THE CHARACTERIZATION OF NOVEL FIBROBLAST GROWTH FACTOR RESPONSE GENES IN *Xenopus Laevis*

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The Characterization of Novel Fibroblast Growth Factor Response Genes in *Xenopus Laevis*

By

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A Dissertation submitted to the School of Graduate Studies in partial fulfillment of the requirement for the degree of Master of Science.

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Abstract

In a search for fibroblast growth factor (FGF)-regulated early response genes, four cDNA fragments were isolated via a polymerase chain reaction-based differential display procedure. The cDNA fragments represent portions of genes that were either activated or repressed in Stage 8 *Xenopus laevis* explants after a 30 minute treatment with 100ng/ml XFGF-2. One 498bp cDNA fragment, named Band V, represents a gene that is downregulated by FGF treatment. This gene is expressed at Stages 8, 9 and 15 in the developing *Xenopus* embryo. A Genbank homology search program revealed similarity of Band V to distinct regions in an alternatively spliced myocyte enhancer factor, the v-j-c region of a T-cell receptor gamma chain, a Meprin A beta-subunit precursor and a myosin I heavy chain-like protein. The three other cDNA bands isolated from the differential display were chosen due to apparent upregulation by FGF. The 449bp Band VIA/B cDNA fragment possesses regions of similarity to the reactive centre of murine serine proteinase inhibitor 2.4 and a contrapsin-related MC-7 precursor. Upregulation of Band VIA/B was not confirmed because gene expression could not be detected via PCR in *Xenopus* explants or throughout development. The third Band 11/12, is a 636bp cDNA fragment whose gene is only expressed at Stage 8 of the developing embryo and was upregulated by FGF-2, in a PCR reaction. A database homology search showed that Band 11/12 was similar to a hypothetical protein. A second, 190bp DNA fragment isolated from Band 12 was a portion of the *c-mos* proto-oncogene. The fourth and final cDNA Band 22 is 517bp in size and represents a previously cloned *Xenopus* 146kDa nuclear splicing factor (Schmidt-Zachmann *et al.*, 1998). This factor is expressed throughout *Xenopus* development, and control of Band 22 gene expression by FGF-2 could not be confirmed.

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List of Abbreviations

AFGF	acidic FGF
AIGF	androgen induced FGF
aMEF-2	alternatively spliced MEF-2
AP1	activator protein 1
BEK	bacterially expressed kinase
BFGF	basic FGF
BHP	bovine hypothetical protein
BMP	bone morphogenic protein
CDK	cyclin dependent kinase
cDNA	complementary DNA
CEK-2	chicken embryo kinse-2
CHR	chemosensory receptor
CKII	casein kinase II
DAG	diacyl glycerol
EGF	epidermal growth factor
ER1	early response 1
ESR	egg sperm receptor precursor
FGF	fibroblast growth factor
FGFR	FGF receptor
FHF	FGF homologous factor
FNK	FGF inducible kinase
GAF	glial activating factor
GSK 3	glycogen synthase kinase 3
H4	histone 4
HI	hypothetical protein HI0867
HP	hypothetical 19.7kDa protein
HST-1	human stomach tumor –1
IG	immunoglobulin
IP_3	phosphatidylinositol
LB	lauria broth
MADS	MCM1-Arg80-agamous-deficens-SRF
MAM	meprin, A-S protein and receptor protein-tyrosine phosphatase micron
MAP	meprin A-β subunit precursor
MAPK	mitogen activated protein kinase
MEF-2	myocyte enhancer factor
MI	mesoderm induction
MIF	mesoderm induction factor
MIHC	myocin I-like heavy chain
MMLV	murine mammary leukemia virus
MMTV	mouse mammary tumor virus
NAM	normal amphibian media
NAP	NCK-associated protein
NDH	NADH dehydrogenase
NP	nuclear protein
ORF	open reading frame
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3'K	phosphoinositide 3' kinase
PIP_3	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLCγ1	phospholipase C gamma1
RNAse	ribonuclease
RSRFC9	related to SRF C9
RT	reverse transcription
SA-PMP	streptavidin coupled paramagnetic particles
SH2	Src homology 2
SMAD2	mothers against *decapentaplegic 2*
SO	Spemann organizer
SOS	son of sevenless
SPI 2.4	serine proteinase inhibitor 2.4
SRE	serum response element
TCR	T-cell receptor
TGF-β	transforming growth factor – β
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
v-j-c	variable-joining-constant
XBRA	*Xenopus* brachyury
XDSH	*Xenopus* disheveled
XeFGF	*Xenopus* embryonic FGF
XFD	dominant-negative *Xenopus* FGF receptor
XFGF-2	*Xenopus* FGF-2
XFZ	*Xenopus* frizzled receptor

Chapter 1

Introduction

1.1 Growth Factors in Development and Oncogenesis

Growth factors are small polypeptide molecules, essential for normal growth and development of living organisms. They direct proliferation and differentiation of cells and are necessary for the survival of specific tissues. Because growth factors are so important for growth and development, lack of control of these molecules may be detrimental to the organism. Many of the genes activated by growth factors are oncogenes (Reviewed by Aaronson, 1991). Therefore, by learning about the roles growth factors play in normal development, we may learn about their potential role in the pathology of diseases such as cancer.

There are a number of growth factor families that have been characterized to date. They include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) and fibroblast growth factor (FGF). All growth factor families are important, however this study is focussed on the role of the FGF family in development.

FGFs are involved in growth and developmental events such as angiogenesis, cell proliferation and differentiation and are expressed in a number of cancers such as breast carcinoma and Kaposi's sarcoma (reviewed in Baird and Klagsbrun, 1991). The current knowledge of FGFs will be reviewed in the next section.

1.2 Fibroblast Growth Factors

Fibroblast growth factors compose a family of polypeptide molecules that possess amino acid similarity, ability to bind FGF receptors (FGFR) and a heparin- binding domain. There are 11 known FGFs to date (Table 1.1). Six other FGF similarity genes have been cloned, however their specific functions are still unknown. Various roles for FGF in normal processes have been delineated, including angiogenesis, wound repair, nervous cell differentiation, and embryonic development. They also play a role in cell proliferation, motility, differentiation and survival. Several FGF family members are also proto-oncogenes, and play a role in proliferation of cancer cells (reviewed in Baird and Klagsbrun, 1991).

The most well characterized members of the FGF family are acidic FGF (aFGF, FGF-1) and basic FGF (bFGF, FGF-2). FGF-1 and FGF-2 possess 55% amino acid similarity and bind to the same FGF receptors, however, they are expressed primarily in different tissues (Baird and Klagsbrun, 1991).

Table 1.1 Members of the FGF Family

Numerical Name	Common Name
FGF-1	aFGF
FGF-2	bFGF
FGF-3	INT-2
FGF-4	kFGF, HST-1
FGF-5	---
FGF-6	HST-2
FGF-7	KGF
FGF-8	AIGF
FGF-9	GAF
XeFGF	---
FGF-10	KGF-2
FGF-11	FHF-3
FGF-12	FHF-1
FGF-13	FHF-2
FGF-14	FHF-4
FGF-15	---

FGF-1 was discovered by Gospodarowicz and colleagues, (1975) on the basis of its ability to cause proliferation and delayed differentiation in myoblasts. It was later shown to stimulate proliferation in endothelial cells. FGF-1 is expressed primarily in the adult brain and retina (reviewed by Gospodarowicz *et al.* 1987).

FGF-2 was discovered in 1974, also by Gospodarowicz, due to its ability to cause proliferation and transformation of BALB/c3T3 cells. It is also very well conserved throughout evolution, and widely expressed in many tissues, suggesting a vital physiological role (Gospodarowicz, 1987). Interestingly, neither FGF-1 nor FGF-2 possesses a conventional signal sequence for release from the cell, however they have both been isolated within the extracellular space (Baird and Klagsbrun, 1991). Currently, their method of secretion remains unknown. Since FGF-2 is the FGF used in this study, more about its role in development will be covered in following sections.

FGF-3 is also known as INT-2. The gene was first discovered by Peters *et al.*, (1983) due to its transcriptional activation in cells by the mouse mammary tumor virus (MMTV) and is therefore a proto-oncogene. *Fgf-3* is normally expressed in a variety of stages in the developing embryo and is thought to play a role in development of tail, brain derived structures and other epithelia in mouse (Tannahill *et al.*, 1992). It is rarely expressed in adult tissues and it has not displayed mitogenic potential, but has shown ability to transform certain NIH3T3 cell lines (Dickson *et al.*, 1991).

FGF-4 is also known as human stomach tumor (*hst-1*). Hst-1, a known oncogene, was originally identified from a human gastric tumor tissue (Sakamoto *et al*, 1986). Interestingly, it is found only 35kbp downstream of the *int-1* gene on chromosome 11 and

they have shown to be co-amplified in several types of cancers. *Fgf-4* is also known to be selectively expressed during development, in germ cell tumors, cancers such as Kaposi's sarcoma and gastric cancers and mouse embryos (Yoshida *et al*, 1988).

Upon conducting transfection assays, to search for human oncogenes, Zhann and colleagues, (1988) discovered FGF-5. *Fgf-5* is expressed in mouse embryos at two stages, 51/4 –8 days post implantation and 9½ –14½ days post implantation (Hebert *et al.*, 1990). Expressional studies comparing *fgf-4* and *fgf-5* indicate that even though they are both expressed in myocytes and other tissues during development, they have a distinct role in development (Drucker and Goldfarb, 1993). *Fgf-5* is expressed in the central nervous system only of adult mice (Haub *et al*, 1990).

FGF-6 was first isolated due to its similarity to FGF-4, thus it was initially called *hst-2* (Marics *et al.*, 1989). FGF-6, like FGF-4 demonstrates transforming ability. It was shown to transform NIH3T3 cells and form a well-vascularized tumor in nude mice, suggesting a role for FGF-6 in angiogenesis. It was also expressed in some human leukemia cell lines (Iida *et al.*, 1992).

Keratinocyte growth factor (KGF) is the common name for FGF-7. It was initially identified as a mitogen in the mouse NIH/3T3 cell line (Rubin *et al.*, 1989). FGF-7 is thought to play a role in normal epithelial cell growth via a paracrine effect (Aaronson *et al.*, 1991).

An FGF thought to be important in mesoderm induction was cloned in *Xenopus* by Isaacs, Tannahill and Slack, 1992. It is highly similar to mammalian FGF-4 and FGF-6, however is not thought to be the *Xenopus* homologue of either one. It was named

Xenopus embryonic FGF (XeFGF), is a maternal transcript in *Xenopus* embryos and contains a known signal sequence (Isaacs *et al.*, 1992).

Androgen induced growth factor (AIGF, FGF-8) was originally identified as a growth factor in a mouse mammary carcinoma cell line (Tanaka *et al*, 1992). *Fgf-8* is differentially spliced to produce 8 different isoforms in the mouse and 4 different isoforms in the human. The differential splicing is due to the presence different amino terminal exons (Gemel *et al.*, 1996).

Glia-activating factor (GAF), a protein known for its ability to promote glial cell proliferation, was the tenth member of the FGF family to be cloned (Miamoto *et al.*, 1993). Like other FGFs, FGF-9 has demonstrated transforming ability. When transfected into mouse BALB/3T3 cells, the cells demonstrated the ability to grow on soft agar, and increased saturation density. When the transformed cells were implanted into nude mice, tumors formed. FGF-9 is expressed in human glioma tumor as well as stomach carcinoma cell lines (Matsumoto-Yoshitomi *et al*, 1997). FGF-9, like FGF-1 and FGF-2, doesn't contain a conventional signal sequence that is cleaved prior to exiting the cell (Miamoto *et al.*, 1993). The Xenopus homologue of *fgf-9* has been cloned. It is 93% identical to the mammalian gene, suggesting an important role in both species (Song and Slack, 1996).

Fibroblast growth factor 10 was originally cloned by Yamasaki *et al.*, 1996. It has been mapped to chromosome 5p 12-p13 (Emoto *et al*, 1997) and was discovered due to its similarity to *Fgf-7*, and originally named *Kgf-2*. However, FGF-10 doesn't appear to have the same function in wound repair as FGF-7. *Fgf-10* mRNA is relatively

abundant in mouse lung, skin, brain, and heart and has been shown to be repressed in fibroblasts by transforming growth factor -β (TGF-β) and tumor necrosis factor-α (TNF-α) (Beer *et al.*, 1997). FGF-10 has displayed mitogenic activity in fetal rat keratinizing epidermal cells, but not in NIH/3T3 cells or fibroblasts (Emoto *et al.*, 1997).

1.2.1 The FGF Homologous Factors and FGF-15

Recently, four genes encoding FGF homologous factors (*fhf*) have been cloned (Smallwood *et al.*, 1996). They were originally cloned via random cDNA sequencing, data base searches and degenerate polymerase chain reaction (PCR). FHFs possess 58-71% amino acid identity to each other, but less than 30% identity to other FGFs, therefore they are thought to be a sublineage of the FGF family, and have been named FHF-1 (FGF-12), FHF-2 (FGF-13), FHF-3 (FGF-11) and FHF-4 (FGF-14). They are expressed in the developing murine and adult central and peripheral nervous system as well as heart and connective tissue (Hartung *et al.*, 1997). Human *fgf-11* and *fgf-12* have been mapped to chromosomes 17 p12 and 3q28-q29, respectively (Verdier *et al*, 1997).

The final FGF identified thus far has been named FGF-15 (McWhirter *et al.*, 1997). It was discovered via subtractive cDNA cloning to identify targets of heterodimeric transcription factors Pbx1 and E2A. FGF-15 yields a 15-30% homology to other FGFs within the 120 amino acids conserved region and does contain an amino-terminal signal sequence indicating that it is secreted. Like other secreted FGFs, FGF-15

was able to transform 3T3 cells via soft agar colony formation and focus formation assays, but did not display oncogenic activity. *Fgf-15* is expressed in murine neuroectoderm shortly after neurulation and is contained mostly within the nervous system throughout development (McWhirter *et al*, 1997).

1.2.2 The FGF Receptor Family

FGF binds two classes of cellular receptors, a high-affinity tyrosine kinase receptor (FGFR) and a low affinity heparin sulfate receptor. The high affinity receptors appear to be most important for carrying out FGF signal transduction and will be discussed further.

There are four *fgf* receptor (*fgfr*1-4) genes, which are alternatively spliced to create several isoforms of each receptor. The *fgfr1* was initially described as a fms-like gene (*flg*) (Ruta *et al*, 1988). FGFR1 binds FGF-1 and FGF-2 with high-affinity (Dionne *et al.*, 1991). FGFR2 was originally named bacterially expressed kinase (bek), and binds FGF-1, FGF-2 and FGF-3 with high affinity (Dionne *et al.*, 1991, Mathieu *et al.*, 1995). FGFR3 is also known as chicken embryo kinase-2 (CEK-2) and binds FGF-1 and FGF-2 with high affinity (Keegan *et al.*, 1991; Mathieu *et al.*, 1995). The final FGFR, FGFR4 was discovered by Michael *et al.*, 1992, and binds FGF-1 with high affinity, but not FGF-2 (Mathieu *et al.*, 1995).

FGFRs are tyrosine kinase receptors, which possess an external domain with three immunoglobulin- like regions (Ig loops) and a hydrophilic "acid box" between the first and second Ig loops, a transmembrane domain, a juxtamembrane region and an internal tyrosine kinase domain. A high affinity interaction between FGF and FGFR is facilitated by heparin. The ligand binding activates dimerization of the receptor and autophosphorylation, whereby the tyrosine kinase of one receptor molecule phosporylates the other. This initiates signal transduction pathways within the cell.

Differential expression of receptor isoforms and differing affinity of FGFs to FGFRs leads to a complex pattern of receptor activation and signal transduction within the developing embryo and adult organism.

1.3 *Xenopus laevis* Development as a Model System

For obvious ethical and logistical reasons, it is not possible to use humans for developmental research of the nature being studied here. Development of many species has been studied over time, however a select number of animal species have become common experimental models due to various favorable characteristics. The mouse, for example, is a common mammalian model. However for our research *Xenopus laevis*, the African clawed-toed frog, is the model system of choice. This species offers many advantages over mammalian models and it has been shown that the regulatory molecules and pathways of development are highly conserved throughout vertebrate species

(reviewed by Dawid, 1992). Therefore, even though amphibians and humans are quite far apart on the evolutionary scale, basic observations made about *Xenopus* development are usually directly applicable to humans.

Xenopus laevis is an ideal model system to study development for several reasons. Firstly, females may be injected with chorionic gonadotrophin to induce ovulation within twenty-four hours. Hundreds of eggs are laid at one time, enabling one to obtain a great amount of material for experimentation. Then, the eggs may be fertilized externally and they develop synchronously so that the exact time of fertilization is known and they are easily manipulated throughout development. Due to the nature of mammalian reproduction, only a small number of offspring are available per female. Also, *Xenopus* embryos are large (1-2mm diameter), making them favorable for microdissection and microinjection experiments. Finally, due to decades of research, there is already a vast amount of information available about *Xenopus* development.

1.4 Early *Xenopus* Development

As previously mentioned, *Xenopus* has become a model system for studying vertebrate development. Landmark experiments with *Xenopus* date back 70 or more years. The purpose of this section is to describe the current understanding of early *Xenopus* development as well as experiments that have led researchers to this understanding.

1.4.1 Fertilization to Metamorphosis

Xenopus laevis reproduce via external fertilization, as previously discussed. At fertilization, a rotation of the outer membrane of the zygote occurs (cortical rotation), and the dorso-ventral poles of the embryo are determined. The point opposite sperm entry becomes the dorsal pole (reviewed in Gilbert, 1994). The darkly pigmented, upper half of the embryo is termed the animal half, and the lighter, yolky bottom half is the vegetal half. The developmental stages of *Xenopus* have been given a numerical value such that they may be compared (Figure 1.1), (Nieuwkoop and Faber, 1967).

Fertilization initiates a rapid period of synchronous cell cleavages, which are cellular divisions without overall growth. After 12 cleavages, the embryo reaches the blastula stage (5 hours post fertilization, stage 8), named so due to hollow cavity within the center of the embryo, the blastocoel. At this point, the embryo is composed of two distinct cell types, the upper animal cells containing the presumptive ectoderm and the lower vegetal cells containing presumptive endodermal cells (Figure 1.2).

It is known that embryonic development initiates the formation of three basic germ layers. The outermost ectodermal layer forms the skin, nervous system and sense organs in adults. The innermost endodermal layer will form the lining of the digestive system and associated organs such as the liver and pancreas. The middle layer is the mesodermal layer. It will form the skeletal, circulatory, excretory and reproductive system in adults. A diagram of the developmental fate of the three germ layers is shown in Figure 1.2.

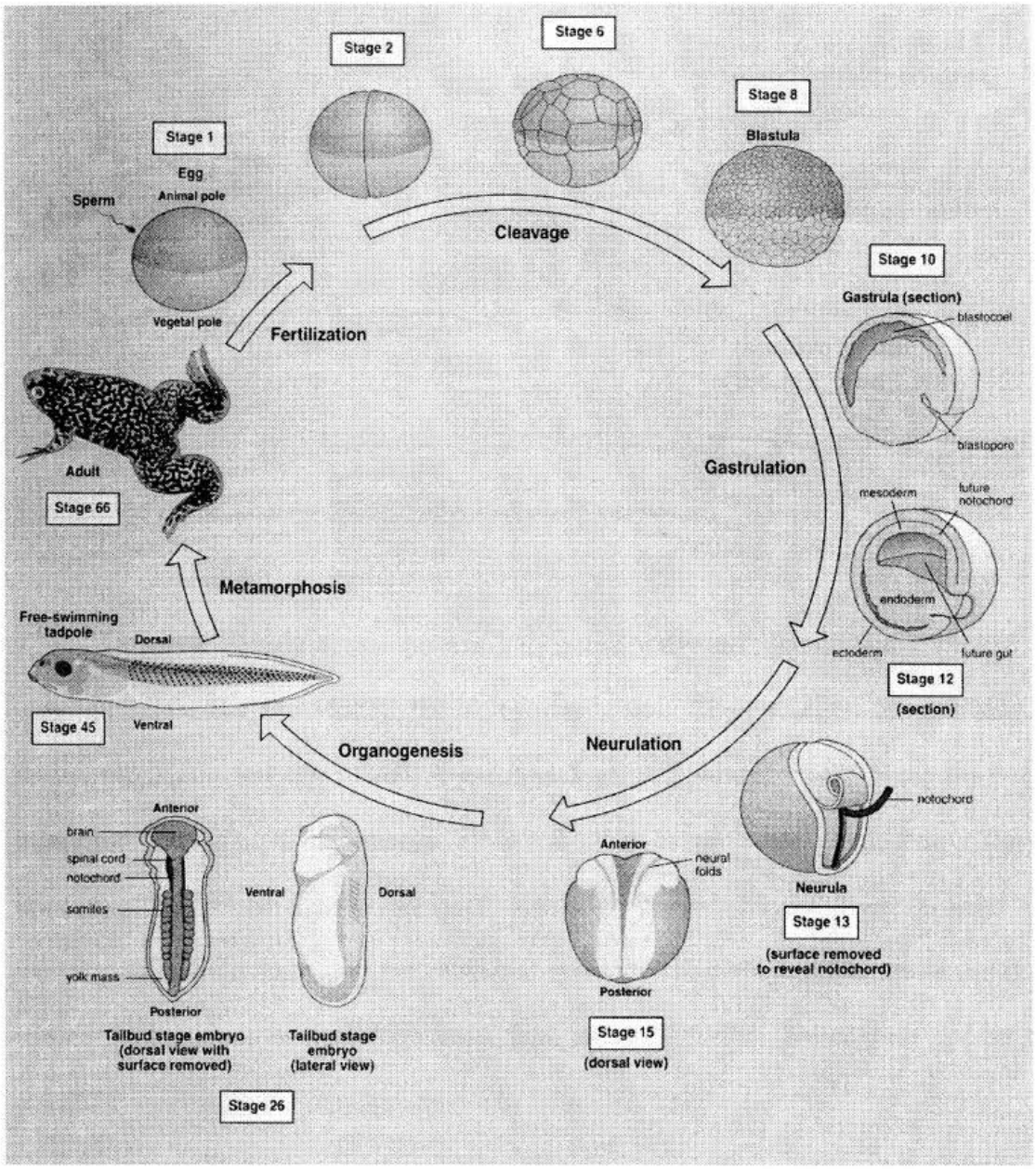


Figure 1.1 The Stages of *Xenopus laevis* Development (Reproduced from Wolpert *et al.*, 1998).

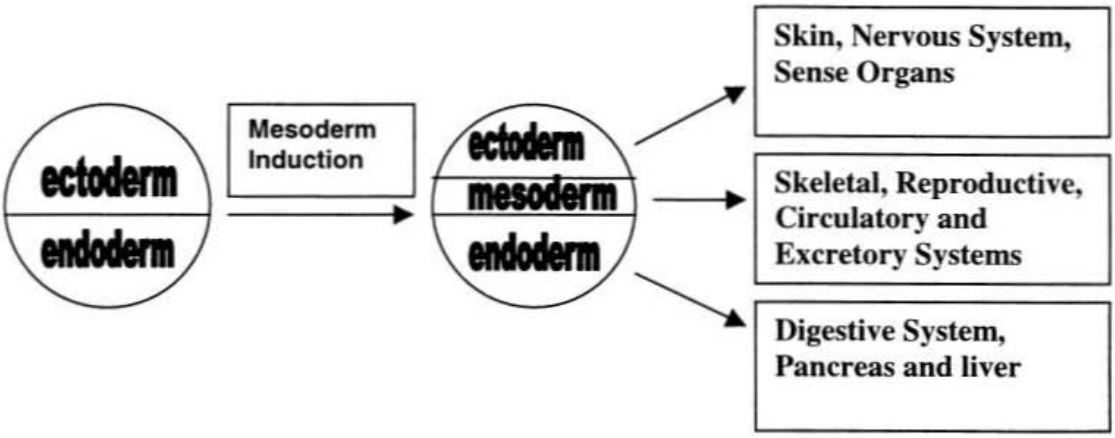


Figure 1.2 Fate Map of the Developing *Xenopus* Embryo.

Up until blastula stage, there is only presumptive ectodermal and endodermal tissue present. The mesodermal tissue is formed later. The formation of mesodermal tissue, mesoderm induction (MI), is a complex process whereby cells composing the equatorial ectoderm of an embryo are acted upon by molecules (induced). These molecules are released from adjacent equatorial vegetal cells. Thus, a band of mesodermal cells is formed at the equatorial region of the embryo. The molecules that induce the formation of mesoderm, mesoderm induction factors, are of great interest to researchers in the field. They will be discussed further later.

Gastrula is the developmental stage directly after blastula stage. An embryo is in gastrula stage (stages 10-13) from 11-14 ¾ hours post fertilization. This stage is named so due to the major cellular movements that take place, termed gastrulation. During gastrulation, the mesodermal tissue migrates upwards along the ectodermal tissue such that they lie adjacent (Figure 1.1). At this point the dorsal-ventral axis is determined.

Neural induction is the second major induction event to take place in the developing embryo. Neurula stage (stages 13-19) occurs between 14 3/4 and 20 hours post fertilization. Neural induction is the process whereby the mesodermal tissue releases molecules onto the adjacent ectodermal tissue to induce the formation of the nervous system. Spemann organizer (S.O.) tissue (a region of dorsal mesodermal tissue) is thought to be responsible for neural induction as well as mesodermal patterning. S.O. tissue is thought to release inducing factors onto overlying ectodermal cells, changing their developmental fate and inducing them to develop into neural plate (reviewed by Dawid, 1992). During neural induction the anterior-posterior axis is formed.

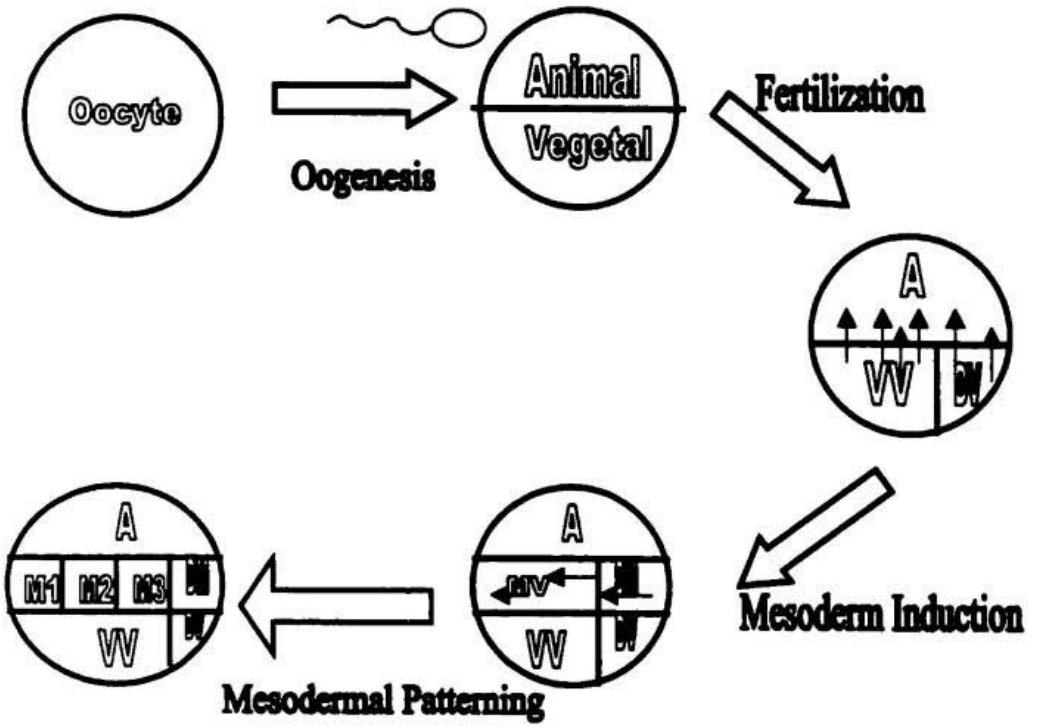
The next major stage after neurulation is organogenesis (stages 22-45, 1-4 days post fertilization). During organogenesis, further tissue differentiation occurs. Differentiation is the process whereby the developmental fate of a cell is changed by the stimulation of new protein species in a pattern different from that previously found in that cell (reviewed by Slack, 1983). Cellular differentiation leads to the formation of muscle, cartilage and neurons. This is the point at which major organs begin developing. The next stage in *Xenopus* development is metamorphosis (stages 45-66, 5-60 days post fertilization), whereby the tadpole develops into an adult frog (reviewed by Wolpert *et al.*, 1998).

Vertebrate development is a very intricate and complex process. The focus of this study is the process of development during which the presumptive mesodermal tissue is created, mesoderm induction. Therefore, this stage of development will be discussed further in the next section.

1.4.2 Mesoderm Induction

In 1971, Sudarwati and Nieuwkoop first described *Xenopus* mesoderm induction. They conducted an experiment whereby the top quarter of the embryo (animal cap, or animal explant) was cut from the *Xenopus* embryo. When the animal cap was cultured in isolation, the tissue developed into ectodermal tissue. However when cultured adjacent to vegetal tissue, it developed into mesoderm. It was thought that something within the vegetal half of the embryo was inducing the ectodermal tissue to become mesoderm. From this and other pioneering experiments, Slack and colleagues proposed the three-signal model to explain mesodermal induction (Smith *et al.*, 1985; Dale *et al.*, 1989; Smith and Slack, 1983; Slack and Forman, 1980 and Dale and Slack, 1987), (Figure 1.3).

The three-signal model proposed that mesoderm induction was the result of three different types of mesoderm induction factors (MIFs) acting on the equatorial ectodermal cells. Two MIFs were thought to be released from the vegetal cells. One signal was released from the dorsal vegetal mass (named the Nieuwkoop center in honour of its discoverer) to act upon the adjacent dorsal animal cells, while a second signal was released from the ventral vegetal mass to act upon the ventral animal cells (Figure 1.3).



Symbol	Legend
A	Animal
VV	Ventral vegetal
DV	Dorsal vegetal
DM	Dorsal meso.
MV	Ventral mesoderm
M1	Ventral meso.
M2	Intermed. Ventral meso.
M3	Intermediate meso.

Figure 1.3 The Three Signal Model of Mesoderm Induction (Reproduced from Slack *et al.*, 1987).

The third signal was thought to act after the dorsal and ventral MIFs. This signal was released from the Spemann organizer and resulted in a gradient of dorsalizing factors from the dorsal equatorial region to the ventral equatorial region. This signal was responsible for patterning of the dorsal-ventral axis (Heasman, 1997). S.O. was discovered by Spemann and Mangold (1924) for its ability to induce the formation of an ectopic dorsal-ventral axis when transplanted into the ventral side of a gastrula stage embryo. S.O. itself was induced by messages from the Nieuwkoop center.

This model was widely accepted for years, and is quite accurate. The main fault of the model is that it over-simplifies the phenomenon of mesoderm induction. The current understanding is that there are more than three factors involved in mesoderm induction, along with a host of competence factors and other response molecules. Competence factors are molecules that make cells able (competent) to respond to other signals.

There is also evidence that MIFs act synergistically to induce mesoderm formation. For example, TGF-β increases bFGF's ability to induce muscle actin expression (Kimelman and Kirchner, 1987).

The complete process of mesoderm induction is still far from being completely understood. The current understanding of the factors responsible for mesoderm induction will be described in the next section.

1.5 Mesoderm Induction Factors

Generally, induction is the process whereby the developmental fate of one cell population is changed by signals released from another population. There are three main criteria used to identify mesoderm induction factors. Firstly, the factor must demonstrate activity, that is, ability to induce animal cap tissue to form mesoderm in vitro. Secondly, the factor must be expressed at the correct stage in development. Thirdly, if the factor's activity is inhibited *in vivo*, there will be a disruption of induction (reviewed by Slack, 1993).

There are two main families of MIFs; the transforming growth factor β (TGF-β) family, and the fibroblast growth factor family. There are also several factors that cannot induce mesoderm formation themselves, however they may complement or impede the actions of MIFs. As described in the three-signal model, there are two major groups of MIFs, dorsal and ventral. The two classes of MIFs interact closely in vivo, but for simplicity, they will be discussed separately in the following sections.

1.5.1 Dorsal Mesoderm Induction Factors

When ultra-violet light is applied to the vegetal side of the embryo at fertilization, it is effective in blocking the dorsal pathway (Holwill *et al.*, 1987). This blockage is

caused by a disruption in the cortical movements that occur at fertilization, thereby preventing the dorsalizing factors from being in the correct location to act upon competent cells during gastrulation. Therefore, using UV irradiated embryos is an effective means of examining molecules expressed in early embryos that are able to rescue the ventralized phenotype (dorsalizing factors).

Several TGF-β family members appear to play an important role in dorsal mesoderm formation. Two important factors are activin A and vegetally localized factor Vg1. Vg1 mRNA is expressed maternally in vegetal cells in an inactive form (Rebagliati *et al.*, 1985; Weeks and Melton, 1987). Injection of a mutant ligand that appears to selectively block Vg1 signaling results in *Xenopus* embryos that lack dorsal mesoderm and axial structures (Joseph and Melton, 1998). However, the method of Vg1 activation has yet to be elucidated.

Activin A is the most potent mesoderm inducer in the TGF-β family. It is able to induce the formation of different forms of mesoderm in animal cap explants, in a concentration dependent manner. When tissue is exposed to 0.1-2.0 ng/ml of activin, ventral mesoderm is induced. However at concentrations of 1.6-4.0 ng/ml, intermediate mesoderm is formed, and at concentrations above 4.3 ng/ml, the tissue becomes dorsal mesoderm and demonstrates organizing ability (Cooke, 1989; Green *et al.*, 1992).

There are several receptors known to interact with various TGF-β family members. Different receptors have been shown to bind more than one ligand, similar to FGFRs. Dominant negative forms of the activin type II receptor reduce mesoderm formation in the embryo (Hemmati-Brivanlou and Melton, 1992).

It has been shown that activin and Vg1 induce expression of *mix2*, a homeobox transcription factor (Vize, 1996). The signal transduction pathway leading to *mix2* expression has been elucidated (Figure 1.4), (Chen *et al.*, 1996). Activin activation of its type 1 receptor leads to phosphorylation and activation of SMAD2 (mothers against *decapentaplegic*). This activated protein then localizes to the nucleus and binds to the forkhead activin-sensitive transcription factor (FAST1), inducing *mix2* transcription (reviewed by Heasman, 1997).

Another signal transduction pathway that is thought to be important in dorsal mesoderm induction is the β-catenin pathway (Figure 1.4). β-catenin is a membrane associated and cytoplasmic protein which, when depleted in *Xenopus* embryos, causes severe ventralizing effects (Heasman et al., 1994).

It has been proposed that activation of a cell surface receptor homologous to the *Drosophila* frizzled receptor (Xfz), results in activation the *Xenopus* homologue of the *Drosophila* disheveled gene (*Xdsh*). In turn, XDSH inhibits glycogen synthase kinase (GSK3). Normally GSK3 phosphorylates β-catenin and initiates its degradation. Therefore, inhibition of GSK3 allows β-catenin to be present in the cell to dimerize with the transcription factor XTcf-3. The heterodimer is thought to localize to the nucleus and initiate transcription of specific genes involved in perpetuation of the dorsal mesoderm induction pathway, such as goosecoid and siamois. This is the proposed pathway, however the exact mechanism is not known (reviewed by Heasman, 1997).

It was previously thought that Xwnt acted as a ligand for Xfz. Xwnt has a proposed involvement in ventral mesoderm patterning (Christian and Moon, 1993). Gain

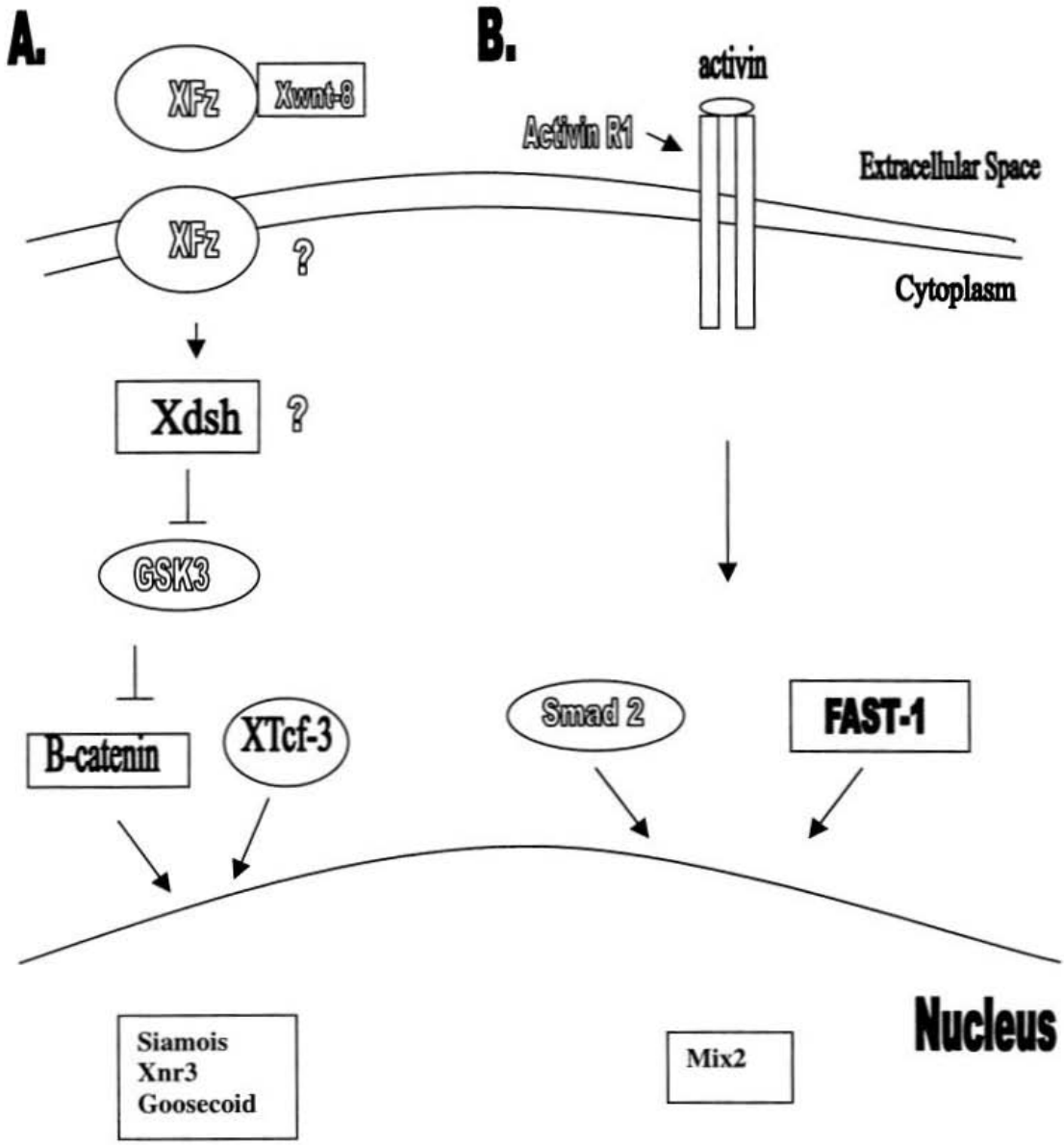


Figure 1.4 Proposed Dorsal Mesoderm Induction Pathways. (A) Xwnt/β-Catenin pathway. (B) The Activin Pathway

and loss-of-function experiments by Hoppler and colleagues support a model in which Xwnt-8 (a member of the Xwnt family) induces expression of genes involved in specification of both ventral and somatic mesoderm (Hoppler *et al.*, 1996). Studies donewith Frzb (a member of the Frz family) indicate that it is a soluble protein expressed in Spemann organizer and is involved in dorsalization of *Xenopus* embryos. It is now thought that Frzb binds and inactivates Xwnt-8, thus preventing Xwnt-8 ventral signaling in dorsal tissues (Wang *et al.*, 1997). However, further study must be done in this area.

1.5.2 Ventral Mesoderm Induction Factors

The bone morphogenic protein (BMP) members of the TGF-β family are thought to be important factors in the formation of ventral mesoderm in *Xenopus* embryos. *Bmp2*, *bmp4* and *bmp7* mRNAs are present in the early embryo due to transcription of the maternal genes (Koster *et al.*, 1991; Nishimatsu *et al.*, 1992; Dale *et al.*, 1992; Jones *et al.*, 1992). BMP4 is first observed at gastrula stage in animal and vegetal equatorial cells. Expression experiments with BMPs in the mesodermal tissue demonstrated induction of ventral mesoderm, such as mesenchyme, blood islands and muscle in *Xenopus* (reviewed in Heasman, 1997). Inactivating this pathway causes dorsal mesoderm to form instead of ventral mesoderm. Dominant negative mutants of BMP receptor results in restoration of axial structures in β-Catenin depleted embryos (Wylie *et al*, 1996). Distinct roles for the three BMPs in mesoderm induction have not yet been delineated.

1.5.3 The Role of FGF in Mesoderm Induction

Currently, five FGFs have been cloned in *Xenopus laevis*. They are; FGF-1 FGF-2, FGF-3, eFGF and FGF-9. FGFs 1-6 (human FGF 4-6 were used) along with XeFGF have demonstrated ability to induce mesoderm formation in animal cap explant experiments (reviewed by Slack, 1994, and Slack *et al.*, 1996). Ability to induce mesoderm in explants, however does not indicate that they do induce mesoderm *in vivo*. As previously mentioned, the inducer must be expressed at the correct time in development, it must be active at that stage and loss of function must have a negative effect within the embryo.

Dominant negative FGF receptor (XFD) studies in which mRNA for an inactive receptor is injected into *Xenopus laevis* indicate that FGF is an important factor in mesoderm induction (Amaya, 1991). Since the FGFR dimerizes when activated, if most of the receptors are inactive, they will bind the normal FGFR making it impossible for the receptor to carry out the signal.

Fgfr1 is expressed throughout development (Musci *et al.*, 1990; Friesel and Dawid, 1991), mostly within the equatorial region (Gillespie *et al.*, 1989). XFD embryos develop abnormally, having reduced levels of mesodermal derivatives, they appear anteriorized with reduced posterior structures (Amaya, 1991). This information does not indicate which FGF(s) are active at this stage in development, however.

Studies with XFGF-2 demonstrate that it is able to induce mesoderm formation in blastula stage animal cap explants (Slack *et al.*, 1987). It is present in the embryo at

intermediate levels during mesoderm induction. As previously mentioned, the secretion mechanism of FGF-2 is yet unknown, as it doesn't have a common signal sequence.

XeFGF is thought to be involved in mesoderm induction *in vivo*. It is expressed maternally and zygotically at peak levels in the gastrula stage embryo. The protein is present in vivo at sufficient levels to induce mesoderm and it is efficiently secreted from the cell (Slack *et al.*, 1996).

The role of FGF in mesoderm induction may be further understood by elucidating the signal transduction pathways initiated by FGF during this time period. Discovering the genes activated by these pathways will lead to a further understanding of the process. The purpose of the following sections is to outline the known pathways and genes activated by FGF during mesoderm induction.

1.5.4 The Role of FGF in Activin Induced Mesoderm Formation

It has been proposed that FGF plays a role as a competence factor for other MIFs during mesoderm induction (reviewed by Isaacs, 1997). Equatorial cells are only able to respond to mesoderm induction signals at a select time during development. XFGF-2 has been suggested as a competence factor for activin during mesoderm induction (Cornell and Kimelman, 1994; LaBonne and Whitman 1994).

Animal cap explants containing dominant negative FGFR, c-RAS or c-RAF were treated with activin. The defective members of the FGF pathway inhibited expression of

some mesodermal markers, such as *muscle actin*, *Xbra*, *Mix1* and *Xnot* which are normally expressed in response to activin treatment. The expression of other genes, including head organizer genes *goosecoid* and *Xlim-1* was also reduced (Cornell and Kimelman, 1994; LaBonne and Whitman 1994). The 'trunkless head' phenotype was created, similar to that observed in dominant negative FGFR embryos without activin. It has been proposed that FGF may act within the equatorial region of Xenopus embryos as a competence factor for mesoderm induction by activin-type signals (Cornell *et al.*, 1995).

1.6 FGF and Signal Transduction

Signal transduction pathways initiated by FGF within activated cells have not yet been fully elucidated. However, FGF has shown to activate several well-known cascades. As mentioned previously, FGF binds and activates its cell surface receptor. The activated receptor then undergoes dimerization and autophosphorylation on tyrosine, which is a high-affinity binding site for src-homology 2 (SH2) containing proteins. The activated receptor possesses tyrosine kinase activity and therefore phoshporylates specific molecules. This results in the initiation of a signal transduction cascade within the cell.

FGFR1 has been shown to act through at least three different signaling pathways: phospholipase C gamma 1 (PLCγ1), Ras, and phosphoinositide 3' kinase (PI3'K) (Ryan

et al., 1998). The three general pathways will be reviewed in the next sections (Figure 1.5), followed by the specific pathways activated by FGF in *Xenopus* embryos.

1.6.1 The Phospholipase Cγ1 Pathway

PLCγ1 is a signal transduction molecule which, when activated, initiates hydrolysis of phosphatidylinositol 4,5- bisphosphate (PIP_3) to diacylglycerol (DAG) and phosphatidylinositol (IP_3). DAG is a protein kinase C (PKC) activator, and IP_3 initiates the release of cellular calcium stores. The second messengers activate several molecules and thereby bring about the desired cellular processes such as transcription (reviewed by Kamat and Carpender, 1997).

1.6.2 The RAS Pathway

The *ras* pathway has been well characterized for other growth factors, such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF). A similar pathway is thought to be initiated by FGF. The pathway is activated by receptor autophosphorylation. Phospho-tyrosines on the activated receptor bind the SH2 domain of growth factor receptor binding protein 2 (GRB2). GRB2 is a cytoplasmic molecule

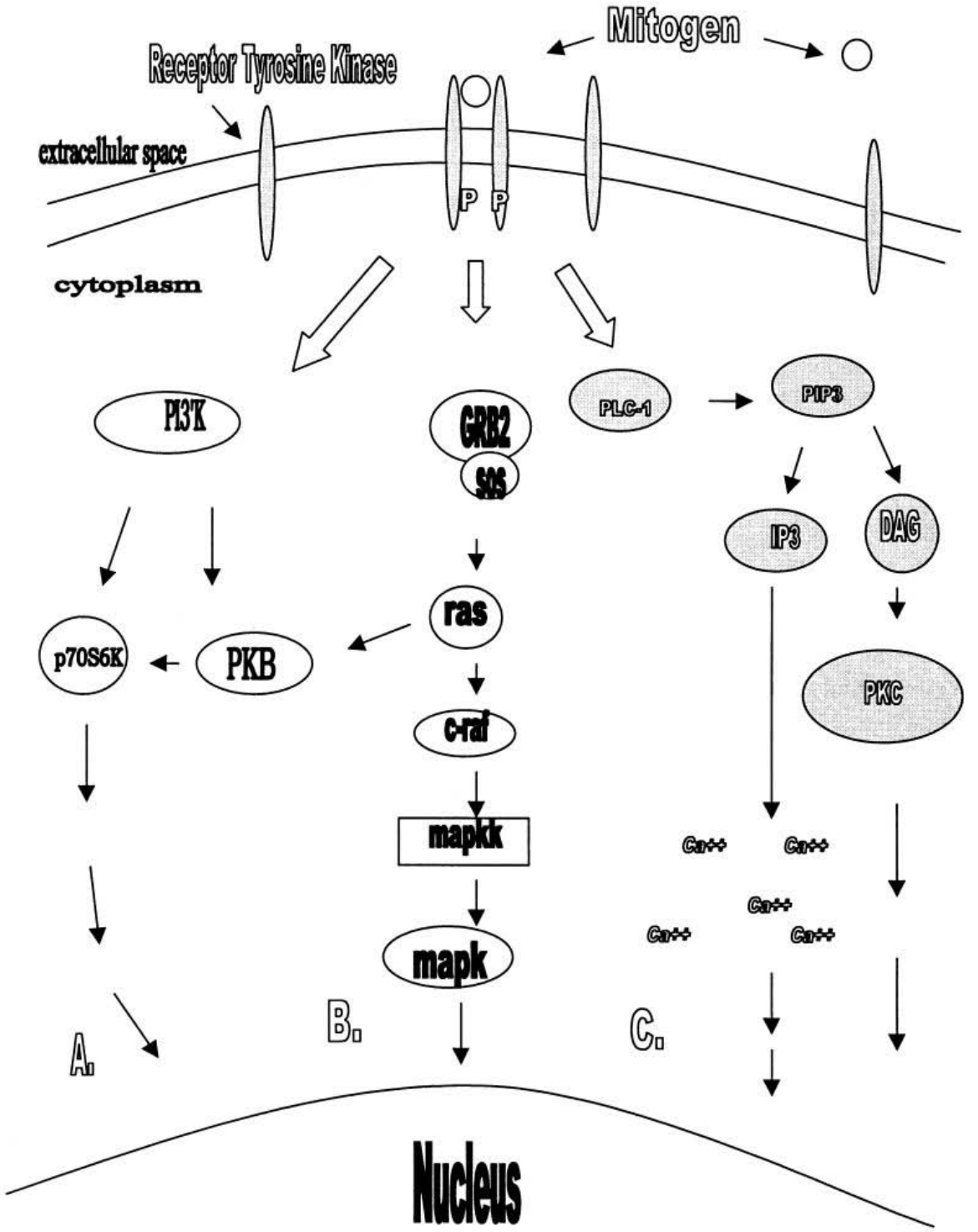


Figure 1.5 Signal Transduction Pathways Initiated by Various Growth Factors. (A) PI3'K, (B) RAS, (C) PLC.

associated with son of sevenless (SOS), a guanine nucleotide exchange factor. Binding of GRB2-SOS to the receptor leads to Ras activation. Ras perpetuates the pathway via subsequent activation and/or phosphorylation of c-Raf, mitogen activated protein kinase (MAPK) kinase, then other kinases, which in turn travel into the nucleus and activate other molecules, initiating transcription of desired proteins (reviewed in Lewin, 1994).

1.6.3 The Phosphoinositide 3' Kinase Pathway

The last known signal transduction pathway initiated by FGF is the PI3'K pathway. This pathway plays various roles in many systems, including; cytoskeletal rearrangements, cellular migrations, mitogenesis, differentiation and protection from apoptosis. PI3'K phosphorylates hydroxyl groups of phosphotidylinositols on the third position of inositol thereby activating inert membrane phospholipids and initiating a signal transduction pathway. Many of the phospholipids involved in the PLCγ pathway may also be phosphorylated by PI3'K.

The exact signal cascade in the PI3'K is not as well understood as the others, however, the understanding of the general mechanism are as follows. The p85 protein is part of PI3'K which binds active phosphotyrosine receptors via SH2. PI3'K is also activated by RAS proteins. PI3'K in turn, phosphorylates various membrane phosphoinositols which activate particular proteins. Proteins activated by this pathway include; protein kinase B (PKB or AKT), p70 ribosomal S6 kinase ($p70^{S6K}$) and PKC.

PKB activates glycogen synthase kinase 3 (GSK3) *in vivo*, and is also thought to act upstream of $p70^{S6K}$ (reviewed by Vanhaesebroeck *et al.*, 1997).

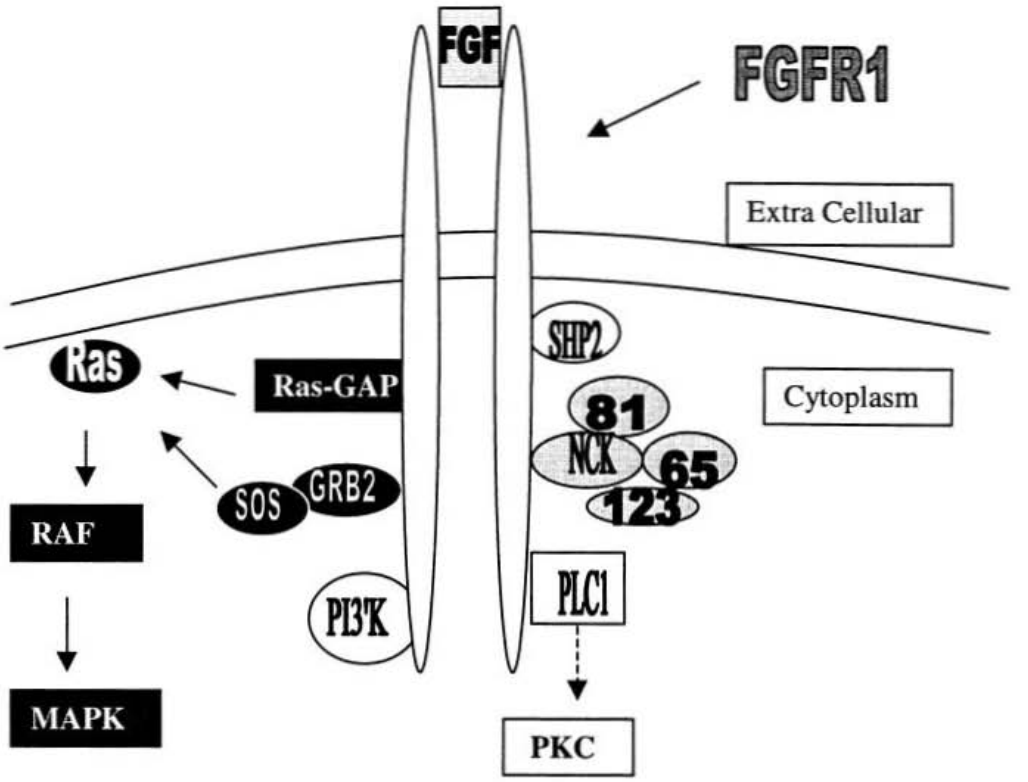
1.6.4 Signal Transduction Molecules Activated by FGF in *Xenopus*

There are several components of the three fore-mentioned signal transduction pathways, as well as molecules of other pathways that are known effectors of FGF in *Xenopus* embryos. There is also evidence of cross talk between different pathways. It has been shown that the PLCγ1 pathway activates mitogen-activated protein (MAP) kinase from the Ras pathway in FGF-2 treated *Xenopus* animal cap explants (Rose and Busa, 1998).

Activated FGFR1 forms a complex with at least ten other proteins (Ryan *et al.*, 1998; Figure 1.6). The FGFR1 associated proteins include: PLCγ1, NCK, SOS, GRB2, Ras-GTPase associated protein (ras-GAP), p85 of PI3'K and SHP2 as well as 3 NCK associated proteins (NAP65, NAP81 and NAP123).

Several of the FGFR1 binding proteins are known effectors of transduction pathways initiated by growth factors and cytokines in other models. For example, GRB2 and SOS bind when stimulated by EGF and are thought to signal via a ras dependent pathway as in Figure 1.5 (Vidal *et al.*, 1998). SHP2 (tyrosine phosphatase) and p85 of PI3'K co-immunoprecipitate in response to interleukin 3 stimulation (Craddock and

A.



B.

Full Name	Abbreviation
Phospholipase C gamma 1	PLCγ1
	NCK
Son of Sevenless	SOS
Growth factor Receptor-Binding protein 2	GRB2
Ras-GTPase Activating Protein	Ras-GAP
P85 subunit of Phosphoinositol 3' Kinase	p85 of PI3'K
	SHP2
123 kDa NCK Associated Protein	123/NAP123
81kDa NCK Associated Protein	81/NAP81
65kDa NCK Associated Protein	65/NAP65

Figure 1.6 FGFR1Associated Proteins A. Schematic diagram of proteins isolated with activated FGFR1 dimer, along with known effectors of the FGF induced signal transduction pathway. B. Table of FGFR1 associated proteins.

Welham, 1997). GRB2 and ras-GAP co-precipitate with insulin receptor, and are thought to interact in the RAS pathway (Hwang *et al.*, 1997).

NCK is an oncoprotein and mediator of several mitogenic effects, composed primarily of SH_2 and SH_3 domains and phosphorylated on tyrosine while associated with FGFR1 (Ryan *et al*, 1998). The function of NAPs is yet unknown.

Through loss of function assays, it has been demonstrated that RAS, RAF, and MAP kinase are all needed to perpetuate FGF signaling (LaBonne *et al.*, 1995; Kim *et al*, 1998). In particular, dominant negative RAF blocks the mesoderm inducing activity of FGF (Demo *et al.*, 1994). In other experiments, PKC inhibition has also shown to inhibit mesoderm induction via FGF (Gillespie *et al.*, 1992). PLCγ1 was shown to be associated with FGFR1 during mesoderm induction in *Xenopus* (Ryan and Gillespie, 1994).

1.7 Immediate-Early Genes

Immediate-early genes (or early response genes) are the first genes to be transcribed differentially in response to growth factor treatment of a cellular population. In order to classify a gene as immediate-early, it does not require *de novo* protein synthesis for transcription and is usually expressed within thirty minutes of mitogen action. Many early response genes are transcription factors, receptor molecules, transmembrane proteins or secretory molecules. Conversely, gene transcription may be terminated by growth factors.

The transcription factors induced as immediate-early genes, in turn, initiate (or terminate) transcription or activation of new genes and proteins, which carry out the desired cellular response. For example, the *mix1* homeobox transcription factor is an immediate-early activin-induced gene (reviewed by Godsave and Gillespie, 1991). Understanding gene regulation via the signal transduction cascades is important for elucidation of the mechanisms whereby FGF elicits cellular actions. However very few FGF response genes have been identified and characterized, a small number of genes activated by FGF have been elucidated.

Mesoderm induction via FGF is inhibited by dominant negative mutant c-jun (Dong *et al.*, 1996). C-jun is an immediate-early oncogene and transcription factor, which forms homodimers and heterodimers with c-fos. The dimer arrangement composes activator protein 1 (AP1). Further studies have shown that c-fos is also necessary for mesoderm formation in *Xenopus* via FGF, therefore it is thought that the heterodimer AP1 is a downstream target of FGF. (Kim *et al.*, 1998).

The *Xenopus* homologue of the Brachyury (Xbra) gene is a DNA binding protein upregulated by FGF. Xbra is able to induce mesoderm formation: ventral mesoderm at low concentrations and intermediate mesoderm at higher concentrations (Cunliffe and Smith, 1994). Xbra and eFGF are able to activate transcription of each other and are thought to act in an autocatalytic manner during mesoderm induction (Isaacs *et al.*, 1994).

An immediate-early gene upregulated by FGF-induced differentiation in rat hippocampal neuronal cells has been cloned, named *pip92* (Chung *et al.*, 1998). The human gene is early TPA responsive 101 (*etr 101*). ETR 101/PIP92 contains 2 nuclear

localization signals, and a region of similarity to JUNs and therefore was originally proposed to be a transcription factor, however its function remains unknown (Shimizu *et al.*, 1991). The serum response element (SRE) of the *pip92* promotor is activated in response to FGF via the transcription factor Elk1/TCF (a member of the Ets family of transcription factors). Elk is activated by phosphorylation via MAPK dependent and independent pathways the detailed mechanism is as yet unknown (Chung *et al.*, 1998).

Another early response gene, upregulated by FGF-1, has been named *fnk* for FGF inducible kinase. It was identified by targeted differential display in mouse NIH/3T3 cells and is also induced in an immediate early fashion in response to FGF-2. FNK is a member of the polo subfamily of serine/threonine kinases (Donohue et al., 1995).

A gene cloned in our laboratory that is upregulated in response to FGF treatment of *Xenopus* animal cap explants is early response 1 (ER1) (Paterno *et al.*, 1997). ER1 contains two nuclear localization signals, a putative DNA binding domain, and has demonstrated transcriptional activity. It is thought that ER1 contains a negatively regulating sequence that, when removed allows it to activate transcription. Therefore, the novel gene, *er1* may act as a transcription factor during mesoderm induction in *Xenopus laevis* (Paterno *et al.*, 1997).

1.8 Purpose of the Project, Hypothesis and Goals

As reviewed in previous sections, FGF appears to play a significant role in normal animal development, especially during mesoderm induction. It is therefore important to elucidate where, when and how FGF molecules elicit their action within various tissues.

The area of FGF research that I have chosen to study is the molecular mechanism of mesoderm induction by FGF, in particular, differential expression of immediate-early genes in response to FGF treatment. As previously described, most immediate-early genes differentially expressed in response to growth factors are transcription factors. These transcription factors, in turn, induce other genes required to perpetuate the anticipated response.

The early response gene ER1 was cloned in this lab via differential display technology. There were four other genes that appeared to be differentially expressed in response to FGF treatment in the same differential display experiment. The goal of this study was to characterize the other four genes isolated from the differential display gel. This was done by determining the nucleotide and potential amino acid sequences of the cDNA fragments, searching for potential functional domains, and examining expression of the representative genes in response to XFGF-2 and throughout development.

CHAPTER 2

Materials and Methods

2.1 Plasmid Preparations

The purpose of this procedure was to isolate plasmid DNA from bacterial cells on a large scale for further analysis of DNA inserts. The plasmid was isolated from bacterial cells, then purified by cesium chloride gradient as described in Sambrook, Fritsch and Maniatis, 1989. This procedure is also known as a "maxi prep".

2.1.1 Materials

Most of the materials required for the plasmid prep were supplied from BDH Inc, and include; agar, glycerol, butanol, peptone from meat (pepsin digested), extract of yeast powder, and sodium chloride. The organic chemicals, including isopropanol, phenol, chloroform, isoamylalcohol, and ethanol were purchased from Fisher Scientific. Ampicillin was obtained from Sigma. Cesium Chloride was purchased from Gibco BRL, and Ethidium Bromide from BioRad. Boeringer Mannheim supplied the RNAse A.

2.1.2 Methods

Glycerol stocks had been made previously from bacterial colonies grown on 50μg/ml ampicillin in luria broth (LB) (10g/l peptone, 5g/l extract of yeast powder, 10g/l sodium chloride), agar plates. A colony was removed from the plate and grown in LB + ampicillin media at 37°C 16 hours, then 800ul of culture was added to 200μl glycerol. Stocks were stored at –70°C until needed.

The glycerol stocks were removed from –70°C and streaked on LB agar plates containing 75μg/ml ampicillin. The bacterial cells were allowed to divide 14-16 hours at 37°C. Distinct colonies were removed and restreaked, as above. A pure colony was then chosen from the restreak plate, and used to inoculate 500 ml of LB broth containing 75μg/ml ampicillin. The culture was grown 14-16 hours with shaking at 230 rpm, 37°C.

The culture was divided into two pre-cooled 250ml bottles and was incubated on ice for 15 minutes and centrifuged at 4000rpm for 5 minutes. The supernatant was poured off and bacterial pellet was resuspended, with pipetting, in 13ml of plasmid prep solution #1 (see Table 2.1 for compositions of Plasmid Prep Solutions). The solution was transferred to a 50ml Oakridge tube and allowed to sit at room temperature for 5 minutes. Next, cells were lysed in 26 ml of plasmid prep solution #2, on ice, for 5 minutes. 13 ml of plasmid prep solution #3 (at 4°C) was added to precipitate protein and chromosomal DNA. Solutions were left on ice for 10 minutes, then centrifuged at 12000rpm for 10 minutes.

The supernatant was carefully pipetted into a new Oakridge tube and 60% of initial volume of isopropanol was added. Precipitation of the plasmid DNA occurred at

room temperature over 5 minutes. The DNA was pelletted by centrifugation at 8000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 3ml of solution #4. The volume was made up to 4.2 ml, and 42µl of 10mg/ml RNAse A was added to digest RNA. 4.7g of cesium chloride was dissolved in the solution, along with 0.5ml of 10mg/ml ethidium bromide to aid in visualization of the DNA. Any remaining lipids or proteins were removed by centrifugation at 8000rpm for 10 minutes, and the preparation was injected into a quick seal tube. The tubes were balanced, heat-sealed (one per preparation), and centrifuged at 45000rpm, 20°C, under vacuum, for 18-20 hours.

Table 2.1 Contents of Plasmid Prep Solutions

Solution Number	Chemical	Final Concentration
1	Glucose 1M Tris, pH 8.0 0.5M EDTA, pH 8.0	0.050M 0.025M 0.010M
2	10% SDS 10N NAOH	 0.002M
3	5M CH_3COOK Glacial Acetic Acid	3M 11.5%
4	Tris, pH 8.0 EDTA, pH 8.0	0.050M 0.001M

The tubes were carefully removed from the rotor and the plasmid DNA (visible as a dark band, but fluorescent under ultra violet light) was removed with a 16-gauge needle into a 15ml polypropylene tube. The ethidium bromide and other products were extracted from the DNA with water saturated butanol. The extraction was conducted 5-8 times with equal volumes of butanol until the aqueous layer appeared colourless to very pale orange. The aqueous phase was then diluted 1:3 with plasmid prep solution #4 and extracted twice with equal volume of phenol:chloroform:isoamylalcohol (25:24:1).

Precipitation of the plasmid DNA was conducted with 10% 3M sodium acetate and 2 times volume of 100% ethanol for 1 hour at room temperature. The precipitate was centrifuged at 8000rpm for 30 minutes, washed with 70% ethanol (EtOH), dried under vacuum and resuspended in 200ul of plasmid prep solution #4. The concentration and integrity of the plasmid was tested by running 1ul of sample on a 1% agarose gel. The remainder was stored at 4°C.

2.2 Sequencing Plasmid DNA

The Chain Termination sequencing method was used to sequence the DNA fragments, which had been previously inserted into pCR 2.0 plasmid DNA at the multiple cloning site. The basic method used in this procedure is as follows: A plasmid specific primer was annealed to the plasmid, then extended with T7 DNA polymerase and

nucleotides, including [α^{35}S]-dATP. The reaction was terminated at varying sites with dideoxynucleotides and the reactions (one for each nucleotide) run on a sequencing gel.

2.2.1 Materials

The Sequenase Version 2.1 Sequencing Kit was purchased from Amersham Life Science. The following reagents were supplied with the kit; 0.1M Dithiothreitol, ddA, ddC, ddG and ddT Termination Mix, Stop Solution, Enzyme Dilution Buffer, Sequenase Buffer, and Sequenase T7 DNA Polymerase. The [α^{35}S]-dATP was supplied by Mandel Scientific. NaOH was purchased from JT Baker and Company. EDTA ($C_{10}H_{14}O_8Na_2$ $2H_2O$) was purchased from Fisher Scientific. Primers SP6, T7, pCR-T and pCR-SP were synthesized by Gibco BRL (see Table 2.2).

2.2.2 Methods

The following protocol was used to sequence the insert DNA from both ends. 10µl (10µg) of plasmid DNA was added to 2µl of 2N NAOH/2mM EDTA and 8µl of distilled water. The mixture was incubated at 37°C for 30 minutes to denature the DNA. The sample was then precipitated with 2.2µl 3M sodium acetate pH 5.2 and 60µl of 100% ethanol at -70°C for 20 minutes. The precipitate was centrifuged at 4°C. for 20 minutes. The DNA was then washed with 70% ethanol, centrifuged again, dried under vacuum, and resuspended in 15µl distilled water.

Table 2.2 Base Pair Sequences of Primers used for Sequencing and PCR

Primer Name	Primer Sequence 5' – 3'
SP6	AT TTA GGT GAC ACT ATA
T7	AT ATA CGA CTC ACT ATA GGG
pCR-SP	AT CCA CTA GTA ACG GCC GCC
pCR-T	AG CGG CCG CCA GTG TGA TGG
5'MEFB	CCA CAC TAT CTC TAT TCC AG
3'MEFB	GGT AAG TAT TGA AGT AGC AC
S14-1	GCT ATA GAG ACG TAT ATG AC
S14-2	CAG TGT ACT GGA AAC TCC AG
ChemR-1	GAA GAT GCC CAG CAG TAA CG
ChemR-2	TGG CAA AGA GCT TGC TTA AC
S175'	GCC TAA ATC ATA CAT CTG CC
S173'	CTT CTA GCA GGC ATG AGT GT
S305'	AAC CTG ATG ATA CTC AGT AC
S303'	ATC AAT ATC TGG TCG CAT AG
T3	ATT AAC CCT CAC TAA AG
PACT2-for	GTT CCA GAT TAC GCT AGC TTG GG
PACT2-rev	CAC AGT TGA AGT GAA CTT GCG G
8858	TCC AGG GTA AAA AGC AAA AGA ATT C
8861	GCC TGG TTA AGT CCA AGC TGA ATT C

The annealing reaction was carried out separately for each primer, the 5' plasmid primers used were SP6 and PCR-SP (a primer designed to anneal very close to the 5' vector-insert join), and the 3' primers used were T7 and PCR-T (again, designed due to close proximity to the 3' join). To 7µl of denatured DNA, 2ul of Sequencing reaction buffer, and 1µl of 10ng/µl primer was added. The mixture was denatured at 65°C for 2 minutes and then allowed to cool slowly in a water bath to 30°C (approximately 45 minutes).

Next, the sequencing reaction was performed. To the annealed DNA, the following components were added:

1µl	Dithiothreitol
2µl	labeling mix (previously diluted 1:5 with water)
1.75µl	sequencing dilution buffer
1µl	3000uCi [α^{35}S]-dATP
0.25µl	Sequenase enzyme

The components were mixed by pipetting and allowed to incubate 5 minutes at room temperature. 0.6µl microcentrifuge tubes were labeled for each nucleotide A, C, G, and T. Previously, 2.5µl of didioxy-terminal mix were added to the appropriate tube, the tubes were heated to 37°C for 2 minutes. 3.5µl of sequencing reaction were added to each tube, and reaction was allowed to proceed for 5 minutes. The reaction was stopped with 4µl of stop buffer. The samples were stored at -20°C until sequencing gel was run.

2.3 Polyacrylamide Sequencing Gel Preparation and Electrophoresis

2.3.1 Materials

Materials for polyacrylamide gel electrophoresis, such as the gel chamber and support, along with the acrylamide solution and TEMED (N,N,N'N' Tetramethylethylenediamine) were purchased from BioRad. Ammonium Persulfate was obtained from BRL (Bethesdia Research Labs). Tris, Boric Acid, EDTA, Urea, Glacial Acetic Acid, and Methanol were purchased from Fisher Scientific.

2.3.2 Methods

A 6% polyacrylamide sequencing gel was used to run the sequencing reactions. The gel plates and chambers were cleaned thoroughly with detergent and ethanol prior to use to eliminate bubbles during pouring of the gel due to dirt and dust. The apparatus was assembled according to manufacture's instructions.

The sequencing gel mix was made with:

40ml water
10ml 10X Tris/Boric acid/EDTA buffer
48g Urea
15ml 40% acrylamide solution (1:19 bisacrylamide:acrylamide)

The urea was dissolved in solution by swirling flask containing mixture under warm tap water. The solution was then filtered using no. 1 Whatman filter paper and degassed under vacuum. 400µl of freshly prepared 10% ammonium persulfate and 40µl of TEMED were added to the gel mix to initiate polymerization. The gel was then poured and allowed to polymerize at least 2 hours before running.

The gel chamber was filled with pre-warmed (to approximately 45°C) 1XTBE buffer and pre-run at 60 volts for 20 minutes to allow equilibration of ions. The sequencing reactions were denatured at 80-90°C for 3 minutes, then 3ul of sequencing reaction was loaded onto the gel. The gel was run at 60 volts until the first, bromophenol blue dye front had run to the bottom of the gel (approx. 2 hours). This was sufficient for sequencing from the vector-insert join inward. The gel was run longer according to where the desired sequence was located relative to the primer.

After running, the gel apparatus was disassembled, the gel was fixed with a 10% methanol/10% glacial acetic acid solution and dried under vacuum. The gel was then packed with X-ray film and developed after approximately 16-20 hours exposure.

2.4 Fertilization of *Xenopus* eggs

2.4.1 Materials

Xenopus laevis were purchased from Nasco. Human Chorionic Gonadotrophin and Cysteine were supplied by Sigma. NAM salts were purchased from the following companies; NaCl (BDH Inc), KCl, $Ca(NO_3)_2 4H_2O$, $MgSO_4\ 7H_2O$, EDTA (all from Fisher Scientific), HEPES (Sigma Chemicals).

2.4.2 Methods

Mature eggs were isolated and artificially fertilized as per Godsave *et al.*, (1988). Approximately 16 hours before eggs were needed, 750 I. U. of Human Chorionic Gonadotrophin was injected subcutaneously into the upper hind leg of a female *Xenopus laevis*, just above the cloaca. Within 12-18 hours, the female began to lay eggs, which were collected on a petri dish.

Next, eggs were fertilized with sperm extracted from a male testis. A male frog was previously sacrificed, testes were removed and stored a 4°C in 1 X NAM (Normal Amphibian Medium, see composition of the NAM solutions in Table 2.3; Slack and Forman 1980). Approximately 100µl of testes were mixed with a large drop of distilled water. The mixture was released by pipetting over the prepared eggs. A period of ten minutes was allowed for fertilization to take place and eggs were covered in water. A visible rotation of zygotes to animal (dark) side up indicated that the eggs had been fertilized.

The embryos were dejellied in a solution of 2.5% cysteine, pH 7.9. The embryos were swirled in cysteine for 20 seconds, then rinsed thoroughly with deionized water, 1l per 200 embryos. Water was removed and eggs were submerged in NAM/20. First cleavage begins 1-1.5 hours after fertilization.

Table 2.3 Composition of NAM (Normal Amphibian Medium) used for *X. Laevis* Experiments.

10X NAM Salts

	grams/litre (in 10X stock)	**mMolar (in 1X solution)**
NaCl	65	110
KCL	1.5	2
$Ca(NO_3)_24H_20$	2.4	1
$MgSO_47H_20$	2.5	1
EDTA (0.5M, pH 8)	2ml	0.1
Hepes (1M, pH 7.5)	100ml	10

For 100 ml - 1X Solutions

	NAM (ml)	**NAM/20 (ml)**
10X NAM Salts	10	0.5
Gentamycin	0.25	0.25
NaH_2CO_3	1	0.0
Sterile H_20	88.75	99.25

2.5 Microdisection and Treatment of *Xenopus* Explants

2.5.1 Materials

Recombinant XFGF-2 was expressed and purified according to Kimelman *et al.* (1988). Bovine Serum Albumin (BSA) was purchased from Sigma Chemicals. Trizol Reagent was purchased from Gibco BRL.

2.5.2 Methods

Xenopus embryos were allowed to incubate in NAM/20 for approximately 5 hours at room temperature to develop to Stage 8 according to Nieukoop and Faber Normal table of *Xenopus Laevis*, (1967). Once they had reached Stage 8, they were transferred to a petri dish lined with 1.5% agar and filled with 1X NAM solution. The vitelline membrane was removed manually with forceps, and the top ¼ of the embryo (animal cap explant) was cut out using a tungsten wire needle. The explants were cut in groups of approximately 20. The experimental set of embryos were placed in 200µl of NAM/2 + 1mg/ml BSA containing 100 ng/ml XFGF-2 in a petri dish. The control explants were treated with NAM/2 + 1mg/ml BSA without XFGF-2. Treatments were for 30 minutes. Explants were then pipetted into microfuge tubes, 0.5 ml of Trizol reagent was added, pipetted and vortexed to homogenize tissue, then solutions were placed at –70°C until the RNA extraction was performed.

Two sets of explants were cut to examine effectiveness of the FGF treatment. One set was placed in NAM/2 + BSA and the other set in 100ng/ml XFGF-2 in NAM/2 + BSA, and allowed to incubate 20-24 hours. The explants were then flooded with NAM/2

and observed for another 2-3 days. Induced explants appear balloon shaped after 2-3 days, large hollow balls with some grey tissue inside. Uninduced explants, however do not change over the course of incubation, they remain as small dark grey spheres of tissue.

2.6 Isolation of RNA from *Xenopus* embryos and explants

2.6.1 Materials

The Chloroform and Isopropanol were supplied by Fisher Scientific. Diethyl-pyrocarbonate (DEPC) was purchased from Sigma Chemicals, and RNA Guard from Pharmacia Biotech.

2.6.2 Methods

RNA was isolated from *Xenopus* tissue using the Trizol Reagent method of extraction. The same volumes of reagents were used for 5 embryos or 20 explants. The tissue was homogenized in 0.5 ml of Trizol reagent, as previously mentioned. At this point, the homogenate was either stored at –70°C or the extraction was conducted immediately. If frozen, the samples were thawed at room temperature and then treated as unfrozen samples.

Homogenates were incubated at room temperature for 10 minutes to allow separation of nucleoprotein complexes. 100µl of chloroform were added to extract the protein. The tubes were vortexed for 10 seconds, then allowed to incubate for 10 minutes

at room temperature. Samples were centrifuged for 10 minutes at 14000 rpm to allow separation of layers. The upper aqueous layer was pipetted into a new tube and 250µl isopropanol was added. The samples were vortexed for 10 seconds and incubated at room temperature for 7-8 minutes to allow for precipitation of RNA. The samples were then centrifuged at 4°C and washed with 1 ml 75% ethanol. The RNA was dried in a dessicator under vacuum and resuspended in DEPC- treated water. 0.5µl of RNA Guard was added to help protect RNA from degradation. The integrity of the RNA was tested by running 0.5µl of sample on a 1% agarose gel.

2.7 Isolation of Messenger RNA from Total RNA

Many of the gene products studied in this project were expressed at very low levels. Therefore, for Northern blotting it was favourable to use polyA+ RNA instead of total RNA. A PolyATract mRNA Isolation System for small scale mRNA isolation was used for this purpose. The basic protocol involved annealing polyA+ RNA to a biotinylated-oligo (dT) probe. The solution was then added to streptavidin coupled paramagnetic particles (SA-PMPs). The probe bound the particles, and the particles were captured using a magnetic stand. All unbound RNA was washed away, then polyA+ RNA was eluted with water.

2.7.1 Materials

The mRNA isolation kit, PolyATract mRNA Isolation System III was purchased from Promega. Reagents were supplied with the kit, including; 20XSSC (Sodium Chloride, Sodium Citrate, see 2.1.2 for concentrations), Biotinylated-Oligo (dT) Probe, Streptavin-Paramagnetic Particles, Magnetic stand, and nuclease free water. The lyophilizer is a Jouan model from Canberra Packard Inc. and the spectrophotometer is from Beckman.

2.7.2 Methods

Total RNA was isolated from approximately 200 embryos as per protocol above (approximately 800µg total RNA). The RNA was resuspended in 500µl of DEPC-treated water. The RNA was heat denatured at 65°C for 10 minutes, 3µl of Biotinylated-Oligo (dT) Probe and 13µl 20X SSC buffer were added and the mixture was allowed to cool to room temperature. The SA-PMPs were prepared by washing with 0.5X SSC as per protocol. The annealing reaction was added to the particles and the biotin-streptavidin binding was allowed to take place over 10 minutes. The unbound RNA was washed off with 4 washes of 0.3ml 0.1X SSC and each time the particles were captured with the magnetic stand. The mRNA was then eluted with 0.25ml of nuclease free water. A spectrophotometric reading was taken for each elution step, as well as for the mRNA to ensure efficient isolation. The mRNA (approximately 10µg) was frozen at -70°C.

The samples were then lyophilized to concentrate the RNA to appropriate volumes for loading on Northern gels (10µl). The frozen samples (in 1.5ml microfuge tubes) were

quickly loaded into a pre-cooled lyophilizer. The samples were allowed to spin under vacuum for 2.5 hours. The RNA pellets were then resuspended in 10µl of DEPC treated water and stored at -70°C until needed.

2.8 Agarose Gel Electrophoresis

2.8.1 Materials

Agarose was purchased from Gibco BRL. Ethidium Bromide was purchased from BioRad. Gels were observed under ultra-violet transilluminator from Hoefer Scientific, and photographed using a photodocumentation system from Stratagene. Analysis and photographs of agarose gels were obtained using ChemiImager 4000 low light imaging system from Canberra Packard Inc.

2.8.2 Methods

All electrophoresis gels were prepared with 1% agarose (Gibco BRL). Agarose was dissolved and the gels were run in 1X TBE buffer (10.8g tris base, 5.5 g boric acid, and 4 ml 0.5M EDTA, pH 8.0 in 1L deionized water). 6.6µl of 10mg/ml ethidium bromide were added per 100ml gel, when gel was ready to be poured. This was added to increase visibility of the nucleic acids under ultraviolet light. Normally, 9µl of sample were added to each well, along with 1µl of 10X loading dye. Gels were run at 100V for 45 –90 minutes. The nucleic acid was visualised by using an ultraviolet transilluminator and photographed using a photodocumentation system.

2.9 Reverse Transcription

2.9.1 Materials

For Reverse Transcription, the Random Primer, First Strand Buffer, and Murine Maloney Leukemia Virus (MMLV) RNA polymerase were purchased from Gibco BRL. dNTP's and RNA Guard were supplied by Pharmacia Biotech. Dithiothreitol (DTT) and Trichloroacetic Acid (TCA) were purchased from Sigma and Fisher, respectively.

2.9.2 Methods

Reverse transcription (RT) was performed on total RNA isolated as above. The volume of RNA solution used for each reverse transcription was adjusted such that each reaction tube contained 200-500ng of RNA. The RNA was diluted in DEPC-treated water to a final volume of 15µl. Two microlitres of 0.1µg/µl Random Primer was added and the mixture was heated to 70°C to denature RNA, and then quickly cooled on ice. 8µl of First Strand Buffer (supplied with enzyme) was added, along with 8µl dNTP (2.5mM each nucleotide), 4µl dithiothreitol, 1µl RNA Guard and 2µl Murine Moloney Leukemia Virus RNA polymerase. The mixture was incubated for one hour in a 37°C water bath.

A tracer experiment was performed at the same time as the reverse transcription as a measurement of efficiency of enzyme activity. 5µl of the reverse transcription sample were removed from the mixture before the incubation step and were added to 0.5µl of [^{35}S]-dCTP. The mixture was allowed to incubate for 1 hour at 37°C and then cooled on ice. Next 500µl of 4°C, 10% trichloroacetic acid was added to precipitate the DNA and the mixture was allowed to sit on ice for 10 minutes. The precipitate was then filtered with a glass filter and

rinsed with more 10% trichloroacetic acid solution. The filter, along with the DNA was placed in a scintillation vial, scintillation fluid was added and the radioactivity was counted on a Beckman liquid scintillation system.

2.10 Polymerase Chain Reaction (PCR)

2.10.1 Materials

The following reagents for PCR were purchased from Promega; Taq DNA Polymerase Enzyme and Buffer, and Magnesium Chloride. PCR Primers (see Table 2.2) and dNTP's were supplied from Gibco BRL. Mineral oil was purchased from Fisher. A Perkin Elmer Thermal Cycler was used to amplify DNA. Kodak X-OMAT AR film was purchased from InterScience.

2.10.2 Methods

A master mix was prepared by adding water to Taq buffer (see Table 2.4 for concentrations and volumes), dNTP's and $MgCl_2$ were added and the contents vortexed. In most cases, more than one set of primers was used at a time. In such cases the Taq enzyme was added next, the mixture vortexed, and a proportional volume aliquoted into a 0.5ml microcentrifuge tube for each set of primers, depending on the number of primers needed for PCR. The particular primer volume was added in proportion to the volume of original master mix in the microcentrifuge tube. 24µl of the respective primer master mix was added to 0.5ml microcentrifuge tubes. 1µl of the reverse transcription sample containing the first

strand DNA was pipetted in and then vortexed. 25µl of mineral oil was added to minimize product loss due to evaporation.

The PCR program used was as follows:

1 cycle:	denaturation	94°C for 4 min
36 cycles:	annealing	50°C for 30 sec
	elongation	72°C for 30 sec
	denaturation	94°C for 30 sec
1 cycle:	polishing step	50°C for 30 sec
		72°C for 10 min.

Histone control reactions had 25 cycling steps. The sample was then brought to room temperature. PCR products were run on a 1% agarose gel. Remaining product was stored in 4°C cooler.

Table 2.4 Reagent Volumes and Concentrations in 1000µl PCR

Volume for 1000 uL Total	Final Concentration
100µl 10x TAQ Buffer	1mg/ml
677µl Deionized Water	---
80µl 10 mM dNTP	0.8 mM
60µl 25 mM $MgCl_2$	1.5 mM
20µl 100 ng/ul Primer #1	2ng/µl
20µl 100 ng/µl Primer #2	2ng/µl
3µl TAQ Enzyme (5µ/µl)	1.5 U/ng
40 µL Reverse Transcription	

PCR were also run using radioactive [α-^{32}P] dATP in the dNTP in the master mix. The only differences from those previously described are the addition of radiolabeled dATP and the number of thermocycles in the PCR, which were: 19 cycles if the histone primer was used, and 26 cycles with the other primers.

PCR products from [α-^{32}P] dCTP labeled PCR were run on 6% acrylamide/bisacrylamide gel as described in section 2.3.2. The gel was exposed X-ray film for a period of 1-3 days inside a cassette with an intensifying screen.

2.11 PCR Product Quantitation

The relative levels of PCR products for each sample were analyzed differently depending on whether radioactivity was used in the reactions. Non-radioactive samples were run on an agarose gel containing ethidium bromide. A photograph of these gels was taken and stored on the ChemiImager 4000 computerized system, which was also used to perform image analysis. Radioactive samples run on a polyacrylamide gel were exposed to film as described above and the film was analyzed using the ChemiImager 4000 software. Radioactive samples were occasionally packed with a phosphoimaging screen (multipurpose or super sensitive) and then analyzed using the Optiquant acquisition and analysis software.

2.12 Northern Blotting

2.12.1 Materials

The agarose used for RNA gels was "Ultrapure" from BRL Labs. Fisher supplied other reagents needed, including; MOPS (Morpholinopropanesulfonic acid), sodium acetate trihydrate, formaldehyde (37% w/v), and formamide. SDS was from Sigma chemicals, Xylene Cyanol purchased from BioRad, and Bromophenol Blue dye from Electran, BDH Chemicals.

Gene Screen hybridization membrane was purchased from NEN Research Products, Dupont. Whatman paper was from Whatman International. SSC and SSPE buffer contents, sodium citrate, and sodium dihydrogen orthophosphate were from Fisher and BDH chemicals, respectively (other chemicals already given). Ultraviolet cross-linker used was UV Stratalinker from Strategene. Methylene blue stain was from Fisher Scientific.

Restriction enzymes for probe preparation were purchased form Gibco, BRL (buffers supplied with enzyme. Ultrafree-MC Centrifugal Filter Units were obtained from Millipore Corp.

Random Primer DNA Labeling kit was purchased from Gibco BRL. Sephadex G-50 was purchased from Pharmacia. The scintillation counter used was Beckman LS 3801, and scintillation fluid was from Amersham Life Sciences.

Salmon sperm DNA and ficoll for hybridization buffer were obtained from Pharmacia Biotech. Polyvinyl-pyrolidone was obtained from BDH Chemicals. Multipurpose and supersensitive phosphoimaging screens were purchased from Canberra- Packard. The images were scanned from the screens using the Cyclone

Storage Phosphor System and analysed using the Optiquant image analysis software also from Canberra-Packard Canada Ltd.

2.12.2 Methods of Northern Blotting

2.12.2.1 Running a Denaturing RNA Gel

An RNA grade gel was prepared with formaldehyde as a denaturant in the following manner. An agarose gel chamber, tray and comb were thoroughly cleaned as to eliminate any RNAses. 1.4g of RNA grade agarose was added to 84.8ml of DEPC-treated water and was heated in a microwave to dissolve agarose. After cooling to approximately 65°C, 12ml of 10X MOPS buffer (0.4M Morpholinopropanesulfonic acid, 0.1M Sodium Acetate Trihydrate, 0.01M Disodium EDTA dihydrate, pH 7.4) and 23.2ml of 37% formaldehyde were added to gel, it was poured into casting tray and allowed to solidify for 30 minutes.

RNA to be loaded onto gel was previously equilibrated to approximately 4μg/μl in DEPC-treated water.

For each sample, the following were added:

10μl	RNA
4.4μl	5X MOPS
7.8μl	Formaldehyde
22.2μl	Deionized Formamide

An RNA marker was used to estimate RNA sizes, and was treated the same as experimental RNA except only 2μl of marker was loaded onto gel with volume made up with DEPC treated water. The RNA was denatured by heating to 70°C for 10 minutes and quick-

cooling on ice. The samples were centrifuged briefly to settle out condensation. 4.4µl of 10X loading buffer (0.5%SDS, 0.25%Glycerol, 0.025M EDTA, Bromophenol Blue and Xylene Cyanol dyes) was mixed with RNA samples

The buffer chamber was filled with 1X MOPS, the samples were loaded onto gel, and it was run at 80V for 2.5-3 hours, or until the bromophenol blue dye front had migrated 10cm. Periodically during running the gel, the buffer was mixed between separate sides of the chamber to prevent accumulation of ions at the electrodes.

2.12.2.2 Blotting

Gene Screen membrane was hydrated with distilled water, and then equilibrated in 6X SSPE buffer (for 20X buffer; 3M Sodium Chloride, 5M Sodium Dihydrogen Phosphate Dihydrate, and 0.020M EDTA, pH 7.4). The blotting tray was filled with 6X SSPE and covered with saturated no.1 Whatman paper. The gel was rinsed with DEPC treated water, inverted, and placed on Whatman paper. Membrane was placed on gel (cut to size of gel), air bubbles were removed and the membrane was covered with three pieces of 6X SSPE saturated Whatman paper and paper towels. The gel was allowed to blot overnight. After 16-18 hours, the blot was disassembled and rinsed briefly with 6XSSPE. The RNA was UV cross-linked to the membrane and then dried under vacuum at 80 °C for 2 hours.

The marker lane portion of the membrane was cut from the remainder, and stained with methylene blue to examine the integrity of the RNA. The blot was stored in a hybridization bag.

2.12.2.3 Isolation of DNA Probe

DNA to be used for probing Northern blots were created from specific fragments which had previously been inserted into pCR2.0, (or another plasmid as specified), amplified by plasmid prep and sequenced as described in section 2.2.1. Histone was used as a control. These DNA fragments for probing were typically 400-600bp in size, and were removed from the plasmid by restriction enzyme digestion, and agarose gel electrophoresis.

The digestion was conducted with restriction enzymes, which cut only at the multiple cloning site in the plasmid. Typically, EcoRV or a mixture of Apa1 and Kpn1 was used. For the Apa1/Kpn1 digestion, 10μg of plasmid DNA was incubated with 1X React 4 buffer and 2μl (10U/μl) of Apa1 at 30°C for 1 hour. Then 2μl (10U/μl) of Kpn1 was added and the reaction was incubated at 37°C for 1 hour. For the EcoRV digestion, 1X React 2 buffer was used, along with 2μl of 10U/μl enzyme at 37°C for 2 hours. The reaction was diluted from 50 to 200μl and extracted twice with an equal volume of phenol:chloroform: isoamylalcohol (25:24:1) and precipitated with ethanol. The DNA was resuspended in 20μl of water, 2μl of 10X loading buffer was added and 11μl of sample was run in 2 wells of a 1% agarose gel. After the gel was run at 100V for 45 minutes, the gel was observed with a hand-held UV source. The band of correct size was cut from gel, gel was macerated, and centrifuged through a filter unit at 8000rpm for 5 minutes which allow passage of the DNA through the membrane, while retaining the agarose within the upper chamber of the filter. The DNA fragment was phenol:chloroform:isoamylalcohol extracted and ethanol precipitated as above, 1μl was re-run on an agarose gel to estimate concentration and remainder stored at -20°C until needed.

2.12.2.4 Labeling DNA Probe

The labeling reaction was conducted using the Random Primers DNA Labeling System (GibcoBRL). The DNA fragment was digested and isolated from plasmid DNA as per protocol. 50-75ng DNA was added to distilled water to a final volume of 20µl. The DNA was denatured by boiling for 5 minutes and quick-cooling on ice. Radioactivity was obtained as either [α^{32}P]-dATP or [α^{32}P]-dCTP, used interchangeably, substituted for the corresponding nonradioactive nucleotide. The following components (supplied with kit, except [α^{32}P]-dATP and [α^{32}P]-dCTP) were added on ice:

2µl 0.5mM dATP solution
2µl 0.5mM dGTP solution
2µl 0.5mM dTTP solution
15µl Random Primers Buffer Mixture
4µl distilled water
5µl [α^{32}P]-dCTP, 3000Ci/mmol, 10µCi/µl

Components were mixed with pipetting and centrifuged briefly. Added 1µl Klenow Fragment, mixed with pipetting, and incubated 1-1.5 hours at room temperature.

Labeled probe was isolated from unincorporated nucleotides using a Sephadex G-50 spin column. Column was made fresh using a 1ml syringe plugged at bottom with glass wool and filled with Sephadex G-50 pre-swollen in Tris-EDTA buffer. Column was packed by centrifuging in a clinical centrifuge at 4 for 2 minutes. Column was refilled until resin volume after centrifugation was 1ml.

The labeling reaction was stopped with 5µl of stop buffer (supplied with kit) and 45µl distilled water was added to take volume up to 100µl. The labeling reaction was loaded onto column and centrifuged in a clinical centrifuge for 2 minutes at setting 4. The column

was spun in a 15ml snap cap tube to catch elutant. The elutant volume was measured and was placed in a 1.5ml microcentrifuge tube. Incorporation of radioactivity was measured with a scintillation counter. 1µl and 2µl of probe were aliquoted into scintillation vials containing scintillation fluid. The radioactivity was counted for 30 seconds and the reading from 0 to 1000 was obtained. From this value, the total radioactivity (CPM) and specific activity (CPM/ug DNA) was calculated. The minimum activity used for probing blots was $1X10^8$ CPM/ug DNA, but generally $2 X 10^9$ CPM/ug DNA was obtained. The probe was stored at 4°C while blot was prehybridizing.

2.12.2.5 Prehybridization and Hybridization

For the first hybridization, each membrane was moistened with 2X SSC (sodium chloride and sodium citrate) buffer (see Table 2.5). Once blots were probed, they were stripped and stored at 4°C in 2XSSC buffer. Once the required image was received from the blot, the old probe was removed so that the membrane could be probed again, with a different probe. In the procedure of stripping the blot, the bond between the RNA on the membrane and the DNA probe was broken and the probe washed away. The bonds were broken by adding it to a boiling solution of 0.5% SDS, and boiling for 10 minutes. The bath was then removed from heat and allowed to cool for 10 minutes. The blot was then washed and stored in 2XSSC until ready to probe.

Prior to probing the membrane, a prehybridization step was conducted. The purpose of this step was to allow DNA (from salmon sperm) to anneal to all portions of the

membrane that did not contain RNA, thereby preventing nonspecific binding of the probe to the membrane. Prehybridization also sets favourable conditions for hybridization.

The volume of hybridization solution needed was calculated according to the size of the membrane, 50µl/cm^2 was needed for each step (prehybridization and hybridization). The solution was made (see Table 2.5) and placed in a 42°C water bath. The salmon sperm was sheared by sonication, boiled 5 min to denature, quick-cooled and then added to the solution. For prehybridization, the solution was added to the membrane, sealed in a hybridization bag, and incubated at 42°C with light shaking for 2-4 hours.

To prepare for hybridization, the amount of probe to be added to the blot was calculated. Typically 25ng of DNA was added with maximum of 5X 10^{6}cpm/ml hybridization solution. The probe was boiled for 5 minutes to denature, and quick cooled on ice. 1ml of 42°C hybridization buffer was added to a 1.5ml tube and the remainder was added to blot (prehybridization buffer removed). The appropriate volume of probe was added to the 1ml hybridization buffer, mixed, and pipetted onto blot. Air bubbles were removed, and the bag was heat-sealed. Hybridization occurred over night in the 42°C water bath with light shaking.

Table 2.5 Composition of Hybridization Solution

Volume Added (for 10 ml Solution)	**Reagent**	**Reagent Composition**
5 ml	Deionized Formamide	
2.5 ml	20XSSC	Sodium Chloride Sodium Citrate
0.20ml	50X Denhardts Solution	1%w/v Ficoll type 400 1%w/v polyvinyl-pyrolidone 1% w/v BSA fraction 5
0.20ml	1M Tris-Cl pH 7.5	
1.0ml	10% SDS	
1.0ml	Sterile water	

2.12.2.6 Washing, Exposing and Analysis of Northern Blot

After allowing the labeled probe to hybridize to matching RNA sequences, the blot was washed to remove excess and nonspecifically bound probe. Three wash solutions were made and heated to 42°C:

Wash Buffer 1 – 2XSSC and 0.5 % SDS

Wash Buffer 2 – 0.5XSSC and 0.5% SDS

Wash buffer 3 – 0.1XSSC and 0.1% SDS

The hybridization solution was drained from the blot and it was rinsed with wash buffer 1. More wash buffer 1 was added to the blot, and it was washed with shaking for 10–15 minutes. The temperature was increased during this washing step from 42-58°C. Wash buffer 1 was poured off and wash buffer 2 added. The blot was incubated again for 10-15 min with shaking and the temperature was allowed to reach 65°C. Wash buffer 2 was then removed and the radioactivity of the blot was tested with a geiger counter. If the blot was still 'hot', it was washed 1- 2 more times with wash buffer 3. This washing step was to a maximum of 68°C for 20 – 30 minutes, or until the blot appeared clean (very little response from the geiger counter).

The blot was then packed with either x-ray film with intensifying screen at -70°C, or with a phosphoimaging screen (multipurpose or supersensitive) at room temperature for approximately 7 days. The films were developed and analyzed as in Section 2.11.

2.13 Ribonuclease Protection Assay

The ribonuclease (RNAse) protection assay is another method used to examine differential expression of specific genes in various tissues or in response to tissue manipulation. The theory behind the RNAse protection assay is that only double stranded RNA, created by the transcript of interest binding to an antisense probe, will be protected from degradation by RNAses. Therefore, RNA is isolated from cells and purified. A radiolabeled antisense probe is created and allowed to bind to its complementary RNA sequence. The RNA is then treated with RNAses that digest single stranded RNA. The digests are then run on a sequencing gel and the relative amount of RNA in various tissues is quantitated. The protocol used for this procedure is similar to that described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1993).

2.13.1 Materials

The dNTPs were purchased from Pharmacia Biotech. Radiolabeled [α-^{32}P] dATP and [α-^{32}P] rUTP were from Mandel. ϕX174 Hinf1 dephosphorylated markers and RQ1 RNAse free DNAse were from Promega. RNAse T1, RNA Polymerase (SP6 or T7), rNTPs, Klenow (Large Fragment of DNA Polymerase 1) and 10X Klenow Buffer were supplied from Gibco BRL. LiCl, and MgCl2 supplied by Fisher Scientific. RNAse A, Proteinase K, and transfer RNA (tRNA) were from Boeringer Mannheim and Sigma supplied the Dithiothreitol, SDS, Piperazine-N,N'- bis[2-ethanesulfonic acid] (PIPES) and Spermidine.

2.13.2 Methods of RNAse Protection Assay

2.13.2.1 Creating an Antisense Probe

Probes were designed to contain a 5' overhang or blunt end and were 100-350bp in size. Each fragment used for creating probes in this experiment was cloned into pCR 2.0, containing a T7 promoter on one end and SP6 on the other. Therefore, endonucleases were used which cut the DNA insert in such a way that when they were amplified with SP6 or T7 polymerase, the correct size probe was created.

20-30μg of DNA was linearized with the appropriate restriction enzyme and resuspended in water to give a 1μg/μl concentration (similar protocol to that used for northern probe preparation, Section 2.12.2.3). The probe was radioactively labeled according to the following protocol.

The following reagents were added to a 1.5ml centrifuge tube:

1μl	10X Transcription Buffer (400mM Tris-Cl, pH7.5, 60mM MgCl2, 20mM Spermidine)
1μl	10mM of rATP, rGTP and rCTP mixture
0.5μl	250mM Dithiothreitol
0.5μl	RNA Guard
0.8μl	57.5μM rUTP
4.2μl	3000Ci/mmol α-^{32}P rUTP
1μl	19U/μlRNA Polymerase (SP6 or T7)
2ul	1μg/μl DNA template

The contents were mixed and incubated at 37°C for 90 minutes. 1μl of 2.5mg/ml RQ1 RNAse-free DNAse was added and the contents was incubated at 37°C for a further 15min. 40μl of DEPC-treated water and 1μl of 10mg/ml tRNA was added. The probe was then

precipitated with; 5µl 0.2M EDTA, pH8, 6.25µl 4M LiCl, and 200µl 100% EtOH for 1 hour at -20°C.

The probe was then centrifuged at 4°C for 30 minutes, washed with 70% EtOH, air dried and resuspended in 50µl Tris/EDTA (TE). The specific activity of the probe was counted in a scintillation counter. An activity of 0.5-1.0 X 10^6 CPM is needed for addition to each experimental sample.

2.13.2.2 Hybridization and Ribonuclease Digestion

The next step in this protocol was hybridization of the radioactive RNA probe to its complementary mRNA sequence. Total cellular RNA was isolated as per section 2.6.2. The following reagents were added to a 1.5ml microcentrifuge tube for each RNA sample:

22µl	Hybridization mix (8.3ml Deionized Formamide, 0.8ml 5M NaCl, 0.8ml 0.5M PIPES, pH 6.4 0.1ml 100mM EDTA)
1µl	RNA Probe
2µl	RNA Sample (~5µg depending on level of expression within tissue)

The reagents were mixed, and the tubes were heated to 80°C for 5 minutes to denature RNA. The samples were then allowed to hybridize at 50°C for 16 hours. Next, the single stranded RNA was digested using the following digestion mix;

10mM Tris-Cl, pH 7.5
5mM EDTA
300mM NaCl
0.1µl/ml 10mg/ml RNAse A
2µl/ml 2µg/mlRNAse T1.

350µl of digestion mix was added to each hybridization sample and incubated 10min at 37°C. Next, the protein was digested with 10µl 20% SDS and 2µl of 25µg/ml Proteinase K

for 10 min at 37°C and extracted with phenol. The undigested RNA was then precipitated using the same method as for the probe and resuspended in 2µl of TE. 2µl of running buffer was added to each sample (formamide with xylene cyanol and bromophenol blue dyes).

A 6% polyacrylamide gel was previously prepared and pre-heated as in section 2.3.2. The samples and molecular markers (see below) were heated to 80°C and loaded onto the gel which was run until bromophenol blue dye front had reached the bottom of the chamber. The gel was dried and packed with X-ray film overnight. Analysis of film was conducted using the ChemiImager system.

2.13.2.3 Labeling Molecular Markers

Dephosphorylated DNA markers, digested with Hinf1, were used to identify the size or the assay products. The markers were radiolabeled and then run on the polyacrylamide gel adjacent to the experimental samples. The protocol used to label the markers is as follows; the following reagents were added to a 1.5ml microcentrifuge tube and allowed to incubate at room temperature for 20-30 minutes:

3µl φX174 Hinf1 dephosphorylated markers
1µl 10X Klenow Buffer (20mM Tris-Cl, pH8, 7mM $MgCl_2$)
1µl 3.3mM dCTP
1µl 3.3mM dGTP
1µl 3.3mM dTTP
1µl α-^{32}P dATP
4µl dH_2O
1µl Klenow (Large Fragment of DNA Polymerase 1)

The enzyme was then heat-inactivated at 70°C for 10 min. 100µl of dH_2O was added, along with 12µl 3M NaOAc and 250µl 100% Ethanol. The DNA was allowed to

precipitate at -70°C for 1 hour. The precipitate was then centrifuged at 4°C for 30 minutes, supernatant was decanted, washed with 70% ethanol, dried and resuspended in 50µl of Tris/EDTA. 1µl of marker was added to 50µl of running buffer and 4µl was run on the polyacrylamide gel.

2.14 Gene Cloning from cDNA Libraries

The following protocol was used to clone larger fragments of the genes isolated from the differential display gel (see Figure 2.1). A Stage 8 *Xenopus* embryo cDNA library cloned into the Bluescript plasmid, a Stage 17 *Xenopus* library clone into a λ gt10 vector and a human testis library cloned into pACT2 library were used.

2.14.1 Materials

cDNA libraries were prepared previously as described in Paterno *et al.*, 1997. All PCR reagents, LB, agar and agarose were supplied as above. The primer sequences are listed in Table 2.2. The TA Cloning ligation kit and the "One Shot" transformation kit were supplied from Invitrogen. Kanamycin was purchased from Boeringer Mannheim.

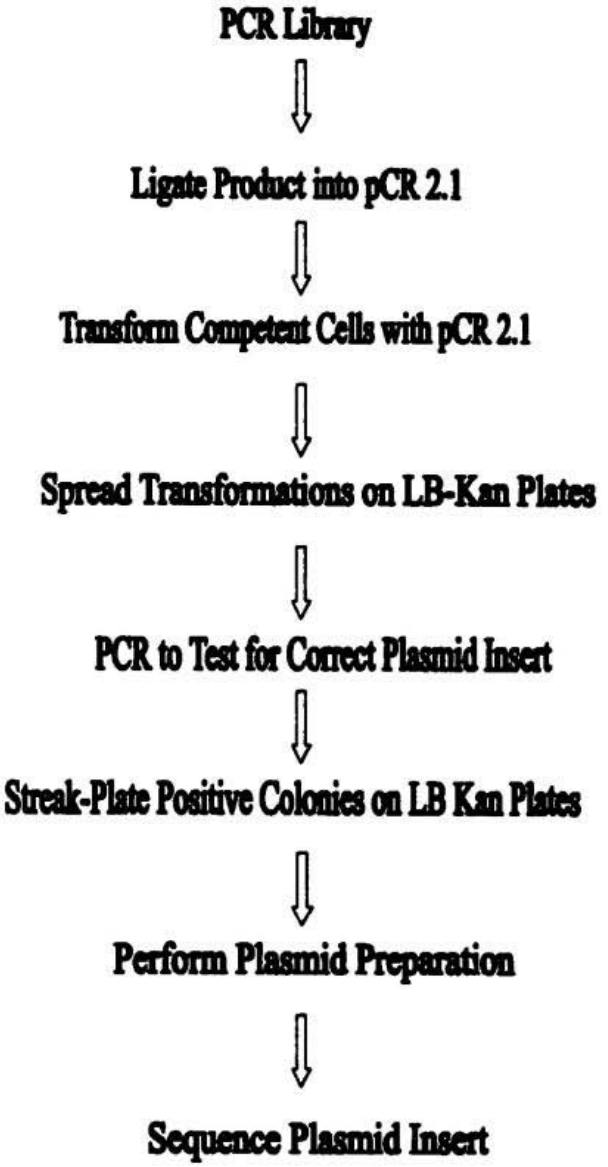


Figure 2.1 Strategy for Cloning from cDNA Libraries

2.14.2 Methods of Gene Cloning

2.14.2.1 Polymerase Chain Reaction on Libraries

The 5' to 3' orientation of the DNA fragments was unknown, therefore the library was tested in both orientations. PCR was conducted on the library with one internal primer and one external (vector) primer (see Table 2.2 for sequences). The following vector primer sets (5' and 3') were used for each library;

Bluescript - T3 and T7

λ GT10- 8858 and 8861

pACT2 – pACT2-for and pACT2-rev.

For example, when cloning Sample 16, the ChemR-1 primer was tested with both 3' and 5' primers for each library plasmid. Serial dilutions were made for each library. PCR was conducted for each dilution. For the genes tested, approximately 1 X 10^8 to 1 X 10^7 plaque forming units (PFU)/ µl DNA was optimum. Reactions were duplicated for each dilution using only the 3' and 5' internal primers to ensure that the correct product was being amplified. A master mix was made as per section 2.10.2, and the PCR program was the same, except 33-36 cycles were used.

PCR products (10µl) were run on a 1% agarose gel. Samples positive for internal sequence, as well as yielding a larger fragment from the library, were ligated into a plasmid.

2.14.2.2 Ligation

The next step in this protocol was to ligate PCR product into a new vector for amplification. The Invitrogen cDNA cloning kit with the pCR 2.1 vector was used. The

protocol is as per kit instructions, and all reagents were supplied. 3:1 ratio between the estimated PCR product and vector was calculated. The following were added to a 0.5ml microcentrifuge tube to 10µl total:

1-2µl	PCR product
1µl	Ligation Buffer
2µl	pCR 2.1 vector
4-5µl	Sterile water
1µl	DNA ligase

The reaction was stored at 14°C for 16-18 hours. Next the plasmids were transformed into bacterial cells.

2.14.2.3 Transformation

The plasmid, containing the new cDNA fragment was then transformed into bacterial cells using a transformation kit. All reagents were supplied with kit. A water bath was set to 42°C and the SOC media was thawed from -70°C to room temperature. The competent "one shot" cells and 0.5M β-mercaptoethanol were thawed gently on ice. 2µl of β-mercaptoethanol and then 2µl of ligation reaction were added to the competent cells and reaction was allowed to continue on ice for 30 minutes. The cells were then heat shocked for 30 seconds at 42°C and placed on ice for 2 minutes. 250µl of SOC media was added to the cells and they were grown at 37°C with shaking for 1 hour.

Two aliquots; 50µl and 200µl of the transformed cells were spread-plated on LB-agar plates containing 100µg/ml kanamycin and allowed to incubate at 37°C 16-18 hours. Bacterial colonies were tested for plasmid insert by PCR with plasmid primer (pCR-SP or pCR-SP) and internal primers (for sample 14, ChemR-1 and ChemR-2). The protocol was as

per section 2.10.2, however, instead of adding 1µl of DNA to each reaction, the colony was touched with a sterile pipette tip and swirled in the reaction tube. Again, both orientations were tested. Bacterial colonies that tested positive for correct insert were streaked on an LB-agar plate containing kanamycin and grown 16-18 hours at 37°C. A plasmid preparation was conducted on the colonies as per Section 2.2.2 and the plasmid insert was sequenced as per Section 2.3.

Chapter 3

Results

3.1 Differential Display

FGF elicits its cellular response by triggering a signal transduction cascade, resulting in differential expression of target genes. The purpose of the differential display (Liang and Pardee, 1992) conducted in this laboratory was to isolate cDNA fragments representing genes activated or repressed by FGF.

The PCR-based differential display was performed by Yu Li (Paterno *et al.*, 1997). A basic overview of the procedure is as follows. Animal cap explants were cut from *Xenopus laevis* embryos at blastula stage (stage 8). Five different experimental sets, each composed of 40 animal cap explants, were used in this experiment. Half of the explants in each set were treated with 100ng/ml XFGF-2 for 30 minutes, and the other half were treated with control media for the same length of time. This was thought to be the optimum time frame to observe differences in expression patterns of immediate-early genes.

RNA was isolated from both sets of explants, and reverse transcription was conducted using a $T_{(11)}AC$ primer to transcribe polyA+ RNA only. The CA dinucleotide was made to anchor the primer at the 5' end of the poly A+ tail, which binds the poly T

region of the primer. A poly T primer without an anchor could prime anywhere along the poly A+ tail and result in cDNA species of multiple sizes, and thus a smear on the differential display gel (poly A+ tails may be as long as 300 nucleotides) (Liang and Pardee, 1995).

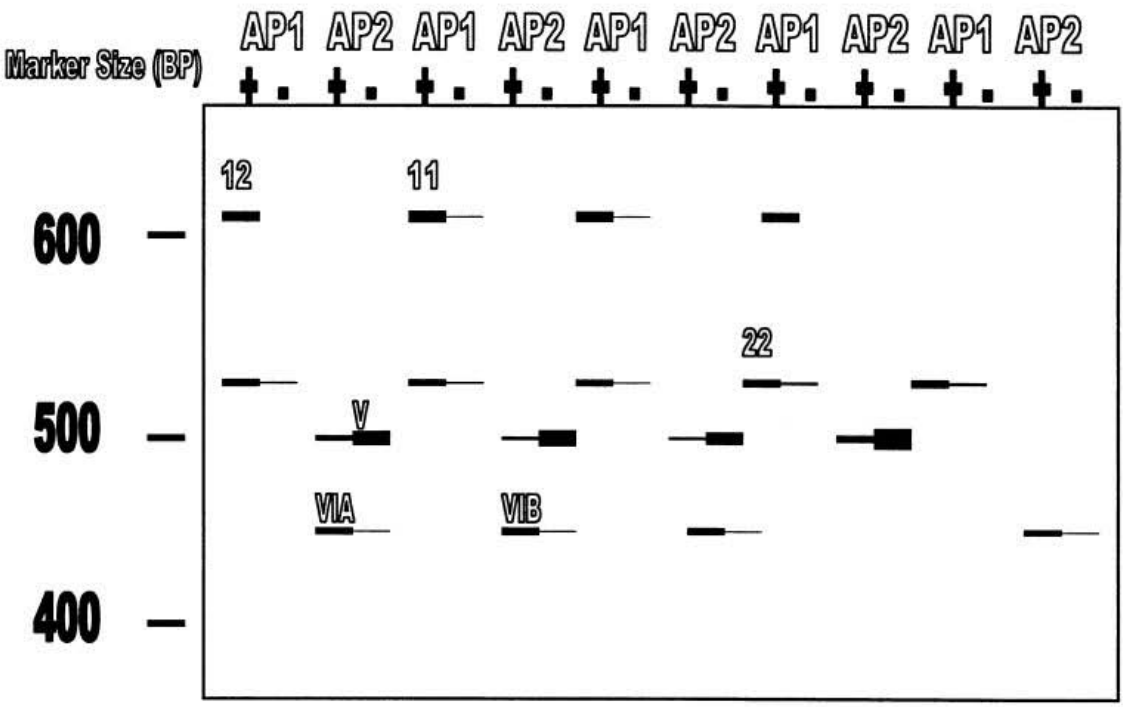
Next, PCR was conducted on the cDNA using the same $T_{(11)}AC$ primer and an arbitrary random primer AP1 or AP2, along with [α^{35}S]-dATP. The amplified products from the 5 matching sets of explant RT-PCRs were separated side by side on a sequencing gel (FGF treated and control) and analyzed by autoradiography. Bands appearing to be either upregulated or downregulated in at least 4 of the 5 sets were eluted from the gel.

Six bands were isolated, all between approximately 400 and 600bp long. The number assigned to each band, PCR primers used to amplify, relative position, and size are given in Figure 3.1. Next, non-radioactive PCRs were conducted to further amplify the DNA fragments isolated from the gel. They were then ligated into pCR 2.0 plasmid vector and transformed into bacterial cells. From there, five cultures were grown for each of the six bands and glycerol stocks were made and stored at -70°C.

An additional differentially expressed band from the differential display, named early response 1 (*er1*), was cloned and characterized prior to this project (Paterno *et al.*, 1997).

The overall goal of this project was to sequence the bands amplified from the differential display gel, examine their regulation by FGF and to determine their expression pattern throughout *Xenopus* development (Figure 3.2).

A.



B.

Band	PCR Primers	Glycerol Stock Numbers	Response to FGF
V	AP2/T_{11}AC	1-5	Downregulated
VIA	AP2 /T_{11}AC	6-10	Upregulated
VIB	AP2 /T_{11}AC	11-15	Upregulated
11	AP1 /T_{11}AC	16-20	Upregulated
12	AP1 /T_{11}AC	21-25	Upregulated
22	AP1/ T_{11}AC	26-30	Upregulated

Figure 3.1 Differential Display. (A) Schematic diagram of differential display gel. + signifies the FGF treated lane, and – signifies the untreated lane. AP1 and AP2 indicate the random primers used for PCR. The bands cut from the gel have been numbered accordingly. (B) Table summarizing bands from the differential display gel.

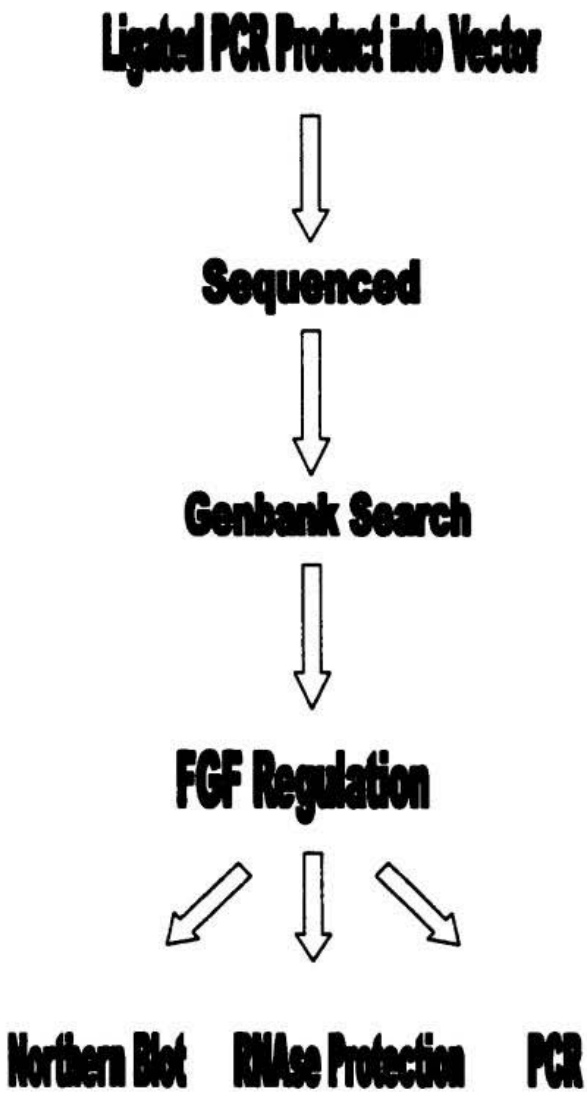


Figure 3.2 Flow Chart of Methodology Used to Investigate Potential Early Response Genes.

3.2 Sequencing and Genbank Searches

The goal for this part of the project was to sequence at least two of the five cDNA fragments from each of the 6 differential display bands. The number of cDNA species in each band was not known, however the dominant species was most likely to represent the FGF regulated gene. Therefore, the more representative bands sequenced, the better. However, for practicality, two bands representing the same cDNA were cloned from each band. Cloning two bands was also useful for confirming the nucleotide sequence between them.

I proceeded with isolating a pure colony from the glycerol stocks, performing a large-scale plasmid preparation and sequencing the chosen stocks. Each glycerol stock was given a sample number from 1-30 for easy identification, for example, glycerol stocks from the first band, Band V were named 1-5, (Figure 3.1).

Next, the sequence of each fragment was sent to the National Center for Biotechnology Information BLAST Network Service and analyzed using the BLASTX 1.4.1.1 program (Genbank), which compares the amino acid sequence in all six reading frames to all other submissions. The reading frame gives reference to the position assigned to the first nucleotide for the first amino acid in a DNA sequence. In the case of unknown sequences, the reading frame is assigned according to the nucleic acid sequence. For a reading frame numbering example, see Figure 3.3.

The Genbank search was to determine whether the DNA fragment from the potential FGF response gene was part of a novel gene, a homologue of a previously

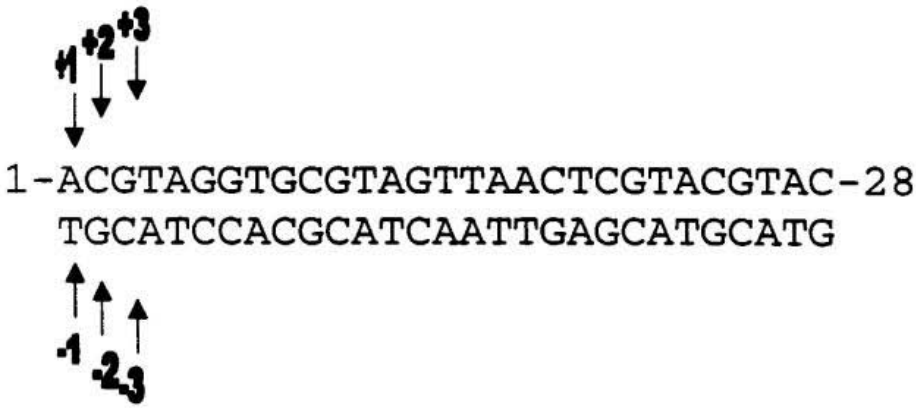


Figure 3.3 Schematic Diagram Referencing the Six Reading Frames. The positive reading frames reference the top sequence reading 5' to 3', and the negative reading frames represent the bottom sequence reading 3' to 5' (left to right).

cloned gene from another species, or a known *Xenopus* gene. The Genbank identifies regions of similar genes by the percent identity and percent positive matches between corresponding amino acids. Percent amino acid identity is calculated from the number of identical amino acids, and positives include identical amino acids and conservative changes in amino acid sequence between the two genes. For example, a change from isoleucine and valine (two hydrophobic amino acids) would be considered a conservative change.

A variable number of matches were obtained for each sequence sent to the Genbank. I have selected Genbank sequences that revealed the largest number of consecutive matches (similar or identical) to the new sequences, or that have functional regions that match with the new sequences. Any amino acid domains similar to a known gene might suggest a possible function of the gene and methods of further investigation.

The combination of numbers and letters following the name of each gene obtained from the Genbank is the accession number and is used to identify genes for databank searches. The Genbank search results for each sequence are given in the following sections.

3.2.1 Sequencing Band V

For Band V, the only band that appeared to be downregulated by FGF treatment, I sequenced stock numbers (Samples) 1 and 3. The nucleotide sequences of the 499bp (Sample 1) and 498bp (Sample 3) vector inserts are shown in Figure 3.4. The sequences are the same with the exception of 7 bp changes, and one extra base pair insert in Sample 1, which may have been due to PCR or sequencing errors.

A Genbank search with Samples 1 and 3 revealed similarity to the small regions of several proteins including:

> Alternatively spliced region of the transcription factor Human Myocyte- Specific Enhancer Factor 2 (aMEF 2) (X68503),
> T-cell receptor (TCR) gamma chain V-J-C region (clone 197G1) from *Ovis aries* (Z12964),
> *H. sapiens* Meprin A Beta-Subunit Precursor (Endopeptidase-2) (X81333),
> *Bos taurus* Myosin I heavy chain-like protein (MIHC) (J02819), and
> Bovine hypothetical 11kDa protein (BHP) upstream of parathyroid hormone gene (X12515).

The regions of similarity between the unknown sequence and the Genbank matches are shown in Figure 3.5. The significance of the search results will be discussed in Chapter 4.

```
  1-CTGCTCTCAG ATGCAATGAC AACACTATCT CTATTCCAGG ATGACTTAAA
    .......... .......... .......... .......... .......C..

 51-GTCAAATGTT GATGTTGTTT AGTTGCTAAG TTATGTCTGA CTCTTTTGCA
    .......... .......... .......... .......... ..........

101-ACCCCATGGA CTATAGCCCA CCTCTGTCCA TAGGATTTCC CAGGCAAGAA
    .......... .......... .......... .......... ..........

151-TACTGGATGG TTTGCCACTT CTCTAGGAAA TCTTTCCAAC CCAGGGACTG
    .......... .......T.. .......... .......... ..........

201-AACCCACATC TTGTGCTTGG CAACCGATTC TTTACCACTG AGCCACTAGG
    .......... .......... .......... .......... ..........

251-GAAGCCCTTA AAGTCATATA AAGTAATGTT AATTTCAGAA TGCTTTCATA
    .......... .......... .......... .......... ..........

301-TCGAAGTTAA GAGCCCAGAT AAATTTTAAA TAGCAGTGAA TCCATTGCAG
    ..A....... .......... .......... .G..T..... ..........

351-CTATTCCCAC CAAGAGTTGG AGTCTATTTT CAACACTCTC CCCTTGACTC
    .......... .......... .......... .......... .....-....

401-TGGGCTGAAT CTATGGTTTT CTTTGGCCAA CAGACTGTGC TACTTCAATA
    .......... .....AC... .......... .......... ..........

451-CTTACCTTCT TACCAGACAC TTCTATCTTG TGAAGGAGCC TGAGAGCAG
    .......... .......... .......... .......... .........
```

Figure 3.4 Nucleotide Sequence of Sample 1 (top row) and Sample 3 (bottom row). Only the nucleotide changes are shown for Sample 3; a dot represents an identical nucleotide and a dash represents a deletion. The number to the left of each line indicates the nucleotide number.

A. Human Myocyte- Specific Enhancer Factor 2

```
S3  326-LNGCES-343        360-PRVGVYFQHSPLT-398
       LNGCES                P     YF+HSPL+
aMEF  7-LNGCES-12          13-PDADDYFEHSPLS-25
```

IDENTITY	13/19 amino acids	68%
POSITIVES	15/19 amino acids	79%
Reading Frame	+2, +3	

B. T-cell receptor gamma chain V-J-C region

```
S1    95 FATPWTIAHLCP 130      S1   121 PLSIGFPRQEYW 156
         FATPWT+A   P                   PL + F RQEYW
TCR    2 FATPWTVACQAP 13       TCR   13 PLPMEFSRQEYW 24
```

IDENTITY	16/24 amino acids	66%
POSITIVES	18/24 amino acids	75%
Reading Frame	+2, +1	

C. Human Meprin A beta-subunit precursor

```
S3   187-PGTEPTSV-211        S3   319-ESIAAIPTKSWSLF-360
        GTEPT V                     E I  IPT SW L+
MAP  218-NGTEPTIV-225        MAP  375-EEIKEIPTGSWQLY-388
```

IDENTITY	11/18 amino acids	61%
POSITIVES	14/18 amino acids	77%
Reading Frame	+2	

D. Myosin I heavy chain-like protein

```
S1    268-YDFKGFPSGSVVKNRL-221   S1    115-YSPWGCKRVRHNLATKQ 65
         +DFKGFP G V+ N L                + W CK+ R  L+ KQ
MIHC  167-FDFKGFPLGGVITNYL-182   MIHC  824-FHQWKCKKFRDQLSPKQ 840
```

IDENTITY	17/33 amino acids	51%
POSITIVES	22/33 amino acids	66%
Reading Frame	-3	

E. Bovine hypothetical 11kDa Protein upstream of Parathyroid Hormone

```
      S3  92-SFATPWTIA-118        S3  120-TSVHRISQARILD-158
            +  TPWT+A                    +SVH   +ARIL+
      BHP 43-TLVTPWTVA-51         BHP  54-SSVHGFFRARILE-66
            S3  154-WMVCHFSRKSFQPRD*THILCLA-222
                  W+   FS +S Q R+ T + C+A
            BHP  67-WVAISFSGESSQSRNQTQVSCIA-89
```

IDENTITY	21/45 amino acids	47%
POSITIVES	31/45 amino acids	69%
Reading Frame	+2, +3, +1	

Figure 3.5 Amino acid similarities between Genbank matches and Sample 3 (and 1). **A.** Myocyte Specific Enhancer factor 2 (aMEF 2), **B.** T-cell receptor gamma chain V-J-C region (TCR), **C.** Human Meprin A beta-subunit precursor (MAP), **D.** Myosin I heavy chain-like protein (MIHC), and **E.** Bovine hypothetical Protein (BHP). The numbers to the left and right of each amino acid sequence reveals the corresponding nucleic acid number for S3 and the amino acid number for each known gene from the Genbank.

3.2.2 Sequencing Bands VIA and VIB

The second and third Bands (VIA and VIB) isolated from the differential display gel represented the same Band cut from different experimental sets (Figure 3.1), therefore one would expect that the DNA sequences would be the same. Both Bands were upregulated by FGF treatment in the original differential display.

Two clones were sequenced from Band VIB, Samples 14 and 15. They contained the same 449bp sequence, with only 1 base pair substitution (Figure 3.6). The 5' end of Samples 6 and 7 from Band VIA were sequenced. Sample 6 and 7 showed identity to the 3' and 5' end of Sample 14, respectively. Since Samples 6 and 7 appeared to represent the same sequence as Samples 14 and 15, I focussed my analysis on the latter.

Upon sending the sequence to the Genbank for analysis, Samples 14 and 15 demonstrated similarity to a mouse serine proteinase inhibitor 2.4 (Spi2.4) (clone 3E46) (X69832) and, a mouse contrapsin-related protein MC-7 precursor (X55148), (Figure 3.7). The serine proteinase inhibitor and contrapsin- related precursor possess the same sequence within the region of similarity to Sample 14, which is partially within the reactive centre of the enzyme. These results, as for all Genbank results, will be discussed in Chapter 4.

```
  1-CTGCTCTCAG AAAAATGCTA TAGAGACGTA TATGACATAA ATAATCTGTG
 51-ATGAAACAAT TTAGGTTTCA TTAGCTTTTA CAAAAATGGA AAAAGTATGA
101-CCATGGTTGC ACAGTTTGGC AAACCATTTT TTCTATCATT CCTACAAAAT
151-ACTACTGAGT GTTACTGGAC ACTGATATGA TTATTAAAGA TATTTTCTTT
201-ATATAAATTG TATATCAATA AATTATAAAT ATGCAAGAGG TAGGTTGCAG
251-TTACCTACTT ACAGAAGCAA TTATCACTAA ACTGCTGACA TGCCAGTTTG
301-GTTGTTCAGC ATACTTCAGT ACAAACAAGA AGCTTCTGGA GTTTCCAGTA
351-CACTGCATTT TATACAAATG TAACGTATAG GCTCATAAAC CTAAAGCACA
401-CTAGTTATTT AGATTTACTA CATACATAAA GATACACAGC TGAGAGCAG
```

Figure 3.6 Nucleotide Sequence of Sample 14. Sample 15 has the same sequence, except nucleotide 261 is G instead of A.

Serine Proteinase Inhibitor and Contrapsin-Related Precursor

```
S14    4-KCYRDVYDIXXXXXXXXGFISFYKNGKSMTMVAQFGKPFF-99
         K   DV +          GFI  +++ +  TM  QF +PF
SPI    4-KAVLDVAETGTEAAAATGFIFGFRSRRLQTMTVQFNRPFL-43

S14  100-LVVQHTSVQTRSF-139
        +V+ HT VQT  F
SPI   44-MVISHRGVQTTLF-56
```

IDENTITY	19/54 amino acids	35%
POSITIVES	28/54 amino acids	52%
READING FRAME	+1	

Figure 3.7 Amino acid similarities between Serine Proteinase Inhibitor (SPI) and Contrapsin-Related Precursor and Sample 14 (and 15). The common amino acids are printed between the two sequences, whereas conservative amino acid differences are denoted as '+'. The values to the left and right of Sample 14 sequence are nucleotide number, however the numbers given for SPI represent the amino acid number.

3.2.3 Sequencing Bands 11 and 12

Bands 11 and 12 were also cut from the same migration position in different experimental sets on the differential display gel (Figure 3.1). Three clones were sequenced from Band 11, Samples 16, 17 and 18. Samples 16 and 18 yielded a 211bp fragment with only 3 base pair differences (Figure 3.8). Upon searching the Genbank, the Samples 16 and 18 yielded regions of similarity to the following proteins (Figure 3.9):

C. elegans Chemosensory Receptor,
S. trpu Egg Sperm Receptor Precursor and,
H. influenzae Hypothetical Protein HI0867 (U32768).

The 636bp sequence of Sample 17 was not similar to the other samples isolated from band 11 (Figure 3.10). Upon searching the Genbank, there was only one gene with comparable similarity to Sample 17, a hypothetical 19.7 kDa protein (open reading frame 2) from a fungus mitochondrion plasmid (X15982). The region of similarity is shown in Figure 3.11.

Samples 22 and 24 were sequenced from Band 12. Sample 22 was found to be identical to Sample 17, with only 2 base pair differences (Figure 3.10) and showed similarity to the same genes (Figure 3.11). This was expected, as Bands 11 and 12 represent the same DNA fragment from different experimental sets of the same differential display gel.

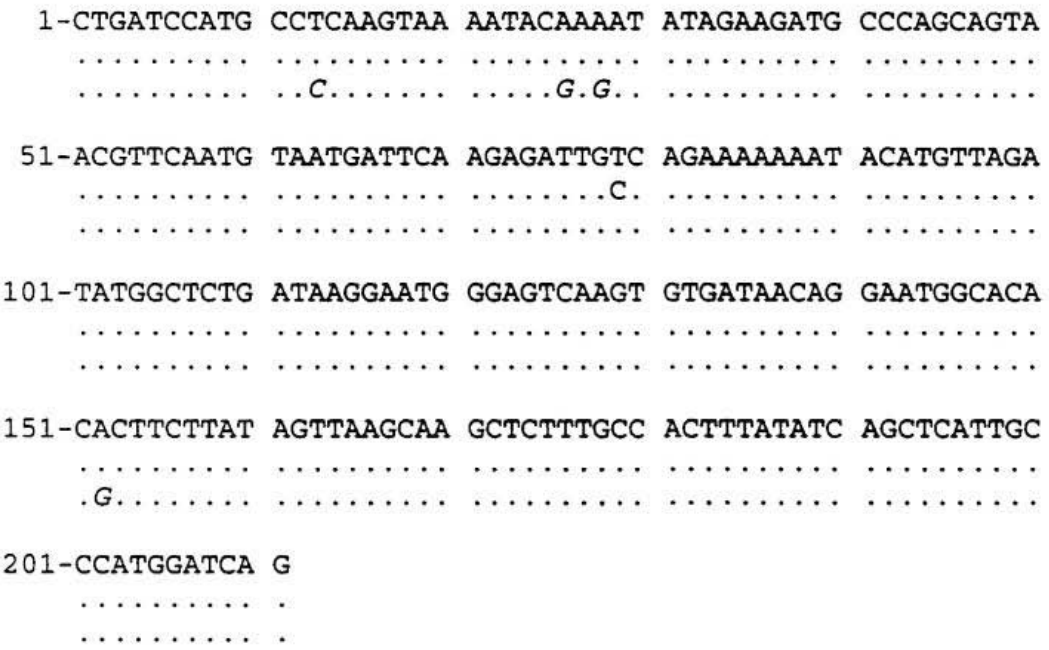


Figure 3.8 Nucleotide Sequence of Sample 16 (top), Sample 18 (middle) and Sample 28 (bottom). The nucleotide changes for Samples 18 and 28 are given. The dots represent identical nucleic acids.

A. Hypothetical protein HI0867

```
S16 155-KCVPFLL-135   S16 149-VPFLLSHLTPIPYQSHI*YVFFSG-78
        KCVPFLL                + FLLS    IPY   + Y+F+ G
HI   18-KCVPFLL-24     HI 320-IVFLLSTSLTIPYLFLVNYLFYHG-343
```

IDENTITY	17/31amino acids	55%
POSITIVES	21/31 amino acids	68%
Reading Frame	-3	

B. Egg sperm receptor precursor

```
S16  64-ITLNVTAGHLLYFVFYLRH-8
       +TLN+ +  LL+FV Y RH
ESR 909-LTLNICSAILLFFVLYARH-927
```

IDENTITY	10/19 amino acids	52%
POSITIVES	14/19 amino acids	73%
Reading Frame	-2	

C. *C. elegans* Chemosensory Receptor

```
S16  187-YKVAKSLLNYKKCVPFLLSH*LPFLI-110
         Y +   L +   C  F      +PF +
CHR   63-YFICNLLFSMALCFAFFYQFLIPFFV-88

S16   97-NMYFFLTI-74    40-HLLYFVFYL-14
        NMYFF+ +           HLL+F FY+
CHR  187-NMYFFVLL-194 244-HLLFFGFYV-252
```

IDENTITY	17/13 amino acids	40%
POSITIVES	25/43 amino acids	58%
Reading Frame	-3	

Figure 3.9 Amino Acid similarity between Sample 16 and genes from Genbank search. **A**. Hypothetical protein HI0867 (HI), **B**. Egg sperm receptor precursor (ESR), **C.** Chemosensory Receptor *C. elegans* (CHR). The numbers to the left and right of each amino acid sequence reveals the corresponding nucleic acid number for Sample 16 and the amino acid number for each known gene.

```
  1-CTGATCCATG GTTTAAGTAT AAATAATTGT TCACTTATAT CTGTTTCAAT
    .......... .......... .......... .......... ..........

 51-CACCTGTCAT TGTAGTTCCC AAAATCTCGC CTAAATCATA CATCTGCCCA
    .....T.... .......... .......... .......... ..........

101-ACCAACCTTC TAACAGCAAT GTTAGGGATG GATTCAAAAA GATCTTTGAG
    .......... .......... .......... .......... ..........

151-GAAATTGGGT GGCAGATACG CGCTAACAAA GATGAGTGAT AGAAATACAA
    .......... .......... .......... .......... .....C....

201-TGGTGATTAC TCCCAATCAG TATAATTCAA ATAGTATAAT GGGTATAACA
    .......... .......... .......... .......... ..........

251-GTAATAGAGT ACATGACATG TTAGGCACTT ACTTTGCTGT GCCAAAGGTA
    .......... .......... .......... .......... ..........

301-TTCCCATCAC TTTGTCTCTC AGAGACACCA ACAGATAGCT GTGGCCTAAT
    .......... .......... .......... .......... ..........

351-CCCTATCTGT GTACCCTGCT TTAACCCAAA CTAATTGACA AACTCGAAAT
    .......... .......... .......... .......... ..........

401-CGATGGTGCT AATTCACCAC CCCCATCTAT TGAGAGTACA TGCTCTCCAT
    .......... .......... .......... .......... ..........

451-GTTATGTTAG CAATAGGATA AATCCTTATT TTCTTTTTCC TATCTCCCTC
    .......... .......... .......... .......... ..........

501-TGGACTCCCC ATGATCTCTA TTTTCCCAAT CGTCGGTTTC TTGCATCCTA
    .......... .......... .......... .......... ..........

551-AGTAATATCC TCTTCAGGAT ACACTCATGC CTGCTAGAAG GATTAACAAA
    .......... .......... .......... .......... ..........

601-TGAATTAGGC ATGATAACGA TTATTGCATG GATCAG
    .......... .......... .......... ......
```

Figure 3.10 Nucleotide Sequence of Sample 22 (top) and Sample 17 (bottom). The different nucleotides for Sample 17 are given. The dots represent identical amino acids. The nucleic acid number is given at the left of each line.

Hypothetical 19.7kDa Protein

```
S17 267-VMYSITVIPIILFELY*LGVITIVFLSLIFVSAYLPPNFLKDLFESIPNI-118
        V+ S+ V  +ILF +Y L +    +FL   I + A     NFL ++F+SI +I
HP   25-VIKSVNVFELILFGIYKLSIFIPLFLIFIRLLATNILNFLFEVFKSIYSI-74
```

IDENTITY	19/50 amino acids	38%
POSITIVES	30/50 amino acids	60%
READING FRAME	-1	

Figure 3.11 Amino acid similarity between Hypothetical 19.7kDa Protein (HP) and Sample 17 (and 22). The numbers to the left and right of each amino acid sequence reveals the corresponding nucleic acid number for Samples 17 and 22 and the amino acid number for HP.

Sample 24 yielded a smaller insert than Sample 22, which was 190bp (Figure 3.12). The two DNA fragments did not share sequence similarity. A search of the Genbank yielded two matching regions between Sample 24 and the NADH Dehydrogenase (NDH) Subunit 2 from *Melospiza georgiana* (AF061652), (Figure 3.12).

A. Nucleic Acid Sequence

```
  1-CTGATCCATG CGTATAGCCT TGAATAATAA AGCTTTGCTC CCTCTAAATG

 51-ACAAATACCA CAATCCACTA CTACCACCTA TGACTGCACT TGAACTTACA

101-AGTAACTAAG GGAACAAGAG GGGGATAAGA AAACAGAAGT ACAGAACTAT

151-CGCAATGACT GCTTTGTGAT CTTATTTCCT ACATGGATCA G
```

B. Amino acid similarity with NADH dehydrogenase subunit 2

```
S24    8-MRIALNNKALLPLNDKYHNP-67
         M + +N  A+LPL  K H+P
NDH   21-MGLEINTLAILPLISKSHHP-40

S24   71-LPPMTALELTS-103
         LPP+T L +TS
NDH  126-LPPITLLYMTS-136
```

IDENTITY	16/31amino acids	52%
POSITIVES	22/31amino acids	71%
READING FRAME	+2	

Figure 3.12 Nucleic acid Sequence and Genbank Match for Sample 24. **A.** Nucleic acid sequence of Sample 24. **B.** Sequence similarity between Sample 24 and an NADH dehydrogenase subunit 2 (NDH) from a Genbank search. The numbers to the left and right of each amino acid sequence reveals the corresponding nucleic acid number for Sample 24 and the amino acid number for NDH.

3.2.4 Sequencing Band 22

Three clones were sequenced from Band 22, Samples 28, 29 and 30. Sample 28 was a 211bp nucleotide (Figure3.8), whose sequence was identical to Sample 16 with the exception of 5bp substitutions. The genebank search results were the same as for Samples 16 and 18 (Figure 3.9). One would not expect Sample 24 to be the same as Sample 16 because they were cut from different sized bands in the differential display gel. This will be discussed in the next section.

Samples 29 and 30 possessed the same 517bp sequence (Figure 3.13), with only minor differences (8 base pair changes). The initial Genbank search revealed that Sample 30 possessed 84% identity (aa) and 94% similarity to a hypothetical protein; *C. elegans* Cosmid TO8A11.2. Due to a high degree of conservation between this fragment and the *C. elegans* gene, it was assumed that this gene plays an important role within both organisms.

Upon a second search of the Genbank, almost a year later, the full *Xenopus laevis* gene had been sequenced (Schmidt-Zachmann *et al.*, 1998). Sample 30 yields 97% amino acid identity to a portion of the newly cloned 146kDa nuclear protein (NP) (Figure 3.14). There are only three amino acids between the two sequences that are not identical or similar. The new nuclear protein is localized within nuclear spliceosomes and is thought to play a role in nuclear splicing (Schmidt-Zachmann *et al.*, 1998).

```
  1-CTGATCCATG AAAAGTGTTA GTGACCAACC TTCTGGAAAT CTTCCATTCC
    .......... .......... .......... .......T.. ..........

 51-TGAAACCTGA TGATATTCAG TACTTTGACA AATTATTGGT CGATGTTGAT
    C......... .....C.... .......... .......... T.........

101-GAATCTACAC TAAGTCCAGA AGAACAGAAA GAAAGAAAAA TAATGAAATT
    .......... .......... .......... .......... ..........

151-ATTGTTAAAA ATAAAAAATG GCACACCTCC AATGAGGAAG GCTGCCTTAC
    .......... ......G... .......... .......... ........G.

201-GACAAATAAC TGATAAAGCT CGTGAGTTTG GAGCCGGTCC ACTATTCAAT
    .......... .......... .......... .......... ..........

251-CAGATCCTGC CTCTGCTGAT GTCGCCAACA CTTGAAGATC AAGAAAGACA
    .......... .......... .......... .......... ..........

301-CTTGCTTGTT AAAGTTATTG ATGGAATTTT GTATAAATTG GATGACTTGG
    .......... .......... ..A....... .......... ..........

351-TCCGCCCATA TGTACATAAG ATTCTTGTCG TTATTGAACC ACTTCTGATT
    .......... .......... .......... .......... ..........

401-GATGAAGACT ATTATGCCAG AGTGGAAGGC AGAGAAATCA TATCTAATTT
    .......... .......... .......... .......... ..........

451-AGCCAAGGCT GCTGGTTTAG CTACAATGAT TTCAACTATG CGACCAGATG
    .......... .......... .......... .......... .........A

501-TTGATAACAT GGATCAG
    .......... .......
```

Figure 3.13 Nucleotide Sequence of Sample 29 (top) and Sample 30 (bottom). The different nucleotides for Sample 30 are given, the dots represent identical nucleic acids. The nucleic acid numbers are at the left of each line.

```
30    4-SMKSVSDQPSGYLPFPKPDDTQYFDKLLVDVDESTLSPEEQKERKIMKLLL
        SMKSVSDQPSG LPF KPDD QYFDKLLVDVDESTLSPEEQKERKIMKLLL
NP  455-SMKSVSDQPSGNLPFLKPDDIQYFDKLLVDVDESTLSPEEQKERKIMKLLL

30  157-KIKDGTPPMRKAALRQITDKAREFGAGPLFNQILPLLMSPTLEDQERHLLV
        KIK+GTPPMRKAALRQITDKAREFGAGPLFNQILPLLMSPTLEDQERHLLV
NP  506-KIKNGTPPMRKAALRQITDKAREFGAGPLFNQILPLLMSPTLEDQERHLLV

30  310-KVIDRILYKLDDLVRPYVHKILVVIEPLLIDEDYYARVEGREIISNLAKAA
        KVIDRILYKLDDLVRPYVHKILVVIEPLLIDEDYYARVEGREIISNLAKAA
NP  557-KVIDRILYKLDDLVRPYVHKILVVIEPLLIDEDYYARVEGREIISNLAKAA

30  463-GLATMISTMRPDIDNMDQ-517
        GLATMISTMRPDIDNMD+
NP  608-GLATMISTMRPDIDNMDE-626
```

IDENTITY	166/171 amino acids	97%
POSITIVES	168/171 amino acids	98%
READING FRAME	+2	

Figure 3.14 Amino acid similarity between Sample 30 and a 146kDa Nuclear Protein (NP). The numbers to the left and right of each amino acid sequence reveals the corresponding nucleic acid number for Sample 30 and the amino acid number for NP.

3.2.5 Summary of Sequenced Samples and Genbank Matches

The following Table (3.1) is a summary of the Genbank matches for each cDNA fragment sequenced. Sample numbers are matched together according to their sequence identity. The BLASTX program used here was effective for identifying similar proteins, but would not pick up regions of similarity with the 5' or 3' untranslated regions (UTRs) of mRNAs or identify artifacts, such as amplification from ribosomal RNA (rRNA). It is possible that the cloned cDNA fragments represented one of these. Therefore, as a final search, all sequences were sent to the National Center for Biotechnology Information BLAST Network Service and analyzed using the BLASTN program, which searches a nucleotide databank.

The search results did not reveal sample similarity to any rRNA sequences. One interesting result however was that the sequence of Sample 24 is identical to the *Xenopus c-mos* proto-oncogene sequence with the exception of only one substitution and one base pair deletion (in Sample 24), indicating that they may represent the same gene or a 99% identical region from different genes. This similarity did not show up in the protein sequence search; C-MOS may not have been entered in that Genbank. The new search did not yield any new matches for the other Samples.

The sequences of all samples representing the same band, but different in size were compared, to ensure that one sample was not a truncated version of the other (Samples 16 and 17 for example). This test revealed that all of the smaller sized fragments were not simply truncated versions of the larger fragment. Therefore,

sequencing the four unique bands from the differential display yielded sequences for six different cDNA fragments in total. The next decision to be made was regarding which of the six fragments to study further.

Table 3.1 Summary of Sequenced Samples and Genbank Matches

Band #	Sample #'s	Sequence Similarity
V	1, 3	T-cell receptor gamma chain, Myocyte- Specific Enhancer Factor 2, Meprin A Beta-Subunit, Myosin I Heavy Chain-like protein, and Bovine Hypothetical 11kDa protein
VIA, VIB	14, 15	Mouse serine proteinase inhibitor and contrapsin-related protein
VIA, VIB, 22	16, 18, 28	*C. elegans* Chemosensory Receptor, *S. Trpu* Egg Sperm Receptor Precursor, *H. influenzae* Hypothetical Protein HI0867
11,12	17, 22	Hypothetical 19.7kDa protein
12	24	NADH Dehydrogenase Subunit 2 from *Melospiza georgiana*, and *Xenopus c-mos* mRNA
22	29,30	*Xenopus* 146kDa nuclear protein

3.3 Choosing Clones for Further Exploration

Once at least 2 of 5 samples had been cloned from each band, a decision was to be made regarding which samples to investigate further. There were several criteria to be met in order for samples to be researched further. At least two of the five samples for each band must possess primarily the same sequence. Also, the insert must have the appropriate primer sequences on each end. For example, samples amplified (by PCR) with AP1 and $T_{(11)}$ AC should possess either AP1 or $T_{(11)}$ AC sequence at either end. This shows that the sequenced plasmid insert was a product of the PCR. Finally, the insert sequences should be approximately the same size as the band cut from the differential display gel.

Samples 1, 3, 14, 15, 17, 22, 29 and 30 met the above criteria, but Samples 16, 18, 24 and 28 did not. Even though Samples 16 and 18 are almost identical and possess the correct primer sequences at both ends, they are the incorrect size. They are only 211bp, but they represent Band 11, which was approximately 600bp. Further to this, it was discovered that Sample 28 was composed of almost the same sequence as Samples 16 and 18. However, Sample 28, which was 211bp, came from Band 22, which was approximately 500bp. The same observation was made regarding Sample 24. Sample 24, when sequenced, was shown to be a 190bp fragment, but it was supposed to represent Band 12, which was approximately 600 base pairs.

Due to the observations made above, and time restrictions, many of the incorrectly sized samples were excluded from further experimentation. From the correct

sized sequences, one clone was arbitrarily chosen from two similar sequences (example; Sample 3 was chosen from 1 and 3). The following DNA samples were chosen for further research; Samples 3, 14, 17, and 30 (Table 3.1).

Table 3.2 Differential Display Samples to be Studied Further

Band # (Colony #'s)	**Approximate Band Size (BP)** (estimated from the bp markers on the original differential display gel)	**Representative Sample**	**Sample Size (BP)**
V (1-5)	500	3	498
VIA and VIB (6-15)	450	14	449
11 and 12 (16-25)	>600	17	633
22 (26-30)	>500	30	516

3.4 XFGF-2 Regulation of Gene Expression

Upon collecting all of the sequences, the next task was to confirm that the genes partially cloned in the previous sections are regulated by XFGF-2. This task proved to be quite difficult. Many genes that play a specific role in development are present within the cell for a short period of time. This is contrary to what would be observed for housekeeping genes, which are important with in the organism throughout its life span and present in abundant levels.

Firstly, the experimental conditions for FGF treatment were repeated from the differential display protocol. Animal cap explants were cut from *Xenopus laevis* embryos at Stage 8 (according to Nieuwkoop and Faber, 1967), and treated with 100ng/ml XFGF-2 or control NAM/2 +BSA solution for 30 minutes. RNA was then extracted from the explants. In order to ensure that there was a response to XFGF-2 in animal cap explants, control animal caps were cut and left in NAM/2 solution for 2 days. They were then scored for induction, observed morphologically under a microscope. If the control XFGF-2 treated explants were induced, it was accurate to assume that the XFGF-2 was effective on the experimentally treated explants.

Northern blotting was the first of three protocols used for FGF regulation studies. Besides giving information regarding regulation by FGF, Northern blots give information about the size of the gene being examined as well as whether there are multiple genes (gene family). This method is also effective in quantitating relative levels of RNA from different tissues.

3.4.1 Northern Blot Analysis

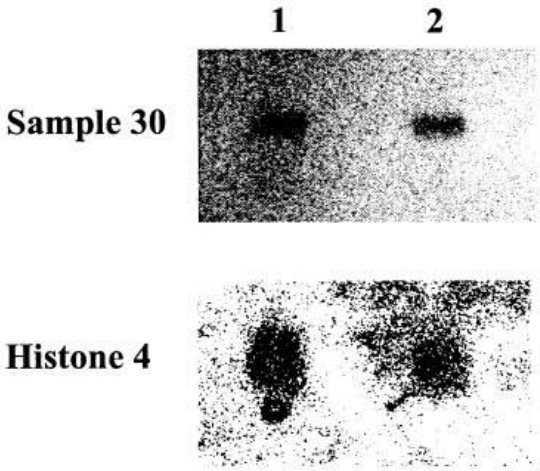
Northern blots were conducted using RNA from FGF treated and untreated animal cap explants as described above. DNA probes were made by labeling sample cDNA fragments with [α^{32}P]-dCTP. Northern blots are very effective for comparing differential expression of genes, and also give the size of the complete mRNA product, as mentioned. However it is insensitive relative to PCR, and is dependent upon the level of mRNA made from the gene of interest compared to the total RNA within the cell, of which only 3-5% is messenger RNA.

Samples 3, 14, 17 and 30 were used to probe Northern blots. The RNA was isolated as described, treated and untreated explants were used from the same experiment, loading levels were normalized using histone 4 (H4) probe as a control. *Xenopus* H4 is an important nuclear protein expressed at constant steady state levels in all cells.

I was unable to detect mRNA representing Samples 3, 14, or 17, even when using 'supersensitive' phoshorimaging screens or X-ray film packed with intensifying screens and exposing up to two weeks. This may have been due to low expression of the genes represented.

Sample 30 was detected by this method, however (Figure 3.15). The Northern blot contained the untreated explant RNA in lane 1 and the FGF-2 treated explant RNA lane 2. Distinct 4.4kb bands were obtained, the same size as those in blots of the nuclear protein cloned by Schmidt-Zachman *et al.*, 1997. When the values were normalized with

A.



B.

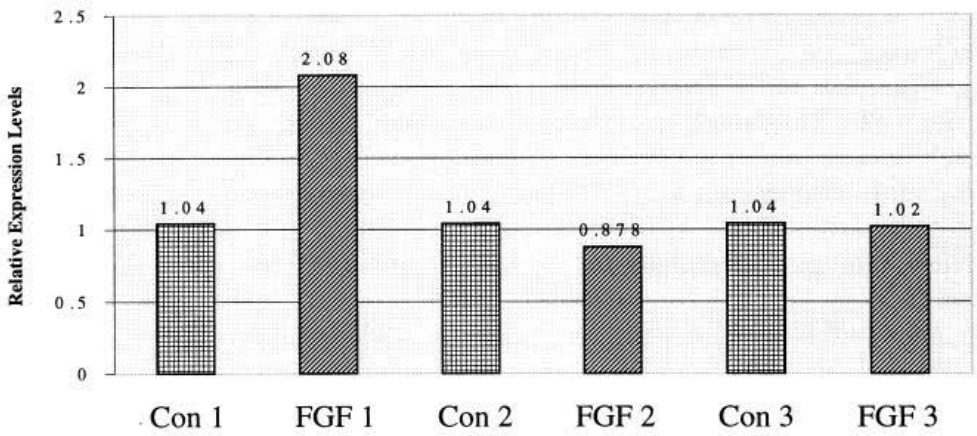


Figure 3.15 Northern Blot showing regulation of Sample 30 by XFGF-2. Northern Blot was created with RNA isolated from animal cap explants. **A**. Top panel; Sample 30 probe. Bottom Panel; Histone 4 Probe. Lane 1; Untreated control explant RNA. Lane 2; XFGF-2 treated explant RNA (30 min.). **B**. Histogram of relative expression levels of Sample 30 from the above Northern Blot (Con 1 and FGF1) and two other Northern Blots Con 2, 3 and FGF2, 3). Quantity of RNA used: Set 1-13µg; Set 2-28µg and Set 3-50µg. The values were obtained by spot densitometry using the ChemiImager System normalized with histone.

H4, it was shown that there was a 2-fold increase in the steady state levels of Sample 30 mRNA after treatment with FGF. However, in two other Northern blots, the level of Sample 30 was approximately equal or slightly decreased in response to FGF treatment (Figure 3.15 (B)). Thus no conclusion could be made regarding regulation of Sample 30 gene expression by FGF.

A second type of Northern blot was made using PolyA+ RNA from FGF treated and untreated explants. This method is more sensitive for detecting genes that are expressed at low levels. With only mRNA being loaded onto the gel, a much higher amount of mRNA could be loaded into each lane. The blot was probed with Sample 17 and exposed using double intensifying screens for up to 13 days, but there was no visible product for Sample 17 at any exposure.

Although precautions were taken to prevent RNA degradation (described in Chapter 2), it is possible that the RNA was degraded before or after it was loaded onto the gel. This could be a reason for the lack of signal form Sample 17. Probing the Northern Blot with a control marker such as Histone, would test the integrity of the RNA, to ensure it was not degraded. However, neither control markers or other experimental samples have been used to probe this Northern blot, this should be done in the future.

3.4.2 Ribonuclease Protection Assay Analysis

Ribonuclease (RNAse) protection assays were attempted next, because it is a more sensitive method than Northern Blotting. As mentioned, the genes being examined may be expressed at low levels, therefore they may be visualized by using RNAse protection assays.

The principle behind RNAse protection is that of all the RNA isolated from a cell population, only double stranded RNA, which is created by RNA annealing to a radiolabeled antisense probe, will remain intact in the presence of RNAses that degrade single stranded RNA. This double stranded RNA is run on a polyacrylamide gel and relative levels of product for each experimental condition is quantitated.

The protocol was initially tested using an antisense probe against a portion of FGFR1 mRNA as a control. This assay worked quite well, the same protocol was tested using FGF treated and untreated explant RNA and an antisense probe created from Sample 3 cDNA. The antisense direction for Sample 3 was chosen because four out of five genes from the genebank search with high similarity match in positive reading frames. Therefore the antisense probe was created to anneal to Sample 3 in the 5' to 3' orientation shown in Figure 3.5 (Page 81).

The RNAse protection for Sample 3 did not work. There was no specific protected fragment observed. A control RNAse protection assay was conducted in order to test that the RNA probe was not degraded and was able to bind its complementary sequence under the experimental conditions. Since RNA is susceptible to degradation, it

is possible that the probe (or cellular mRNA) was degraded before it annealed to its complementary sequence, however this was not observed in the controls.

A control assay was conducted by creating both sense and antisense mRNA from Sample 3 cDNA. The sense mRNA represented the cellular mRNA and was expected to bind to the antisense probe, and to remain intact during RNAse treatment. The antisense RNA was a control that would not bind Sample 3, because they are the same sequence, therefore it would be degraded.

A 209bp fragment was created from the Sample 3 probe, but since a portion of this probe was composed of uncomplimentary vector RNA, degraded by RNAses, the double stranded fragment (protected fragment) was 142bp. It was shown that the antisense probe was able to protect the sense mRNA (representing the cellular mRNA) from RNAse degradation. However the antisense RNA, as expected, did not anneal to the antisense probe and was effectively degraded (Figure 3.16).

In the same experiment, *Xenopus* Stage 18 RNA and *Xenopus* adult lung RNA were also probed with Sample 3 antisense RNA. No protected product was observed on the polyacrylamide gel.

The assay was shown to work with the FGFR1 under cellular conditions (data not shown) and with Sample 3 using control RNA, but did not work with Sample 3 using RNA from Xenopus explants, Stage 18 embryos or adult lung. This was most likely due to the relatively low abundance of message, or may also be due to incorrect orientation of the probe. This would mean that the probe was sense, not antisense and the cellular mRNA was the same, not complementary to the probe, as will be discussed further in Chapter 4. The other samples have not been tested by this method.

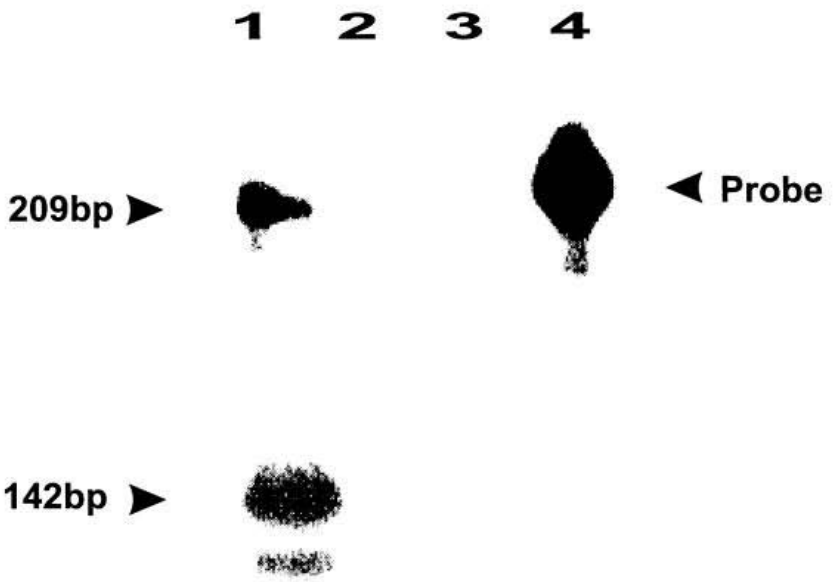


Figure 3.16 Control RNAse Protection Assay. Sense and Antisense RNA was produced from Sample 3 cDNA. A radiolabeled Antisense Sample 3 Probe of 209bp was created to anneal to complementary RNA. Lane 1, Sample 3 Sense RNA(100ng) and Antisense Probe. Lane 2, Antisense Sample 3 RNA(100ng) and Antisense Probe. Lane 3, RNAse treated Antisense Sample 3 Probe. Lane 4, Untreated Antisense Sample 3 Probe. Upper left and right Arrowheads-209bp Probe. Lower left arrowhead- 142bp Protected Fragment.

3.4.3 RT-PCR Analysis

The most sensitive method for detection of low levels of RNA is RT-PCR because the specific sequence is amplified at an exponential rate. However, this method was used for the initial differential display, and it was favorable to verify the observations regarding FGF responsiveness using a different protocol. RT-PCR was chosen only after the mRNA levels were not detected via northern blotting or RNAse protection assay.

PCR primers were designed for the newly cloned genes. One 5' and one 3' 20-mer primer were made for Samples 3, 14, 17 and 30 (sequences shown in Table 2.2, Page 40). The primers were shown to effectively amplify the sample cDNA in control experiments, therefore they were working as expected. Explants were FGF-treated and RNA isolated as previously described. Reverse transcription and PCR were conducted on several sets of control and FGF treated explants.

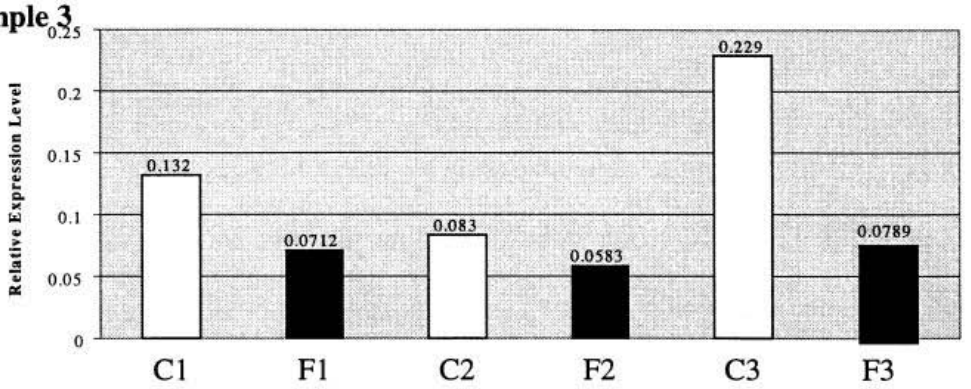
The amount of cDNA added to each PCR reaction was normalized with H4. Equivalent levels of histone 4 represent equivalent cDNA (and input RNA) concentrations. Therefore, differences in PCR product levels between samples represent differences in expression, not differences in overall cDNA levels added to the reaction.

The PCRs were repeated several times, varying the number of cycles, to ensure that the reaction conditions were not creating saturated amounts of products, especially for H4 and Sample 30. If the DNA products were at saturating levels, one would be unable to accurately quantitate relative expression.

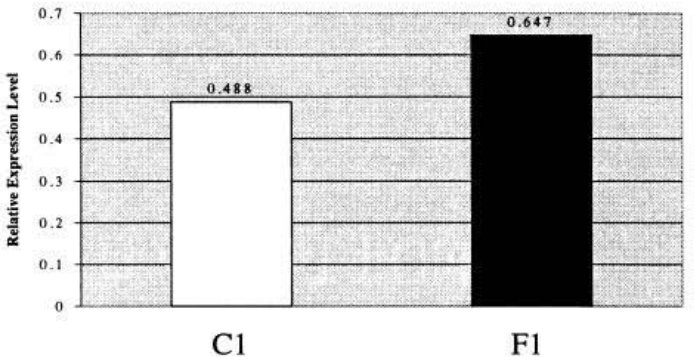
The amount of PCR product increases exponentially with every cycling. Sample 14 was not detectable even with a 38 cycle PCR program which is thought to be the upper limit for Taq DNA polymerase activity. After this time, the enzyme loses activity, and is no longer efficient for additional cycling. The absence of Sample 14 in FGF-treated explants indicates that either the representative gene is expressed at very low levels or it is a product of contamination that is not actually expressed at all in *Xenopus laevis* explants.

Samples 3, 17 and 30 were visible by PCR. Regulation by FGF was determined by using spot densitometry (ChemiImager 4000) to compare relative levels of PCR products for control and FGF explants. Sample 3 was downregulated in varying amounts by FGF treatment in all three of the experiments shown (Figure 3.17). Sample 17 was upregulated by FGF treatment in the one experiment, but the PCR must be repeated in order to conclude FGF regulation. Sample 30 was upregulated by FGF treatment in three of four experiments. Thus, again the experiments did not allow confirmation of upregulation of Sample 30 by FGF-2. These results as well as those for the Sample 30 Northern blots will be discussed further in Chapter 4. For all three samples, further experimentation and statistical analysis must be performed, to confirm or rule out FGF-2 regulated expression.

A. Sample 3



B. Sample 17



C. Sample 30

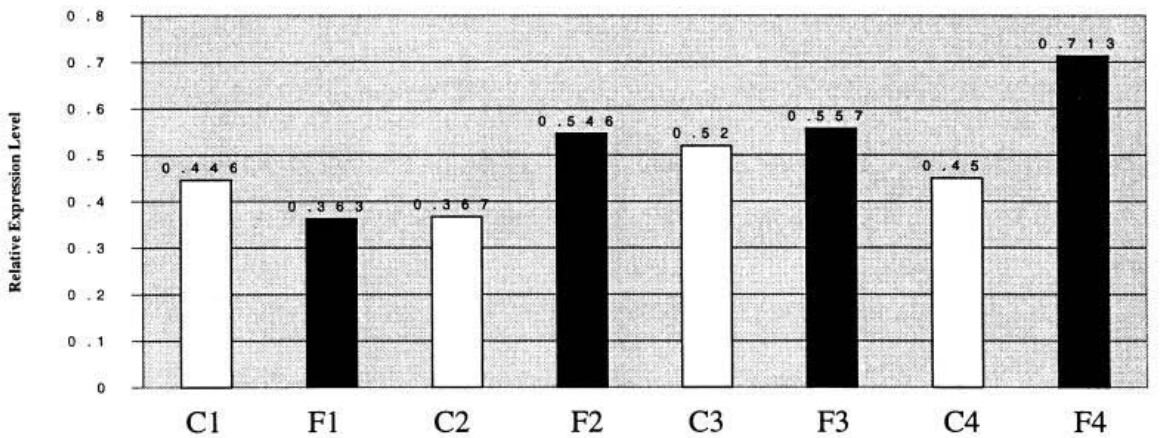


Figure 3.17 Samples 3, 17 and 30 Response to FGF: PCR Results. Xenopus Explants were treated with 100ng/ml XFGF-2 or control for 30 min., RNA was isolated and RT-PCR was conducted according to the Materials and Methods section. Results were quantitated with spot densitometry using the ChemiImager 4000 system and normalized by calculating ratios with histone values. Control (C), explants; white, FGF (F) treated explants; black. Numbers on X-axis indicate matching pairs for different experiments. A. Sample 3 PCR results. B. Sample 17 PCR results. C. Sample 30 PCR results.

3.5 Developmental Expression of Potential FGF Response Genes

Studying the expression levels of genes throughout development is often very useful. Since the genes being studied here are potential FGF-2 response genes, they should be expressed when FGF is active. Since FGF-2 is a proposed mesoderm inducer (Slack *et al.*, 1987), one would expect that genes activated/repressed by FGF-2 are important for mesoderm induction. The stages of development at which genes are expressed indicate the time-points during which they are important. Expression of the genes throughout *Xenopus* development was examined using Northern blotting and PCR.

RNA was isolated from whole embryos at the following general developmental stages; first cleavage, mid-cleavage, early blastula, late blastula, early gastrula, mid gastrula, early neurula, neurula, late neurula, tailbud, late tailbud (Figure 1.1, Page 12). The RNA was used for Northern blotting and RT-PCR. Northern blotting was the first protocol investigated due to favourable properties mentioned earlier. Since most samples were difficult to detect in explant Northerns, RT-PCR was used as an alternative test.

3.5.1 Developmental Northern Blots

Northern blots were made using RNA from the developmental stages listed in the previous section. Samples 3, 14, 17 and 30 were used to probe blots containing RNA from various developmental stages. Again, Sample 30 was the only one detected on a

Northern blot, revealing a 4.4kbp band the same size as obtained by Schmidt-Zachmann and colleagues for the 146kDa nuclear protein (1997) (Figure 3.18).

Sample 30 is expressed throughout development. The relative levels of Sample 30 mRNA were quantitated using spot densitometry (Optiquant Analysis Program) from three different Northern blots. The values given for each developmental stage have been normalized by dividing by the densitometry reading for histone 4 (Figure 3.19). There appears to be some variation in Sample 30 expression between developmental stages and there was a general trend as follows: expression increases slightly from first cleavage to Stage 8-9, and then decreases at gastrula Stage 10-10.5. There is an increase in expression after gastrulation and then a decrease again at mid to late tailbud, Stages 30-35.

The Northern blots used to this point contained approximately 20µg total RNA per lane. Since this method was not sensitive enough to pick up signals from Samples 3, 14 and 17, again poly A+ RNA was isolated. Approximately 7µg of messenger RNA was run on a Northern gel isolated from Stage 8-8.5 and Stage 8.5-9 embryos. As a control, 10µg of remaining RNA (primarily ribosomal RNA) which was eluted as waste during the poly A+ RNA isolation was also loaded onto the gel. The blot was first probed with Sample 3 (Figure 3.20). The probe has bound to the poly A+ RNA (first two lanes) without binding to ribosomal RNA (last two lanes).

There were three detectable bands in the mRNA lanes, approximately 4.8kbp, 3.4kbp and 2.7kbp. The upper and lower bands are approximately the same intensity, and are darker than the middle band. Expression at Stage 8-8.5 is higher than at Stage 8.5-9.

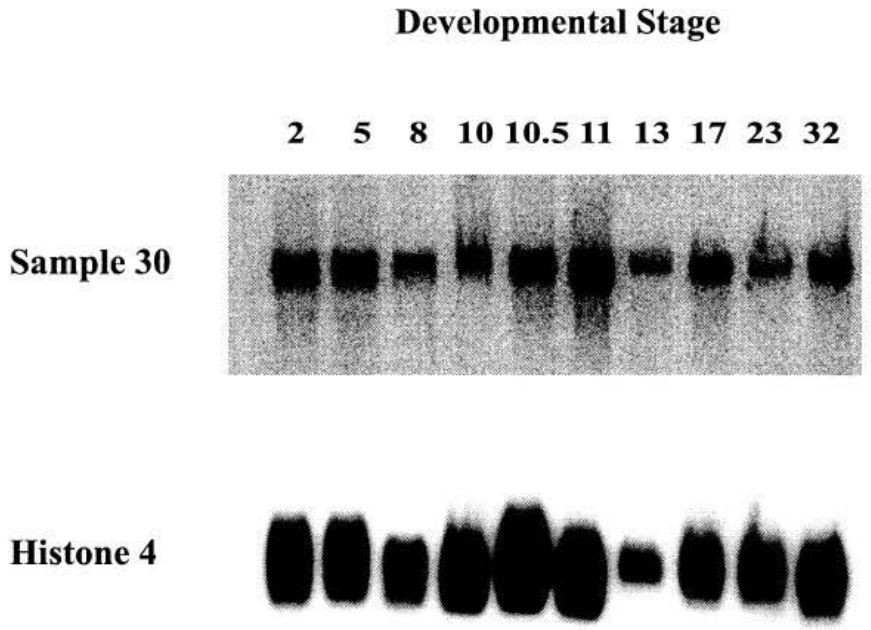


Figure 3.18 Northern Blot Containing RNA from Developing *Xenopus* embryos. Developmental Stages are shown above top panel. 30ug RNA was loaded into each well. Northern was probed with Sample 30 Radiolabeled DNA Fragment (top panel), and Histone 4 Radiolabeled Probe (bottompanel). Sample 30 band was 4.4kbp.

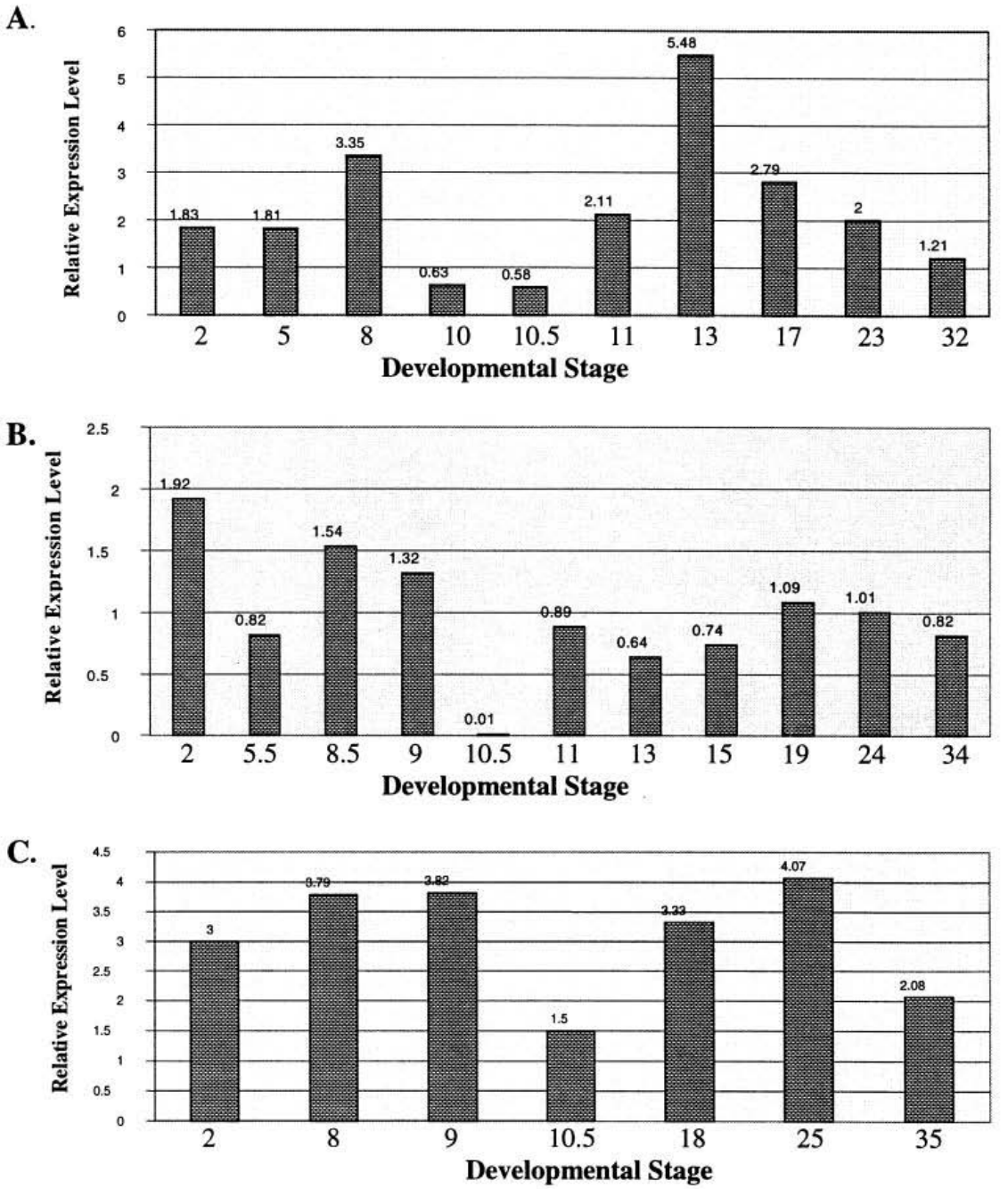


Figure 3.19 Sample 30 Expression in *Xenopus* Embryos Throughout Development. Northern blots containing RNA from developing embryos (Stages shown on horizontal axis) were probed with Sample 30 and then H4. Spot densitometry was conducted and the values were divided by H4 values to normalize. The normalized values are given. **A.** Northern Blot 1 (from Figure 3.19), (30μg RNA each lane) **B.** Northern Blot 2, (40μg RNA each lane) **C.** Northern Blot 3 (20μg RNA each lane).

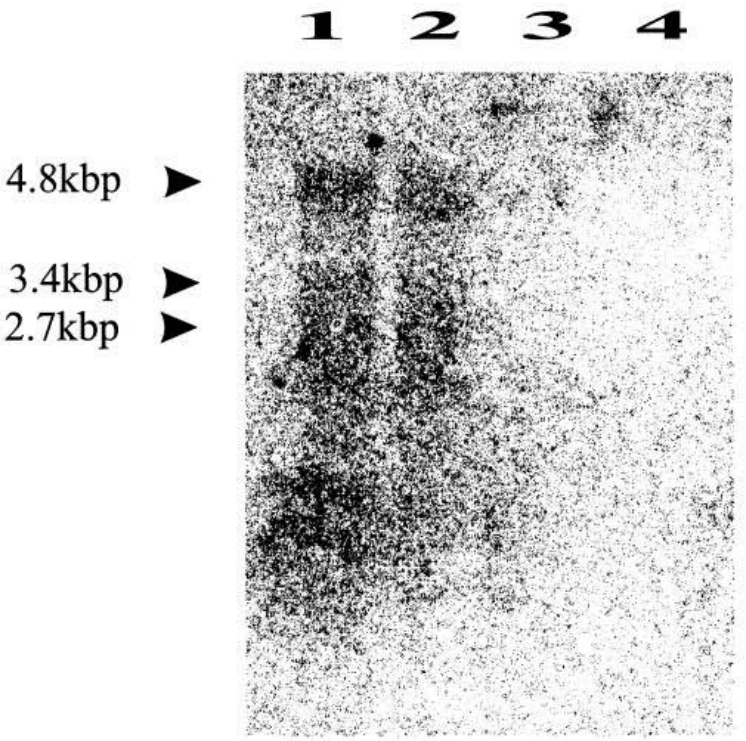


Figure 3.20 Northern Blot Containing *Xenopus* Stage 8 and Stage 9 mRNA. Sample 3 radiolabeled cDNA was used to probe. Lane 1, Stage 8-8.5 mRNA. Lane 2, Stage 8.5-9 mRNA. Lane 3, Stage 8-8.5 Poly A- RNA.. Lane 4, Stage 8.5-9 Poly A- RNA..Lanes 1 and 2 contain 8ug PolyA+ RNA. Lanes 3 and 4 contain 80ug Poly A- RNA.

The appearance of three bands on the Northern blot indicates that Sample 3 is a member of a gene family, or is a single gene that can be alternatively spliced. This blot was also probed with Sample 17 however, there was no product visible, even for exposure times up to 13 days.

3.5.2 PCR Analysis of Expression Throughout *Xenopus* Development

PCRs were conducted using primers for Samples 3, 14, and 17. Again, H4 was used as a control. The PCR was set up according to protocol and the optimum number of cycles were used so that the products were visible when run on a gel, but not too high such that the products were no longer within the linear range on the exponential curve (saturated). Thirty-six cycles were used for all experimental sets and 27 cycles were used for H4. The developmental stages tested in this experiment were as follows; 2, 5, 8, 9, 10.5, 11, 13, 15, 18, 25, and 33.

The results show that Sample 3 was expressed at Stages 8 and 15 (Figure 3.21 (A)). Again, there was no PCR product observed for Sample 14 at any stage in development. No product at a high number of cycles indicates that either the gene is expressed only due to FGF induction at very low levels during development or not at all. Sample 17 was expressed at Stage 8 only (Figure 3.21 (B)) which coincides with the developmental stage during which mesoderm induction is known to occur. H4 was also used as a control for relative cDNA levels in this experiment (Figure 3.21 (C)).

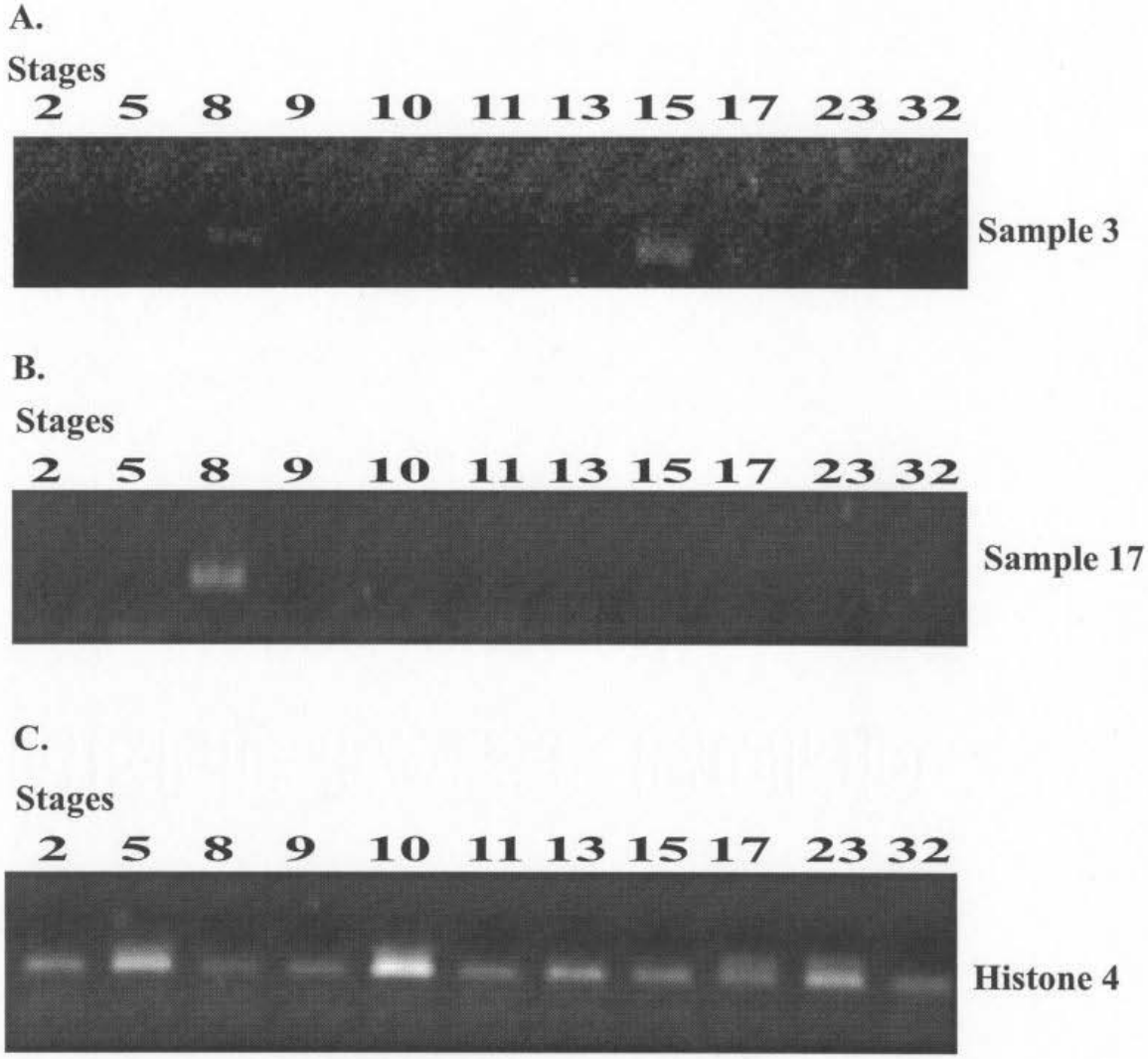


Figure 3.21 Developmental PCR from *Xenopus* Embryos. PCR was conducted on RNA extracted from embryos throughout development (Stages are listed on top of image). RNA was isolated, reverse transcription and PCR were performed according to the protocol in Materials and Methods section. The PCR products were run on a 1% agarose gel and photographed with the ChemiImager camera.. Panel A. Sample 3 PCR products; Panel B. Sample 17 PCR product; Panel C. Histone 4 Control PCR products.

3.6 Cloning Larger cDNA Fragments

The next set of experiments in this project involved attempting to clone larger fragments of the proposed FGF early response genes. There are many advantages to obtaining the full gene sequence. If this were done, the full amino acid sequence could be obtained, and various domains could be identified, thereby indicating possible protein function. A number of useful experiments could be conducted, such as overexpression and mutational analysis, which would again give information regarding the function of the unknown genes.

I attempted to clone larger fragments from Samples 3 and 14. Due to low expression of the gene represented by Sample 3 and inability to detect Sample 14 at all, the cloning methodology used was such that the experimental conditions were optimized. A Stage 8 *Xenopus* cDNA library was used for cloning. The stage 8 library was a logical choice since it was the stage from which RNA was isolated for the differential display and Sample 3 is expressed at this stage.

Initially, a PCR was conducted with the internal PCR primers to test whether the cDNA was present in the library. Internal primers are the primers designed specifically to anneal to the 5' and 3' ends of the sequence being studied. Several dilutions of the library were tested. The initial PCR was repeated several times, each time modifications were made to further optimize the conditions. For example, a 'hot start' was conducted whereby the Taq DNA polymerase was added after the reaction had reached 94°C in order to prevent primer annealing to and amplifying non-specific DNA. The number of

cycles used for the PCR were also increased (see Section 2.14.2, Page 70). The specific gene products for Sample 3 and 14 were not observed in this cDNA library, therefore it was not productive to proceed further with cloning.

A Stage 17 neurula cDNA library was also tested using these cloning techniques. Sample 3 is expressed at Stage 15, expression at Stage 17 was not tested prior to this. Research with the neurula library did not yield any more sequence of Sample 3 or 14.

FGFR was used as a positive control for the cloning techniques. When a PCR was conducted on the library using internal FGFR primers, one distinct band of the correct size was obtained, thus showing that the FGFR was present in the library and undegraded. Secondly, when cloning with the internal and external primers, in order to clone the full 5' or 3' piece, one distinct band was obtained that was also the expected size. Since the control experiments worked, the problem with cloning the Samples 3 and 14 was thought to be due to low level expression, not technique.

3.7 Summary

The goal of sequencing two samples for each of the bands cut from the differential display gel was met, and clones were chosen for further study. The results are summarized in Table 3.3. DNA representing band V (Samples 1 and 3) has shown similarity to Myocyte- Specific Enhancer Factor 2, T-cell receptor gamma chain, Meprin A Beta-Subunit, Myosin I Heavy Chain-like protein, and BovineHypothetical 11kDa

Table 3.3 Summary of Cloning, and Expression Results for Potential FGF Response Genes.

Band #	Sample #	Sequence Similarity	Conformation – FGF regulated	Developmental Expression (Stages)
V	3	T-cell receptor gamma chain, Myocyte- Specific Enhancer Factor 2, Meprin A Beta-Subunit, Myosin I Heavy Chain-like protein, and Bovine Hypothetical 11kDa protein	Yes-Downregulated	8 and 15
VIA, VIB	14	Mouse serine proteinase inhibitor and contrapsin- related protein	--	--
11,12	17	Hypothetical 19.7kDa protein	Inconclusive	8
22	30	*Xenopus* 146kDa nuclear protein.	Inconclusive	Throughout Development

protein. Sample 3 is expressed in normal embryos at Stage 8, 9 and 15 and was downregulated by XFGF-2 as observed via RT-PCR. Results from an mRNA Northen blot, indicate that Sample 3 may be a member of a gene family, with products of approximately 4.8kbp, 3.4kbp and 2.7kbp in size.

From Bands VIA and VIB, Samples 14 and 15 possessed amino acid similarity to a mouse serine proteinase inhibitor Spi2.4 and a mouse contrapsin-related protein MC-7 precursor. Expression was not observed via PCR, or Northern blotting. Therefore, this gene appears to be expressed at very low levels, if at all, in both animal cap explants and in whole embryos throughout development.

Samples 17 and 22, the representative clones from Bands 11 and 12, showed similarity to hypothetical 19.7 kDa protein (open reading frame 2) from a fungus mitochondrion plasmid. Of the developmental stages tested, Sample 17 is expressed only at Stage 8, and was upregulated by XFGF-2 treatment in one RT-PCR experiment. This is not enough to conclude that FGF controls Sample 17 gene expression.

Band 22 represents a known *Xenopus* 146kDa nuclear protein. It is expressed throughout development at a fairly constant rate, but decreases slightly at gastrulation and again at mid-tailbud stage. Regulation of the Sample 30 gene by XFGF-2 could not be confirmed via Northern blot and RT-PCR studies.

Chapter 4

Discussion

4.1 FGF and Cancer

As described previously, several *fgfs* were originally discovered due to their expression in various cancers. FGF-1 to FGF-6 have all demonstrated an ability to transform cultured fibroblasts. Aberrant expression of *fgf-2*, or *fgf-4* results in dramatic changes in the genetic stability of cells, leading to increased drug resistance and high rates of gene amplification (reviewed by Wright and Huang, 1996).

Fibroblast growth factors, including FGF-1, FGF-2 and FGF-4 play a role in angiogenesis (Deroanne *et al.*, 1987). FGF-2 is a known angiogenesis factor in breast cancer, for example (Wang *et al.*, 1998). In order for tumors to grow, they must establish a blood supply. Therefore, inhibiting the actions of FGFs, which are expressed at high levels in some tumors, may restrict tumor growth by cutting off its blood supply.

It is obvious that FGF is a very important molecule. In order to understand the mechanism of action of FGF, we must first elucidate the pathways whereby it elicits its action.

4.2 How to Find Early Response Genes

The main interest of this study was to identify and characterize genes activated by FGF. There are several methods used to distinguish differentially expressed genes. These include subtractive hybridization (Duguid *et al.*, 1988), RNA arbitrarily primer PCR (AP-PCR) fingerprinting (Welsh *et al.*, 1992) and PCR- based differentially display (Liang and Pardee, 1992).

The basic protocol for subtractive hybridization is based on comparison of cDNA libraries from two different cell populations. The single-stranded cDNA library from control cells is biotinylated and hybridized to the single stranded experimental library. All double stranded hybrids and single stranded control cDNAs containing biotin are removed. The remaining cDNAs, those that are not present in the control library (differentially expressed), are then transformed into bacterial cells (Duguid *et al.*, 1988).

Bacterial colonies are probed with normal and experimental radiolabeled cDNAs separately (colony hybridization). The differentially expressed cDNA fragments are isolated from the bacterial cells and sequenced (Duguid *et al.*, 1988). Disadvantages of this procedure are that it is quite laborious, requires a large amount of mRNA, and is only able to identify upregulated genes, not downregulated.

AP-PCR fingerprinting is quite similar to differential display. The main differences between the two is that AP-PCR fingerprinting starts with mRNA and only uses one arbitrary primer (20bp) for both the reverse transcription and PCR (Ayala *et al.*, 1995).

Differential display methodology is a popular method for efficiently and effectively identifying differentially expressed genes. The procedure has been successfully used for identification of genes differentially expressed in cancers, heart disease, embryogenesis, and in response to growth factor stimulation, among others (Liang and Pardee, 1995). I have chosen differential display for my study of FGF-2 early response genes; the advantages and disadvantages of such will be discussed in the next section.

4.2.1 Advantages and Disadvantages of Differential Display

Differential display is a means of isolating differentially expressed genes in a fast and relatively uncomplicated procedure. The methodology also allows a potentially large number of genes to be isolated and various experimental conditions to be tested in one experiment.

The differential display protocol was designed to amplify messenger RNA transcripts by using a polyT primer to make cDNA. Both the polyT primer and one of two arbitrary primers (AP1 or AP2) were used for the PCR. Interestingly, all of the clones amplified by PCR in this experiment contained either the AP1 or AP2 primer on both ends, none had the polyT primer and therefore did not contain the polyA+ region. This observation was made in previous studies (Tokuyama and Takedo, 1995; Guimaraes *et al.*, 1995) and makes the protocol similar to AP-PCR fingerprinting.

As a precaution to ensure that the clones that I have sequenced are mRNA, not rRNA or tRNA, the nucleic acid sequences were sent to a nucleotide Genbank. None of the clones showed any similarity to rRNA or tRNA sequences, and are therefore thought to represent mRNA sequences.

As previously described, none of the sequences isolated in this study were anchored at the polyA+ tail, each sequence was flanked by the internal primers. Therefore, the cDNAs may be from any site along the mRNA message including the 5' or 3' untranslated regions or the coding region. Anchored cDNAs would all be from the highly variable 3' untranslated region. Internal primers are favourable for isolation of cDNA coding regions.

Cloning a central coding region of mRNA is very helpful for identifying functional domains in the new clones and are much more useful when searching the Genbank. Therefore, the protocol used for this differential display was very effective in isolating potential translated regions.

Differential display has the drawback of creating false positives however. Since its first introduction, several modifications have been made to the differential display protocol to reduce the number of false positives created. For example, it is important to ensure the RNA is free of chromosomal DNA contamination by treating with a DNAse before reverse transcription. Otherwise, the primers may anneal to and amplify chromosomal DNA, thereby increasing (or creating) a signal aberrantly. Liang and Pardee themselves admit that false positives may never be eliminated from differential

display and the "gold standard" for confirmation that a gene is differentially expressed is Northern blotting (Liang and Pardee, 1995).

4.3 Problems Encountered in this Study

There were several problems encountered in this study that were either unforeseen, or unavoidable. For example, the low level of expression of some of the genes could not be avoided. However PCR contamination was a problem that was avoidable to some degree. The following is a discussion of the problems encountered and how they were managed.

4.3.1 Different DNA Fragments Extracted from the Same Bands

Four distinct bands were initially cut from the differential display gel. When the DNA fragments were cloned, however, there were seven distinct sequences. Band V yielded Sample 3 (1), Bands VIA and VIB yielded Sample 14 (15), Bands 11 and 12 yielded Samples 16 (18 and 28), 17 (22) and 24 and Band 22 yielded Sample 30 (29) (similar samples are given in brackets). It is not uncommon to find more than one DNA fragment represented in a single band (reviewed by Liang and Pardee, 1995).

I chose to examine the DNA fragments that were the same size as the bands cut from the differential display gel (Samples 3, 14, 17 and 30). These fragments most likely represent the differentially expressed bands.

Samples 16, 18 24 and 28 were not thought to represent differentially expressed sequences for the following reasons. Firstly, they were not the correct size. Sample 16 and 18 were 211 base pairs, but Band 11, which they were supposed to represent, was approximately 600bp. Samples 24 and 28 were 190bp and 211bp, respectively, but they represented Bands 12 and 22, which were approximately 600bp and 500bp.

The samples could not be excluded solely on size, however. During the cloning process, the eluted DNA bands were re-amplified by PCR prior to ligation into a plasmid vector. Therefore, there could be several reasons for the samples being the incorrect size. For example, if there was more than one site for the PCR primers to anneal, a smaller portion of the original DNA fragment could have been amplified during the second PCR. Also, bacterial cells sometimes modify foreign plasmids, thereby reducing the insert size. Therefore, upon first consideration, the samples could represent a truncated version of the differential display bands.

If the samples were simply truncated versions of the bands, they would have the same sequence as the correctly sized samples. For example, Sample 16 would simply be a truncated version of Sample 17. This is not the case for any of the smaller samples, as determined by comparing nucleic acid sequence. Also, Samples 16 and 18 represent the same sequence as Sample 28, but they are from different bands. Therefore, it is less likely

that Samples 16, 18, 24 and 28 represent FGF early response genes. This was not confirmed, however.

If these samples were caused by contamination, what could be the source? Since all four samples contained either the AP1 or AP2 primer on both ends, the contamination would have occurred during or prior to the reamplification PCR. If the contamination was initiated during ligation of the fragment into the plasmid, or within the bacterial cells, the DNA would not possess the correct primer sequence on both ends. Therefore the most likely source of PCR contamination would be due to contaminants from the solutions or from the surrounding environment entering the reaction tubes.

4.3.2 Inability to Detect Gene Expression

The protocol used for differential display can allow the amplification of transcripts of genes that are expressed at very low levels. The very nature of PCR is to amplify DNA at an exponential rate. Therefore, when less sensitive methods, such as Northern blotting are used for analysis, the new gene transcripts can be difficult to detect. It is common for differential display bands not to be detected by Northern blotting, due to low expression levels (reviewed in Liang and Pardee, 1995).

Also, many immediate-early genes play specific roles in development and are only expressed for short periods of time. Therefore the time period from which the RNA was obtained for experimentation is crucial. Stage 8 in the *Xenopus* blastula embryo lasts

approximately 2 hours, depending on environmental temperature. The rate of development also varies naturally depending on the male and female frogs used.

The differential display was conducted by Yu Li, whereas I did all of the further analysis. Therefore, if I isolated the RNA at a different point in Stage 8 than Yu Li, this could make a difference in expression levels of the genes being studied. Subjective differences in staging the embryos from person to person and in *Xenopus* developmental rate could not be avoided.

Expression of the gene represented by Sample 14 was not detected by Northern blotting or PCR in FGF-treated explants or in developing embryos. This is unusual, as one would expect at least minimal expression in FGF-treated explants because the original differential display was conducted under the same conditions. So even with subjective differences in embryo staging, Sample 14 should be detected by PCR.

4.3.3 Base Pair Substitutions

None of the DNA fragments cloned from the same differential display bands possessed exactly the same DNA base pair sequence. There was at least one difference in the nucleotide sequence in all matching samples. For example, there are two base pair differences between Samples 17 and 22. Several explanations can be offered for these differences.

During PCR, if the conditions are not stringent enough, a substitution error may occur during the amplification step and result in the further amplification of the fragment with an altered sequence. For instance, if an 'A' is replaced by a 'T' within the first round of amplifications, all of the subsequent products will have that error.

Sequencing errors may also arise, mostly due to incorrectly reading the sequencing film. Errors in sequence reading are due mostly to multiple bands in one lane or aberrations in the polyacrylamide gel.

4.4 Genbank Protein Matches, Potential Functional Domains and Expression

The sequences of the new DNA fragments from the differential display gel were sent to a Genbank in order to determine whether they were novel or previously cloned genes. A Genbank search is beneficial for elucidating catalytic and functional domains, which have been well researched and are often easily recognized. Identifying functional and catalytic domains is quite useful for elucidating possible functions for the cDNA fragments from novel genes. This information, along with expression studies, can offer a great deal of information regarding the potential FGF early response genes. Even though it was not possible to conclude FGF regulation of the new samples, several of them appear to be developmentally expressed (Samples 3, 17, and 30) which is of great interest on its own and warrants further investigation.

4.4.1 Sample 24 is 99% Identical to the *c-mos* Proto-oncogene

As previously described, a nucleotide Genbank search revealed 99% identity at the nucleic acid level between Sample 24 and *Xenopus c-mos*. This observation was made quite late in the study and due to time constraints this matter could not be studied further.

MOS is a serine/threonine kinase that plays a vital role in *Xenopus* oocyte maturation (Reviewed by Gebauer and Richter, 1997). Normally, vertebrate oocytes undergo a growth arrest after prophase I. At this stage, many immature mRNA species are present in the cell, possessing short poly A+ tails. Upon oocyte stimulation with progesterone or insulin, *c-mos* mRNA is polyadenylated, and protein translation begins. MOS initiates a MAPK kinase phosphorylation cascade responsible for polyadenylation of other mRNAs and maturation of the oocyte (Sheets *et al.*, 1995). Injection of antisense *c-mos* RNA into immature oocytes blocks polyadenylation of many important cellular mRNAs such as Histone B4 and cyclins A1 and B1 (DeMoor and Richter, 1997). Normally, MOS is not usually found in somatic cells, only maturing oocytes (reviewed by Vande-Woude, 1994).

The full sequence of the gene represented by Sample 24 must be obtained in order to confirm whether it is *c-mos* or a different gene, which shares a region of identity with *c-mos*. Further work must also be done to determine if Sample 24 is present due to contamination or an actual FGF regulated gene. A PCR reaction to test whether *c-mos* is expressed due to FGF treatment of animal cap explants would be a worthwhile venture to

confirm whether Sample 24 is a product of contamination or it is actually expressed in the developing embryo.

4.4.2 Potential FGF– Regulated Genes

4.4.2.1 Band V, Samples 1 and 3

Genbank Protein Matches and Potential Functional Domains

Samples 1 and 3 from Band V represent a novel gene possessing significant similarity to four known proteins and one hypothetical protein. The Bovine hypothetical 11kDa protein upstream of parathyroid hormone gene was not useful in identifying function, as its own function is unknown.

The four other proteins bearing sequence similarity to Band V were quite useful in suggesting potential functional domains. The highest degree of similarity was to the alternatively spliced region of the Human Myocyte- Specific Enhancer Factor 2 (aMEF2) (Yu *et al.*, 1992).

MEF2 is a member of the MADS (MCM1-Arg80-agamous-deficens-SRF) box transcription factor family, which are defined by a conserved motif (MADS-box) within the DNA binding domain. The amino-terminal MADS box specifies the DNA binding specificity and the carboxy-terminal defines the dimerization domain whereby two MADS-box proteins interact. The MEF and related to serum response factor (RSRF)

proteins form a subfamily of MADS transcription factors (reviewed by Shore and Sharrocks, 1995).

MEF2 binds to muscle specific enhancers and promoters and is induced in non-muscle cells by MyoD. It has been implicated in the formation of muscle cells, but is not sufficient to induce the formation of the full muscle phenotype (Yu *et al.*, 1992). In murine studies, MEF2 has been identified very early in development (day 8.5-9 p.c.) in mesodermally derived regions associated with heart and skeletal muscle and are thought to be important for their formation (Subramanian and Nadal-Ginard, 1996).

In particular, Band V is similar to the alternatively spliced region of MEF2 (see Figure 4.1). Alternative splicing is thought to be important for tissue specific expression of MEF2. The exact function of the alternatively spliced region is unknown, however, in DNA binding tests, the alternative form of MEF shows a several-fold increase in DNA binding than the original form. Therefore the alternative peptides are proposed modulators of DNA binding via the adjacent MADS domain (Yu *et al.*, 1992).

The region of aMEF2 that is similar to Band V is identical to another protein, RSRFC9 (related to serum response factor C9). RSRFC9 is thought to be analogous to aMEF2, except RSRFC9 does not contain the alternatively spliced region labeled B in Figure 4.1 (Pollock and Treisman, 1991). There is no specific functional information available regarding the RSRFC9.

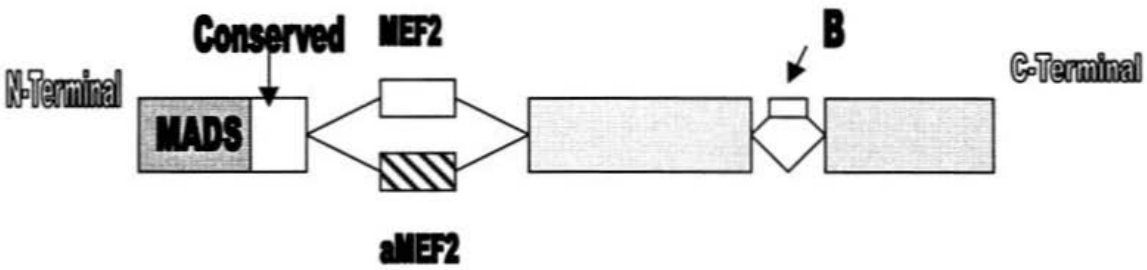


Figure 4.1 Schematic Diagram of MEF2. The diagonally striped region contains the alternatively spliced region bearing similarity to Band V. The Alternatively spliced region found in RSCRFC9 but not MEF2 is the area labeled B.

The gene with the second highest similarity region to Band V is the T-cell receptor (TCR) gamma chain V-J-C region (clone 197G1) from *Ovis aries.* T-cell receptor is an important antigen receptor found on the T-cells of the immune system that regulates activation and growth of T-lymphocytes. Activation of T cell receptor by specific antigens results in activation of protein tyrosine kinases and initiation of several signal transduction cascades, including phospholipase C γ 1 and phosphatidylinositol 3'-hydroxy kinase (reviewed by Cantrell, 1996).

The specific heterodimerization of the TCR chains (α, β, δ and γ) and genetic rearrangements confer antigen specificity (Hein *et al.*, 1990). The δ and γ dimers only form 5% of TCR in adult humans, whereas they are much more abundant in sheep (origin of the Band V matching protein).

Band V is specifically similar to the V-J-C (variable-joining-constant) region of the γ chain which is at the immediate amino-terminal end of the protein responsible for

conferring antigen specificity. The matching region is directly amino-terminal of the constant region conserved between sheep, human and mouse γ chains (Hein et al., 1990).

The third protein with similarity to a region of Band V is *H. sapiens* Meprin A Beta-Subunit Precursor (MAP), also known as, endopeptidase-2. Meprin is a dimeric metalloproteinase from the astacin family composed of α and β chains linked covalently through disulfide bonds. It is anchored to the cell membrane and found primarily in the brush border membranes of the human intestine (Uren and Vaux, 1996).

A basic schematic diagram of the Meprin β-Subunit is shown in Figure 4.2. The Astacin domain is responsible for proteolytic activity, the function of the MAM (found in meprin, A-5 protein and receptor protein-tyrosine phosphatase micron) domain is unknown (Marchand et al., 1996). The TRAFC domain is a region similar to a region of TRAF (tumor necrosis factor receptor-associated factors), whose function is also unknown. Band V possesses similarity to Meprin A precursor in a region of the MAM domain and another region thought to be within the Astacin domain (Uren and Vaux, 1997).

Number	Region
1	Signal Peptide
2	Propeptide
3	EGF-like Domain
4	Membrane Anchor
5	Cytosolic Domain

Figure 4.2 Schematic Diagram of the Meprin β-Subunit. Revised from Uren and Vaux, 1997.

The fifth match to Band V was *Bos taurus* Myosin I heavy chain-like protein (MIHC), or brush border myosin I. It was the only match that occurred in the minus reading frame of Band V as presented in Figure 3.4 (Page 75). MIHC is found primarily in the intestinal brush boarder, its mRNA has been identified at very low levels within other tissues, including liver, testis, lung, brain and kidney. It is thought to play a role in intracellular trafficking. MIHC contains three functional domains; the 78kDa ATPase and actin binding domain, the 12kda calmodulin-binding domain and the 20kDa carboxy-terminal tail that binds phospholipids (Reviewed by Coluccio, 1997).

Band V is similar to MIHC in two regions; the first is 57 amino acids carboxy-terminal of the ATP binding consensus sequence. The second region of similarity is between amino acids 823-840 of 1043 total amino acids. This is the alpha helix region of the protein (Hoshimaru and Nakanishi, 1997).

Although no definite assignment of functional domains can be made to regions of Band V at this point, based on regions of similarity to known proteins, possible functional motifs may be suggested. If one were to consider the 5' to 3' orientation of Band V given in Figure 3.5 (Page 81) as the correct orientation, the domains of similarity to known genes would be as shown on the top region of Figure 4.3.

Similarity of aMEF-2 with Band V is close to the carboxy-terminal of the proposed protein. The region of similarity does not bind DNA itself, but is thought to facilitate DNA binding for aMEF-2 and may play a similar role in Band V protein. The TCR and MAP regions of similarity to Band V overlap with the hypothetical protein (HP) region. Since the region of similarity to the TCR is within the amino-terminal

extracellular immunoglobulin domain of the receptor, the protein represented by Band V may also have a protein-binding domain. Band V is also similar to the extracellular Asticin proteolytic domain of MAP, which is also a membrane bound protein.

These similarities suggest that Band V could possibly possess regions that facilitate DNA binding, bind other proteins and/or have a proteolytic activity. The regions of similarity are not identical to the known proteins, and only suggest possible functional regions.

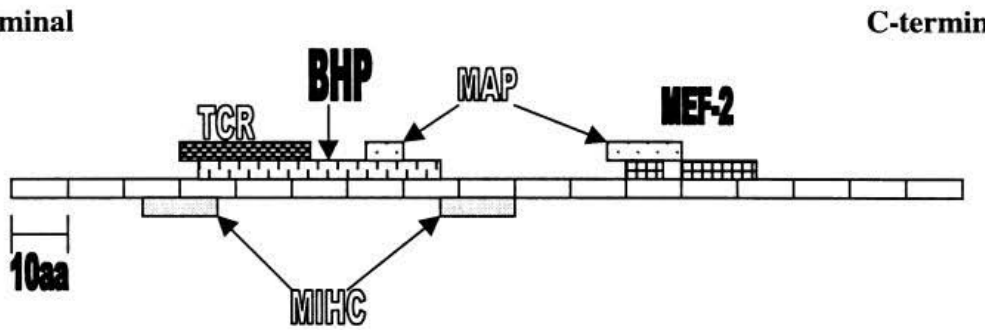


Figure 4.3 Schematic Diagram of Band V Showing Regions of Similarity with Genbank Matches. Legend: T-cell Receptor (TCR), Bovine Hypothetical Protein (BHP), Meprin A Precursor (MAP), Myocyte Enhancer Factor-2 (MEF-2), Myocin I Heavy Chain-like Protein (MIHC).

If the opposite orientation is the actual correct reading frame for Band V, similarity to MIHC reveals potential functional domains. The matching region on the left of Figure 4.3 may represent an alpha-helical region, while the matching region to the

right may be involved with ATP binding as these are the function of the regions in MIHC.

The above observations are to describe regions of similarity only and to suggest possible functional regions. More of the sequence must be determined and more studies done before any definitive function for Band V can be made.

Expression

Band V is unique in this study because it is downregulated by FGF treatment. However, Band V is expressed at Stage 8, when mesoderm induction occurs, and FGF-2 is expressed. It may be that Band V is normally important in development at stage 8, but its gene is repressed by FGF treatment. Downregulation in response to FGF suggests that the FGF signal cascade activates proteins that inhibit Band V gene expression.

Other than Stages 8-9, Band V was only found to be expressed at neurula Stage 15, signifying that the gene product may play a role in neural development.

The appearance of three bands on the mRNA Northern blot probed with Sample 3 suggests that there is more than one mRNA species annealing to the probe. Thus it is possible that Band V is from a member of a gene family. It is also possible that its mRNA is alternatively spliced, in a fashion similar to aMEF-2.

4.4.2.2 Bands VIA and VIB, Samples 14 and 15

Genbank Protein Matches and Potential Functional Domains

Samples 14 and 15 from Band VIB exhibited similarity to a serine proteinase inhibitor 2.4 (SPI 2.4) and contrapsin-related protein MC-7 precursor, which possess the same amino acid sequence within the matching area.

SPI 2.4 is a member of the SPI protein superfamily in mouse. SPIs play roles in blood coagulation, complement cascade and inflammatory response and is abundantly expressed in the liver (Inglis and Hill, 1991). Mouse contrapsin in a major trypsin inhibitor, highly similar to the human α_1-antichymotrypsin (Ohkubo *et al.*, 1991).

Bands VIB and VIA are similar to the reactive centre of the SPI 2.4 (and proposed reactive centre of contrapsin precursor) (Figure 4.4). The reactive centre is the region of the SPI in which proteinases attack; upon attack, the proteinase is inactivated. The proteolytic specificity of the SPI is primarily determined by a single amino acid within the reactive centre, the P1 residue, however the other amino acids in the reactive centre are important for specificity and efficiency of inhibition. Among different family members, the reactive centre itself is also highly variable (Inglis and Hill, 1991).

Since Bands VIA and VIB yield similarity to the reactive centre of a proteinase inhibitor, it may also play a role as an enzyme inhibitor. However, more of the sequence of the protein represented by Bands VIA/B must be obtained before any further conclusions can be made about its function.

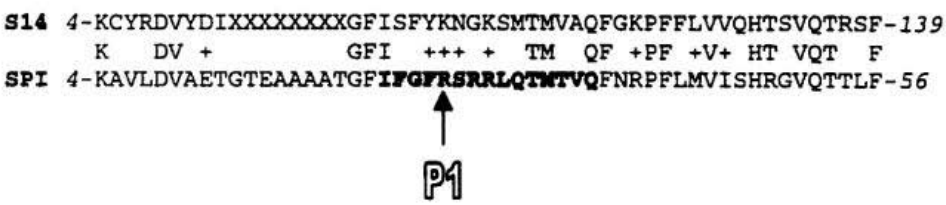


Figure 4.4 Reactive Centre of Serine Proteinase Inhibitor (SPI) Similarity with Sample 14. P1 is the residue that determines enzyme specificity. The reactive centre is in bold.

Expression

None of the methods used in this study were able to confirm expression of Bands VIA and VIB as examined through Sample 14. Due to low expression levels, many of the other samples tested by Northern blot were not detected. However, Sample 14 was not detected in FGF treated animal cap explants by PCR. A gene upregulated by FGF should be expressed at high enough levels to be detected by sensitive PCR methods. The specific Sample 14 primers were able to amplify Sample 14 plasmid cDNA, therefore the problem with PCR amplification was not thought to be due primer design.

There are several possibilities for the problem of Sample 14 detection by PCR. The gene may be expressed at very low levels, even if upregulated by FGF. Another possibility is that Bands VIA and VIB may not be a product of the *Xenopus* genome, but a result of contamination from another source.

4.4.2.3 Bands 11 and 12, Samples 17 and 22

Genbank Protein Matches and Potential Functional Domains

Samples 17 and 22, from Bands 11 and 12 respectively, yield amino acid similarity to a hypothetical 19.7 kDa protein (open reading frame 2) from a fungus mitochondrial plasmid. The 19.7kDa protein from the fungus *Ascobolus immersus* is a proposed DNA polymerase according to open reading frame (ORF) 1, which is in the positive orientation. Similarity to Band 11/12 DNA fragment is in ORF 2, which is a short ORF in a minus reading frame, opposite of the suspected correct reading frame. Thus this information was not useful in determining the function of the matching regions (Kempken *et al.*, 1989).

Expression

Bands 11 and 12 mRNA were not detected by Northern blotting, but were detected in Stage 8 *Xenopus* embryos by RT-PCR. In the PCR experiment, the gene product was shown to be upregulated in response to FGF treatment. This suggests that the protein transcribed from this gene plays a specific role in development at Stage 8. Its upregulation by FGF in animal cap explants adds to the validity of this hypothesis, however this experiment must be repeated several times in order to make a final conclusion regarding FGF regulation.

4.4.2.4 Band 22, Samples 29 and 30; a 146kDa Nuclear Protein

Samples 29 and 30 from Band 22 yielded 97% identity to a 146kDa *Xenopus* widespread nuclear protein (NP). From the high degree of identity, it is assumed that Band V represents the same protein. NP is localized within the nucleus in nuclear speckles, which contain the ribonucleoprotein particles and other splicing factors invloved in processing RNA. It is thought to be a widespread nuclear protein that co-localizes with the mammalian splicing factor SF3b and is thus a proposed component of this factor (Schmidt-Zachmann *et al.*, 1998).

NP possesses a region of threonine-proline phosphorylation sites for cyclin dependent kinase (CDK). CDK is one of a family of kinases that play important roles in the regulation of cell division cycles and gene transcription (reviewed in Morgan, 1997). NP also has phosphorylation sites for PKC, cAMP dependent protein kinase (protein kinase A, PKA) and casein kinase II (CKII) (Schmidt-Zachmann *et al.*, 1998).

PKC plays diverse roles in cell differentiation, growth, cell cycle control and tumorigenesis (Livneh and Fishman, 1997) and is a well-known effector of diacyl glycerol in the PLCγl pathway (Figure 1.5, Page 27). PKA is also involved in a wide variety of cellular functions and is a key molecule in a phosphorylation cascade as an effector of the adenylate cyclase membrane protein (Fantozzi *et al.*, 1994). Casein kinase II is a widely effective serine/threonine kinase required for cell cycle progression and cell viability. CKII is expressed ubiquitously, and at high levels in proliferating cells (Pinna and Meggio, 1997).

As previously described, Band 22 is 84% identical to a region of the *C. elegans* Cosmid TO8A11.2, cloned as a result of the Nematode sequencing project (Wilson *et al.*, 1994). A portion of a corresponding human protein was 91.75% identical to the *Xenopus* protein, suggesting that they are homologous molecules (Schmidt-Zachmann *et al.*, 1998). The high level of similarity suggests that this gene has been well conserved throughout evolution, any may play a vital role in cellular processes.

Immunolocalization studies with NP reveals that it is present in the developing embryos as well as the follicle epithelial cells surrounding the oocytes, fibroblasts and other dermal cells, cardiomyocytes, endothelial cells and erythrocytes. In erythrocytes that are inactive in transcription and replication, NP protein was present suggesting that it is a general component of the nucleus and is not dependent on RNA or DNA synthesis (Schmidt-Zachmann *et al.*, 1998).

Expression

My studies were not able to confirm the upregulation of Band 22 expression by FGF-2 in *Xenopus* animal cap explants. Three of the four RT-PCR experiments revealed that Band 22 was upregulated by FGF treatment. In one Northern blot, a more reliable source for studying expression, Band 22 mRNA doubled in response to FGF treatment. Two other Northern blots revealed that Band 22 expression is approximately the same or decreased in response to FGF. The conflicting results, especially with Northern blots, make it impossible to conclude whether Band 22 is upregulated by FGF.

Schmidt-Zachmann and colleagues (1998) suggest that NP is a ubiquitous protein.

My Northern blotting experiments reveal that Band 22 is expressed throughout *Xenopus* development. There is a slight increase in expression from first cleavage to Stage 8-9, and then a decrease at gastrula stage 10-10.5. There is an increase in expression after gastrulation and then a decrease again at mid to late tailbud, Stages 30-35. Increase in expression at Stage 8-9 would be expected because this is the stage at which the zygotic expression begins, presumably when increased levels of splicing occur. This also marks the onset of mesoderm induction, when NP could also play an important role.

4.5 Future Studies

There are several experimental approaches that may be taken to further study FGF-2 response genes in *Xenopus laevis*. One approach would be to start fresh with a new differential display. This way, one could check whether the same genes are upregulated by FGF in the second experiment as the first.

Performing 'slot blotting' is said to be an effective method of confirming whether genes are differentially expressed, and a large number of genes may be checked quickly, before too much time is spent studying them (Mou *et al.*, 1994). For example, in a differential display conducted by Mou and colleagues, only 8 out of 38 DNA fragments were differentially expressed according to the slot blot. This method saved the researchers a lot of research time that could have been spent working on other methods (Mou *et al.*, 1994).

The slot blot method involves spotting the potential differentially expressed DNA fragments onto a nylon membrane and probing the membrane with radiolabeled cDNA reverse transcribed from mRNA isolated from the different experimental conditions. For example, one could probe with cDNA from FGF treated and untreated explants. This is a reverse of the methodology used for Northern blotting, and many fragments may be blotted on one membrane and tested at one time. However, since a large amount of the potential differentially expressed fragment may be hybridized to the membrane, this process is more sensitive than Northern blotting.

As discussed, it has been difficult to quantitate expression levels of the new genes by PCR. One option to help quantitation of the PCR products would be to amplify known quantities of synthetic DNA and make a standard curve. Thus, one could compare unknown samples with the standard curve.

A major obstacle to overcome with Samples 3 and 17 is to be able to obtain a larger piece of the DNA sequence. One would have to use more specialized cloning methods. An option for Sample 17 (which may also work for Sample 14) would be to create a cDNA library made at the developmental stage at which the samples are expressed, or made from FGF treated explant cDNA. For example, an option for cloning Sample 3 in the future would be to create a new cDNA library of stage 8 embryos and to use a modified, more sensitive cloning technique.

A possibility for future studies would be to continue working on the samples that have already been isolated, Samples 3, 14, 17, and 30. Samples 3, 17 and 30 are quite interesting because they have demonstrated differential developmental expression. One

approach would be to conduct spatio-temporal analyses. One could treat embryos at second cleavage with a vital dye to label the dorsal end. Then at various stages of development, explants could be cut from each region of the embryo. RNA could be isolated and RT-PCR conducted to determine when and where the genes are expressed.

Another experiment that would be of interest would be to examine expression of the genes in adult tissues. One could also search for homologues in other species including human. Examining differential expression in various tissues, normal and cancerous would also be useful. One could also examine expression in response to other mesoderm inducers, such as activin.

It would be worthwhile to further experiment with RNAse protection assay. Since this method is quite sensitive to lower RNA levels, it may be useful for examining differential expression. This time, however, it would be important to use both positive and negative orientations for probing, as one does not know which is the sense strand. Titration of a control RNA at known concentrations would also be useful. Titrating the control RNA to levels that can no longer be detected will allow one to estimate the minimum amount of sample RNA needed for detection on RNAse assay. Thus, the amount of total explant/embryo RNA needed for the experiment may be calculated.

Further experimentation with Sample 24 expression would also be interesting in light of its similarity to *c-mos*. PCRs testing FGF-regulation could be conducted to determine whether *c-mos* is expressed in response to FGF treatment. This could be performed quite quickly.

There is great potential for future studies in developmentally expressed genes and FGF early response genes and a vast amount of knowledge to be obtained in the area. The more information we obtain regarding developmental and FGF response genes, the closer we are to elucidating the mysteries of both normal embryonic development and the pathology of cancer.

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