.

The plasmid DNA pellet was resuspended in 100μl TE, pH8.0 buffer and analyzed by:

1. UV absorption
2. Restriction enzyme digestions
3. Sequencing

For UV absorption analysis, 1μl plasmid IVB^+ was transferred to a quartz cuvette containing 600μl TE buffer. The UV 280nm and 260nm absorption values were measured with a DU®-64 spectrophotometer. The concentration of the plasmid was then calculated according to the formula:

$$plasmid\,concentration = 50\mu g/ml \times OD_{260} \times 600.$$

For restriction enzyme analysis, restriction enzyme digestion reaction was carried out at 37°C for 2 hours. The reaction mixture contained 1μl 10×reaction3 buffer, 3μl plasmid IVB^+, 4μl dH_2O and 2μl EcoRI. The digest was analyzed by agarose gel electrophoresis.

2.2.7 Sequencing Analysis of the Cloned cDNA Fragment

The sequencing reaction was carried out according to the instructions of the Sequenase™ sequencing kit (*Amersham*).

I. Denaturation

Denaturation was carried out at 37°C for 30 minutes. The reaction mixture contained 1μl plasmid IVB^+ (about 4μg), 17μl dH_2O and 2μl denaturing solution (500μl denaturation solution contains 100μl 10N NaOH and 2μl 0.5M EDTA, pH8.0.). The denatured plasmid was precipitated at −70°C for about 15 minutes by adding 22μl 3M NaOAc, pH5.2 and 60μl 95% ethanol. The denatured plasmid was then pelleted by centrifugation at 4°C, 12,000rpm for 15 minutes, followed by washing with cold 70% ethanol and vacuum drying.

II. Annealing

The vacuum dried denatured plasmid was resuspended in 14μl dH_2O. 7μl was used for annealing. Each annealing mixture contained 7μl resuspended denatured plasmid, 2μl 5×sequencing reaction buffer, 1μl 10ng/μl T_7 primer or 1μl 10ng/μl SP_6 primer. Annealing of the primer to the plasmid DNA was carried out by heating the reaction mixture at 65°C for 2 minutes, followed by slowly cooling to room temperature. This cooling step takes about 45 minutes. Then, the cooled tubes are put on ice for about 5 minutes.

III. Labeling

The labeling reaction mixture contained 10μl of the above annealing mixture, 1μl 0.1M DTT, 2μl diluted labeling mix,[2] 1μl α-^{35}S-dATP, 1.75μl enzyme dilution buffer and 0.25μl Sequenase.[3] Labeling involved two steps: firstly, the above labeling mixture was incubated at room temperature for 5 minutes; secondly, 2.5μl each of ddATP, ddCTP, ddGTP, ddTTP were added into 1.5ml eppendorf tubes labeled with A, C, G, T, respectively, incubated at 37°C for 3 minutes, and followed by addition of 3.5μl of the above labeling mixture to each tube. The tubes were then incubated at 37°C for 5 minutes. Reactions were terminated by adding 4μl stop solution to each tube.

[2]per 5μl diluted labeling mix contains 1μl labeling mix plus 4μl dH_2O.

[3]T_7 DNA polymerase.

IV. Electrophoresis

2.5μl of each reaction from step III was denatured at 80°C for 3 minutes, then loaded onto a 6% DNA sequencing gel in the order of A, C, G, T. Electrophoresis was carried out at 55W constant power for 2 to 4 hours according to the region of the sequence want to be read. Then, the gel was fixed with 10% Methanol/10% glacial acetic acid, dried and exposed to x-ray film overnight.

2.2.8 Analysis of the Sequence of the Cloned cDNA Fragment IVB^+

Nucleic acid sequence databases were searched for sequences homologous to the cloned cDNA fragment IVB^+ using the BLASTX program from the *National Center for Biotechnology Information* (Bethesda, MD) using the BLAST network service. We named our novel gene *er1* (early response #1).

2.2.9 Cloning and sequencing the 5'-end of *er1*

Cloning of a 1kb DNA Fragment

I. PCR From a *Xenopus* Stage 8 cDNA Library

Two primers, mta_1 and mta_2, which are specific to *er1* were designed. PCR reactions were carried out as 1 cycle of 94°C for 3 minutes, 35 cycles of 94°C for 50 seconds, 50°C for 50 seconds and 72°C for 1.5 minutes, followed by 1 cycle of 72°C

for 12 minutes. Each PCR reaction buffer mixture contained 0.6μl 10mM dNTP, 3μl 10×PCR reaction buffer, 0.6μl 100ng/μl mta_1 primer or mta_2 primer, 0.6μl 100ng/μl T_3 primer, 1.8μl 25mM $MgCl_2$, 10μl stage 8 cDNAs (2ng/μl), 0.4μl AmpliTaq and 13μl dH_2O.

II. Purification of PCR Products

1. Pretreatment of NA45 cellulose membrane

   NA45 was cut into small rectangles. They were pretreated by washing with 10mM EDTA, pH8.0 for 10 minutes, followed by washing with 0.5M NaOH for 5 minutes, and finally with sterile water several times and stored in dH_2O at 4°C.

2. Electrophoresis

   25μl of each PCR reaction was loaded onto a 1.2% agarose gel. Electrophoresis was carried out in 1×TBE buffer, 90V for about 45 minutes. The largest PCR product amplified with T_3 and mta_1 primers was selected. A slit was cut just behind the 1kb band and a piece of NA45 membrane was inserted into the slit. The agarose gel was then put back into the electrophoresis bed in the opposite direction. Electrophoresis was then carried out under the same conditions for 7 minutes. Thus, the 1kb DNA fragment was transferred onto the NA45 membrane.

3. Elution and Precipitation of the 1kb DNA Fragment

The NA45 membrane was washed with 600μl rinse solution (0.15M NaCl, 0.1mM EDTA, pH8.0, and 20mM Tris, pH8.0.) three times and put into a 1.5ml eppendorf tube containing 700μl Elution solution(1.0M NaCl, 0.1mM EDTA, pH8.0 and 20mM Tris, pH8.0.). The tube was then incubated at 68°C for 20 minutes. Thus, the 1kb DNA fragment was eluted from the membrane. The 1kb DNA fragment was precipitated at -70°C overnight by adding 700μl isopropanol. The next day, the pellet was collected by centrifugation at 4°C, 12,000rpm for 15 minutes, followed by washing with cold 85% ethanol and vacuum drying. The dried pellet was dissolved in 10μl dH_2O. 2μl of this was used for examining the size and concentration by agarose-gel electrophoresis.

III. Cloning of the Gel Purified 1kb DNA Fragment

5μl of the gel purified 1kb DNA fragment was used for ligation into the pCR™II vector (Figure 3.3). The ligation and transformation methods are the same as described in section 2.2.4 on page 33. Ten white transformants were selected and analyzed by PCR with either SP_6 and T_7 or mta_1 and mta_2 primer pairs. Clone 3 and 7 were selected for plasmid extraction and sequencing with primers mta_1, mta_2, MT_2 and T_3.

2.2.10 Cloning and sequencing the 3'-end of *er1*

Total RNA was extracted from 4 stage 8 embryos. First strand cDNA was obtained by reverse transcription as described in section 2.2.3 on page 31 except that 2μl 250ng/μl PO_1 primer was used instead of 2μl 100ng/μl 5'-T_{11}AC primer.

The PCR reaction was carried out as follows: firstly, a PCR reaction mixture was added into a 0.5μl eppendorf tube. The mixture contained 1.5μl 10×PCR reaction buffer, 0.3μl 10mM dNTP, 0.3μl 250ng/μl PO_2 primer, 0.3μl 100ng/μl MTU primer, 0.9μl 25mM $MgCl_2$, 2μl RT-mix, and 9.5μl dH_2O. The tube was then capped and put into the PCR reaction block; secondly, the PCR reaction was initiated at 95°C for 5 minutes, followed by 72°C for 50 seconds. While maintaining the 72°C temperature, 0.2μl AmpliTaq and 30μl 72°C mineral oil was added into the mixture; thirdly, the PCR reaction was continued as 1 cycle of 55°C for 5 minute, 72°C for 40 seconds; 40 cycles of 95°C for 40 seconds, 55°C for 1 minute and 72°C for 3 minutes; 1 cycle of 72°C for 12 minutes. PCR products were analyzed by agarose-gel electrophoresis. The largest band was selected, gel purified, and cloned. Clone 4 was selected for plasmid extraction and sequencing.

2.2.11 Cloning the 2.3kb fragment of *er1*

A 2.3kb fragment was cloned by PCR from the stage 8 cDNA library using *er1* specific primers. The PCR reaction was carried out as 1 cycle of 94°C for 5 minutes; 35 cycles of 94°C for 50 seconds, 64°C for 50 seconds, 72°C for 2 minutes; 1 cycle of 72°C for 12 minutes. The reaction mixture contained 0.6μl 10mM dNTPs, 3μl 10×Taq reaction buffer, 0.6μl 100ng/μl mta9 primer, 0.6μl 100ng/μl mta10 primer, 1.8μl 25mM $MgCl_2$, 13μl dH_2O, 10μl stage 8 cDNA library and 0.4μl AmpliTaq.

A 2.3kb PCR product was obtained and cloned as described in section 2.2.4 on page 33; clone 19 was selected, followed by plasmid extraction and sequencing.

2.2.12 Analysis of Gene Expression by Northern Hybridization

Northern hybridization was performed as described in Sambrook *et al.*, 1989.

I. Preparation of the Denaturing Agarose Gel

2.16g of molecular biology grade agarose was added into a flask containing 127ml of DEPC-dH_2O, and heated until all the agarose gel was dissolved. The agarose gel was then cooled to 60°C in a 60°C water bath, followed by addition of 36.8ml 37% formaldehyde and 18ml of 10×MOPS (0.2M MOPS, pH7.0, 0.05M NaOAc and 0.01M EDTA.) in a fume hood. The flask was covered and re-heated in a 60°C water bath to remove any bubbles. Next, the gel was poured into a 15cm×15cm tray using a 15 well comb. The gel was then cooled to room temperature for about 45 minutes.

II. Electrophoresis

Total RNA extracted from 12 whole embryos was dissolved in 5μl DEPC-dH_2O, followed by addition of 15μl denaturing sample buffer(15ml denaturing sample buffer contained 2ml 10×MOPS, 3ml 37% formaldehyde and 10 ml deionized formamide.). The total RNA samples were then denatured at 75°C for 5 minutes, and quickly put on ice for 5 minutes. Next, 2μl of 10×gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in dH_2O.) was added into each sample. The samples were subsequently loaded onto the denaturing gel. Electrophoresis was carried out at 1×MOPS buffer, 90V for about 6 hours.

III. Transfer of Denatured RNA from the Denaturing-gel to a Nylon Membrane

After electrophoresis, the gel was transferred to a plastic box, washed with DEPC-H_2O 3 times and soaked in 20×SSC buffer (1L 20×SSC contained 175.3g NaCl and 88.2g $Na_3Citrate$, final pH7.0.) for about 45 minutes. The Gene Screen™ hybridization transfer membrane was moistened with DPEC-H_2O and soaked with 20×SSC for 5 minutes before use. Two pieces of 3MM paper were treated with 2×SSC for about 2 minutes before use. Northern transfer was carried out as shown in Figure 2.1 for about 20 hours at room temperature. After northern transfer, the membrane was rinsed with 6×SSC for 1 minute, followed by air drying for about 15 minutes. The RNA ladder lane was cut from the blot and stained with a solution containing 0.5M Sodium Acetate, pH5.2, and 0.04% methylene blue. The rest of the blot was then put between two pieces of 3MM paper and baked at 80°C for 2 hours in a vacuum oven.

Prehybridization

The blot was moistened with 6×SSC and put into a plastic bag containing 12ml hybridization buffer (12ml hybridization buffer contains 9ml Gene Screen buffer, 3ml 20% SDS and 300μl 10mg/ml ssDNA. 375ml Gene Screen buffer contains 50g dextran sulphate, 166ml 1M sodium phosphate solution, pH7.0 and 100ml 50mM EDTA. Sodium phosphate solution contains 0.6M Na_2HPO_4 and 0.4M NaH_2PO_4. ssDNA needs to be boiled for 5 minutes before use.). The bubbles were removed, the plastic bag sealed and prehybridization was carried out at 65°C for 4 hours.

V. Preparation of the Northern Hybridization Probe

The Northern hybridization probe was prepared with the random primer labeling method as described in section 2.2.5 on page 37. The 2.3kb DNA fragment was cut from the plasmid with BstXI and gel-purified as described on page 43. 100ng of the 2.3kb DNA fragment was used as a template for random primer labeling. The probe was then denatured by heating to 100°C for 5 minutes before use. Histone H_4 probe was also prepared using the same method and used as a loading control.

VI. Hybridization

After 4 hours of prehybridization, the blot was taken out of the plastic bag and put into another plastic bag with 12ml fresh hybridization buffer and the denatured probe. The bubbles were removed and the bag sealed. Hybridization was carried out at 65°C for about 20 hours.

VII. Washing

The blot was washed with 2×SSC, 2% SDS at room temperature for 30 minutes, followed by 0.1×SSC, 0.1% SDS at 65°C for 40 minutes.

VIII. Autoradiography

The blot was exposed for 3 days to X-ray film (Kodak X-AR) at −70°C with an intensifying screen.

2.2.13 Analysis of Gene Expression by Quantitative RT-PCR

Quantitative RT-PCR method was carried out as described in Niehrs *et al.*, 1994.

Total RNA, extracted by the "SDS" method from either five control animal caps or five FGF-treated animal caps, was used for reverse transcription using random primers as described on page 31. The PCR reaction for *er1* was carried out as 1 cycle of 94°C for 5 minutes, 28 cycles of 94°C for 50 seconds, 57°C for 50 seconds, and 72°C for 50 seconds; 1 cycle of 72°C for 12 minutes. Each PCR reaction mixture for *er1* contained 0.6μl 10mM dNTPs, 3μl 10×Taq buffer, 0.6μl 100ng/μl MTU primer, 0.6μl 100ng/μl mta_1 primer, 1.8μl 25mM $MgCl_2$, 2μl RT-mix, 0.3μl AmpliTaq, 0.1μl α-^{32}P-dATP and 21μl of dH_2O.

EF-1α was used as an input control. Amplification of EF-1α was carried out as 1 cycle of 94°C for 5 minutes, 28 cycles of 94°C for 50 seconds, 55°C for 50 seconds, and 72°C for 50 seconds; 1 cycle of 72°C for 12 minutes. The PCR mixture contained 0.3μl 10mM dNTPs, 1.5μl 10×PCR reaction buffer, 0.9μl 25mM $MgCl_2$, 3μl 3μM EF-1α primer pairs mix, 7μl dH_2O, 0.2μl AmpliTaq, 0.1μl α-^{32}P-dATP, and 2μl RT-mix. 2μl of each PCR product was mixed with 2μl loading buffer and loaded into a 6% DNA sequencing gel. Electrophoresis was carried out at 1×TBE, 60W for about 4 hours. The gel was then fixed, transferred to 3MM paper, dried at 80°C for 2 hours. Autoradiography was carried out by exposing the gel to a pre-flashed[4]

[4]The pre-flashing of the X-ray film is carried out by a quick exposure of the film to a magnesium light flash.

Kodak X-LS film at −70°C with an intensifying screen.

2.2.14 *In vitro* coupled transcription-translation

In vitro transcription-translation was carried out using the TNT™ coupled Reticulocyte Lysate system. The reaction mixture contained 25μl TNT rabbit reticulocyte lysate, 2μl TNT reaction buffer, 1μl 1mM amino acid mixture minus methionine, 1μl RNAguard, 1μl T_7 RNA polymerase, 4μl ^{35}S-methionine, 2μg plasmid 19 DNA (see section 2.2.11) and nuclease-free H_2O to final volume 50μl. The reaction mixture was incubated at 30°C for 90 minutes. The translation products were analysed by SDS-polyacrylamide gel electrophoresis.

2.2.15 SDS-polyacrylamide gel electrophoresis

5μl of above translation products and 10μl of $1.5 \times SSB$ (3 parts $2 \times SSB$[5] + 1 part dH_2O) were mixed and denatured by boiling for 5 minutes, followed by loading onto a 8% SDS-polyacrylamide gel [6]. Electrophoresis was carried out for about $1.5hrs$ in 1×electrophoresis buffer ($1L$ 1×electrophoresis buffer contains $6g$ Tris base, $28.8g$ glycine and $0.9g$ SDS.) at $30mA$ constant current until the front blue dye migrated to the bottom. The gel was then fixed for 10 minutes with 45% methonal/10% glacial acetic acid, destained for 10 minutes with 20% methonal/6% glacial acetic acid, and soaked for 30 minutes with *Amplify* (Amersham). The gel was then transferred to

[5] $2 \times SSB$: $5ml$ stacking gel buffer ($0.5M$ Tris, pH6.8), $5ml$ 20% SDS, $2.5ml$ β-mercaptoethanol, $5ml$ glycerol, $5ml$ dH_2O and a few crystals of bromophenol blue.

[6] $2.64ml$ 30% acrylamide, $2.5ml$ running gel buffer ($1.5M$ Tris, pH8.0), 100μl 10% SDS, $4.66ml$ dH_2O, 66μl 10% ammonium persulfate, and 4μl TEMED.

3*MM* paper, dried and exposed overnight to a Kodak X-LS film.

Chapter 3

Results

3.1 Identification of Differentially Expressed cDNA Fragments by Differential Display

To identify potential FGF response genes, I used the differential display method as outlined in section 1.7. cDNA fragments were amplified from five individual sets of FGF-treated and untreated animal caps . Only those cDNAs that were differentially expressed in all five sets were chosen for further analysis. This way I could minimize the number of false positives and false negatives. For this amplification, I used the primer $T_{11}AC$, which hybridizes to the polyA tail of the mRNAs, and either primer AP_1 or AP_2 which randomly hybridizes to the first strand cDNAs (Liang and Pardee, 1992). PCR products from the five sets were separated on a sequencing gel.

With the primer pair, $T_{11}AC$ and AP_1, about 60 cDNA fragments ranging in size from 100bp to 700bp were amplified. With the other primer pair, $T_{11}AC$ and

AP_2, about 70 cDNA fragments in the same size range were obtained. (Figure 3.1). As can be seen in Fig. 3.1, the pattern of the amplified fragments differed markedly from those amplified with different primer pairs. A total of eleven differentially expressed bands were identified and one of the fragments, designated IV, was consistently expressed at a higher level in all five experimental groups (Figure 3.2) and, therefore, was chosen for further analysis.

Band IVB^+ was recovered from the gel for cloning. The TA cloning kit was used to clone the IVB^+ fragment. The linearized vector supplied in this kit, pCR™ II vector, is specifically designed for cloning PCR products. The principle is that Taq polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine (dA) to the 3'-end of PCR products. The pCR™ II vector has 3' deoxythymidine (dT) residues which allows PCR fragments to ligate efficiently into the vector (Figure 3.3). In addition, the pCR™ II vector contains T_7 and SP_6 promoters and primer sites, which can be used for *in vivo* or *in vitro* transcription of sense and antisense RNA as well as DNA sequencing.

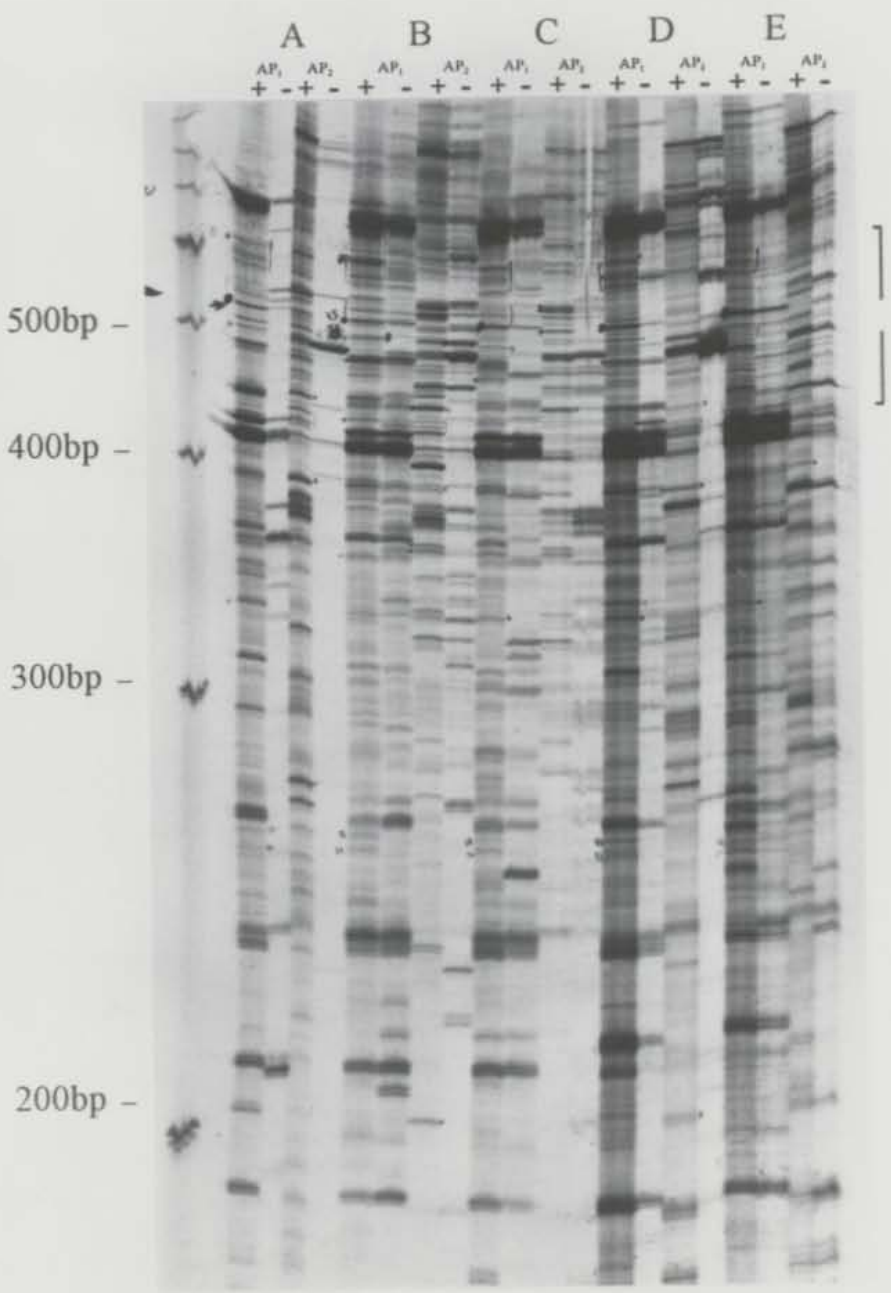

A
B
C
D
E
AP1 AP2
+ - + - + - + - + - + - + - + - + - + -
500bp –
400bp –
300bp –
200bp –

Figure 3.1: **Differential display of FGF treated (+) and control (−) animal caps** Five individual sets of explants (5 per sample) from stage 8 *Xenopus* blastulae were treated for 30 min in the presence (+) or absence (-) of 100ng/ml XbFGF. Total RNA was extracted and reverse transcribed with primer $T_{11}AC$, followed by PCR amplification with primer pair $T_{11}AC$and AP_1, or primer pair $T_{11}AC$ and AP_2. The PCR products were separated on a sequencing gel. The five sets are labeled A, B, C, D, E, respectively, at the top of the lanes. AP_1 or AP_2, located on the top of every two lanes indicate that those bands were amplified from either $T_{11}AC$ and AP_1 or $T_{11}AC$ and AP_2, respectively. DNA molecular size markers are shown on the left, and brackets indicate the region that is enlarged in Figure 3.2.

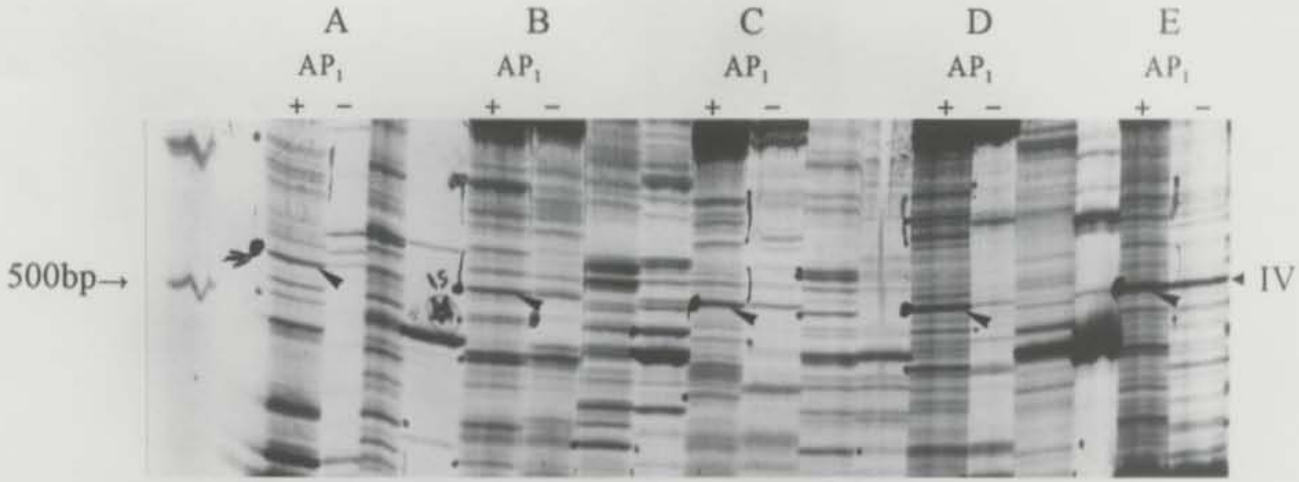

A
B
C
D
E
AP1
AP1
AP1
AP1
AP1
+ −
+ −
+ −
+ −
+ −
500bp→
IS
IV

Figure 3.2: **A differentially expressed band IV is shown in all five experimental groups** This figure is enlarged from the bracketed region of Figure 3.1. The five replicates are labeled A, B, C, D, E. DNA molecular size markers are shown on the left. +: indicates the FGF treated samples; - indicates the control samples. AP_1 indicates that the bands below were amplified from primer pair $T_{11}AC$ and AP_1. Unlabeled lanes are PCR products amplified from primer pair $T_{11}AC$ and AP_2. Positions of bands IV are indicated with arrow heads. It is clearly shown that the expression levels of the IV cDNA are higher in the FGF-treated samples than in the control samples in all five individual sets.

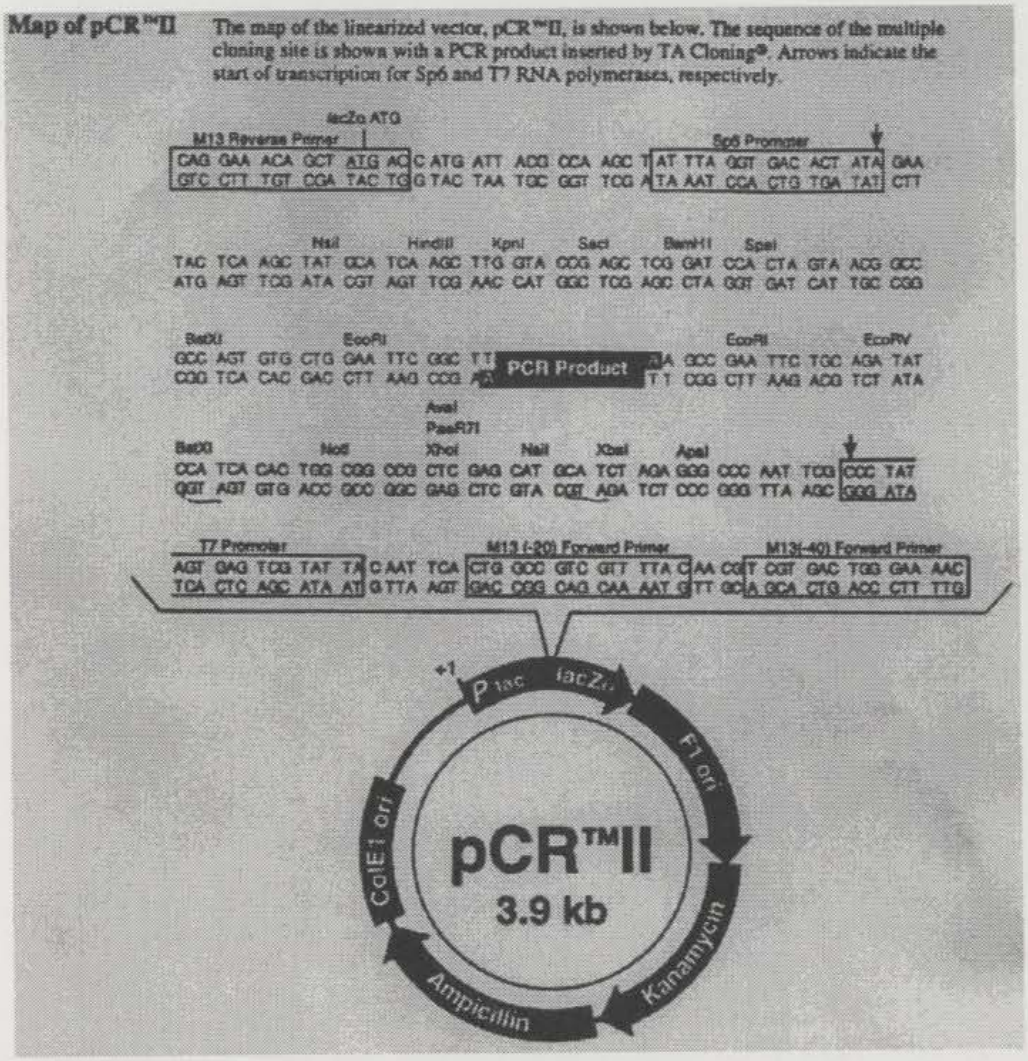


Figure 3.3: **The map of the PCR™ II vector** The sequence of the multiple cloning site is shown with the PCR product inserted by TA cloning (*Invitrogen*). PCR products usually have a single deoxyadenosine (dA) in the 3'-end because of Taq polymerase's nontemplate-dependent activity, and the PCR™ II vector contains 3'dT residues. Arrows indicate the start of transcription for Sp6 and T7, respectively.

3.2 Verification of Differential Expression by DOP-PCR-Southern Analysis

Before determining the identity of the cloned cDNA fragment IV, I verified the differential expression between the FGF-treated and the nontreated animal caps. This is because differentially displayed bands do not always indicate differential expression of the corresponding cDNAs; instead, the difference may be due to technical discrepancies or experimental errors. Northern hybridization is usually used to verify differential expression with the differentially displayed cDNA fragments as probes. However, I encountered a detection problem in using Northern hybridization for verification. First of all, these probes are short (usually 200–500bp) and this decreases the sensitivity of Northern hybridization. Secondly, total RNAs were extracted from manually dissected animal caps and it was difficult

to obtain the amount of total RNAs required for Northern hybridization.(usually 20μg$\equiv$at least 50 animal caps.) This combined with the fact that mRNA encoding regulatory proteins are usually present as low abundance mRNAs meant that it was not practical to screen the differentially expressed cDNA fragments by Northern hybridization. In order to get around these difficulties, I have developed a new method to screen the differentially expressed cDNA fragments. The strategy of this method is to reverse transcribe the mRNAs using random primers or an oligodT primer, followed by non-specific amplification of all first strand cDNAs by PCR using a commercially available degenerate oligonucleotide primer (DOP) (Boehringer). The amplified cDNAs are then used to carry out Southern hybridization. This method is referred to as "DOP-PCR-Southern" method. In our case, only five animal caps

were required for each sample.

DOP-PCR-Southern analysis showed that the expression level of IVB^+ was higher in FGF-treated animal caps than in control animal caps (Figure 3.4). Histone H_4 was used as a loading control. This gave me confidence that IVB^+ expression is regulated by FGF and I proceeded with the analysis of this gene.

3.3 Sequencing of the IVB^+ sequence

The next step was to sequence the IVB^+ cDNA to determine the identity of this clone. The 535bp nucleotide sequence of IVB^+ is shown in Figure 3.5. Unexpectedly, both ends of the IVB^+ fragment were flanked by the AP_1 primer sequence. Thus, the IVB^+ fragment is a PCR product amplified by primer pair $AP_1 \sim AP_1$ instead of $T_{11}AC \sim AP_1$, and it is not located at the end of the full-length cDNA. A search of the database for similarity to known sequences revealed that this cDNA represented a novel *Xenopus* gene, which I have named *er1* (early response 1).

3.4 Cloning and sequencing of the full length *er1* cDNA

For further analysis of *er1*, I set out to obtain a full-length *er1* cDNA. This can be done using either of two methods: The first would involve screening a cDNA library and the second would involve PCR to amplify it from a cDNA libray. I chose the latter method because of the ease and speed with which PCR cloning can be

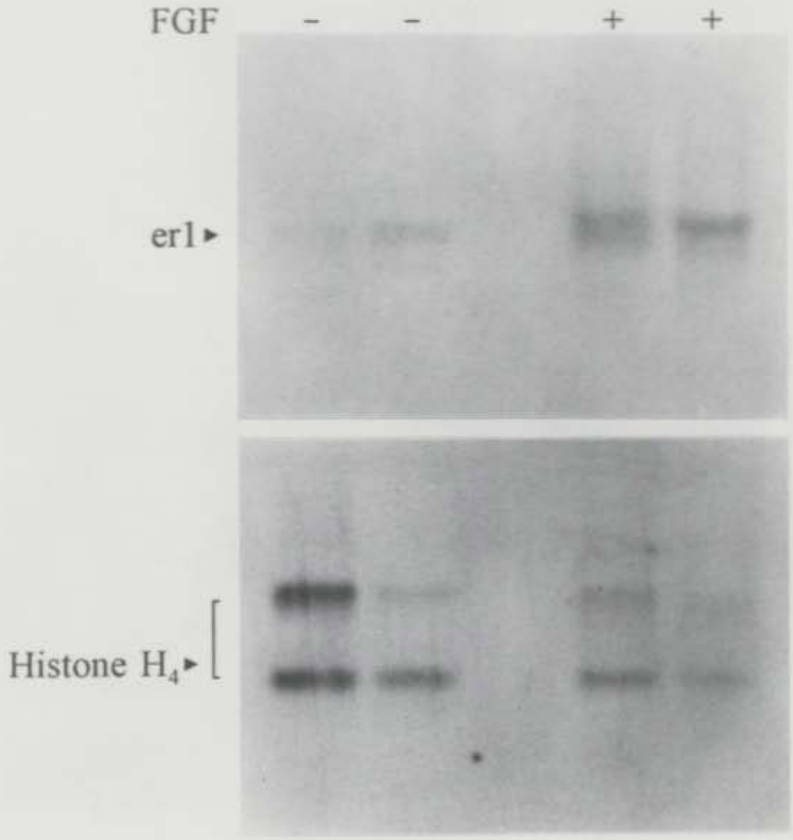

FGF
–
–
+
+
er1
Histone H_4

Figure 3.4: **DOP-PCR-Southern analysis of expression levels of *er1* in FGF-treated and control animal caps** Explants (5 per sample) from stage 8 *Xenopus* blastulae were treated for 30 min in the presence (+, lane 3, 4) or absence (-, lane 1, 2) of 100ng/ml XbFGF. Total RNA was extracted and reverse transcribed with either oligodT (lane1,3) or random primer (lane 2, 4), followed by DOP-PCR. PCR products were separated on a 1.5% agarose gel, transferred to a nylon membrane, followed by Southern hybridization analysis using ^{32}P-labeled IVB^{+} CDNA as probe. Then the blot was stripped and reprobed with ^{32}P-labeled Histone H_4 cDNA. It is clearly shown that the expression levels of IV cDNA were higher in the FGF-treated samples than in the control samples.

```
AP1--------->
  1  CTGATCCATG TTCCAGGCTG AAATTCCAGT TGGTATTTGC AAATACAGAG
 51  AAACAGAGAA AGTATATGAA AATGATGATC AGCTCCTCTG GAATCCAGAA
101  TATGTAATGG AAGAAAGAGT AATAGACTTC TTAAATGAGG CATCCAGAAG
151  GACTTGTGAA GAGAGAGGGC TAGATGCTAT TCCTGAAGGA TCCCACATAA
201  AGGACAATGA GCAGGCCCTA TATGAACATG TAAAATGCAA TTTTGACACA
251  GAAGAGGCAT TGAGAAGACT AAGATTTAAT GTCAAAGCCG CCAGAGAAGA
301  ACTTTCCGTT TGGACTGAAG AAGAATGTAG AAATTTTGAG CAAGGTCTAA
351  AAGCTTATGG CAAAGATTTC CACTTGATTC AGGCTAACAA GGTAAGGACA
401  AGGTCTGTTG GAGAATGTGT GGCATTCTAC TACATGTGGA AAAAATCAGA
451  ACGTTATGAC TTCTTTGCCC AACAAACACG ATTTGGAAAA AAGAAGTATA
501  ATCTACATCC TGGTGTAACG GATTACATGG ATCAG
                                  <----------AP1
```

Figure 3.5: **Nucleotide sequence of the IVB^+ PCR product** The nucleotide sequence numbers of IVB^+ cDNA are shown on the left. The first ten nucleotides are coincide with the AP_1 primer sequence, and the last ten nucleotides are complementary to the AP_1 primer sequence. Thus, the IV cDNA was amplified from primer pair AP_1 and AP_1, but not primer pair $T_{11}AC$ and AP_1.

performed. In order to clone the full length *er1* cDNA, I designed an experiment which consists of the following three steps: The first step is to clone the 5'-end of the *er1* cDNA, the second step is to clone the 3'-end of the *er1* cDNA, and the third step is to clone the entire length of the *er1* cDNA. The strategy for cloning the 5'-end cDNA fragment of *er1* was to PCR amplify it from a *Xenopus* stage 8 λ ZapII cDNA library. In this cDNA library, all the cDNAs are oriented with their 5'-ends close to the T_3 promoter and 3'-ends close to the T_7 promoter. The 5'-end was cloned by using T_3 primer and an *er1* specific primer to carry out PCR (Figure 3.6).

The 3'-end of *er1* was cloned using the 3'-RACE method . The strategy is explained by Frohman (Frohman, 1990) (Figure 3.7). In short, first strand cDNAs were synthesized by reverse transcription using a 35-mer primer PO_1, which is composed of 15 oligodT and 20-mer adaptor. The 3'-end of the specific gene is amplified from above the RT product by PCR using a specific gene primer and the 20-mer adaptor primer.[1]

Mta_1 and mta_2 primers were designed according to the known *er1* sequence (Figure 3.8A). No PCR product was obtained with primer pair T_3 and mta_2, but a 1kb cDNA fragment was obtained with primer pair mta_1 and T_3 (Figure 3.8B), thus, the orientation of the cDNA fragment was determined. It was subsequently cloned into the pCR™ II vector, and sequenced. The 1kb cDNA fragment contains a 508bp overlap with IVB^+ and contains additional sequence at the 5'-end of the *er1* cDNA (Figure 3.8B). Among the three reading frames, there is one completely

[1] PO_2 primer.

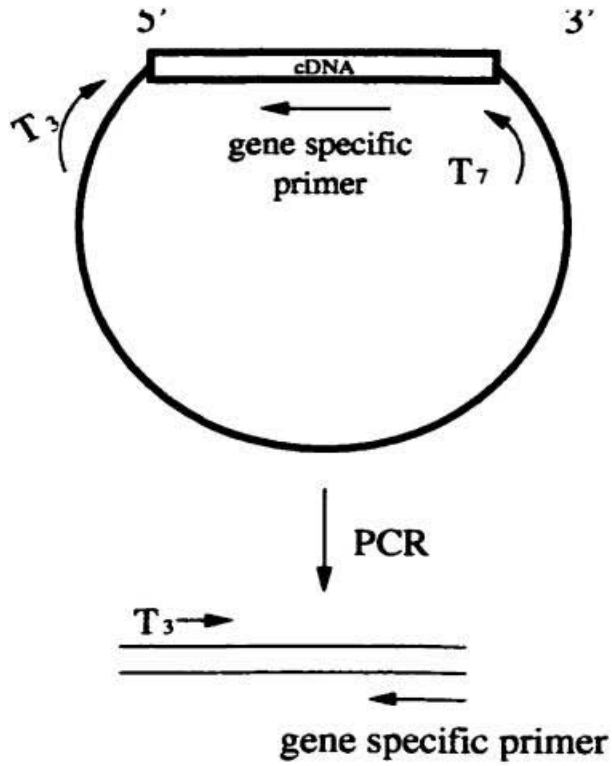


Figure 3.6: **Strategy for cloning the 5'-end of *er1* from stage 8 λ ZapII cDNA library** The vector has two promoters: T_3 and T_7. All the cDNAs in the library are orientated with their 5'-end close to the T_3 promoter and 3'-end close to the T_7 promoter. 5'-end of *er1* was cloned by PCR with the T_3 primer and an *er1* specific primer.

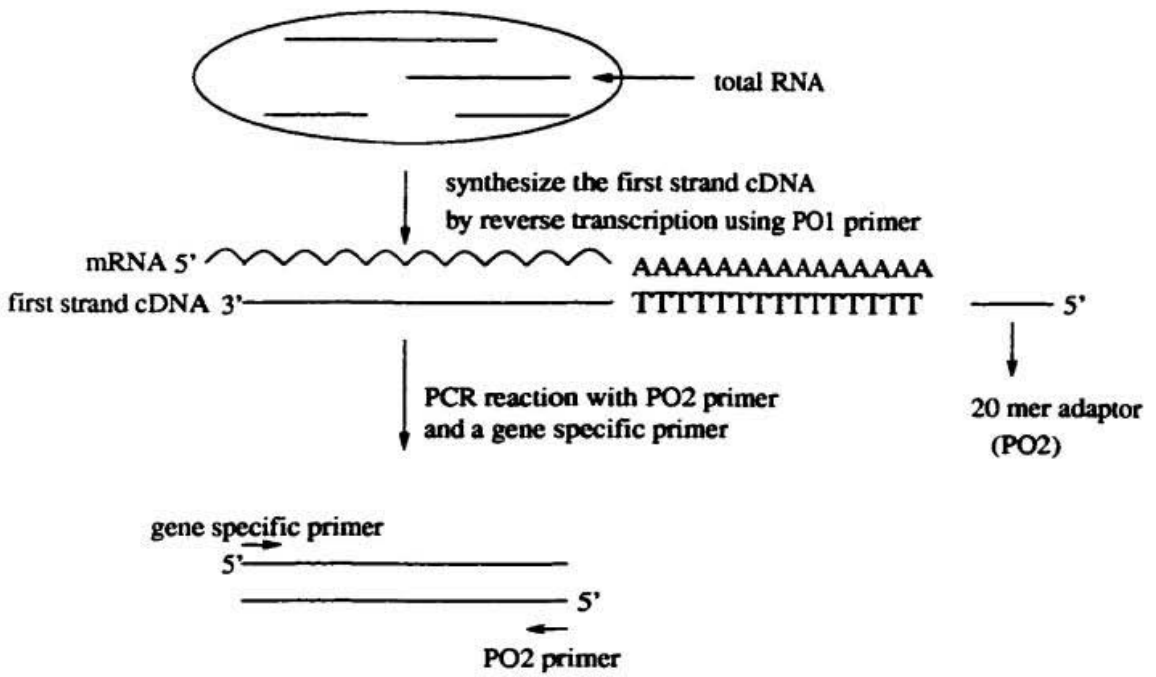


Figure 3.7: **Strategy for cloning of the 3'-end of *er1*** First strand cDNAs were synthesized with the 35-mer primer PO_1, which is composed of 15 oligodT and a 20-mer adaptor PO_2. Thus, all the 3'-end of the cDNAs have the 20-mer adaptor PO_2. The 3'-end of *er1* was cloned by PCR with the primer pair PO_2 and the gene specific primer.

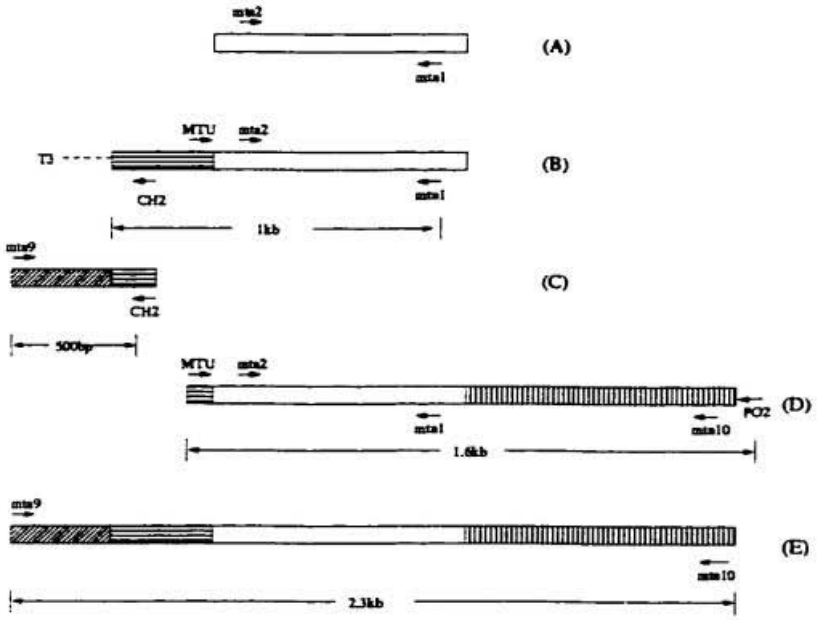


Figure 3.8: **cDNA map of *er1*** (A): Open rectangle represents the sequence of IVB^+. The positions of primer mta_1 and mta_2 are indicated . (B): A 1kb cDNA was cloned by PCR with primer pair T_3 and mat_1. The open rectangle represents the overlap of the 1kb cDNA with IVB^+ cDNA. The 1kb cDNA contains additional sequences at the 5'-end represented by a horizontal rectangle. (C): An additional 500bp cDNA was cloned by PCR with primer pair T_3 and CH_2. It contains 122bp overlap with the 1kb cDNA (horizontal lined rectangle), and additional sequences at the 5'-end (shaded rectangle). (D): A 1.6kb cDNA was cloned by PCR with primer pair MTU and PO_2. It contains overlaps with the previous clones and additional sequences at the 3'-end (vertical lined rectangle). (E): A 2.3kb cDNA was cloned by PCR with primer pair mta_9 and mta_{10}.

open reading frame (ORF). Since an entire ORF usually follows a 5'-untranslated region which usually has stop codons in all three reading frames, it was likely that the entire ORF had, at this point, not been cloned. Thus, the next step is to clone more of the 5'-end sequence of *er1* in order to obtain the entire ORF.

A primer CH_2, which is complementary to the 5'-end sequence of the 1kb cDNA, was designed to clone more of the 5'-end *er1*. An additional 500bp cDNA fragment was obtained by PCR using the $T_3 \sim CH_2$ primer pair (Figure 3.8C). It was cloned into the pCR™ II vector and sequenced. As expected, it contained 122bp overlap with the 1kb DNA fragment. In addition, several 5'-end stop codons were found in all three frames, confirming that the translated 5'-end had been cloned. The 3'-end of *er1* was cloned using the 3'-RACE method with a *er1* gene specific primer MTU and a PO_2 primer (Figure 3.8D). A 1.6kb fragment was obtained, cloned and sequenced. As expected, it contains 644bp overlap with the 1kb fragment, and 528bp overlap with the IVB^+ fragment. The first in frame stop codon TAA was found at the 3'-end of *er1*.

Several 5'-end and 3'-end stop codons were found in all three frames, strongly suggesting the entire coding region of *er1* was cloned. Primers mta_9 and mta_{10}, representing the 5' and 3' ends of the cloned *er1* sequence respectively, were designed to clone the entire cDNA from the stage 8 *Xenopus* cDNA library by PCR(Figure 3.8E). A 2.3 kb cDNA fragment was cloned.

Sequence analysis revealed that among the six possible reading frames, the 2.3kb cDNA contained a 1479bp single open reading frame (frame+2) (Figure 3.9),

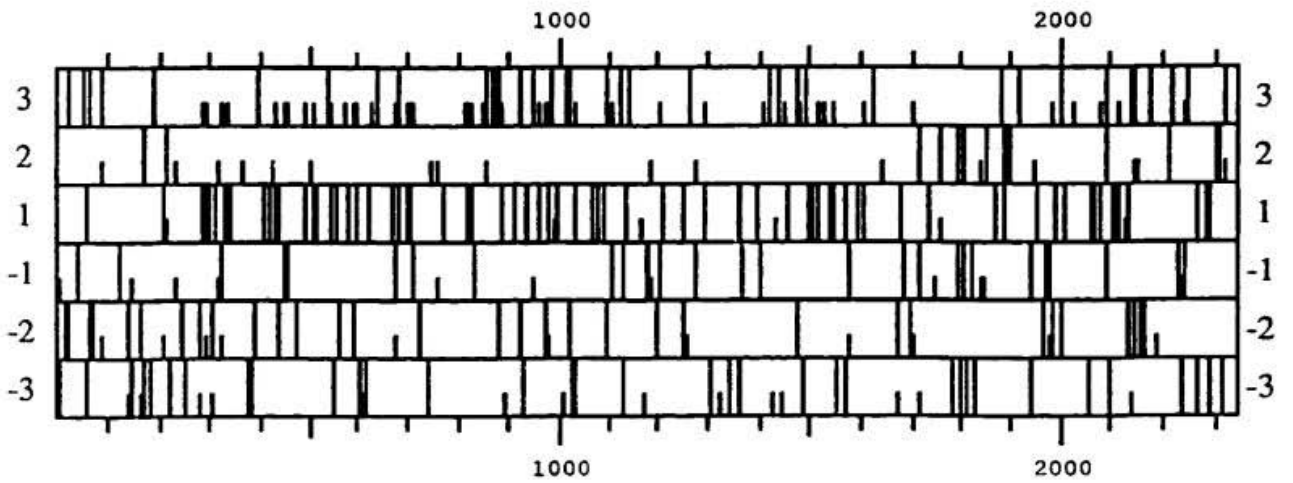


Figure 3.9: **Open reading frame map of *er1*** The nucleotide numbers are shown in the top and the bottom.The stop codons are indicated with complete vertical lines. The methionines are indicated with the incomplete vertical lines. The reading frames are shown on the left and right. It is shown that there are six possible reading frames, and only the frame +2 contains a large open reading frame.

bracketed by a 232bp 5'-untranslated region, and a 629bp 3'-untranslated region. The ATG initiator codon is predicted to be at nucleotides 233-235, as this site is positioned within a Kozak consensus sequence for the start of translation (Kozak,1986), with a purine in the -3 position and a G in the +4 position. The single open reading frame is predicted to encode a protein of 493 amino acids, beginning at nucleotide 233 and ending with an in-frame TAA stop codon at position 1712 (Figure 3.10).

A computer-aided search for motifs within the predicted amino acid sequence using MOTIFS and PSORT software programs revealed that the *er1* protein: ① does not contain an N-terminal signal sequence for transfer into the endoplasmic reticulum, ② does not contain a hydrophobic domain characteristic of transmembrane proteins, ③ contains a putative nuclear localization signal(NLS)KKSERYDFFAQQTRFGKKK (Figuer 3.11), which conforms to the consensus sequence for a bipartite NLS (Robbins *et al.*,1991). This suggests that the *er1* protein is not secreted nor is it a transmembrane protein but instead may be localized to the nucleus. Additional examination of the sequence revealed the presence of a cluster of acidic boxes located in the N-terminal region of the protein (Figure 3.11), a feature characteristic of acidic activation domains present in a number of transcription factors, such as GCN4 and VP16. In addition, there is a proline-rich sequence near the C-terminal regin of *er1* which corresponds to the PXXP motif found in all high affinity SH_3-domain binding ligands (Cohen *et al.*, 1995) (Figure 3.11), suggesting that this protein may also be involved in signal transduction.

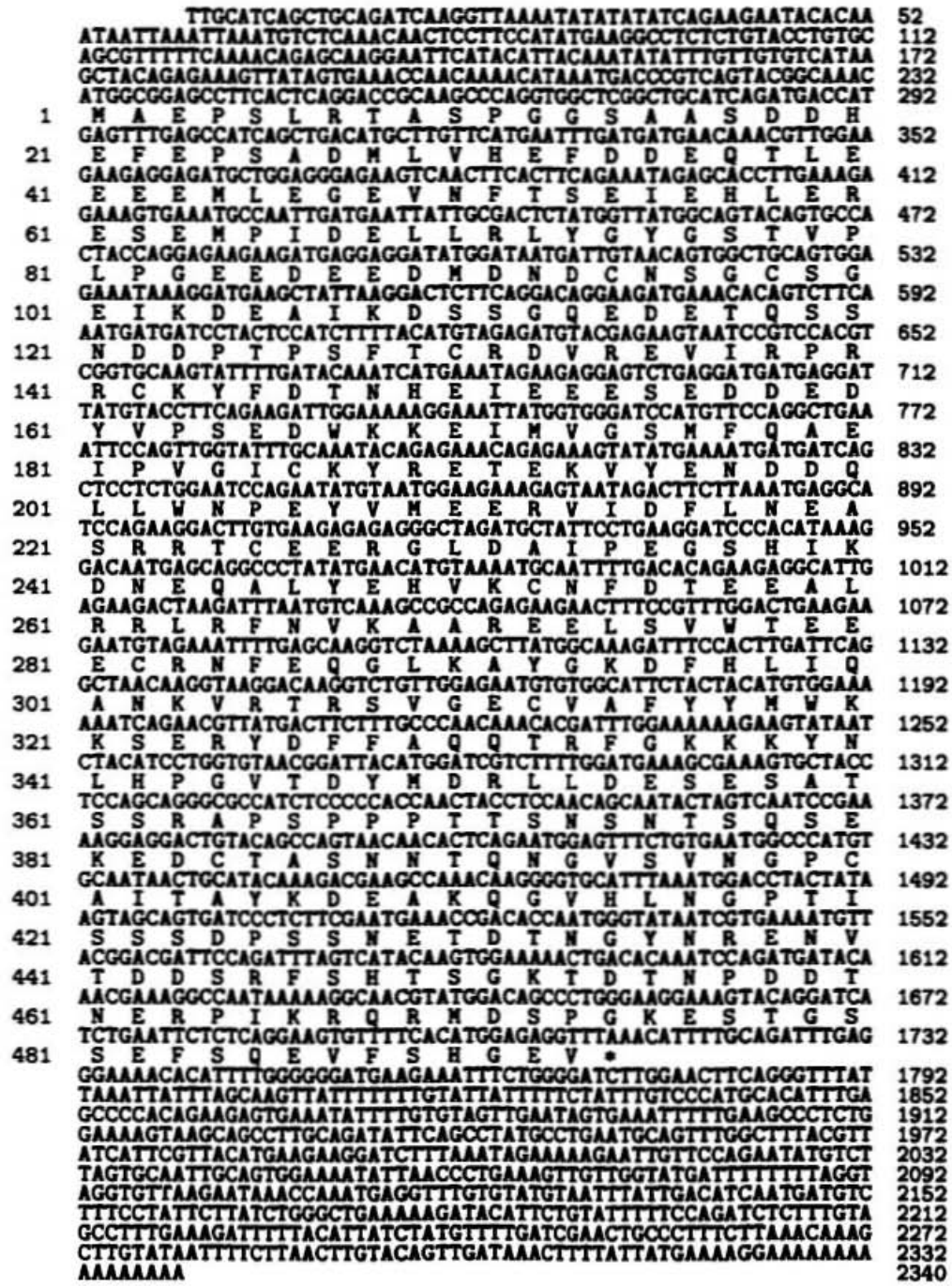


Figure 3.10: **Nucleotide and predicted amino acid sequence of *er1*** The nucleotide sequence numbers of *er1* cDNA are showm on the right and the amino acid sequence numbers of the predicted *er1* protein are shown on the left. The TAA termination codon is indicated by an asterisk.

```
1   M A E P S L R T A S P G G S A A S D D H
21  E F E P S A D M L V H E F D D E Q T L E
41  E E E M L E G E V N F T S E I E H L E R
61  E S E M P I D E L L R L Y G Y G S T V P
81  L P G E E D E E D M D N D C N S G C S G
101 E I K D E A I K D S S G Q E D E T Q S S
121 N D D P T P S F T C R D V R E V I R P R
141 R C K Y F D T N H E I E E E S E D D E D
161 Y V P S E D W K K E I M V G S M F Q A E
181 I P V G I C K Y R E T E K V Y E N D D Q
201 L L W N P E Y V M E E R V I D F L N E A
221 S R R T C E E R G L D A I P E G S H I K
241 D N E Q A L Y E H V K C N F D T E E A L
261 R R L R F N V K A A R E E L S V W T E E
281 E C R N F E Q G L K A Y G K D F H L I Q
301 A N K V R T R S V G E C V A F Y Y M W K
321 K S E R Y D F F A Q Q T R F G K K K Y N
341 L H P G V T D Y M D R L L D E S E S A T
361 S S R A PSPPP T T S N S N T S Q S E
381 K E D C T A S N N T Q N G V S V N G P C
401 A I T A Y K D E A K Q G V H L N G P T I
421 S S S D P S S N E T D T N G Y N R E N V
441 T D D S R F S H T S G K T D T N P D D T
461 N E R P I K R Q R M D S P G K E S T G S
481 S E F S Q E V F S H G E V *
```

Figure 3.11: **Potential functional domains of *er1*** The amino acid sequence numbers of the predicted *er1* protein are shown on the left. Four stretches of predominantly acidic residues are underlined, the proline-rich region is in boldface and the putative nuclear localization signal (NLS) is enclosed in framebox.

3.5 Amino acid comparison between full-length *er1* and known proteins

Amino acid sequence comparison between *er1* and known proteins revealed that *er1* contains three regions of similarity to the product of the rat metastasis-associated gene, *mta*1 (Figure 3.12), a gene that was isolated by differential cDNA library screening and whose expression was associated with a metastatic phenotype (Toh, *et al.*, 1994).

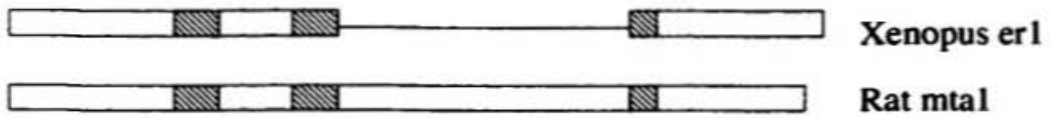
3.6 Northern analysis of the temporal expression of *er1* in the whole embryos

Northern hybridization analysis was subsequently carried out to study the temporal expression of *er1* at different developmental stages. An mRNA of approximately 2.8kb was detected predominantly during cleavage and blastula stages, with a slight increase at blastula stage, and little or no mRNA present during subsequent stages of development (Figure 3.13), as determined by densitometric analysis and normalization to histone mRNA.

3.7 Quantitative RT-PCR analysis of *er1*

Quantitative RT-PCR (Niehrs, *et al*, 1994) was carried out to further verify the differential expression of *er1* between FGF-treated and normal animal caps. EF-1α was used as the loading control. Densitometric analysis revealed that the expression

A



B

```
            168                                                 215
Xenopus er1: KKEIMVGSMFQAEIPVGICKYRETEKVYENDDQLLWNPEYVMEERVID
             K EI VG+ +QA+I   +    E +     +  +W     + ++ ID          45%
rat mta1   : KGEIRVGNRYQADITDLLKDGEEDGRDQSKLETKVWEAHNPLVDKQID
             164                                                 211

             271                                                       325
Xenopus er1: REELSVWTEEECRNFEQGLKAYGKDFHLIQANKVRTRSVGECVAFYYMWKKSERY
             R+E+  W+  E   FE+ L+ YGKDF  IQ + +  +S+   + +YYMWK ++RY  63%
rat mta1   : RDEMEEWSASEANLFEEALEKYGKDFTDIQQDFLPWKSLTSIIEYYYMWKTTDRY
             282                                                       336

             360              379
Xenopus er1: TSSRAPSPPPTTSNSNTSQS
             T  R P P P  S+S+  S                                      50%
rat mta    : THPRPPKPDPVKSSSSVLSS
             543              562
```

Figure 3.12: **Amino acid comparison of *Xenopus er1* to the rat *mta1*** A: Schematic illustrating alignment of the predicted *Xenopus er1* protein sequence with the rat *mta1*. The N-termini were aligned and a gap (line) was introduced in the *Xenopus* protein in order to align the regions of similarity (hatched) identified by the BLAST program. White boxes indicate unique regions. B: Alignment of the predicted *er1* amino acid sequence with the *mta1* amino acid sequence in the regions of similarity illustrated in A. The amino acid sequence numbers of the *er1* protein are shown on the top. The amino acid sequence numbers of the rat *mta1* protein are shown on the bottom. Identities are indicated by the one-letter amino acid code, conservative changes are indicated by plus sign (+) and dashes (-) indicate non-conservative changes. The percentages of the similarity are shown on the right.

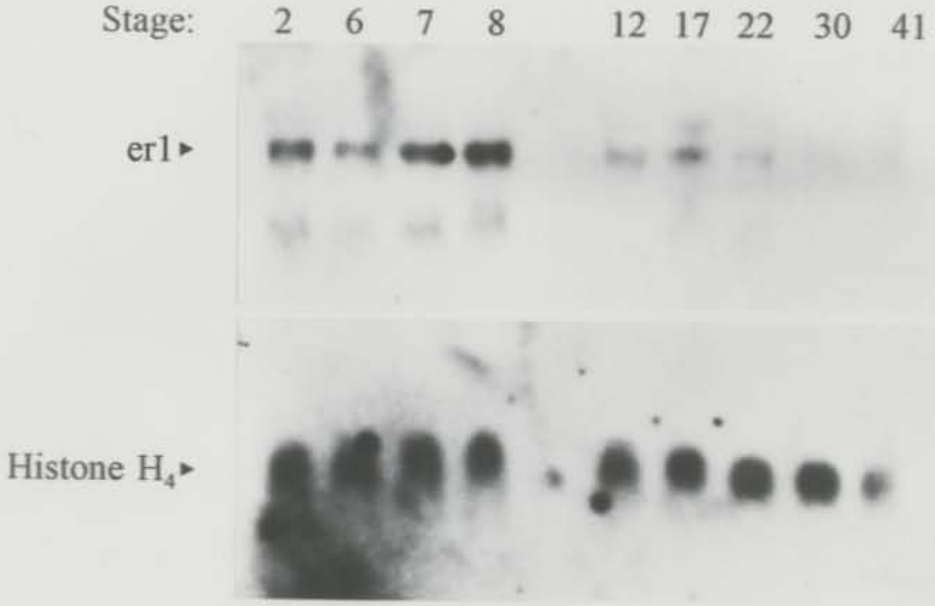

Stage:
2
6
7
8
12
17
22
30
41
er1
Histone H_4

Figure 3.13: **Northern analysis of the temporal expression of *er1* in the whole embryos** Total RNAs were extracted from the following developmental stages: stage 2 (2-cell stage; lane 1), stage 6 (64-cell stage; lane2), stage 7 (early blastula; lane 3), stage8 (mid-blastula; lane4), stage12(mid-gastula; lane5), stage 17 (neurula; lane6), stage 22 (tailbud; lane 7), stage 30 (lane 8), and stage 41 (tadpole; lane 9), and loaded onto a 1.2% denaturing gel for electrophoresis, followed by transfer of the RNAs to the nylon membrane. The blot was probed with ^{32}P–labeled *er1* cDNA, then stripped and reprobed with ^{32}P–labled Histone H_4 cDNA..

level of *er1* in the FGF-treated (half an hour) animal caps is three to four fold higher than in the normal animal caps (Figure 3.14). This data confirmed that *er1* level was increased by treatment with FGF.

3.8 Coupled *in vitro* transcription/translation analysis of *er1*

Using a coupled transcription-translation rabbit reticulolysate system, I demonstrated that *er1* can be translated *in vitro*. A single major translation product of apparent molecular mass of 74kDa was obtained (Figure 3.15). FGFR's translation product was used as a control. FGFR is a previously cloned and characterized gene in this laboratory and its *in vitro* translation protein product is known to be 90kDa.

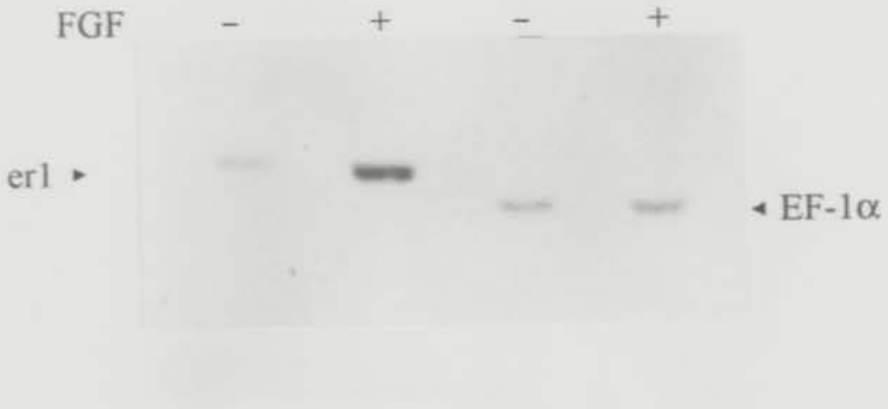

FGF
-
+
-
+
erl ▸
◂ EF-1α

Figure 3.14: **FGF-stimulated increase in steady-state levels of *er1*** Explants (5 per sample) from stage 8 *Xenopus* blastulae were treated for 30 min in the presence (+; lane 2, 4) or absence (-; lane 1, 3) of 100ng/ml XbFGF. Total RNA was extracted and RT-PCR analysis was performed by using *er1* gene specific primer pairs mta_1 and mta_2 (lane 1, 2) or EF-1α gene-specific primer pairs (lane 3,4). PCR products were separated on a 6% polyacrylamide urea gel and visualized by autoradiography. Steady-state levels of *er1* were estimated by densitometry and normalization to EF-1α .

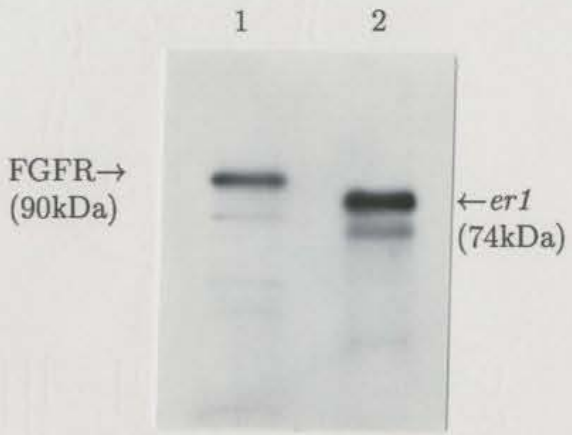


Figure 3.15: **Coupled *in vitro* transcription/translation analysis of *er1*** FGFR protein (lane 1) or *er1* protein (lane2) were synthesized *in vitro* using the TNT^{tm} coupled reticulocyte lysate system with the incorporation of ^{35}S-methionine. Protein products were analyzed by SDS-PAGE, and visualized by autoradiography. The synthesized protein bands are indicated by arrows, and molecular size of the proteins are in brackets.

Chapter 4

Discussion

4.1 *er1* is a novel, potential FGF response gene

FGF has previously been demonstrated to be able to induce animal caps, which normally form ectoderm, to form mesoderm. In this thesis I have used the PCR-based differential display method to isolate cDNAs representing novel genes inducible by FGF in animal caps from *Xenopus* embryos in order to gain insight into the role of FGF during mesoderm induction. A 532bp differentially expressed cDNA fragment was isolated and used to clone a 2.3kb cDNA from a *Xenopus* blastula library (Gillespie, *et al.*, 1995), which I have named *er1*. The *er1* cDNA contains a single open reading frame (ORF) predicted to encode a protein of 493 amino acids. The estimated size of this protein is about 54kDa.

DOP-PCR-Southern and quantitative RT-PCR analysis revealed that *er1* levels ranged from three to four fold higher in FGF-treated sample. These data confirm

that *er1* levels were increased by treatment with FGF. The amino acid sequence of *er1* was found to be unreported in *Xenopus* by database homology search, demonstrating that *er1* is a novel *Xenopus* FGF-response gene.

Using a coupled transcription-translation rabbit reticulolysate system, I demonstrate that *er1* can be translated *in vitro*. A single major translation product of apparent molecular mass of 74 kDa was obtained, which is greater than the estimated size. This discrepancy could be due to the abnormal migration on SDS-PAGE, as has been observed in other proteins. Aberrant mobility on SDS-PAGE is usually linked to conformation resulting from distinct features in the primary structure (Armstrong and Roman, 1992; Traub, *et al.*, 1993). In this case, aberrant electrophoretic mobility may be the function of *er1*'s high content of acidic residues which account for 111 of the 493 residues.

A database search for homology revealed that *er1* contains three regions of similarity to the product of the rat metastasis-associated gene, *mta1* (Toh *et al.*, 1994), a gene that was isolated by screening a differential cDNA library. Subsequently, the human and *C. elegans* homologues have been isolated. [1] The expression level of *mta1* is 4-fold higher in the highly metastatic rat mammary adenocarcinoma cell lines compared to non-metastatic cell lines (Toh *et al.*, 1994). In addition, examination of the expression of the *mta1* gene in human breast cancer cell lines demonstrated that the expression level of this gene is directly related to metastatic potential: the ratio of

[1]The full length human and *C. elegans mta1* cDNAs have not been published yet; I obtained the sequences from the Genbank.

mta1 mRNA among non-metastic, invasive, and metastic cell lines is 1:2:4 (Toh *et al.*, 1995). Within regions of similarity, *er1* shares 45%, 50%, and 63% similarity at the amino acid level, respectively, with three regions of the rat *mta1*, Although the overall percent similarity between *er1* and rat *mta1* was only 13%. Therefore, it is not clear whether *er1* represents the *Xenopus* homologue of *mta1*. More likely, *er1* is a related member of a family of proteins, or simply a protein containing some of the same functional domains.

Those three regions of similarity are interesting since highly conserved regions may represent functional domains of the proteins. FGF mediated signal transduction has previously been demonstrated to influence cellular migration (KLämbt *et al.*, 1992) and tumor cell metastasis (Egan *et al.*, 1995). For instance, cell migration is required during the formation of the tracheal system in Drosophila. Mutants of the *breathless* gene – a Drosophila FGF receptor homologue, result in tracheal cells failing to migrate (KLämbt *et al.*, 1992). In addition, NIH3T3 cells transformed by Ras or Raf , whose activation is necessary for FGF-mediated signal transduction, are capable of forming metastatic tumor cells (Egan *et al.*, 1995).

FGF signalling is also important in gastrulation movements since microinjection of a dominant negative mutant of FGFR into 4-cell stage *Xenopus* embryos causes them to have gastrulation movement defects (Amaya, *et al.*, 1991). The molecular mechanisms for FGF signalling in tracheal cell migration, tumor cell metastasis, or gastrulation movements are not clear. Identification of *er1*, whose expression level is inducible by FGF, and which shares some similarity with a metastasis-associated

protein, may offer an important clue to the above mechanisms. Clearly there is still much work to be done to investigate *er1*'s role in the above processes.

Computer-assisted analysis of the deduced amino acid sequence predicts that the N-terminal region of *er1* includes several highly acidic stretches, characteristic of acidic transactivation domains, and that *er1* also contains a potential nuclear localization signal (NLS). FGF signals ultimately are transmitted to the nucleus, resulting in transcription of a selected group of immediate-early genes. The protein products of these genes are synthesized in the cytoplasm, and many of these proteins are translocated into the nucleus to function as transcription factors involved in activating the expression of other genes. The presence of the putative NLS and acidic transactivation domains in the predicted *er1* protein suggests a potential role as a transcription factor. A proline-rich stretch was also found at the C-terminal end which corresponds to consensus sequence for the SH_3-binding motif (Cohen *et al.*, 1995). Growth factor receptor (GFR) complexes, such as the FGFR complex, EGFR complex, PDGFR complex etc., are formed by recruiting additional proteins to the GFRs, mediated in part by SH_3 domains (see review in Pawson and Schlessinger, 1995). The presence of the putative SH_3- binding motif in the predicted *er1* protein suggests a direct involvement of *er1* in some signal transduction pathways.

4.2 Temporal expression of *er1*

Northern analysis of the temporal pattern of *er1* during embryonic development revealed a single *er1* mRNA. The estimated 2.8-kb size of the message was

slightly larger than that of the cDNA clone. There are two possible explanations for this discrepancy. Either *er1* mRNA has a fairly long poly A-tail (greater than 300bp), or some sequence at the 5'-end of the untranslated region is missing. It may be useful to determine whether I am missing some of the 5'-sequence by carrying out primer extension. However, for further experimentation on the function of this gene, I have all the sequence I need, namely the entire coding region. *er1* transcripts were detected predominantly during cleavage and blastula stages, with a slight increase at blastula stage, and little or no mRNA present during subsequent stages of development. The presence of *er1* mRNA in embryos prior to the start of zygotic transcription, which occurs at mid-blastula transition (Newport and Kirschner, 1982), indicates that *er1* is a maternal message. The expression level of *er1* was highest during blastula stage, coinciding with mesoderm induction *in vivo*.

4.3 Future investigation of *er1*

4.3.1 Is *er1* a immediately-early response gene to FGF?

The expression level of *er1* in the cytoplasm was increased 30 min after addition of FGF, suggesting a transcriptional activation of the gene within 10 min (Rosa, 1989). This demonstrated that the increase in *er1* occurs early during the cellular response to FGF. The possibility that *er1* is a FGF early response gene will be investigated further. By definition, the expression of early response genes are not dependent upon protein synthesis, and so are not inhibited by the protein synthesis inhibitor cyclohexmide. Therefore, whether *er1* is an early response gene will be de-

termined by comparing the induction levels of *er1* when the animal caps are induced by FGF in the presence and absence of cycloheximide.

4.3.2 Is *er1* expression necessary for mesoderm induction?

Northern results revealed that *er1* was expressed during mesoderm induction and gastrulation stages, with the highest expression level during the blastula stage, suggesting a possible relationship between *er1* expression and mesoderm induction. Besides temporal expression, spatial expression also usually coincides with a gene's endogenous function. For instance, *siamois*, a *Xenopus* homeobox gene, is expressed in the marginal zone and vegetal pole, but not in the animal pole (Lamaire *et al.*, 1995). Furthermore, in situ hybridizatiion results revealed that it is mainly expressed in the dorsal-vegetal cells of the stage 10 *Xenopus* embryos. These correspond with its endogenous function-to be able to induce a complete secondary axis (Lemaire *et al.*,1995). Therefore, it is helpful to study *er1*'s spatial expression pattern, namely, to compare *er1*'s mRNA level among animal pole, marginal zone, and vegetal pole; and to detect *er1*'s spatial expression by performing in situ hybridization.

Animal caps can be induced to form mesoderm by both FGF and activin as well as by endogenous signals emitted from the vegetal poles. Some previously identified mesodermal genes , such as Xbra, can be induced by all three types of inducers (Smith *et al.*,1991). To date, genes induced by both FGF and activin, such as Xnot (Von Dassow *et al.*, 1993), or solely induced by activin , such as *goosecoid* (De Robertis *et al.*), have been identified. However, no genes have been identified that are induced by FGF alone. Further experiments will be carried out to investigate whether *er1*

can be induced by activin or the vegetal pole cells. The results may help us to understand more about the relationship among the three types of inducers.

4.3.3 Prospective investigation of *er1*'s endogenous function

mRNAs can be translated faithfully *in vivo* by microinjecting them into 2-cell or 4-cell *Xenopus* embryos. Some genes' functions have been identified by microinjecting their mRNAs into the *Xenopus* embryos at 2-cell or 4-cell stages. For instance, microinjection of *goosecoid* mRNA into two ventral blastomeres of 4-cell embryos results in the formation of the secondary axis, which mimics the properties of Spemann's organizer (De Robertis *et al.*, 1992). This indicates that *goosecoid* may play an important role in organizer activity. Therefore, *er1*'s endogenous function can be studied by this method.

When embryos are injected with bFGF or kFGF at the 2-cell stage, followed by cutting the animal caps at blastula stage, the animal caps undergo elongation, and cardiac actin is activated , even when the animal caps are only cultured in salt solution (Thompson and Slack, 1992). Mesoderm induction is concentration-dependent. Recently, it has been shown that over expression of Xbra, a FGF response gene, in the animal pole causes mesoderm formation, with different types of mesoderm being formed in response to different concentration of Xbra (O'Reilly *et al.*, 1995). Thus, it will be helpful to determine whether animal caps from *er1* mRNA injected embryos undergo morphological changes and gene activation, as well as concentration-dependent mesodermal cell differentiation.

Blocking FGF intracellular signal transduction should effectively inhibit the expression of FGF response genes. For instance, microinjection of dominant negative mutant of FGFR (XFD) inhibits the Xbra expression (Schulte-Merker and Smith, 1995). Therefore, we will investigate whether the same happens to *er1* when XFD mRNA is injected into *Xenopus* embryos. Isaacs and his colleagues have shown that gastrulation movements on the dorsal side of the embryos are more dependent on FGF than the ventral side of the embryos (Isaacs *et al.*,1994). This result seems to be contradictory to the traditional view that FGF induces ventral mesoderm, and activin induces dorsal mesoderm. Injection of *er1* into the equatorial region of the two ventral blastomeres or to the two dorsal blastomeres of the embryos at 4-cell stage will help to determine *er1*'s function in dorsal and ventral mesoderm differentiation.

As mentioned earlier, the predicted *er1* protein contains several putative functional domains. Functional domains usually play very important roles in genes' function. Microinjection of the mRNA transcribed from the constructed cDNAs, whose functional domains are deleted, into the *Xenopus* embryos usually results in normal development disruption. For instance, a mutant cDNA of Xbra was constructed by replacement of the transcriptional activation domains, located at the C-terminal regions of Xbra, with the repressor domain of the Drosophila engrailed protein (Schulte-merker and Smith,1995). Microinjection of mRNA transcribed from this chimeric cDNA generated *Xenopus* embryos with posterior mesoderm and axis development defects. Thus, for further investigation of *er1*'s endogenous function, It will be important to construct a series of functional domain deleted *er1* cDNAs,

inject their RNAs to the 2 or 4-cell stages and study the development of these embryos.

In summary, I have cloned a cDNA encoding a novel *Xenopus* FGF response gene with the RT-PCR based differential display method. This is the first time that this method has been used to clone mesodermal response gene. Our results demonstrate that the differential display is a powerful strategy to isolate genes which are inducible by FGF or other inducers.

References

1. Amaya, E., Musci, T. J. and Kirschner, M. W. (1991). *Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos.* Cell 66, 257-270.

2. Amaya, E., Stein, P. A., Musci, T. J. and Kirschner, M. W. (1993). *FGF signalling in the early specification of mesoderm in Xenopus.* Development 118, 477-487.

3. Armelin, H. (1973). *Pituitary Extracts and Steroid Hormones in the Control of 3T3 Cell Growth.* Proc. Natl. Acad. Sci. U.S.A. 70, 2702-2706.

4. Armstrong DJ. Roman A. (1992). *Mutagenesis of human papillomavirus types 6 and 16 E7 open reading frames alters the electrophoretic mobility of the expressed proteins.* Journal of General Virology. 73, 1275-1279.

5. Burgess, W.H., Dionne, C. A., Kaplow, J., Mudd, R., Friesel, R., Zilberstein, A., Schlessinger, J. and Jaye, M. (1990). *Characterization and cDNA cloning of phospholipase C-γ, a major substitute for heparin—binding growth factor 1 (acidis fibroblast growth factor)-activated tyrosine kinase.* Molecular and Cellular Biology 10, 4770-4777.

6. Cardellini, P., Polo, C. and Coral, S. (1994). *Suramin and herapin: aspecific inhibitors of mesoderm induction in the Xenopus Laevis embryo.* Mechanism of Development 45, 73-87.

7. Cho, K. W. Y and De Robertis, E. M. (1990). *Differential activation of Xenopus homeobox genes by mesoderm-inducing growth factors and retinoic acid.* Genes and Development 4, 1910-1916.

8. Cho, K. W. Y., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). *Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid.* Cell 67, 1111-1120.

9. Cohen GB. Ren R. and Baltimore D. (1995). *Modular binding domains in signal transduction proteins. Review* Cell 80, 237-248.

10. Cooke, J. (1989). *Mesoderm-inducing factors and Spemann's organizer phenomenon in amphibian development.* Development 107, 229-241.

11. Cornell, R. A. and Kimelman, D. (1994). *Activin mediated mesoderm induction requires FGF.* Development 120, 453-462.

12. Dale, L. and Slack, J. M. W. (1987a). *Fate map for the 32-cell stage of Xenopus Laevis.* Development 99, 527-551.

13. Dale, L. and Slack, J. M. W. (1987b). *Regional specification within the mesoderm of early embryos of Xenopus Laevis.* Development 100, 279-295.

14. Dale, L., Howes, G., Price, B. M. J. and Smith, J. C. (1992). *Bone morphogenetic protein 4: a ventralizing factor in early Xenopus development.* Development 115, 573-585.

15. Dawid, I. B., Taira, M., Good, P. J. and Rebagliati, M. R. (1992). *The role of grwoth factors in embryonic induction in Xenopus Laevis.* Molecular Reproduction and Development 32, 136-144.

16. Delli Bovi, P. and Basilico C. (1987a). *Isolation of a rearranged human transforming gene following transfection of Kaposi's Sarcoma DNA.* Proc. Natl. Acad. Sci. U.S.A. 84, 5660-5664.

17. Delli Bovi, P., Curatola, A. M., Kern, F. G., Greco, A., Ittmann, M. and Basilico, C. (1987b). *An oncogene isolated by transfection of Kaposi's Sarcoma DNA encodes a growth factor that is a member of the FGF family.* Cell 50, 729-737.

18. De Robertis, E. M., Blum, M., Niehrs, C. and Steinbeisser, H. (1992). *Goosecoid and the organizer.* Development 1992 Supplement, 167–171.

19. Dickson, C. and Peters, G. (1987). *Potential oncogene product related to growth factors.* Nature 326, 833.

20. Egan, S. E., Wright, J. A., Jarolim, L., Yanaglihara, K., Bassin, R. H. and Greenberg, A. H. (1995). *Transformation by oncogenes encoding protein kinases induces the metastasis phenotype.* Science 238, 202–205.

21. Finch, P. W., Rubin, J. S., Miki, T., Ron, D. and Aaronson, S. A. (1989). *Human KGF is FGF-related with properties of a para crine effector of epithelial cell growth.* Science 245, 752-755.

22. Friesel, R. and Dawid, I. B. (1991). *cDNA cloning and developmental expression of fibroblast growth factor receptors from Xenopus Laevis.* Molecular and Cellular Biology 11, 2481-2488.

23. Friesel, R. E. and Maciag, T. (1995). *Molecular Mechanism of angiogenesis: fibroblast growth factor signal transduction.* The FASEB Journal 9, 919-925.

24. Frohman, M. (1990). *PCR protocols, a guide to methods and applications* Academic Press Inc. 28-38.

25. Gerhart, J., Danilchik, M., Doniach, T., Roberts, S., Rowning, B. and Stewart, R. (1989). *Cortical rotation of the Xenopus egg: consequences for the anteroposterior pattern of embryonic dorsal development.* Development Supplement 37-51

26. Gillespie, L. L., Paterno, G. D. and Slack, J. M. W. (1989). *Analysis of competence: receptors for fibroblast growth factor in early Xenopus embryos.* Development 106, 203-208.

27. Gillespie, L. L., Paterno, G. D., Mahadevan, L. C. and Slack, J. M. W. (1992). *Intracellular signalling pathway involved in mesoderm induction by FGF.* Mechanism of Development 38, 99-108.

28. Gillespie, L. L., Chen, G. and Paterno, G. D. (1995). *Cloning of a fibroblast growth factor receptor 1 splice variant from Xenopus embryos that lacks a protein kinase C site important for the regulation of receptor activity.* The Journal of Biological Chemistry 270, 22758-22763.

29. Gospodarowicz, D. (1974a). *Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth.* Nature 249, 123-127.

30. Gospodarowicz, D. and Moran, J. (1974b). *Effect of a fibroblast growth factor, insulin, dexamethasone, and serum on the morphology of BALB/C 3T3 cells.* Proc. Natl . Acad. Sci. U. S. A. 71, 4648-4652.

31. Gospodarowicz, D., Weseman, F., Moran, J. (1975). *Presence in the brain of a mitogenic agent distinct from fibroblast growth factor that promotes the proliferation of myoblasts in low density culture.* Nature 256, 216-219.

32. Gospodarowicz, D., Neufeld, G. and Schwoigerer, L. (1986). *Fibroblast growth factor.* Molecular and Cellular Endocrinol 46, 187-192.

33. Gospodarowicz, D., Ferrara, N., Schweigerer and Neufeld, G. (1987). *Structrual characterization and biological functions of fibroblast growth factor.* Endocrine Reviews 8, 95-114.

34. Green, J. B. A., New, H. V. and Smith, J. C. (1992). *Response of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm.* Cell 71, 731-739.

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

36. Howard, J. E. and Smith, J. C. (1993). *Analysis of gastrulation: different types of gastrulation movements are induced by different mesoderm-inducing factors in Xenopus Laevis.* Mechanisms of Development 43, 37-48.

37. Issacs, H. V., Tannahill, D. and Slcak, J. M. W. (1992). *Expression of a novel FGF in the Xenopus embryo: a new candidate inducing factor for mesoderm formation and antero-posterior specification.* Development 114, 711-720.

38. Issacs, H. V., Pownall, M. E. and Slack, J. M. W. (1994). *eFGF regulates xbra expression during Xenopus gastrulation.* The EMBO Journal 13, 4469-4481.

39. Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. E. and Hogan, B. L. M. (1992). *DVR-4 (Bone Morphogenetic Protein-4) as a posterior-ventralizing factor in Xenopus mesoderm induction.* Development 115, 639-647.

40. Jones, E. A. and Woodland, H. R. (1986). *Development of the ectoderm in Xenopus: tissue specification and the role of cell association and division.* Cell 44, 345-355.

41. Keller, R. and Danilchik, M. (1988). *Regional expression, pattern and timing of convergence and extension during gastrulation of Xenopus Laevis.* Development 103, 193-209.

42. Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J. and Rhee, S. G. (1991). *PDGF stimulation of inositol phospholipid hydrolysis requires PLC-γ1 phophorylation on tyrosine residues 783 and 1254.* Cell 65, 435-441.

43. Kimelman, D. and Kirchner, M. (1987). *Synergistic induction of mesoderm by FGF and TGF-β and the identification of an mRNA coding for FGF in the early Xenopus embryo.* Cell 51, 869-877.

44. Kimelman, D., Abraham, J. A., Haaparanta, T., Palisi, T. M. and Kirchner, M. W. (1988). *The presence of fibroblast growth factor in the frog egg: its role as a natrual mesoderm inducer.* Science 242, 1053-1056.

45. Klämbt, C., Glaser, L. and Shilo, B. Z. (1992). *Breathless, a Drosophila FGF receptor homology, is essential for migration of tracheal and specific midline glial cells.* Genes & Development 6, 1668–1678.

46. Klint, P., Kanda, S. and Claesson-Welsh, L. (1995). *Shc and a novel 89-kDa component couple to the Grb2-SOS complex in fibroblast growth factor-2-stimulated cells.* The Journal of Biological Chemistry 270, 23337-23344.

47. Kozak, M. (1987). *An analysis of 5'-noncoding sequeces from 699 vertebrate messenger RNAs.* Nucleic Acids Res. 15: 8125–8148.

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

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

50. Lemaire P. Garrett N. Gurdon JB. (1995). *Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary — axis.* Cell 81, 85-94.

51. Lee, S. W., Tomasetto, C. and Sager, R. (1991). *Positive selection of candidate tumor-suppressor genes by substractive hybridization.* Proc. Natl. Acad. Sci.

U.S.A. 88, 2845–2829.

52. Lemmon, S. K., Rielly, M. C., Thomas, K. A., Hoover, G. A., Maciag, T. and Bradshaw, R. (1982). *Bovine fibroblast growth factor: comparison of brain and pituitary preparations.* Journal of Cell Biology 95, 162-169.

53. Liang, P. and Pardee, A. B. (1992). *Differential display of Eukaryotic messenger RNA by means of the polymerase chain reaction.* Science 257, 967-971.

54. Maciag, R., Cerundolo, J., Isley, S., Kelley, P. R. and Forand, R. (1979). *Identification and partial characterization.* Proc. Natl. Acad. Sci. U. S. A. 76, 5674-5678.

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

56. March, C. J., Mosley, B., Larsen, A., Cerretti, D. P., Braedt, G., Price, V., Gillis, S., Henney, C. S., Kronheim, S. R., Grabstein, K., Conlon, P. J., Hopp, T. P. and Cosman, D. (1985). *Sequence and expression of two distinct human interleukin-1 complementary DNAs.* Nature 315, 641-647.

57. Marics, I., Adelaide, J., Raybaud, F., Mattei, M., Coulier, F., Planche, J., de Lapeyriere, O. and Birnbaum, D. (1989). *Characterization of the HST-related FGF.6 gene, a new member of the fibroblast growth factor gene family.* Oncogene 4, 335-340.

58. Mitani, S. (1989). *Retarded gastrulation and altered subsequent development of neural tissues in heparin-injected Xenopus embryos.* Development 107, 423-435.

59. Miyamoto, M., Naruo, K., Seko, C., Matsumoto, S., Kondo, T. and Kurokawa, T. (1993). *Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property.* Molecular and Cellular Biology 13, 4251-4259.

60. Moore, R., Casey, G., Brookes, S., Dixon. M., Peters, G. and Dickson, C. (1986). *Sequence, topography and protein coding potential of mouse int-2: a putative oncogene activated by mouse mammary tumor virus.* The EMBO Journal 5, 919-924.

61. Musci, T. J., Amaya, E. and Kirchner, M. W. (1990). *Regulation of the fibroblast growth factor receptor in early Xenopus embryos.* Proc. Natl. Acad. Sci. U. S. A. 87, 8365-8369.

62. Muslin, A. J., Peters, K. G. and Williams, L. (1994). *Direct activation of phospholipase C-γ by fibroblast growth factor receptor is not required for mesoderm induction in Xenopus animal caps.* Molecular and Cellular Biology 14, 3006-3012.

63. Niehrs C. Steinbeisser H. De Robertis EM. 1994 *Mesodermal patterning by a gradient of the vertebrate homeobox gene goosecoid.* Science 263, 817-20.

item Newport J. Kirschner M. (1982). *A major developmental transition in early Xenopus embryos: I. characterization and timing of cellular changes at*

the midblastula stage. Cell 30, 675-86.

64. Nieuwkoop, P. D. (1969). *The formation of mesoderm in Urodelean amphibians. I. Induction by endoderm.* Wilhelm Roux' Arch. Entw. Mech. Org. 162, 341-373.

65. Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus Laevis.* (Daudin) Amsterdam: North-Holland.

66. Paterno, G. D., Gillespie, L. L., Dixon, M. S., Slack, J. M. W. and Heath, J. K. (1989). *Mesoderm -inducing properties of int-2 and kFGF: two oncogene-encoded grwoth factors related to FGF.* Development 106, 79-83.

67. Pawson, P. (1995). *Protein modules and signalling networks.* Nature 373, 573-580.

68. Robinson, C. J. (1991). *Multiple receptor found for the growing FGF family.* TIPS 12, 123-124.

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

70. Ruiz i Albata, A. and Melton, D. A. (1989a). *Bimodaland graded expression of the Xenopus homeobox gene Xhox3 during embryonic development.* Development 106, 173-183.

71. Ruiz i Albata, A. and Melton, D. A. (1989b). *Involvement of the Xenopus homeobox gene Xhox3 in pattern formation along the antero-posterior axis.*

Cell 57, 317-326.

72. Ryan PJ. Gillespie LL. (1995). *Phosphorylation of phospholipase C gamma 1 and its association with the FGF receptor is developmentally regulated and occurs during mesoderm — induction in Xenopus laevis.* Developmental Biology 166,101-11.

73. Shiurba, R. A., Jing, N., Sakakura, T. and Godsave, S. F. (1991). *Nuclear translocation of fibroblast growth factor during Xenopus mesoderm induction.* Development 113, 487-493.

74. Slack, J. M. W., Darlington, B. G., Heath, J. K. and Godsave, S. F. (1987). *Mesoderm induction in early Xenopus embryos by heparin-binding growth factors.* Nature 326, 197-200.

75. Slack, J. M. W. and Isaacs, H. V. (1989). *Presence of basic fibroblast growth factor in the early Xenopus embryo.* Development 105, 147-153.

76. Smith, J. C. (1987). *A mesoderm-inducing factor is produced by a Xenopus cell line.* Development 99, 3-14.

77. Smith, J. C. (1989). *Mesoderm induction and mesoderm-inducing factors in early amphibian development.* Development 105, 665-677.

78. Smith, J. C., Price, B. M., Green, J. B. A., Weigel, D. and Herrmann, B. G. (1991). *Expression of a Xenopus homology of Brachyury(T) is an immediate-early response to mesoderm induction.* Cell 67, 79-87.

79. Smith, J. C. and Howard, J. E. (1992). *Mesoderm-inducing factors and the control of gastrulation.* Development Supplement, 127-136.

80. Smith, J. C. and Harland, R. M. (1992). *Expression on cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos.* Cell 70, 829-840.

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

82. Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994). *A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo.* Proc. Natl. Acad. Sci. U. S. A. 91, 10255-10259.

83. Tanaka, A., Miyamoto, K., Minamino, N., Takeda, M., Sato, B., Matsuo, H. and Matsumoto, K. (1992). *Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells.* Proc. Natl. Acad. Sci. U. S. A. 89, 8928-8923.

84. Thompson, J. and Slack, J. M. W. (1992). *Over-expression of fibroblast growth factors in Xenopus embryos.* Development 38, 175-182.

85. Toh, Y., Pencil, S. D. and Nicolson, G. L. (1994). *A novel candidate metastasis-associated gene, mta1, differentially expressed in highly metastatic mammary adenocarcinoma cell lines.* Journal of Biological Chemistry 269, 22958-22963.

86. Umbhauer, M., Marshall, C. J., Mason, C. S., Old, R. W. and Smith, J. C. (1995). *Mesoderm induction by Xenopus caused by activation of Map kinase.* Nature 376, 58-62.

87. Van den Eijnden-Van Raaij, A. J. M., Van Zoelent, E. J. J., Van Nimment, K., Koster, C. H., Snoek, G. T., Durston, A. J. and Huylebroeck, D. (1990). *Activin-like factor from a Xenopus Laevis cell line responsible for mesoderm induction.* Nature 345, 732-734.

88. Von Dassow, G., Schmidt, J. E. and Kimelman, D. (1993). *Induction of the Xenopus organizer: expression and regulation of Xnot, a novel FGF and activin-regulated homeo box gene.* Genes and Development 7, 355-366.

89. Wylie, C. C., Brown Derek, Godsave, S. F., Quarmby, J. and Heasman Janet (1985). *The cytoskeleton of Xenopus oocytes and its role in development.* J. Embryol. Exp. Morph. 89, Supplement, 1-15.

90. Zhan, X., Culpepper, A., Reddy, M., Loveless, J. and Goldfarb, M. (1987). *Human oncogenes detected by a defined medium culture assay.* Oncogene 1, 369-376.

91. Zhan, X., Plourde, C., Hu, X., Friesel, R. and Maciag, T. (1994). *Association of fibroblast growth factor receptor-1 with c-Src correlates with association between c-Src and Cortactin.* Journal of Biological Chemistry 269, 20221-20224.