CHARACTERIZATION OF THE EXPRESSION AND FUNCTION OF THE EARLY RESPONSE 1 GENE IN XENOPUS LAEVIS EMBRYONIC DEVELOPMENT

HEMA ARTEE LUCHMAN


CENTRE FOR NFLD. STUDIES
MEMORIAL UNIVERSITY
OF NEWFOUNDLAND

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by

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ABSTRACT

Xenopus early response gene 1 is a maternally-derived immediate-early gene whose expression is activated by FGF during mesoderm induction in *Xenopus* embryos. The purpose of this project was to characterize the expression and investigate the function of ER1 protein during early development in *Xenopus*. Analysis of the expression pattern of ER1 showed that the protein is present in the early embryo but retained in the cytoplasm until mid-blastula stages after which it is translocated to the nucleus, first in the presumptive mesoderm, then in the presumptive ectoderm, and finally in the endoderm. Overexpression of the dominant negative FGF receptor XFD completely blocks translocation of ER1 to the nucleus at mid-blastula suggesting that nuclear translocation of ER1 is dependent on events triggered by FGF signalling. Deletion analysis of stretches of acidic amino acid in the N-terminal region of ER1 showed that the protein has transactivation activity *in vitro*, suggesting that the protein may function as a transcription factor *in vivo*. Overexpression of ER1 in embryos results in embryos with posterior truncations, a phenotype similar to that of embryos overexpressing XFD. RT-PCR analysis of molecular markers expressed during early development showed that overexpression of ER1 downregulates the expression of *Xbra*, BMP-4, and *HoxB9*. These results suggest that ER1 may function as an endogenous, negative regulator of the FGF signalling pathway during *Xenopus* embryogenesis.

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List of Abbreviations

AD	acidic domain
AMP	adenosine monophosphate
AP	alkaline phosphatase
ATP	adenosine triphosphate
bp	base pair
BMP	bone morphogenetic protein
C	Celsius
CAT	chloramphenicol transferase
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
CRD	cytoplasmic retention domain
CTP	cytosine triphosphate
CSF	colony stimulating factor
DEPC	diethyl pyro carbonate
DMEM	Dubelco's modified eagle medium
DMSO	dimethyl sulfoxide
dNTPs	deoxyribonucleotide triphosphates
DV	dorsovegetal
EC	extracellular domain
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis-N,N,N',N'-tetra-acetate
ER1	early-response gene 1
EF1α	elongation factor 1 alpha
ELM2	EGL-27 and MTA1 homology2
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
g	grams
GNEF	guanine nucleotide exchange factor
GDP	guanine diphosphate
GSK-3	glycogen synthase kinase-3
GTP	guanine triphosphate
GV	germinal vesicle
h	hours
HCL	Hydrochloric acid
HMG	high mobility group
HRP	horseradish peroxidase
IκB	I kappa B
JM	juxtamembrane
kb	kilobase pair
kDa	kilodalton
KD	kinase domain
JAK	janus kinase
L	litre

M	molar
MAB	maleic acid buffer
MAPK	mitogen activated protein kinase
MBT	mid-blastula transition
ml	millitres
mm	millimetres
mta1	metastasis associated gene1
μg	micrograms
μl	microlitres
mM	millimolar
min	minutes
mRNA	messenger ribonucleic acid
NAM	normal amphibian medium
ng	nanogram
NLS	nuclear localization signal
NFκB	nuclear factor kappa B
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PI3'K	phospho inositide 3' kinase
PLCγ	phospholipase c gamma
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
RT	room temperature
RT/PCR	reverse transcription ploymerase chain reaction
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulphate
sec	seconds
SH2	src homology domain 2
SH3	src homology domain 3
snRNPs	small nuclear ribonucleic proteins
SRE	serum response element
STAT	signal transducer and activator of transcription
SV40	simian virus 40
TBS-T	tris buffered saline-tween
TPA	12-O-tetradecanoylphorbol 13-acetate
TM	transmembrane
TTP	thymidine triphosphate
U	units
UTP	uridine triphosphate
VV	ventrovegetal
w/v	weight per volume
xbra	xenopus brachyury
XFD	dominant-negative FGFR

SECTION I

CHAPTER 1: GENERAL INTRODUCTION

1.1 FOREWORD

There has been considerable progress in recent years in identifying new genes and in determining the function of their protein products, both in normal biological systems, and in malfunctioning systems. Often, the identification of genes and proteins only gives us a two-dimensional picture of their function in a cellular system. In order to gain a more complete understanding of how a biological system functions, one needs to understand the dynamic interactions that are ongoing at all times in cells, tissues and organisms. These interactions involve a complex network of molecules that are turned on and off at specific times through constant signalling and feedback loops. Model systems, both *in vivo* and *in vitro*, have provided researchers with tools to test hypotheses on how and why genes are expressed and how proteins interact with each other in biological systems. Each model system presents strengths and weaknesses, but together they have provided many insights into the functioning of organisms.

Model organisms, both vertebrate and invertebrate, are used for the study of developmental processes. Reasons for this include: ease of study, familiarity with their development and/or genetics, and because of their biological interest

(Wolpert *et al.*, 2002). Some of these model systems such as the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans,* sea urchins, zebrafish, amphibians, chickens, rats and mice, have been studied widely.

The vertebrate group is comprised of a large number of different organisms, all possessing the characteristic vertebral column. As adults, some of these organisms are very different in their morphology. However, during the early stages of their life cycle, all vertebrate embryos undergo similar developmental stages and pass through a phylotypic stage (where they resemble each other) (reviewed in Galis & Metz, 2001). All vertebrate embryos start by cleavage of the zygote after fertilization. Initially, the embryo repeatedly divides into a number of smaller cells without a matching increase in mass. This process is followed by gastrulation, a stage characterized by cell movements, which results in the laying down of the germ layers determining the body plan. The phylotypic stage occurs after gastrulation, and the vertebrate embryos more or less resemble each other and have characteristic vertebrate features such as the notochord, somites and neural tube. The notochord forms along the antero-posterior axis and subsequently becomes incorporated into the vertebral column. The brain and the spinal column are derived from the neural tube, which is formed from ectodermal tissue lying directly above the notochord. The somites develop into the vertebral column, muscles of the trunk, dermis and into limbs. These structures are representative of all vertebrate classes (reviewed in Galis & Metz, 2001).

The similarity in morphology during the early development of vertebrates has long been used to justify the use of what may seem like a limited number of developmental models to study development in general. The similarity in body plan suggested that the processes that regulate development in different animals might also be similar. In the last few years, molecular evidence has supported the view that early development may be conserved at the gene level. With the recent progress in the cloning of several genomes such as human, *Drosophila melanogaster*, and *Caenorhabditis elegans*, there is evidence that the genes involved in the early development of organisms are conserved throughout evolution not only between vertebrates but, as is the case for certain ancestral genes, even between vertebrates and invertebrates.

Developmental models are often useful for studying cancer. It has been shown that expression of these genes that are very tightly regulated during early development and is up- or down-regulated spatially and temporally in cells and tissues with a high degree of precision may become misregulated in developmental diseases and cancer. The various components of signal transduction cascades, from the ligands and receptors initiating the cascades to the transcription factors which effect a response at the DNA level, are potential oncoproteins and tumor suppressor proteins. Loss of regulation at any step can result in uncontrolled cell growth and in neoplastic growth (Powers *et al.*, 2000).

The work in this thesis deals with the protein product of *er1*, a *Xenopus laevis* gene, and attempts to provide further insight into its expression pattern and its function during early development.

1.2 *Xenopus laevis* as a model organism for vertebrate development.

Xenopus laevis (Xenopus), the South-African clawed frog, has been useful in elucidating some of the cellular, molecular and genetic mechanisms that control cellular processes in vertebrates and has helped advance two important areas in vertebrate biology: early embryonic development and cell biology. In the past, the use of newts and salamanders was very common, and nowadays *Xenopus tropicalis* is becoming more popular among developmental biologists due to its potential for creating transgenics.

Xenopus laevis has been critical in identifying basic embryonic processes such as early fate decisions, patterning of the basic body plan, and organogenesis. *Xenopus* has helped cell biologists and biochemists understand chromosome replication, chromatin and nuclear assembly, cell cycle components, cytoskeletal elements, and signalling pathways. *Xenopus laevis* presents many advantages for the study of development and cellular processes.

1. The frogs are relatively easy to raise and breed in laboratory conditions and develop normally in tap water.
2. Eggs are easy to obtain in large quantities by stripping females induced overnight with the human chorionic gonadotropin hormone (HCG).
3. Fertilization can be performed *in vitro* on a large number of eggs by using sperm from testes removed from *Xenopus* males.

4. The embryos are hardy, highly resistant to infection and easy to maintain in saline solutions.
5. The eggs are large –1 to 2 mm- in diameter and are very easy to manipulate and dissect.
6. The life cycle of *Xenopus* is fully known.

1.3 *Xenopus laevis* development

In this study, *Xenopus* embryos in the early stages of development (stage 1 to 45) were used. This section will highlight some of the major stages in the life cycle of *Xenopus* (Figure 1.1) with emphasis on stages fertilization up to gastrulation. Figure 1.1 shows stages 1, 6.5, 10 (gastrula), 13 (neurula), 22 (tailbud), 28 (late tailbud), 40 (tadpole) and adult frogs.

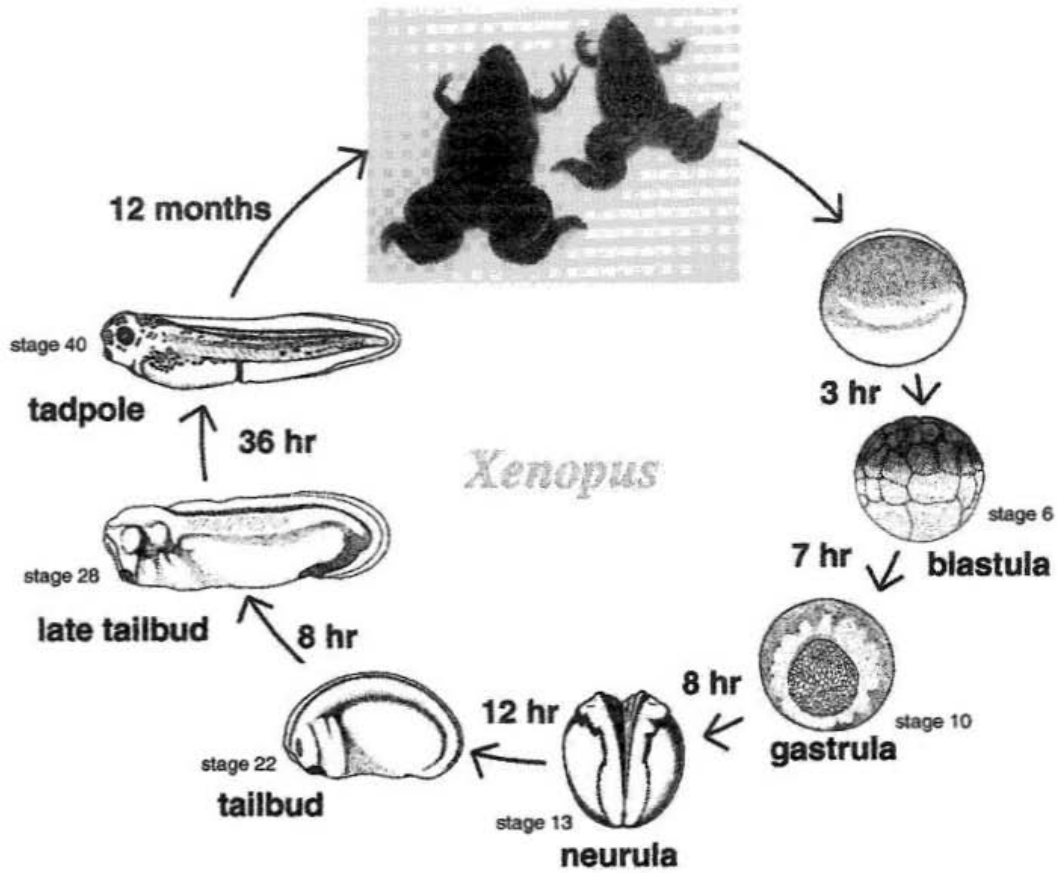


Figure 1.1 The Life cycle of Xenopus laevis
The major developmental stages and the approximate time scale following fertilization at room temperature are shown (reproduced from http:/cbrmed.ucalgary.ca/pvize/html/buttons/XenCycleH.jpg).

1.3.1 Oogenesis

Xenopus females produce gametes or ova through the process of oogenesis. Prior to oogenesis, primordial germ cells undergo mitotic cell divisions and migrate to the genital ridge or future gonad (for review see Saffman & Lasko, 1999). The primordial germ cells differentiate into eggs at the start of meiosis. Adult females have eggs in different stages of development in their ovaries; these stages are often classified according to their size, the smallest being stage I and the largest stage VI. Growth from stage I to stage VI requires about 8 months and during this time oocytes acquire the yolk, enzymes and precursors of DNA, RNA and protein synthesis required by the embryo during early development (Smith *et al.*, 1991a).

It is during oogenesis that the animal-vegetal polarity is specified in the egg. The animal hemisphere in the *Xenopus* egg is darkly pigmented, whereas the vegetal hemisphere is unpigmented. During the early stages of oogenesis, the eggs are asymmetric with respect to their constituents, but as oogenesis proceeds, pigment is localized to the animal hemisphere and largest yolk granules to the vegetal. Yolk synthesis and deposition in the eggs occurs by a process known as 'vitellogenesis" and is triggered by environmental cues. These signals stimulate the release of gonadotropin-releasing hormone from the hypothalamus, which itself stimulates the release of gonadotropins by the pituitary. These gonadotropins signal the follicle cells of the ovary to release oestrogens, which circulate to the liver where vitellogenin is made. The

vitellogenin is carried to the eggs for uptake. Yolk is a highly important constituent of the egg because it is the only food source available to the embryo during early development. Other maternal constituents such as glycogen granules, ribosomes, lipochondria, endoplasmic reticulum and the germinal vesicle (nucleus) translocate towards the animal hemisphere and maternal RNA localizes to different regions of the egg (Danilchik and Gerhart, 1987). The maternal RNA transcribed during oogenesis suffices to meet all the requirements of the embryo until it initiates transcription of its own RNA at the mid-blastula transition (MBT), which occurs after the 12^{th} cleavage cycle (Newport & Kirshner, 1984).

The oocytes arrest in the first meiotic cycle at the G2/M transition at stage VI unless stimulated (Smith *et al.*, 1991). Eggs can remain in this state for several months until environmental cues prompt the follicle cells surrounding the oocytes to produce the hormone progesterone, which causes the resumption of meiosis and ovulation. Unstimulated eggs will usually undergo atresia (Hunt, 1989; for reviews see Sagata, 1988, Ferell, 1989). In the laboratory, females are stimulated to complete ovulation by an injection of HCG, which causes the stage VI oocytes to mature to the second meiotic metaphase followed by ovulation (reviewed in Wolpert *et al.*, 2002). Females can be manually harvested of the mature eggs about 14-18 hours after stimulation with HCG and fertilized *in vitro* with sperm obtained from adult males.

1.3.2 Fertilization

Fertilization is triggered by the sperm entering the egg (Fig. 1.2A). Fusion of the sperm and egg plasma membrane causes a rapid release of free calcium ions from the egg's endoplasmic reticulum into the egg's cytoplasm. As a result of the high levels of intracellular calcium, the egg membrane becomes depolarised and cortical granules are released into the space between the egg and the vitelline membrane (an acellullar membrane surrounding the plasma membrane). The vitelline membrane prevents polyspermy. Calcium release also causes the activation of the enzyme, calmodulin-dependent protein kinase II. This enzyme causes the degradation of the cyclin component of maturation promoting factor (MPF) causing the egg to re-enter and complete meiosis (Nuccitelli, 1991; Wanatabe *et al.*, 1991; Runft *et al.*, 1999).

The sperm and egg pronuclei fuse to form the zygote nucleus. The vitelline membrane lifts off the surface of the egg and the egg rotates under the influence of gravity so that the heavier, yolky, vegetal region is facing downward. (reviewed in Wolpert *et al.*, 2002)

1.3.3 Cortical rotation and establishment of the future dorsal side

Before fertilization, the *Xenopus* egg has a radial symmetry along the animal-vegetal axis, which is broken by sperm entry. The dorsal side forms opposite the sperm entry point (reviewed in Harland & Gerhart, 1997). The

series of events triggered when the sperm enters the egg result in the definition of the dorso-ventral axis of the embryo. Within 90 minutes of fertilization, the plasma membrane and the cortex opposite the sperm entry point perform a 30° rotation in the direction of the sperm entry point relative to the rest of the cytoplasm, a process known as cortical rotation (Fig. 1.2B) (reviewed in Harland & Gerhart, 1997).

Cortical rotation results in the formation of a signalling center in the vegetal region opposite the site of sperm entry. This signalling center has been named the Nieuwkoop center, after the embryologist Peter Nieuwkoop. The Nieuwkoop center is a vegetal dorsalizing center, which signals to the surrounding tissues and determines dorso-ventral polarity in the embryo (Fig. 1.2 C) (reviewed in Harland & Gerhart, 1997).

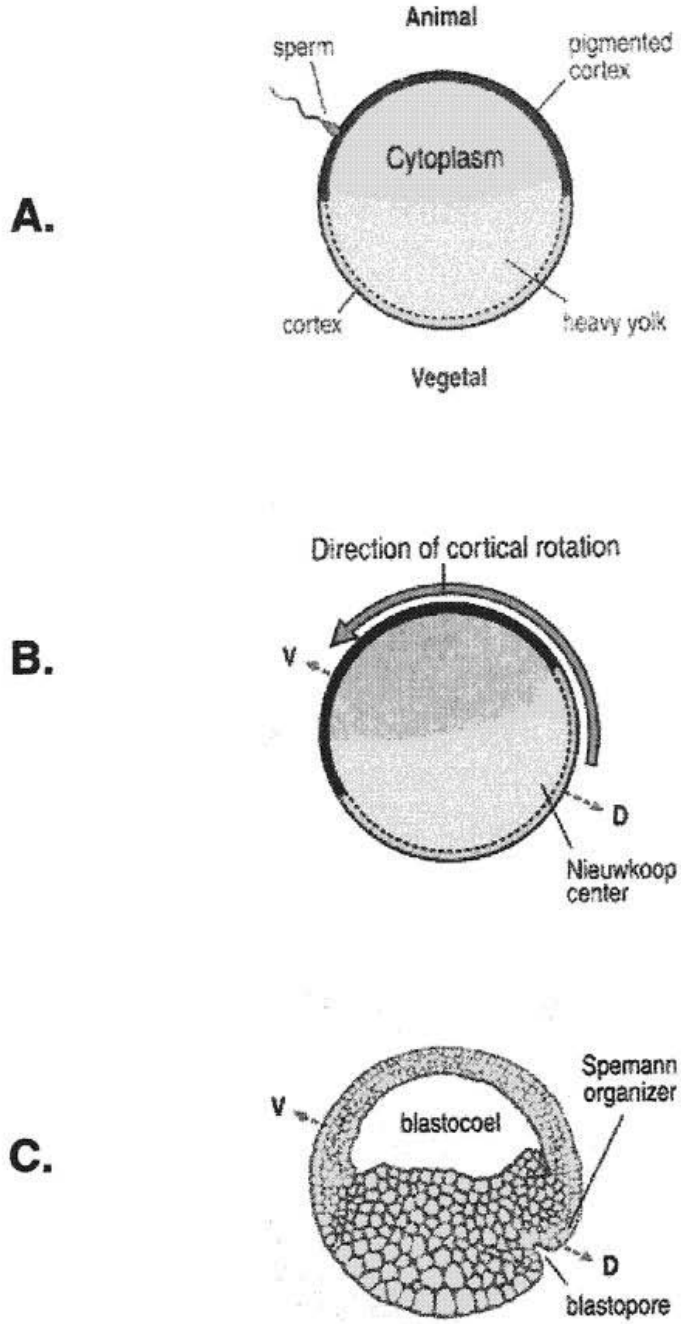


Figure 1.2: Fertilization, cortical rotation and establishment of future dorsal side

After fertilization (**A**), the cortical layer rotates 30 degrees towards the site of sperm entry (**B**), and results in the formation of the Nieuwkoop centre opposite the site of sperm entry. Later, the Spemann organizer and the blastopore form in a region just above the Nieuwkoop centre (**C**). V=ventral; D=dorsal (adapted from Wolpert *et al.*, 1998).

1.3.4 Cleavage events, blastula stages and patterning events

The first cleavage occurs about 90 minutes after fertilization and passes through the point of sperm entry and through the Nieuwkoop center. The embryo divides into two equal halves. Subsequent cleavages occur at intervals of about 20-30 minutes. The second cleavage is also along the animal-vegetal axis but at right angles to the first. The third cleavage is equatorial and at right angles to the first two and results into four smaller animal cells and four bigger vegetal cells known as blastomeres. The difference in size of animal and vegetal cells is a direct result of the unequal distribution of yolk in the embryo. The blastomeres keep dividing at regular intervals without any increase in cell mass. These continued synchronous divisions result in a spherical mass of cells inside which a fluid-filled cavity, the blastocoel, develops. The embryo is now known as a blastula (reviewed in Jones & Smith, 1999).

After about 12 cycles of cell divisions, the embryo reaches a stage known as mid-blastula transition (MBT). This stage is characterized by a slowing of the mitotic rate, the beginning of asynchronous cell divisions, the onset of zygotic transcription and cell motility (Newport and Kirshner, 1984; Masui and Wang, 1998).

1.3.4.1 Patterning of the Xenopus blastula

The different regions in the embryo, the animal region, the marginal region (a belt-like region dividing the animal and vegetal regions), and the vegetal region, give rise to the three germ layers: the ectoderm, mesoderm, and endoderm, respectively (Fig. 1.3A). The ectoderm later covers the entire surface of the embryo and forms the epidermis; ectodermal derivatives also give rise to nervous tissue. The mesoderm differentiates into the notochord, lateral plate (which forms heart, kidney and blood islands), and somites (Boterenhood & Nieuwkoop, 1973). The lining of the gut and gut-associated organs such as the liver and pancreas are derived from the endoderm (Fig. 1.3B). The late blastula stage embryo is a sphere of cells consisting of the future ectoderm, mesoderm and endoderm (Fig. 1.3) (Dale & Slack, 1987; reviewed in Harland & Gerhart, 1997).

By the time the *Xenopus* egg is laid, there are already differences along the animal-vegetal axis. Maternal factors are thought to determine the fates of the ectodermal and endodermal regions. For example, if explants from the different regions of the early blastula are cultured in simple salt medium, explants from the animal region will form an epidermal ball of cells whereas explants from the vegetal region will be endodermal in nature (Nieuwkoop, 1969; Boterenhood & Nieuwkoop, 1973). These results match the expected fates of these regions and there is no evidence that signals from other regions are required. However,

if an explant from the animal region is cultured in combination with an explant from the vegetal hemisphere, it is induced to form mesodermal derivatives, which normally result from explants from the marginal zone only (Boterenhood & Nieuwkoop, 1973; reviewed in Isaacs, 1997).

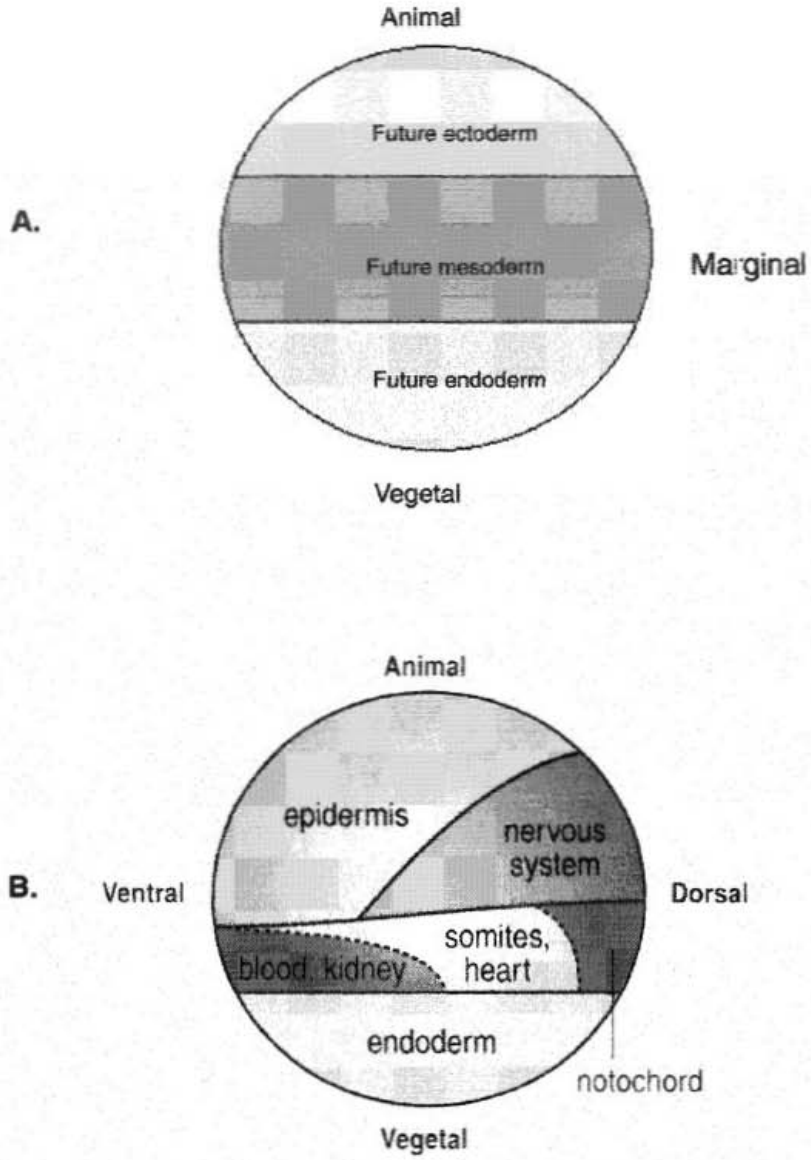


Figure 1.3 Fate map of the *Xenopus* embryo

A. The *Xenopus* embryo showing the animal, marginal and vegetal regions which will develop into ectoderm, mesoderm and endoderm respectively. **B.** A fate map of the *Xenopus* blastula (lateral view) illustrating the normal fates of the different blastula regions (adapted from Keller, 1975; Keller 1976; Dale and Slack 1987; reproduced from Wolpert *et al.*, 1998).

It is now widely accepted that inductive cell interactions between the animal and vegetal hemispheres control the differentiation of the mesodermal embryonic germ layer. Landmark experiments by Nieuwkoop (1969) demonstrated that vegetal cells induced animal cells to form mesoderm. One of the most important and famous experiments demonstrating the presence of inducers during mesoderm induction and embryonic axis formation was performed by Spemann and Mangold in 1924. They transplanted a section of the dorsal marginal zone from one embryo into the ventral side of a second embryo and obtained an embryo with two body axes. These results demonstrated that cells of the dorsal marginal zone were responsible for the formation of dorsal mesodermal structures such as the notochord, in addition to specifying or organizing the antero-posterior body axis. In these experiments, it was effectively shown that cells of one type, dorsal mesoderm, had the ability to induce the formation of mesodermal derivatives in other cells. This region of the dorsal marginal zone subsequently became known as the 'Spemann organizer' (Figure 1.2) (reviewed in Harland & Gerhart, 1997).

It has been proposed that signals are established by general mesoderm inducers shortly after cortical rotation, which is triggered by sperm entry in the egg and causes the division of the vegetal cytoplasm into ventrovegetal and dorsovegetal regions. Cortical rotation involves the establishment of a parallel array of microtubules in the vegetal hemisphere along which the cortex is thought to move (Elinson & Rowning, 1988). Disruption of the microtubule array by

ultraviolet radiation prevents cortical rotation, and results in embryos lacking an embryonic axis (Gerhart *et al.*, 1989). Conversely, manual tipping or centrifugation of egg during the first cycle causes a duplication of the axis (Gerhart *et al.*, 1989). Cortical rotation establishes a dorsal determining activity region opposite the point of sperm entry, the Nieuwkoop centre, which induces the Spemann's Organizer. In turn, the organizer establishes dorso-ventral polarity in the embryo (reviewed in Moon & Kimelman, 1998).

In recent years, a widely accepted model for mesoderm patterning, the Three Signal Model (Smith & Slack, 1983) has been proposed (depicted in Figure 1.4). The Three Signal Model proposes that mesoderm patterning is initiated by early signalling, from general mesoderm inducers released by the vegetal region to the animal region. The first set of signals originates in the dorsovegetal (DV) region (Nieuwkoop centre) and induces the formation of dorsal mesoderm, including the Spemann's organizer. The inducing factors from the ventrovegetal (VV) region induce the marginal zone cells directly overlying them to broadly specify ventral-type mesoderm such as mesenchyme, mesothelium and blood. Subsequently, the third signal, a dorsalizing inductive signal, emanates from the organizer and imposes more dorsal or paraxial (resulting in the formation of notochord) and intermediate fates (such as muscle) on neighbouring ventral mesoderm at gastrula stages (Smith & Slack, 1983; Dale *et al.*, 1985; reviewed in Harland & Gerhart, 1997).

The model described above does not necessarily imply only three distinct signalling molecules. It is possible that each signal represents more than one molecule or one molecule acting at several different concentration gradients. Some of the inducing and competency factors involved in mesoderm induction have been identified and will be discussed further in the next section.

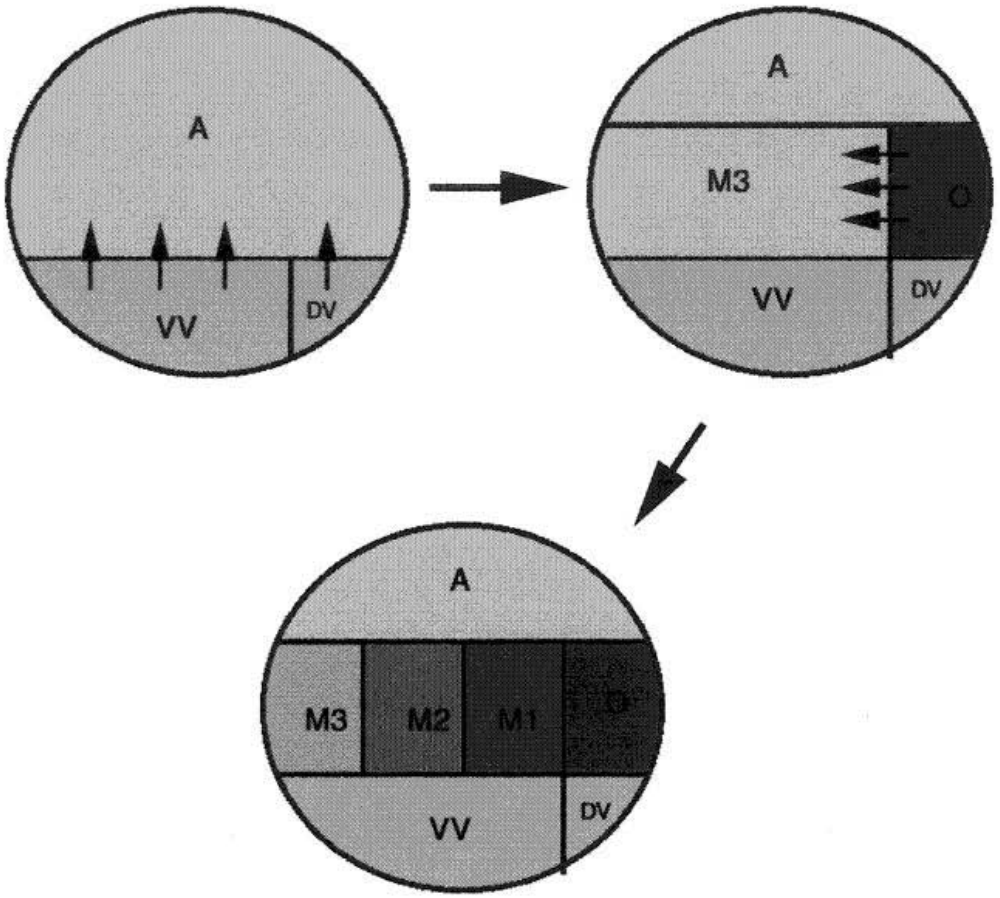


Figure 1.4 The three-signal model of mesoderm induction

Shown are the origins and actions of the three signal types involved in mesoderm induction. During mesoderm induction, the dorsovegetal (DV) region induces the organizer (O) and the ventrovegetal (VV) region induces ventrally specified mesoderm (M3) in the marginal zone. Signals from the organizer region "dorsalize" the ventral mesoderm to give regions of lateral mesoderm (M1 and M2). A = animal hemisphere. (adapted from Smith & Slack, 1983).

1.3.4.2 *Molecules that pattern the mesoderm*

Several molecules have been shown to induce mesoderm in animal cap explants in culture. However, to be qualified as a natural inducer in the embryo, several criteria need to be met by potential mesoderm-inducing proteins. These criteria include the presence of the inducing protein in the right concentration and at the right time in the vegetal region of the embryo; showing that the appropriate cells can respond to the factor; and that blocking the response prevents induction (reviewed in Harland & Gerhart, 1997). Furthermore, it is thought that the mesoderm-inducing molecules are most likely to be secreted. It has been shown that when isolated animal and vegetal cell fragments are cultured together but separated by a filter with pores too small to allow cell contacts to develop, the animal cells still differentiate into mesoderm (Slack, 1991). This demonstrates that mesoderm induction is due to small, secreted diffusible molecules produced by the vegetal region (Slack, 1991). It has also been shown that mesoderm induction starts at around the 32-cell stage and is almost complete by the time gastrulation starts (Dale & Slack, 1987). Nakamura and Takasaki (1970) showed that the equatorial region of the embryo formed mesoderm in culture only after the 64-cell stage; before this they would differentiate into epidermis. Therefore, natural mesoderm inducers are most likely maternally-derived factors that are present in the vegetal hemisphere and induce overlying animal cells to form mesoderm.

Although there is still a debate as to which molecule/s act/s as the general mesoderm inducer released by the VV region, several candidates have been proposed. It is generally agreed that TGF-β proteins play a key role in mesoderm induction (reviewed in Kessler & Melton, 1994; Jones *et al.*, 1995; Smith *et al.*, 1995). The various members of the TGF-β family implicated in mesoderm induction are activin (Asashima *et* al., 1990, Smith *et* al., 1990; Thomsen *et* al., 1990); Vg1 (Thomsen & Melton, 1993); TGF-β2 (Rosa *et* al., 1988) and *Xenopus* nodal-related 1 and 2 (Xnr1, Xnr2) (Jones *et* al., 1995). Bone morphogenetic proteins (BMPs) have also been shown to have ventral-mesoderm inducing activity at high doses (Wilson & Hemmati-Brinvanlou, 1995). Signalling by β-catenin (Heasman *et al.*, 1994) is also important during mesoderm induction (discussed later) and FGF signals have been shown to be necessary for the maintenance of induced mesoderm (Amaya *et* al., 1993;Isaacs *et al.*, 1994).

Although the mature, active protein products of the various molecules listed above can induce mesoderm in animal cap explants *in vitro*, it has been difficult to block the activity of specific proteins in order to show their importance (reviewed in Harland & Gerhart, 1997). All of these molecules are present in the form of mRNA and/or protein in the embryo or are transcribed in the late blastula. When a dominant negative TGF-β family receptor, activin type IIB receptor (XAR1; lacking most of the cytoplasmic portion of the receptor), is injected in the

embryo at an early stage, mesoderm formation is prevented (Hemmati-Brinvalou & Melton, 1992), and signalling by activin (Hemmati-Brinvanlou & Melton, 1992), Vg1 (Shulte-Merker *et al.*, 1994), and BMPs (Hemmati-Brinvanlou & Thomsen, 1995) is blocked. These experiments implicate a TGF-β in the induction of the mesoderm but do not identify a specific family member.

Activin is a potent mesoderm inducer of animal cap explants in culture but is thought to be an unlikely inducer in the embryo since follistatin, an inhibitor of activin, has been shown to block the activity of activin added to animal caps but not to prevent mesoderm induction (Shulte-Merker *et al.*, 1994, Kessler & Melton, 1995). Another attractive candidate for general mesoderm induction is Vg-1, which is expressed from maternal RNA localized to the ventral region of the embryo. Like other members of the TGF-β family, Vg-1 has to be proteolytically cleaved in order to be active. Researchers have shown that mature, processed Vg-1 induces mesoderm when injected into animal cap explants (Thomsen & Melton, 1993; Kessler & Melton, 1994; Forristall *et al.*, 1995). Although processed/active forms of Vg-1 have not yet been detected in the embryo, inhibition of Vg-1 with a dominant-negative mutant leads to defects in dorsal mesoderm formation (Joseph & Melton, 1998).

Using antisense oligodeoxynucleotides to deplete maternal mRNA, it has been also been shown that *VegT*, which encodes a transcription factor containing a T-box, is a maternal determinant required for early embryonic events (Zhang *et al.*, 1998).

Zygotic signals such as *Derriere* and the *Nodal*-related genes *Xnr1, Xnr2* and *Xnr4* are also involved in mesoderm induction (Zhang *et al.*, 1998; Kimelman & Griffin, 1998; reviewed in Kimelman & Griffin, 2000). The expression of *Xnr1, Xnr2,* and *Xnr4* starts at midblastula can be activated synergistically by VegT and Vg-1 (Zhang *et al.,* 1998, Joseph & Melton, 1998; Agius *et al.*, 2000) acting together with the dorsal determinant β-catenin (Heasman *et al.*, 1994; Scheneider *et al.,* 1996). Endogenous *Xnr1, Xnr2,* and *Xnr4* are expressed zygotically in the blastula endoderm in a graded fashion with a maximum in the dorsal endoderm (Agius *et* al., 2000). These zygotic factors can induce both ventral and dorsal mesoderm. It has been proposed that a gradient of Nodal-related signals, released by the endoderm during mesoderm induction, may be acting as the first two signals in the three-signal model (Agius *et al.*, 2000). VegT can also activate Derriere. Derriere is functionally different from the Xnrs as it seems to be required only in trunk and tail development, in contrast to Xnrs, which are required throughout the body (Kofron *et al.*, 1999; Piccolo *et al.*, 1999; Sun *et al.*, 1999).

Members of the FGF family have also long been considered prime candidates as mesoderm inducing factors (Slack,1994). FGF-2 (bFGF) and eFGF (Isaacs *et al.*, 1992; Tanahill *et al.*, 1992; Song & Slack, 1994; Song & Slack, 1996) have been proposed as mesoderm inducers. However, recent work has shown that during blastula stages the FGFs do not act as vegetally localized inducing signals. Instead, they are required in the animal hemisphere as

competence factors that are required in the animal hemisphere for the full range of responses to the vegetally localized molecules (reviewed in Isaacs, 1997). In contrast to the data obtained using the dominant negative activin receptor, which suggest that the TGFβ signalling pathway is required for the expression of all mesodermal genes (Cornell *et* al., 1995), FGF function is required for the correct regulation of only a subset of the genes that are expressed throughout the newly formed mesoderm (reviewed in Harland & Gerhart, 1998).

Several molecules have been identified as the potential dorsal and ventral signals (Figure 1.5). An essential component of the Wnt signalling pathway, the protein kinase, Glycogen synthase Kinase-3 (GSK-3) has been implicated in the normal suppression of dorsal fates. When GSK-3 activity is blocked by injecting dominant-negative GSK-3 mutants in the embryo on the ventral side of the embryo, a secondary axis is induced on the ventral side of the embryo indicating that GSK-3 normally functions to suppress dorsal axis formation (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce & Kimelman, 1995). GSK-3 regulates the level of the dorsal determinant, β-catenin, in the embryo by phosphorylation, which tags β-catenin for proteolysis. β-catenin is a transcription factor which is a member of the Wnt signalling pathway and has been identified as a potential early dorsal determinant involved in inducing activity (reviewed in Moon & Kimelman, 1998). Activation of Wnt signalling or overexpression of the dominant-negative kinase, prevents phosphorylation and stabilizes β-catenin.

Lithium had been shown to dorsalize *Xenopus* embryos when added or injected at early cleavage stages much before the identification of any of the signalling molecules in axis specification (Kao *et al.*, 1989). It has now been shown that lithium acts as a non-competitive inhibitor of GSK-3 *in vitro* (Klein & Melton, 1996; Stambolic *et al.*, 1996) and stablilizes β-catenin in cultured cells and *Xenopus* embryos (Stambolic *et al.*, 1996; Larabell *et al.*, 1997). These results suggest that lithium dorsalizes *Xenopus* embryos by increasing the levels of β-catenin in the embryo (Moon & Kimelman, 1998).

During early cleavage stages, β-catenin becomes enriched in the cytoplasm in the dorsal side of the embryo and by 16- to 32-cell stages is found in the nuclei of the dorsal side (Heasman, 1997; Moon & Kimelman, 1998), where it binds the HMG Box factor, XTcf-3. Expression of a XTcf-3 mutant lacking the β-catenin binding domain blocks the formation of the dorsal axis in *Xenopus* (Molenaar *et al.*, 1996). The association of β-catenin with XTcf-3 in the nuclei is believed to be required for the specific expression of dorsal genes during late blastula stages (reviewed in Moon & Kimelman, 1998). XTcf-3 directly binds the *siamois* promoter and activates the gene. *Siamois* is a homeobox gene encoding a transcription factor (Lemaire *et al.*, 1995). It is thought to play a major role in the specification of Spemann's organizer (Heasman, 1997; Moon & Kimelman, 1998). A dominant- inhibitory mutant of *siamois* created by fusing the repressor domain of the *Drosophila engrailed* gene

to the homeodomain of *siamois* blocks axis development and the expression of organizer-specific genes such as *goosecoid* (reviewed in Moon & Kimelman, 1998). *Goosecoid* in turn activates other organizer genes such as chordin (Sasai *et al.*, 1994).

After the induction of the organizer, further patterning subdivides the mesoderm into mesodermal subdomains along the dorso-ventral axis. Molecules such as BMP-4, Xwnt-8, Derriere and FGFs have been to be proposed molecules, present throughout the marginal zone, which act as ventralizing factors in the mesoderm (Figure 1.5) (reviewed in Moon & Kimelman, 1998). The activity of these ventralizing factors is controlled by dorsalizing signals, emanating from the Spemann's organizer (Figure 1.5). The dorsalizing molecules include noggin, chordin and frizbee, which act as anti-ligands by binding to the ventralizing factors and hence preventing the latter from binding to their receptors. Noggin and Chordin bind with BMP-4 and prevent it from binding to its receptor (Kessler & Melton, 1994; Zimmerman *et al.*, 1996; Piccolo *et al.*, 1996; reviewed in Moon & Kimelman, 1998). Frizbee acts by interacting with Wnt proteins (Leyns *et al.*, 1997; Sokol, 1999). Therefore, the mesoderm is probably patterned in a dose-dependent manner according to the distribution of the ventralizing factors and the localization of their inhibitors. The Spemann's organizer has been proposed to act as an anti-ventralizing zone, releasing dorsalizing factors, which inhibit the ventralizing factors. The cells near the organizer are therefore able to give rise to more dorsal-type mesoderm whereas

the tissues furthest from the organizer, which do not "see" dorsalizing signals, give rise to more ventral-type mesoderm.

For mesoderm induction to occur, the signalling molecules described above must bind specific cell surface receptors and activate signal transduction pathways which mobilize transcription activators and result in transcription of specific genes which specify and pattern mesoderm. Examples of early response zygotic activated genes that encode transcription factors are Xbra, goosecoid, chordin, pintavallis, HNF-3β, Xnot, and Xlim-1 (Smith *et al.*, 1991; Taira *et al.*, 1992; von Dassow *et al.*, 1994; Ang & Rossant, 1994; O'Reilly *et al.*, 1995). The genes activated by these transcription factors remain largely unknown but they are likely to encode secreted proteins such as noggin and frizbee. Goosecoid, for example, has been shown to activate the dorsal signalling gene chordin, (Heasman, 1997; Moon & Kimelman, 1998).

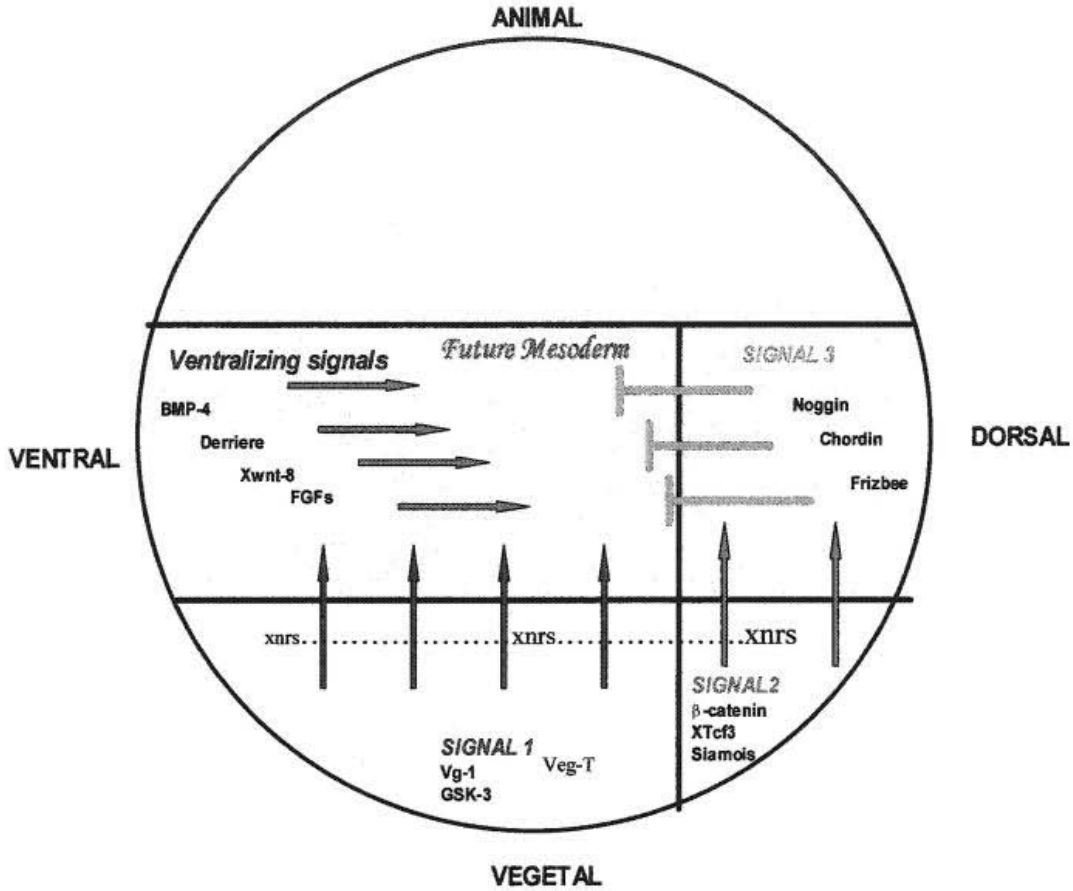


Figure 1.5 A mesoderm induction and patterning schematic showing a revised version of the Three-Signal Model with some of the molecules implicated. Vg-1 may act as the first signal or general mesoderm inducers. β-catenin binds to XTcf-3 and induces the expression of Siamois in the Nieuwkoop Centre on the dorsal side of the embryo. These molecules act as the second signal and induce the formation of the organizer. The Nodal-related Xnrs, which are activated by VegT may also be acting as both signal 1 and 2 in a dose dependent manner. Noggin, Chordin and Frizbee, which are released by the Organizer, act as Signal 3 by inhibiting molecules such as BMP-4, Xwnt-8, Derriere and FGFs which expressed throughout the marginal zone and give rise to ventral type mesoderm. Signal 3 results in more dorsal mesodermal type tissues in the organizer region

1.4 FGFs in mesoderm formation

FGFs are thought to play a two-part role during mesoderm induction. It has been suggested that maternal FGFs act to provide sub-threshold stimulation of the tyrosine kinase pathway in the animal hemisphere. The second function of FGF during mesoderm induction is the maintenance of mesodermal genes.

FGFs act as competence factors which are required for the full range of response to vegetally localized mesoderm inducers (reviewed in Isaacs, 1997). It has been shown that FGFs can induce mesoderm in animal cap explants in culture and that the type of mesoderm induced by FGF is concentration-dependent: at low doses of FGF, there is ventral mesoderm formation (such as mesothelium) while higher doses induce more lateral type mesoderm such as muscle (Slack *et al.*, 1987; Slack *et al.*, 1988). Furthermore, expression of a dominant-negative FGF receptor (XFD) inhibits mesoderm formation in animal caps and causes defects in trunk and posterior development in embryos (Amaya *et al.*, 1991; Kessler & Melton, 1994). It has been proposed that FGFs may potentiate the response of animal cap cells to TGF-β-like molecules and that they are involved in the control of cell movements and gene expression in the early mesoderm (Isaacs *et* al., 1994).

Therefore, rather than being primary inducing factors, FGFs are competence factors that are required for mesoderm induction by general mesoderm inducers. They are also required for the continued expression of a number of mesodermal genes (Dyson & Gurdon, 1997; reviewed in Isaacs,

1997). In support, ablation of FGF signalling affects primarily induction of ventral type mesoderm (Amaya *et al.*, 1991; Kessler & Melton, 1994). The low level of activity of maternal FGFs is necessary for the transcription of the mesodermal gene, *Xbra* (Isaacs *et al.*, 1994; Schulte-Merker & Smith, 1995), the *Xenopus* homologue of the Brachyury gene, an early response gene expressed throughout the mesoderm and encoding a transcription factor (Smith *et al.*, 1991b). *Xbra* acts in an autocatalytic loop to induce eFGF, which may function as a mesoderm secondary inducing factor (reviewed in Isaacs, 1997).

eFGF is necessary for the maintenance of Xbra. Injection of Xbra mRNA in the presumptive ectoderm causes formation of ventral mesoderm and further supports a role for Xbra and FGF in mesoderm patterning (Cunliffe & Smith, 1992; Cunliffe & Smith, 1994).

FGFs are among the major regulators of patterning of the developing embryo. The activities of FGFs during embryonic development appear to depend on FGF regulation of several fundamental life functions: the abilities to survive, replicate, make attachments, move, and attain a characteristic form (reviewed in Szebenyi & Fallon, 1999).

The next three sections will provide a brief overview of the structure and function of FGFs, FGF receptors and FGF signal transduction pathways.

1.4 FGFs – Structure and Function

FGF was first purified from bovine pituitary gland as a mitogen that could stimulate the growth of NIH3T3 cells (reviewed in: Burgess and Maciag, 1989; Mason, 1994; Galzie *et al.*, 1997; McKeehan *et al.*, 1998; Kato and Sekine, 1999; Szebenyi and Fallon, 1999; Ornitz 2000; Powers *et al.*, 2000). Since the initial discovery of FGF, approximately 25 years ago, 20 FGFs (Table 1.1), among which are some FGF homologous factors (FHFs 1-4; also referred to as FGFs 11-14, which comprise a separate branch of the FGF family and have been implicated in the development of the nervous system and limbs), have been identified in vertebrate species. Four FGF receptors (FGFRs) have been identified in vertebrates, two FGFRs and one FGF have been identified in *Drosophila* and one FGF/FGFR has been identified in *C. elegans* (Szebenyi & Fallon, 1999).

Historically, the first FGFs cloned (FGF-1 and 2) were shown to have mitogenic activity in fibroblasts, hence the name fibroblast growth factor. However, structure and heparin-binding ability, not specifically growth promoting activity, is the defining feature of the FGF family (Powers *et al,*. 2000). Although by no means exhaustive, Table 1.1 shows a sample of the functions of FGF family members. Therefore, although the members of the FGF family are collectively referred to as "FGF" followed by a numerical designation (Table 1.1),

each exerts different biological actions through specific high-affinity receptors (FGFRs) (Powers *et al.*, 2000).

Table 1.1 Characteristics of the members of the FGF family (adapted from Powers *et al.*, 2000 and Szebenyi & Fallon, 1999)

Name	Synonym	Species	Signal sequence	Comments
FGF-1	Acidic FGF, aFGF	Human, hamster, bovine, rat, pig, chick, mouse	Absent	1 mRNA form, nuclear localization motif, mitogenic, angiogenic, induces limb bud formation
FGF-2	Basic FGF, bFGF	Human, opossum, bovine, rat, chick, mouse, sheep, *Xenopus*, newt	Absent	4 protein isoforms through the use of alternate start codons, some isoforms have nuclear localization motifs, mitogenic, mesoderm inducing factor in *Xenopus*, angiogenic, induces limb bud formation, may be involved in apoptosis, highly expressed in human gliomas
FGF-3	Int-2	Human, chick, fish, mouse, *Xenopus*	Present	Site of MMTV integration in mouse genome, nuclear localization motif, expressed primarily during development, mesoderm inducing factor in *Xenopus*, involved in induction of inner ear, activated in mouse mammary carcinogenesis and tumorigenic clones of human colon cancer cell lines
FGF-4	Kaposi FGF, KFGF, hst-1	Human, chick, mouse, bovine, *Xenopus*	Present	Identified by screening stomach tumours and Kaposi's sarcoma, induces limb bud formation
FGF-5		Human, mouse, rat	Present	Regulatory factor in gastrulation, expressed in pancreatic cancer-associated macrophages and fibroblasts.
FGF-6	Hst-2	Human, mouse	Present	
FGF-7	KFGF	Human, mouse, rat, sheep, dog	Present	Specific for epithelial cells, mitogenic, angiogenic
FGF-8	AIFGF	Human, mouse, chicken, *Xenopus*	Present	7 isoforms, involved in migration of mesenchymal cells away from the primitive streak in mouse embryos, important in midbrain development and cell patterning of the neural plate, induces limb bud formation
FGF-9	GAF	Human, rat, mouse, *Xenopus*	Absent	Not angiogenic

FGF-10	KGF-2	Human, rat, chick, mouse	Present	Similar in structure and function to FGF-7, involved in lung development
FGFs 11-14	FHFs	Human, mouse (FGF-11), Human, mouse, chicken (FGFs 12-13), mouse (FGF 14)	Absent	All contain nuclear localization motifs
FGF-15		Mouse		Activated by E2A-Pbx1
FGFs 16-19		Rat	Present	
FGF-20	XFGF-20	*Xenopus*		Sequence homology to FGF-9

FGFs have been implicated in numerous cellular processes such as regulation of cell growth, survival, differentiation, migration, angiogenesis, chondrogenesis, morphogenesis, wound healing, skeletal formation, tumorigenesis, and metastasis (Kato and Sekine, 1999; Ornitz, 2000) and are produced at some point during the development of each of the four histological tissue types (epithelial, muscle, connective, and nervous tissue) (Szebenyi and Fallon, 1999).

FGFs form a family of structurally related polypeptide growth factors (Figure 1.6 shows a generic FGF protein), which range in molecular weight from 17 to 34 kDa in vertebrates, and up to 84 kDa in Drosophila (Ornitz, 2000). Members of the FGF family share a strong affinity for heparin and heparan-like glycosaminoglycans (HLGAGs) (Burgess & Maciag, 1989).

The most conserved sequence within the FGFs is a core of 120 amino acids, where FGF orthologs (divergence resulting from speciation) are 71-100% and FGF paralogs (divergence due to gene duplication resulting in several isotypes within a single species) are 22-66% identical (Szebenyi & Fallon, 1999). This core region does not contain a secretory signal. The mechanisms whereby some members of the FGF family such as FGF-1, -2, -9, -11, -12, -13, -14 are secreted by cells is still unknown but may be dependent on novel uncharacterized secretory pathways (Fernig & Galllagher, 1994; Szebenyi & Fallon, 1999; Powers *et al.,* 2000). Other family members such as FGF-3 to -8 and FGF-10 have a consensus signal sequence found near the AUG translation

initiation site (Figure 1.6) and are targeted for secretion through the endoplasmic reticulum.

Some FGF family members, such as *fgf-2* and *fgf-3* make use of alternative 5' CUG translation initiation sites as well as the canonical AUG codon (Figure 1.6) and yield high and low molecular weight proteins. The protein isoforms have different subcellular localization; the high molecular weight forms of FGF-2 contain nuclear localization sequences and localize to the nucleus whereas the low molecular weight form is cytoplasmic. The high and low molecular weight forms associate with distinct sets of proteins and have a different range of biological activities (Bivfalvi *et al.*, 1995; Patry *et al.*, 1997)

Post-translational modifications of FGFs include glycosylation, phosphorylation and ADP-ribosylation (Figure 1.6) (Szebenyi & Fallon, 1999). Glycosylation may have a role in modulating interactions between FGFs and proteases. Mutating the glycosylation site in FGF-4 results in cleavage of FGF-4 prior to secretion resulting in peptides that are biologically more active than the full-length protein (Bellosta *et al.*, 1993). The phosphorylation state of some FGFs such as FGF-2, which is phosphorylated on threonine 121, regulates their binding affinity for their receptors (Baird, 1994).

A number of different FGFs have been identified during the early stages of embryonic development in *Xenopus*. To date, four members of the FGF family have been identified in *Xenopus* (reviewed in Isaacs, 1997). These are FGF-2, FGF-3, eFGF and FGF-9. FGF-2, eFGF and FGF-9 all show maternal and

zygotic expression in *Xenopus*. FGF-3 becomes expressed in the embryo after the onset of zygotic transcription (reviewed in Isaacs, 1997). Both FGF-2 mRNA and protein are present in the early embryo. At blastula stages, FGF-2 is present predominantly in the animal hemisphere (Song and Slack, 1994). At later stages (neurula and tailbud), FGF-2 has widespread expression in the central nervous system and somatic tissue (Song and Slack, 1994). FGF-3 has a posterior domain of expression in the mesoderm of the blastopore region of the *Xenopus* gastrula and in the anterior domain of the ectoderm; at later stages, the posterior domain becomes localized to the tailbud and the anterior domain breaks up into a complex pattern of expression in the head (reviewed in Isaacs, 1997). FGF-9 is present throughout early development with maternal expression being primarily in the animal hemisphere and later zygotic expression throughout the whole of the developing axis (reviewed in Isaacs, 1997). The maternal expression of eFGF is primarily in the animal hemisphere. At gastrula stages eFGF is expressed zygotically in a ring around the blastopore region with a higher expression level on the dorsal side of the embryo (Isaacs et al., 1996). In late gastrula and early neurula stages eFGF expression is seen in the blastopore region and in the notochord. Subsequently, eFGF becomes localized to the extreme posterior of the embryo in the chordoneural hinge and posterior wall of the neuroenteric canal at late neurula and tailbud stages (Isaacs et al., 1996).

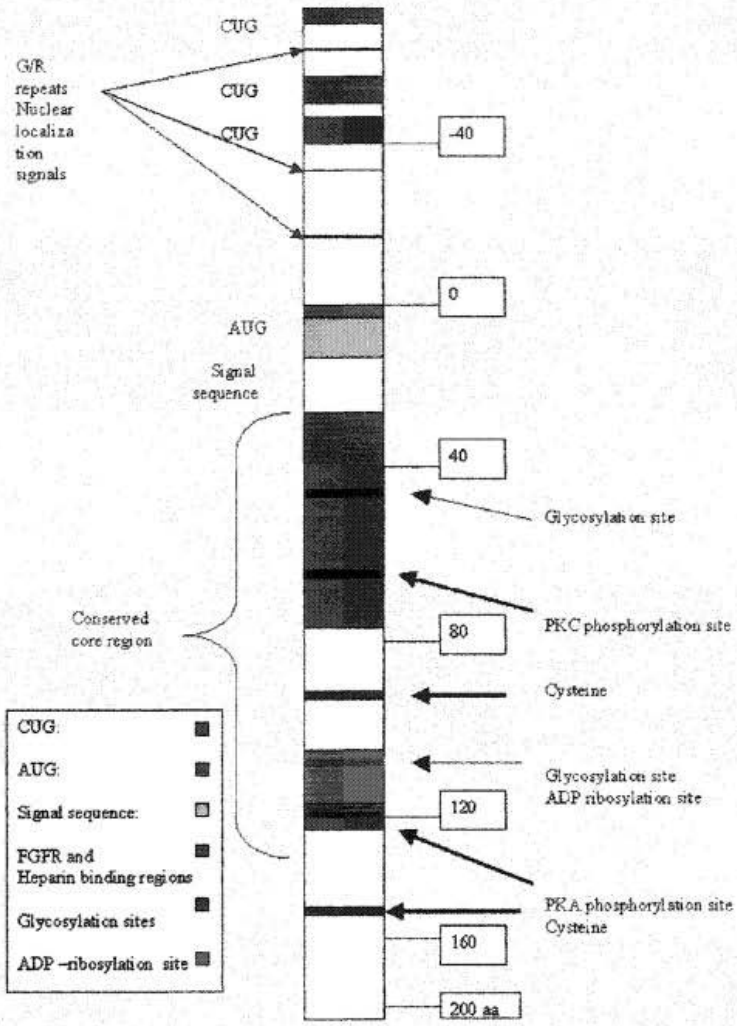


Figure 1.6 The structure of a generic FGF protein
There are four translation initiation sites (three CUGs and one AUG), sites of post-translational modification, and a conserved core region that contains receptor-binding sites. The scale in amino acids is indicated (adapted from Szebenyi & Fallon, 1999).

The FGF system has multiple functions in early development including mesoderm formation, gastrulation movements and anteroposterior patterning (Slack *et al.*, 1996; Song & Slack, 1996; Isaacs, 1997).

Mesoderm induction by FGFs has been demonstrated *in vitro*. FGF-1-6, FGF-9 and eFGF mimic the activity of the presumptive endoderm (vegetal pole) and induce animal cap elongation and the expression of mesodermal markers *in vitro* (reviewed in Isaacs *et al.*, 1997). In addition, FGF overexpression has been shown to suppress anterior development (reviewed in Isaacs, 1997) and a dominant-negative FGFR inhibits the formation of posterior and lateral mesoderm and disrupts the normal dorsoventral pattern (Amaya *et al.*, 1991). As mentioned previously, the type of mesoderm induced by FGFs is concentration dependent and, *in vivo*, FGFs such as FGF-2 and eFGF are probably involved in mesoderm patterning rather than mesoderm induction. As described previously, FGFs are thought to act in synergy with molecules such as BMP-4 and WNT genes as ventralizing factors that pattern the mesoderm and give rise to ventral mesodermal derivatives (Gotoh & Nishida, 1996).

The results of disrupting FGF signalling pathways by either its overexpression or its inhibition are quite striking. Disruption of *fgfr1* results in severe growth retardation and severely misshapen embryos, which often die at gastrula stages. Expression of a dominant-negative FGF receptor (XFD) inhibits mesoderm formation in animal caps and causes defects in trunk and posterior development in embryos (Amaya *et al.*, 1991; Kessler & Melton, 1994).

FGFs are also involved in patterning events, which will not be discussed further here, occurring later in development such as neural induction and patterning of the brain, and patterning of the limbs; Szebenyi & Fallon (1999) and Powers *et al.*, (2000) provide recent reviews of these activities.

1.6 FGF receptors

FGFs bind to at least three distinct types of receptors: fibroblast growth factor receptors (FGFRs), heparan sulphate proteoglycans (HSPGs), and a cysteine-rich FGF receptor (CFR) (reviewed in Szebenyi and Fallon, 1999). FGFRs are receptor tyrosine kinases (RTKs), which are required for most biological activities of FGFs; HSPGs are low-affinity receptors for FGFs, which alter or modulate FGF-FGFR interactions, and CFR participates in FGF intracellular transport (reviewed in Szebenyi & Fallon, 1999).

The diverse activities exhibited by the FGF family reflect a complex process involving potential interactions between multiple FGFs, FGFRs and the side chains of HSPGs (Faham *et al.*, 1998).

HSPGs have been traditionally classified as low-affinity receptors for FGFs. FGFs bind to the extracellular matrix of target tissues by interacting with heparan sulfates and related proteoglycans. HSPGs are sulphated glycosaminoglycans covalently bound to a core protein (reviewed in Szebenyi & Fallon, 1999). A number of roles have been proposed for proteoglycans in FGF-mediated cellular response: protection from proteolysis, localization, and storage and internalization of FGFs (reviewed in Taipale & Keski-Oja, 1997). FGF-stimulated signal transduction involves the dimerization of FGFR and it is probable that HSPGs participate in this process through a direct interaction with FGFR (Kan *et al.*, 1993; Kan *et al.*, 1996) and/or through oligomerization of FGFs (Ornitz *et al.*, 1992; Mach *et al.*, 1993; Spivak-Kroizman *et al.*, 1994; Thompson

et al., 1994). It is thought that HSPGs are important for sustained cellular response to FGFs. This idea is supported by FGF-induced gene expression studies, which have shown that FGF-1 can activate early response genes in the absence of exogenous heparin, but that HSPGs are required for sustained cellular responses (Donohue *et al.*, 1997). HSPGs have also been shown to protect FGF-2 from degradation in the cell matrix; this protection is most probably an important component in ensuring sustained cellular responses (Donohue *et al.*, 1997; Vlodavsky *et al.*, 1996; Yeoman, 1993). The HSPGs implicated in FGF action include syndecans, glypican, and perlecan (reviewed in Szebenyi & Fallon, 1999). FGFs may require different HSPGs with distinct glycosaminoglycans for their activities; for example, glypican has been shown to promote the mitogenic activity of FGF-2, but inhibits responses to FGF-7 (Bonneh-Barkay *et al.*, 1997). Therefore, HSPGs have been proposed as factors that regulate specific FGF-FGFR interaction and determine which biological activity prevails (Szebenyi & Fallon, 1999).

CFR is a 150-160 kDa single transmembrane protein that has an extracellular region with 16 cysteine-rich repeats and a short intracellular tail. FGF binding to CFR and to FGFRs is mutually exclusive, suggesting that the binding sites in FGFs for these two receptors are overlapping, and CFR may regulate intracellular FGF levels and signalling through FGFR by competing with the latter for FGFs (Zhou *et al.*, 1997). CFRs may be involved in intracellular

trafficking and targeting of FGFs but their function and interaction with FGFs have not yet been fully characterized (Szebenyi & Fallon, 1999).

FGF-stimulated signal transduction involves its binding to the FGFR and dimerization of the receptor. FGFRs are members of the RTK superfamily (reviewed in Ornitz, 2000), which also includes other receptors such as platelet-derived growth factor (PDGF) receptor, epidermal growth factor (EGF) receptor, and insulin growth factor (IGF) receptor (Heldin & Westermark, 1989). There are four known FGFR genes, FGFR-1, -2, -3, -4, which have an overall similar structural organization (Figure 1.7). The extracellular domain (EC) contains two (II and III) or three (I, II, III) immunoglobulin (Ig)-like domains, followed by the transmembrane (TM) stretch, the juxtamembrane (JM) domain, the kinase domain (KD) interrupted by a short kinase insert, and a carboxy tail that has several potential autophosphorylation sites; all these regions interact with intracellular substrates (reviewed in Szenbenyi & Fallon, 1999). The FGF-binding site is contained within a 139 amino acid region that includes parts of the Ig-2 and Ig-3 loops (Wang, 1995). Ligand selectivity is determined by Ig-3 loop (Ornitz *et al.*, 1996). The Ig-1 loop is the least conserved region in the FGFR and regulates binding affinity to different FGFs (Szebenyi & Fallon, 1999). Recent reviews of the structure and function of these different domains are provided in Klint & Claesson-Welsh (1999); Szebenyi & Fallon (1999) and Powers *et al.*, (2000).

Alternative splicing of the FGF receptor mRNAs generate several different variants of the receptors, some of which have distinct ligand-binding and signalling properties (reviewed in Johnson and Williams, 1993; Szebenyi & Fallon, 1999). The genomic sequence of the third immunoglobulin-like loop of FGFR-1 to –3 contains two alternative exons (IIIb and IIIc) for the second half of the third Ig-like loop (Figure 1.7) (reviewed in Klint & Claesson-Welsh, 1999). Different FGF family members will activate the receptor subtypes to different extents depending on their abilities to bind with high affinity to each receptor type (Table 1.2) (Ornitz *et al.*, 1996). This explains the ability of a large number of FGFs to generate different cell specific responses through a somewhat limited number of receptors (Table 1.2).

Mutations in FGFR1, FGFR2, and FGFR3 can cause different congenital, autosomal dominant disorders affecting the craniofacial (craniosyntosis) and sketal (chondrodysplasias) development (Hertz et al., 2001).

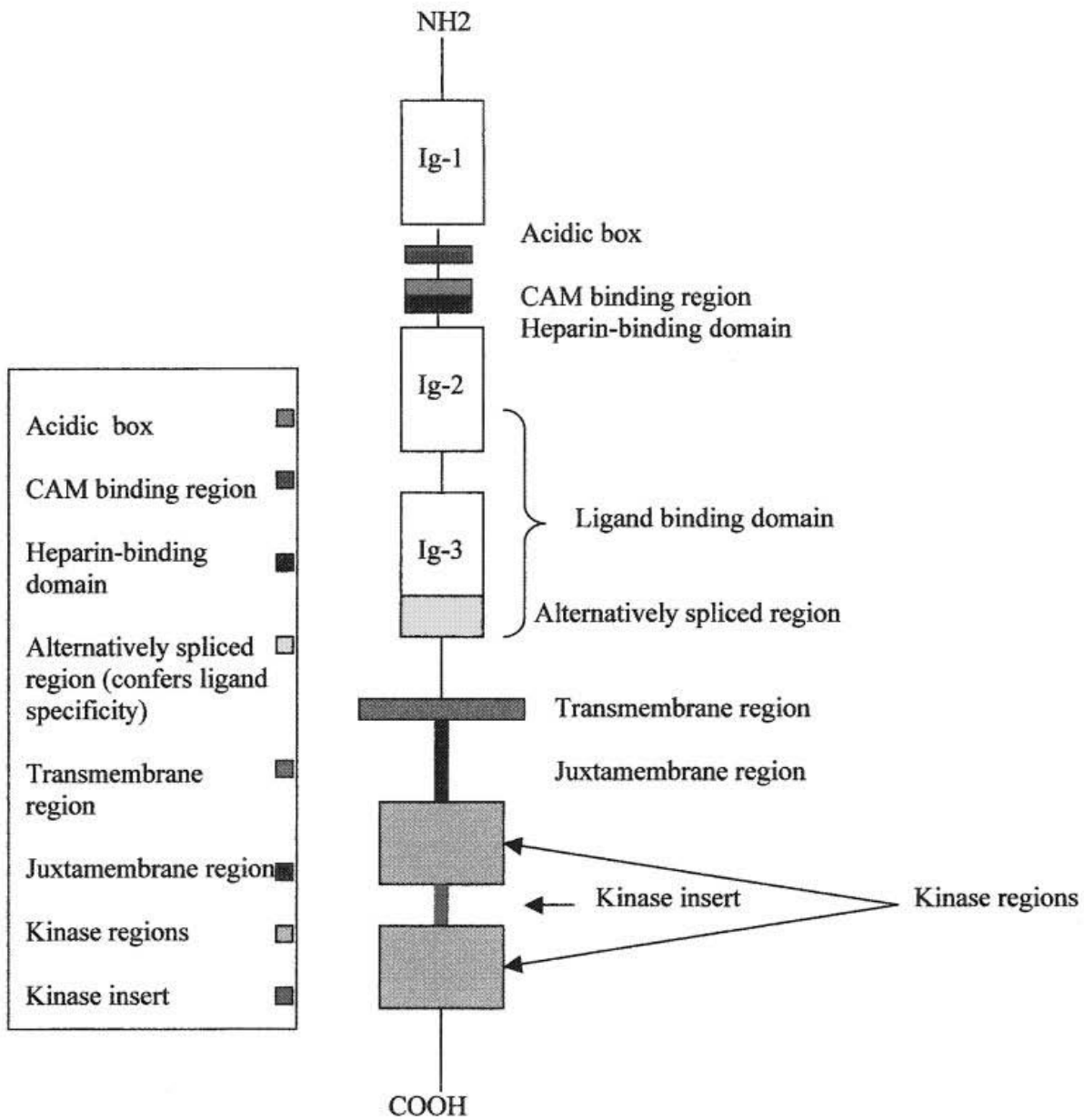


Figure 1.7 The structure of a generic FGFR protein. The major structural features of FGFRs, an acidic box, CAM binding domain, heparin-binding region, Ig loops, transmembrane, juxtamembrane, kinase, and kinase insert regions, are indicated by different colour codes and the three Ig loops are labelled. Ig loop 3 is alternatively spliced in FGFR 1-3 but not FGFR-4 yielding B or C splice variants that differ in their ligand-binding properties.

Table 1.2 FGF signalling through high-affinity FGFR isoforms (adapted from Powers *et al.*, 2000)

FGF	FGFR
FGF-1	FGFR-1, IIIb & IIIc; FGFR-2, IIIb & IIIc; FGFR-3, IIIb & IIIc, FGFR-4
FGF-2	FGFR-1, IIIb & IIIc; FGFR-2, IIIc; FGFR-3, IIIc; FGFR-4
FGF-3	FGFR-1, IIIb; FGFR-2, IIIb
FGF-4	FGFR-1-IIIc; FGFR-2, IIIc; FGFR-3, IIIc; FGFR-4
FGF-5	FGFR1-IIIc; FGFR-2,IIIc
FGF-6	FGFR1-IIIc; FGFR-2,IIIc; FGFR-4
FGF-7	FGFR-2, IIIb
FGF-8	FGFR-1; FGFR-2, IIIc, FGFR-3, IIIc; FGFR-4
FGF-9	FGFR-2, IIIc; FGFR-3, IIIb & IIIc; FGFR-4
FGF-10	FGFR-1, IIIb; FGFR-2, IIIb
FGFs 11-14	Unknown
FGF-15	Unknown
FGFs 16-19	FGFR-1, IIIc; FGFR-2, IIIc
FGF-20	Unknown

1.7 FGF signalling pathways

Ligand binding to receptors initiates signal transduction cascades which propagate the signals and result in the activation of molecules downstream in the cascade. FGF signal transduction cascades are initiated by the binding of FGF to its cell surface FGFR. The activated receptor then undergoes dimerization and autophosphorylation on tyrosines within the dimer. Phosphorylated tyrosine residues recruit other signalling molecules, such as proteins containing src-homology (SH2) domains and phosphotyrosine-binding domains (PTB), to the activated receptors (reviewed in Klint & Claesson-Welsh, 1999). SH2 domain proteins may be substrates for receptor-mediated phosphorylation themselves or may, as is the case for PTB proteins, function as adaptor proteins to recruit other target proteins and hence propagate the signal through many signal transduction pathways (Pawson, 1995).

Although the signal transduction cascades initiated by FGF within activated cells have yet to be fully elucidated, FGFs have been shown to activate several well-known cascades such as the phospholipase C γ (PLCγ) pathway, the mitogen activated protein kinase (MAPK) pathway, and the phosphoinositide 3' knase (PI3'K) pathway (reviews by Hawkins *et al.*, 1997; Kamat & Carpenter, 1997; Powers *et al.*, 2000; Szebenyi & Fallon, 1999). These transduction cascades are initiated by the autophosphorylation of the FGFRs. It should be noted that mitogenic response has been observed in cells in which receptor transphosphorylation is much reduced (Krufka *et al.*, 1996) and that some FGFs

have nuclear localization sequences, so that FGFs may also have signalling pathways that are distinct from those activated on the cell surface (Szebenyi & Fallon, 1999).

The next three sections will briefly review the three main pathways.

1.7.1 *The Phospholipase Cγ Pathway*

PLCγ is a signal transduction enzyme which, when activated, initiates hydrolysis of phosphatidylinositol 4,5-biphosphate to diacylglycerol (DAG) and phosphatidylinositol 3 (IP_3). DAG is an activator of protein kinase C (PKC), a serine-threonine kinase, and IP_3 initiates Ca^{2+} release from intracellular stores. Second messengers like PKC and Ca^{2+} activate several molecules including transcription factors and thereby result in desired cellular responses such as transcription (reviewed by Kamat & Carpenter, 1997).

PLCγ has been identified as being phosphorylated and associated with FGFR following ligand-dependent activation (Burgess *et al.*, 1990; Gillespie *et al.*, 1992; Ryan & Gillespie, 1994; Ryan *et al.*, 1998) by the binding of its SH2 domain to the phosphorylated Tyr^{766} of FGFR-1 (Mohammadi *et al.*, 1991). However, although mutation of the phosphorylated tyrosine residue to phenylalanine demonstrated that phosphatidylinositol hydrolysis was disrupted (Mohammadi *et al.*, 1992; Peters *et al.*, 1992), the mutation did not affect FGFR-mediated mitogenesis, neuronal differentiation (Spivak-Kroizman *et al.*, 1994), or

mesoderm-induction in *Xenopus* animal caps (Muslin *et al.*, 1994). It has been shown that activation of protein kinase C (PKC) alone, a downstream effector of the PLCγ pathway, is not sufficient to induce mesoderm in animal cap explants, although activated PKC has been detected in FGF-treated explants (Gillespie *et al.*, 1992). This implies that either PLCγ is redundant with respect to mitogenesis and differentiation or that the PLCγ pathway is important for other functions of FGFR signalling (Powers *et al.*, 2000). It has been suggested that the activation of PKC during mesoderm activation could be part of a negative feedback mechanism on the FGF mesoderm induction pathway (Gillespie *et* al., 1992).

1.7.2 *The Phosphoinositide 3' Kinase Pathway*

PI3'K, which phosphorylates the inositol ring at the 3' position and thereby activates inert membrane phospholipids and initiates a signal transduction pathway, has been found to be involved in signal transduction of most, if not all, tyrosine kinases (reviewed in Hawkins *et al.*, 1997). The PI3'K pathway is involved in many systems, including: cytoskeletal rearrangements, cellular migrations, mitogenesis, differentiation, and protection from apoptosis (reviewed by Vanhaesebroeck *et al.*, 1997; Wymann and Pirola, 1998; Leevers *et al.*, 1999).

FGF receptors lack optimal binding motifs for PI3'K, and FGF-induced PI3'K activity is difficult to detect *in vitro* as well as *in vivo* (Kanda *et al.*, 1996;

van Weering *et al.*, 1998). However, PI3'K activity has been implicated in both migration and mitogenesis stimulated by a range of other growth factors, and since FGFs are capable of transducing both these responses, it is quite possible that PI3'K initiates some of these responses in FGF-mediated signal transduction (Klint and Claesson-Welsh, 1999). Activated PI3'K has been shown to be associated with the FGFR during mesoderm induction (Ryan & Gillespie, 1994; Ryan *et al.*, 1998). Recent work has shown that PI3'K acts downstream of Ras and in parallel to the MAPK pathway (described in next section) in the FGF pathway during *Xenopus* mesoderm induction (Carballada *et al.*, 2001). Using natural and synthetic inhibitors of PI3'K, the authors found that PI3'K catalytic activity is required for the definition of trunk mesoderm in the FGF signalling pathway.

1.7.3 *The RAS/MAPK Pathway*

Subsequent to receptor autophosphorylation, SH2 (src homology) domain containing proteins and phosphotyrosine-binding domain (PTB) docking proteins bind to specific phosphotyrosines (reviewed in Szebenyi & Fallon, 1999). Some of the docking molecules for different FGFRs are distinct; for example, SHC and FRS2 function as docking molecules for FGFR1 whereas FGFR4 was shown to associate with a p85 serine kinase, and the activated FGFR3-GRB2-SOS complex contains either a novel 66-kDa protein or SHC (reviewed in Szebenyi & Fallon, 1999). The RAS/MAPK pathway is propagated through the recruitment of

SH2 domain proteins and PTB domain molecules to the activated FGFR receptor. Docking molecules such as FRS2 bind to the phosphorylated receptor and recruit the GRB2-SOS complex. GRB2 can also dock directly on the phosphorylated receptors through its SH2 (Src homology 2 domain, which binds to phosphorylated tyrosines). GRB2 contains SH3 (Src homology-3) domains that allow it to bind to a guanine nucleotide exchange factor (GNEF) such as SOS. This puts SOS in close vicinity with RAS, which is membrane bound. SOS promotes the dissociation of GDP from RAS, allowing the protein to bind a GTP molecule and to become activated (reviewed in Ferell, 1996).

The activated membrane-associated RAS then recruits RAF-1, a serine-threonine MAPK kinase kinase (MAPKKK). In turn, RAF-1 activates MAPKK (MEK), which activates MAPK. MAPK phosphorylates transcription factors such as JUN, FOS, and the ribosomal S6 kinase. In addition to nuclear substrates, MAPK has been found to phosphorylate cytosketal proteins, phospholipase, and protein kinases (reviewed in Ferell, 1996). The RAS/MAPK pathway has been associated with a number of different FGFR-mediated cell responses such as cell proliferation, cell responses during gastrulation, migration of certain cell types in *Drosophila*, and activation of the urokinase plasminogen activator, cell motility, and mesoderm formation in *Xenopus* embryos (reviewed in Szebenyi & Fallon, 1999).

Activated forms of MAPK have been shown to induce the expression of the mesodermal marker *Xbra* in animal caps (LaBonne *et al.*, 1995). The

RAS/MAPK pathway has been shown to be necessary for FGF signalling during mesoderm induction using a MAPK-specific phosphatase, which blocked the FGF-mediated formation of mesoderm in animal cap explants (Gotoh *et al.*, 1995; LaBonne *et al.*, 1995). Embryos lacking ventral type mesoderm were produced and were similar in phenotype to embryos overexpressing a dominant negative FGFR, XFD, which ablates FGF signalling in the embryo (Gotoh *et al.*, 1995; LaBonne *et al.*, 1995; Amaya *et al.*, 1991). These results directly implicate the RAS/MAPK pathway in FGF signalling during mesoderm induction.

The MAPK family consists of five distinct groups of related kinases: A. the TEY MAP kinases (include the well studied p42/Erk2 MAP kinase and p44/Erk1 MAP kinase), B. the Hog1/TGY group, C. the Jnk/SAP kinase group, D. the Smk1 group, and E. the Erk3 group (reviewed in Ferell, 1996). The different groups have been implicated in biological processes such as mitogenesis, cell fate determination, differentiation and induction (reviewed in Ferell,1996).

The RAS/MAPK pathway is well characterized in FGF/FGFR cell signalling, but it is becoming clear that there are several proteins acting in parallel with RAS, RAF and MAPK. For example, PKC and PKA can also activate RAF (reviewed in Szebenyi & Fallon, 1999). Several mammalian isoforms of MAPK and parallel MAPK pathways have been found (Ferell, 1996). There is also some evidence of crosstalk between the RAS/MAPK pathway and other pathways such as the phosphoinositide (PI) cycle during mesoderm induction in *Xenopus* (Rose & Busa, 1998). Stimulation of the PI cycle in

explants in the absence of growth factors does not induce mesoderm. However, PI cycle stimulation during treatment of explants with FGF-2 has been shown to increase MAPK activity and potentiate FGF-2-induced expression of the mesodermal marker, *Xbra* (Rose & Busa, 1998). These results suggest that the PI cycle may be acting synergistically with the MAPK pathway during mesoderm induction.

In *Xenopus*, the FGF family seems to be responsible for the full pattern of activated MAPK in early development (Christen and Slack, 1999). LaBonne and Whitman (1997) showed that the presence of activated MAPK, or phosphorylated MAPK, is abolished by XFD injection during early gastrula stages. MAPK activation first starts at stage 8 in *Xenopus* embryos, and XFD has been shown to abolish activated MAPK staining until tailbud stages whereas eFGF activates MAPK only in embryos (Christen and Slack, 1999).

The staining pattern of activated MAPK during embryonic development has been characterized. At stage 7, activated MAPK is not visible in embryos. Activated MAPK is first seen as a dorsal patch in stage 8 embryos in the region of the future blastopore. At stage 10.5, activated MAPK is present around the blastopore and at neurula stages is expressed strongly around the blastoporal ring and in the forebrain, the midbrain/hindbrain junction, dorsal midline and on either side of the neural plate. At tailbud stages, MAPK is active in the tailbud, brachial arch, otic vesicle, stomodeal anlage, forebrain and midbrain junction, heart anlagen and dorsal region of the cement gland (Christen and Slack, 1999).

This staining pattern is indicative of regions of active FGF signalling during mesodermal and posterior patterning in *Xenopus* (Christen and Slack, 1999).

1.8 FGF Target Genes

FGF treatment result in changes in the steady-state levels of many different mRNAs. Some of the targets of the FGF signalling pathway during mesodermal and posterior patterning have been identified. It has been shown that FGF is involved in the maintenance of *Xbra* expression in mesoderm precursor cells (Smith *et al.*, 1991b). *Xbra* and eFGF have been shown to be the components of an autoregulatory loop in which *Xbra* induces eFGF expression, which in turn maintains *Xbra* expression (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). FGF signals also regulate the expression of the caudal gene family member *Xcad*3 (Northrop and Kimmelman, 1994; Pownall *et al.*, 1996), which has a critical function in the regulation of posterior hox genes (Isaacs *et al.*, 1998). It has been shown that *krox20* and *Hoxb9* expression is induced in explants following FGFR1 activation (Umbhauer *et al.*, 2000). FGF-regulated genes also include: homeobox genes, patterning genes, growth factors and their receptors, skeletal muscle regulatory factors, matrix proteins, proteases, and protease activators and inhibitors (Szebenyi & Fallon, 1999).

While the list of FGF-modulated genes is still incomplete, it supports the hypothesis that FGFs have a broad and diverse range of action and affect the expression and function of many diverse groups of proteins. It should be noted

that not all these FGF regulated proteins are expressed at the same time and in the same cell type, although many of them have been observed be to activated simultaneously or sequentially. Furthermore, FGFs have been shown to act either synergistically or antagonistically with other growth factors such as members of the TGF-β family, insulin-like growth factor (IGF), and members of the wingless family (WNT) (reviewed in Szebenyi & Fallon, 1999).

The next section will focus on one class of FGF target genes, the immediate-early genes.

1.8.1 Growth factor-induced immediate-early genes

The first genes to be transcribed in response to growth factor treatment of a cellular population are known as immediate-early genes (or early response genes). Immediate-early genes have also been described as the set of genes linked directly to receptors by transduction mechanisms and include those that are expressed when a cell is stimulated to leave the G_0 phase of the cell cycle and enter G_1 (reviewed in Thomson *et al.*, 1999).

To be classified as immediate-early, a gene must be transcribed without *de novo* protein synthesis and depend solely on factors already present in the cell to enable a quick response, and is usually expressed within thirty minutes of mitogen activation (Thomson *et al.*, 1999). Some immediate-early genes, such as members of the c-*fos* and c-*jun* families, are characterized by their rapid and transient expression in response to extracellular stimuli. In addition, phosphorylation of a number of transcription factors and two chromatin-associated proteins, HMG-14 (high mobility group-14) and histone H3, is tightly correlated to immediate-early gene induction (Mahadevan *et al.*, 1991; Barratt *et al.*, 1994a; Barratt *et al.*, 1994b; Hazzalin *et al.*, 1996). Immediate-early gene response is elicited by three broad classes of biochemical, physical and pharmacological stimuli: (i) polypeptide growth factors such as epidermal growth factor (EGF), FGF, platelet derived growth factor (PDGF) and cytokines; (ii) stresses such UV radiation and heat shock; and (iii) pharmacological compounds

such as 12-O-tetradecanoylphorbol 13-acetate (TPA), okadaic acid and anisomysin compounds (reviewed in Thomson *et al.*, 1999).

Thus, transcriptional activation of immediate-early genes, such as transcription factors, is the first response to the signal transduction pathways initiated by growth factors. In turn, the transcription factors initiate (or terminate) transcription of new genes, which are or are not subsequently translated into proteins. The absence or presence of the latter ultimately carry out the desired cellular response.

To date, only occasional immediate-early FGF response genes have been identified and characterized. One well-characterized FGF immediate-early response gene is the *Xenopus* homologue of the Brachyury gene, Xbra. Expression of Xbra is an immediate-early response to mesoderm induction by FGF (Smith *et al.*, 1991). Xbra is able to induce mesoderm formation: ventral mesoderm at low concentrations and intermediate mesoderm at higher concentrations (Cuncliffe and Smith, 1994). Furthermore, as mentioned previously, Xbra has been shown to activate transcription of eFGF in an autocatalytic manner during mesoderm induction (Isaacs *et al.*, 1994).

1.9 Isolation and cloning of *er1*

Our lab recently identified a novel transcript, early-response gene 1 (*er1*), whose expression levels increased in *Xenopus* embryo explants during mesoderm induction by FGF-2 (Paterno *et al.*, 1997).

As described in Paterno *et al.,* (1997), *er1* was identified through the PCR-based differential display method (Liang & Pardee, 1992) in an attempt to identify and characterize genes that are expressed during early cellular response to FGF. A *Xenopus* blastula library (Gillespie *et al.*, 1995) was used to obtain the full sequence of *er1* cDNA, which is 2.3 kb, and consists of a 1497-base pair single open reading frame predicted to encode a protein of 493 amino acid residues. Expression of *er1* mRNA peaks during the late blastula stage of *Xenopus* (Paterno *et al.*, 1997).

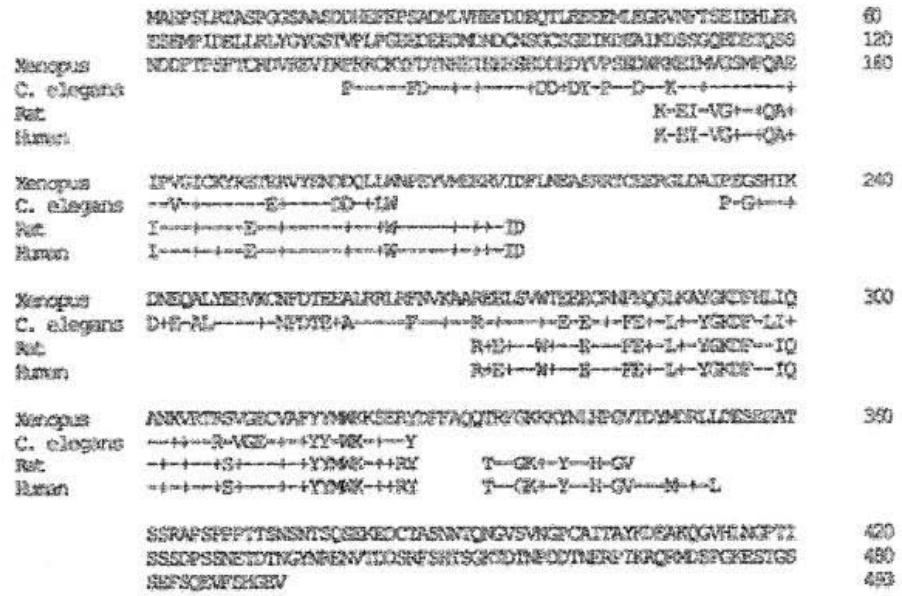
The steady-state levels of *er1* were shown to increase 3-4 fold upon treatment with FGF. However, this increase in *er1* levels was not due to *de novo* protein synthesis because cycloheximide (an inhibitor of protein synthesis) did not affect the FGF-induced increase in *er1* levels. These results effectively showed that *er1* is an immediate-early gene (Paterno *et al.*, 1997).

A database homology search revealed three regions of similarity in the predicted ER1 amino acid sequence to rat and human proteins encoded by the metastasis-associated gene, *mta*1, and two regions of similarity to the *Caenorhabditis elegans* sequence that has similarity to *mta1* (Paterno *et al.*, 1997). However, *er1* is not the *Xenopus* homolog of *mta*1, since a human homolog of *er1* has been found that is distinct from human *mta1* (Paterno *et al.*, 1997; Paterno *et al.*, 1998).

The expression of rat *mta*1 has been associated with metastatic phenotypes (Toh *et al.*, 1994). Human *er1* is expressed in breast-carcinoma cell

lines and breast tumour tissue while being undetectable in normal tumour cell lines and tumour tissue suggesting that *er1* expression is associated with the neoplastic state in human breast carcinoma (Paterno et al., 1998). Figure 1.8A shows amino acid homology between *Xenopus* ER1 and rat and human MTA1 and figure 1.8B shows the amino acid homology between *Xenopus* and human ER1.

A.



B.

```
Human    MAEPSVESSSPGGSATSDDHEFDPSADMLVHDFDDERTLEEEEMMEGETNFSSEIEDLAR 60
Xenopus  •••••LRTA•••••••A•••••••E•••••••••E•••••Q••••••••L•••V••T••••H•E•

Human    EGDMPIHELLSLYGYGSTVRLPEEDEEEEEEEEEGEDDEDADNDDNSGCSGENKEENIKD 120
Xenopus  •SE•••D•••R•••••••••P••G•ED••D-----------M•••C••••••••I•D•A••••

Human    SSGQEDETQSSNDDPSQSVASQDAQEIIRPRRCKYFDTNSEVEEESEEDEDYIPSEDWKK 180
Xenopus  ••••••••••••••••TP•FTCR•VR•V•••••••••••••H•I•••••••••••V••••••••

Human    EIMVGSMFQAEIPVGICRYKENEKVYENSDQLLWDPEYLPEDKVIIFLKDASRRTGDEKG 240
Xenopus  ••••••••••••••••••K•••T•••••••D••••••••••VM•ER••D••NE•••••CE•R•

Human    VEAIPEGSHIKDNEQALYELVKCNFDTEEALRRLRFNVKAAREELSVWTEEECRNFEQGL 300
Xenopus  LD••••••••••••••••••H•••••••••••••••••••••••••••••••••••••••••••

Human    KAYGKDFHLIQANKVRTRSVGECVAFYYMWKKSERYDFFAQQTRFGKKKYNLHPGVTDYM 360
Xenopus  ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••

Human    DRLLDESESAASSRAPSPPPTASNSSNSQSEKEDGTVSTANQNGVSSNGPGILQMLLPVH 420
Xenopus  ••••••••••T••••••••••T•••NT•••••••C•A•NNT•••••V•••CAITAYKDEA

Human    FSAISSRANAFLK*                                                 432
Xenopus  KQGVHLNGPTISSDDPSSNETDTNGYNRENVTDDSRFSHTSGKTDTNPDDTNERPIKRQR

Xenopus  MDSPGKESTGSSEFSQEVFSHGEV*
```

Figure 1.8 Amino acid homology between ER1 and MTA1 and *Xenopus* and human ER1

A. Homology between *Xenopus* ER1 and *C. elegans*, rat and human MTA1

B. Homology between *Xenopus* and human ER1

lysis of the ER1 amino acid sequence revealed four regions that contain putative nuclear localization signals (NLSs) (Paterno *et al.*, 1997; Post *et al.*, 2001). These are ^{138}RPRRCK^{143} (NLS1), ^{261}RRLR^{264} (NLS2), ^{320}KKSERYDFFAQQTRFGKKK^{338} (NLS3) and ^{463}RPIKRQRMDSPGK^{475} (NLS4) (Post *et al.*, 2001). NLS4, which contains a core region (^{463}RPIKRQRN|MD^{471}) similar to the core NLS directing c-MYC to the nucleus, has been found to be the only *bona fide* NLS necessary and sufficient for targeting ER1 to the nucleus (Post *et al.*, 2001). Of the other putative three NLSs, only NLS1 has been shown to function only as a weak NLS (Post *et al.*, 2001).

A proline-rich sequence corresponding to the PXXP motif found in all high affinity SH3 (src homology 3) ligands was identified near the C terminus. The N terminus of ER1 contains four highly acidic regions, characteristic of the acidic activation domain of many transcription factors (Paterno *et al.*, 1997). There is also a highly conserved region in the middle region of ER1 (amino acid 287-349), the SANT domain (Paterno *et al.*, 1998). The SANT domain is a motif with potential transcription and chromatin-related functions initially found in Swi3, Ada2, the co-repressor NcoR, and TFIIB, and conserved in a number of transcription factors and oncogenes such as *mta*1 and members of the myb family of oncogenes (Aasland *et al.*, 1996). Although the function of the SANT motif is itself unclear, SANT homologous repeats in the Myb oncoprotein have been implicated in DNA binding (Howe *et al.*, 1990; Ogata *et al.*, 1994). Recent reports have indicated that a SANT-containing region of several co-repressors

can bind (You *et al.*, 2001; Guenther *et al.*, 2001) and activate (Guenther *et al.*, 2001) histone deacetylases, indicating a chromatin-related role. Analysis of the amino acid sequences of both ER1 and MTA1 has revealed that the similarity between ER1 and MTA1 lies in the SANT domain (Paterno *et al.*, 1998). Another conserved region, ELM2 (EGL-27 and MTA1 homology2), has been found in ER1 (amino acid 169-229) (Solari *et al.*, 1999). As discussed in Solari et al., (1999), the ELM2 region is not similar to any domains of known function but is present in many proteins containing the SANT domain including MTA1.

Figure 1.9 shows a schematic of the putative functional domains in ER1.

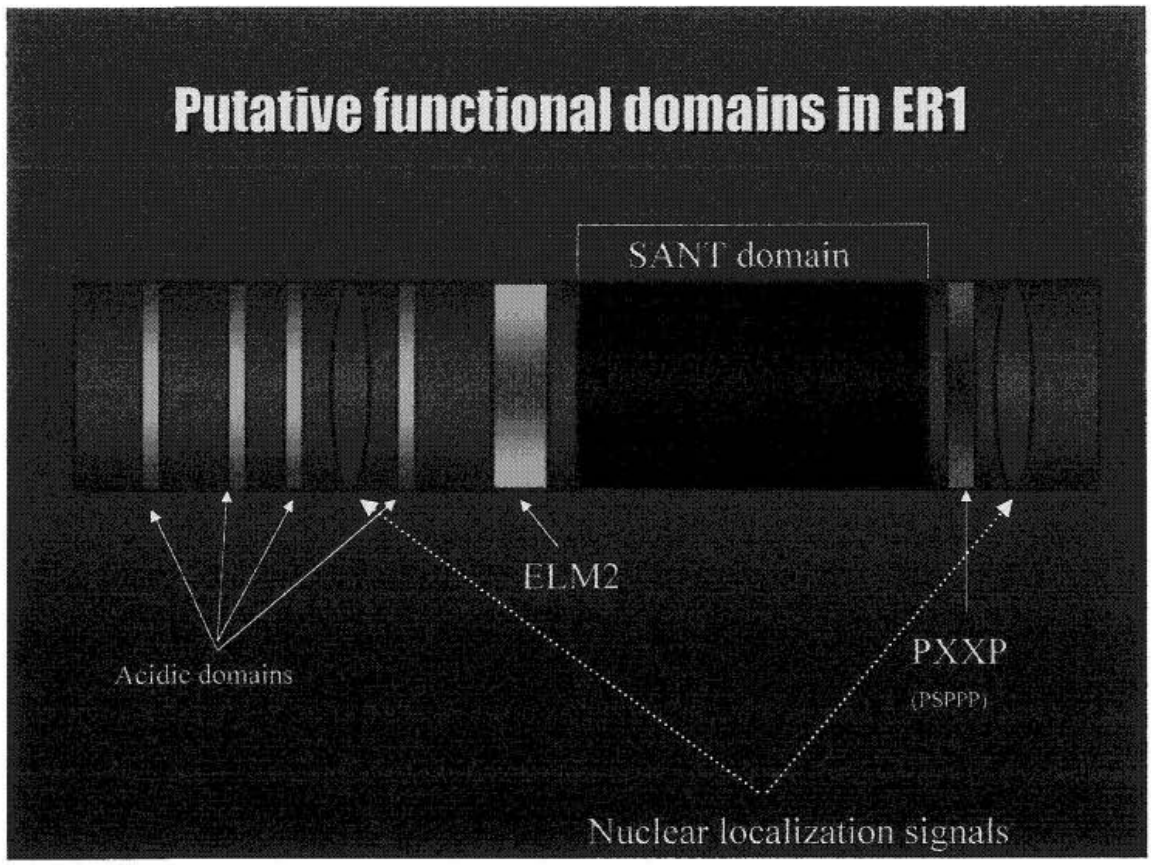


Figure 1.9 A schematic showing putative functional domains in ER1

There are four regions of acidic amino acids in the N-terminal region. The ELM2 region is found in the middle of the protein and the SANT domain is found in the C-terminal region. There is a PXXP motif in the C-terminal region and two NLSs. The NLS in the N-terminus functions as a weak NLS and the NLS in the C-terminus has been found to be the *bona fide* NLS that is sufficient for targeting ER1 to the nucleus (Post *et al.*, 2001).

1.10 Purpose of this study

At the time this study was undertaken, *er1* was a newly cloned gene whose expression pattern and function were unknown. The purpose of this study was to characterize the expression and function of ER1 protein during early development of *Xenopus* and gain an insight into its function.

Objective 1: Characterization of the expression pattern of ER1 protein during early development in *Xenopus*.

The mRNA levels of a gene give a good indication of the expression of transcripts, but ultimately it is the expression of the protein products of the gene that are most useful in determining the tissues and time frame of the expression of a particular gene. The spatio-temporal expression pattern of proteins is critical to understanding their function. At the start of this project, Northern blotting had given us an indication of the levels of *er1* transcripts during early development. It was shown that *er1* was a maternal transcript, whose steady-state levels were relatively constant during early cleavage stages, increased 2-fold at blastula stages, and then decreased 6-fold during gastrula, neurula and tailbud stages and remained below detection level during subsequent development (Paterno *et al.*, 1997). The first objective of this project was therefore to characterize the expression pattern of ER1 protein during early developmental stages in *Xenopus*.

Objective 2: Identification of mechanisms regulating the expression of ER1.

Spatio-temporally restricted expression pattern is a characteristic of many genes that are expressed during early development, and is often an indication of a distinct and defined role in embryogenesis. The mechanisms that regulate the differential expression of these genes often vary. Resolving the processes that regulate the expression of a gene is often crucial to determining their function in a biological system. Once the expression pattern of ER1 protein was characterized, the second objective was to determine how this expression pattern was regulated during development.

Objective 3: Characterization of the function of ER1.

Analysis of the DNA sequence of genes gives clues to the function of the protein products of these genes. At the start of this project, computer assisted analysis of the DNA sequence of *er1* had identified several domains, including stretches of acidic amino-acid residues, nuclear localization signals, and protein-protein interaction domains (Paterno *et al.*, 1997). Identification of these domains, as well as characterization of the expression pattern of ER1 (Objective 1), helped design experiments to determine the function of ER1. A common strategy used when working with new genes, like *er1*, whose functions are unknown, is to use the information present in the DNA sequence to identify putative function. The third objective of this project was to characterize the

function of ER1 using *in vitro* assays involving deletion constructs of putative functional domains of ER1.

Objective 4: Characterize the function of ER1 during early development in *Xenopus*.

A common technique used to elucidate the function of genes and gene products in developmental studies is to misregulate their expression by overexpressing or inhibiting them *in vivo*. In embryological studies, cRNA is injected in newly fertilized embryos and their development is followed to detect morphological abnormalities. By misregulating the expression of a gene, and studying the effects of this misregulation, one can attempt to define the normal function of the gene and its product. The fourth objective of this project was to characterize the function of ER1 in embryos by studying the effects of ER1 overexpression.

SECTION II ER1 IS DIFFERENTIALLY LOCALIZED TO NUCLEI DURING EARLY DEVELOPMENT IN XENOPUS EMBRYOS

CHAPTER 2 CHARACTERIZATION OF THE EXPRESSION PATTERN OF ER1 DURING EARLY DEVELOPMENT IN XENOPUS EMBRYOS

Note: Part of chapter 2 was previously published in Luchman *et al.*, (1999)

2.1 INTRODUCTION

There is little or no transcription during early cleavage stages in amphibian development. As a result, early embryos depend on proteins translated from maternally inherited mRNAs (transcribed during oogenesis and stored in the maternal cytoplasm) and on maternal proteins (which are already present in the egg) for the numerous cellular processes that take place in the embryo before the onset of zygotic transcription (Seydoux, 1996). Maternal mRNAs and proteins provide essential factors for growth and cell viability and regulate embryonic polarity and cell fate. It is critical, therefore, to regulate precisely the

accumulation of both maternal proteins and of the proteins translated from maternal mRNAs after fertilization as a means of regulating their function (Seydoux, 1996). The expression pattern of a protein is the gateway to understanding its function during development.

*er*1 is a *Xenopus* immediate-early response gene, cloned in our laboratory, whose expression is activated by FGF-2 (Paterno et al., 1997). It was shown that *er*1 is a maternally derived message whose expression is restricted to stages prior to mid-gastrula. The first objective for this study was to characterize the expression pattern of ER1 protein during early development. Western blotting, whole-mount staining and immunocytochemistry techniques were used.

2.2 MATERIALS AND METHODS

2.2.1 *In vitro* fertilization of mature *Xenopus* oocytes

Female *Xenopus* frogs (Nasco) were induced to ovulate with a subcutaneous injection in the dorsal side in the upper portion of one of the hind legs towards the cloaca with 400-500 I.U. (0.4-0.5ml) of HCG (Sigma-Aldrich) and left at room temperature (RT) for 12-14 hours until they started to ovulate.

A male frog (Nasco) was sacrificed by a subcutaneous injection in the dorsal side in one of the hind legs with a lethal dose of the anaesthetic

Methanesulfonate (MS222, Sigma-Aldrich) and the testes were removed. The testes were cleaned by rinsing in 1 times Normal Amphibian Medium (1xNAM; 110mM NaCl (BDH), 2mM KCl (Fisher Scientific), 1mM $Ca(NO_3)_2.4H_2O$ (Fisher Scientific), 1mM $MgSO_4.7H_2O$ (Fisher Scientific), 0.1mM EDTA pH 8.0 (Fisher Scientific), 10mM HEPES (Fisher Scientific), pH 7.5, 1mM $NaHCO_3$ (Fisher Scientific), and 12.5mg Gentamycin (Sigma-Aldrich) in dH_2O) and stored at 4^0C in 1xNAM.

Before fertilization, a small portion of testes was macerated in a drop of distilled water (dH_2O) and the released sperm were examined under a compound microscope for viability. Eggs were squeezed from an ovulating female into a 100x15mm Fisher brand disposable petri dish. A small portion of testes was rinsed in dH_2O and macerated in fresh dH_2O before being pipetted on the eggs. The eggs and sperm were mixed by rocking gently for a few seconds and left at RT for 5 minutes to allow fertilization to take place. The eggs were flooded with dH_2O to dilute the sperm mixture. After 15 minutes, successfully fertilized eggs rotated so the dark pigmented side of wild-type eggs was on top. Albino eggs were also left for 15 minutes to rotate. The dH_2O was poured off and 2.0% Cysteine-HCL (Sigma-Aldrich) in dH_2O, pH 7.8-8.1, was added to the embryos, the mixture was transferred to a 250 ml glass beaker and shaken for a few minutes to remove the jelly coat from the embryos. Once the outer jelly coat was removed, eggs became close packed. Dejellying was stopped by gently diluting the embryo-cysteine mixture with dH_2O and rinsing with at least five changes of

dH_2O and two changes of NAM/20 (NAM/20; 5.5mM NaCl, 0.1mM KCl, 0.05mM $Ca(NO_3)_2.4H_2O$, 0.05mM $MgSO_4.7H_2O$, 0.005mM EDTA pH 8.0, 0.5mM HEPES pH 7.5, 0.05mM $NaHCO_3$; 1.25mg Gentamycin in dH_2O). Embryos were cultured in NAM/20 at RT, 14°C or 18-20°C, and staged according to Nieuwkoop and Faber (1967).

2.2.2 Western blot analysis of ER1 protein at different embryonic stages

Extracts from embryos at different developmental stages (stage 2, 6.5, 8, 8.5, 10, 13, 27, and 45) were prepared for Western blotting by homogenizing whole embryos in ice-cold solubilization buffer (1% Triton-X100 (Fisher Scientific), 10mM Tris-Cl (Fisher Scientific), pH 7.5, 10mM EDTA, plus protease inhibitors: 1mM PMSF (Sigma-Aldrich), 25 μg/ml aprotinin (Sigma-Aldrich), 25 μg/ml leupeptin (Sigma-Aldrich), and 5 μg/ml TLCK (Sigma-Aldrich) in dH_2O). Ten embryos were processed per treatment. After 30-min solubilization at 4°C, the samples were centrifuged at 10 000*g* for 5 min. The clear middle layer was removed with a 1ml syringe leaving the bottom pellet of insoluble material and the top lipid layer behind. The process was repeated if the middle layer was cloudy. The extracts were vortexed with an equal volume of Freon (Sigma-Aldrich) to separate yolk proteins from the other soluble proteins and the upper aqueous layer was removed. Total protein was precipitated out of the aqueous layer with 100% acetone at –20° C for 25 min and centrifuged at 10 000*g* for 20 min. The pellet was washed once by vortexing with ice-cold 70% acetone and centrifuging for 20 min. The pellet was air-dried for 5 min and

resuspended in sample buffer [50mM Tris pH 6.8, 2%Sodium dodecyl sulfate (SDS) (Fischer Scientific), and 800mM beta-Mercapto Ethanol (BME) (Bio-Rad Laboratories) in dH_2O]. Protein measurements were performed using the Bio-Rad protein assay (Bio-Rad Laboratories), to ensure equal loading of protein.

The samples were subjected to SDS-PAGE through 8% polyacrylamide gels, followed by Western blotting onto Hybond^{TM}-ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Bio-Rad prestained molecular weight standards (12.5 μg/lane) (Bio-Rad Laboratories) were included on each gel. The blots were blocked for 1 hour with 5% non-fat milk powder in TBS-T buffer (20mM Tris, pH 7.6, 137mM NaCl, and 0.1% (v/v) Tween-20 (Bio-Rad) in dH_2O) plus 0.02% sodium azide) and stained with anti-ER1 antiserum at a 1:1000 dilution in TBS-T (Paterno et al., 1997) overnight at RT. The blots were washed with four changes of 100ml TBS-T over a one-hour period followed by a one-hour incubation with a 1:3000 dilution of a 1mg/ml HRP-labelled goat anti-rabbit secondary antibody (Invitrogen Life Technologies Inc.) in TBS-T. The wash step was repeated. The detection step was performed using the ECL system (Amersham Pharmacia Biotech), as per manufacturer's directions. The experiments were repeated three times.

2.2.2 Embryo staging, sectioning, and ER1 immunocytochemistry and nuclear and antibody staining of embryos

Albino *Xenopus laevis* embryos were obtained as described above and staged according to Nieuwkoop and Faber (1967). Antibody staining of whole-mount embryos, immunocytochemistry and nuclear staining of sectioned embryos was performed according to the procedure described by Harland (1991) with some modifications. Ten embryos at each different developmental stage (stage 6.5, 8, 8.5, 10, 13, 27, and 45) were fixed for one hour with rotation in freshly prepared MEMFA (100 mM MOPS (Fisher Scientific), 2mM EGTA (Fisher Scientific), 1mM $MgSO_4$, and 0.37% formaldehyde (Fisher Scientific) in dH_2O) at RT. The fixed embryos were stored in 100% Methanol at -20^{0}C until required for staining. Embryos were bleached under white light for one hour in bleaching solution (1:3 (v/v) of 30% H_2O_2 and two parts Dent's fixative (20% dimethyl sulfoxide (DMSO) (Sigma-Aldrich), 80% methanol)). Bleaching decreases pigmentation and optimizes visualization of the stain. The bleaching solution was replaced with maleic acid buffer (MAB; 100 mM maleic acid (Sigma-Aldrich) and 150mM NaCl in H_2O, pH 7.5). The embryos were washed with three changes of MAB and placed into an 8 well polystyrene (Costar) insert dish. The embryos were incubated on a rotator for one hour in blocking buffer (2% (w/v) blocking reagent for nucleic acid hybridization and detection (BMB) (Boehringer Manheim), 5% (v/v) DMSO, 5 % (v/v) heat-inactivated goat serum (Invitrogen Life Technologies Inc.,) and 0.02% (v/v) sodium azide (Sigma-Aldrich) in MAB) at RT.

The blocking buffer was replaced with a 1:200 dilution of anti-ER1 antibody (Paterno et al., 1997) in blocking buffer. The embryos were incubated overnight on a rotator at 4°C after which they were washed with MAB every 30 minutes for 6 hours. The embryos were then incubated with a 1:200 dilution of an alkaline phosphatase-coupled goat-anti-rabbit secondary antibody (Invitrogen Life Technologies, Inc.) in blocking buffer and the embryos were rotated at 4^0C overnight. The following day, the embryos were washed as before followed by two 5 min washes with alkaline phosphatase (AP) buffer (100mM Tris, pH 9.5, 5mM $MgCl_2$ (Fisher Scientific), 100mM NaCl, 0.5% (v/v) Tween-20, and 5mM Levamisole (Sigma-Aldrich) in dH_2O). Embryos were stained with a mix of 4-nitro blue tetrazolium chloride (NBT; 33.75 mg/ml; Boeringher Manheim) and X-phosphate/5-bromo-4-chloro-3-indoyl-phosphate (BCIP; 17.5 mg/ml; Boeringher Manheim) in AP buffer until a dark purple colour developed. The staining reaction was stopped by removing the staining solution and replacing it with MEMFA. The stained embryos were kept at 4^0C. For long-term storage, the embryos were kept in MAB plus 0.02% sodium azide at 4^0C. The experiment was repeated three times.

For sectioning, ten embryos at each of stage 6.5; 8, 8.5, 10, 13, 27, and 45 were fixed in MEMFA and stored in 100% methanol at 4^0C. Polyester wax (PEG 400 distereate, Aldrige Chem Co.) and embedding moulds were warmed at 40^0C in a water bath a few hours before use. Embryos were transferred to 100% ethanol then transferred into moulds in a 1:1 ethanol:wax solution and some of the ethanol wax

solution was removed and replaced by fresh molten wax. This was repeated several times over one hour to allow the wax to penetrate. The mould containing the embryo and wax was removed from the water bath and cooled to RT, a mould holder was placed on top of the mould while the wax was still molten. The embryo was oriented in the wax using two forceps until the wax solidified. The wax was allowed to harden at RT for 2-3 hours after which the moulds were kept at 4^0C. All sectioning instruments were cooled at -20^0C for 5 minutes before sectioning. The block of wax was carefully removed from the mould and trimmed to expose the embryo. The holder was placed in a microtome, 8μm sections were made, and sections were collected on gelatin subbed glass slides. A solution of 0.1% Triton-X100 in dH_2O was carefully pipetted on the slides to spread the sections, after which the solution was removed and the slides left to air dry at RT. The sections were de-waxed using a 100%, 95%, 80%, 75%, 50%, 30% ethanol rehydration series in MAB and a MAB rinse). Antibody staining was performed as for whole mount staining except slides were incubated in small plastic boxes and washes were performed in Coplin jars. Nuclear staining was performed by incubating the slides in a 1:500 dilution of a live-cell nucleic acid stain (Molecular Probes). The experiment was repeated three times.

2.3 RESULTS

Northern blotting by Paterno *et al.* (1997) had previously shown that *er1* mRNA is expressed as a single message, which is detectable during early cleavage stages prior to the onset of zygotic transcription. Densitometric analysis showed that *er1* levels stay constant during these early cleavage stages, increase slightly at blastula and decrease during gastrula, neurula and tailbud stages. This pattern of expression indicates that *er1* is a maternally derived RNA, since the mRNA is present in the embryo before the onset of zygotic transcription at mid-blastula stages.

Although mRNA expression gives an idea of the expression pattern of a gene, it does not always give an accurate picture of the spatio-temporal expression pattern of a protein since amphibian embryos often contain maternal stores of protein as well as mRNA. Some maternal mRNAs, such as CRM1, have been shown to be under negative translational control during early development in *Xenopus* (Kudo et al., 1997). Other maternal RNAs, such as xlgv7/xlcaax-1, have been shown to be redundant during early development. When the store of xlgv7/xlcaax-1 maternal RNA is depleted through antisense technology, the embryos still develop normally due to the presence of a maternal store of xlcaax-1 protein (Reddy *et al.*, 1991a, 1991b).

The spatio-temporal expression pattern of ER1 protein was therefore examined in order to get a better indication of the tissues in which ER1 was functional.

2.3.1 The expression level of ER1 protein is constant during early developmental stages in *Xenopus*

The expression pattern of ER1 protein was analysed by Western blotting during stages similar to those examined by Paterno *et al.*, (1997) for RNA expression by Northern blotting. Stages 2, 6.5, 8, 8.5, 10, 13, 27 and 36 were examined. The Western blot revealed that ER1 protein is detectable and that expression levels are similar for all stages examined; staining pattern for stages 6.5, 8, 8.5, and 10 are shown in Fig. 2.1.

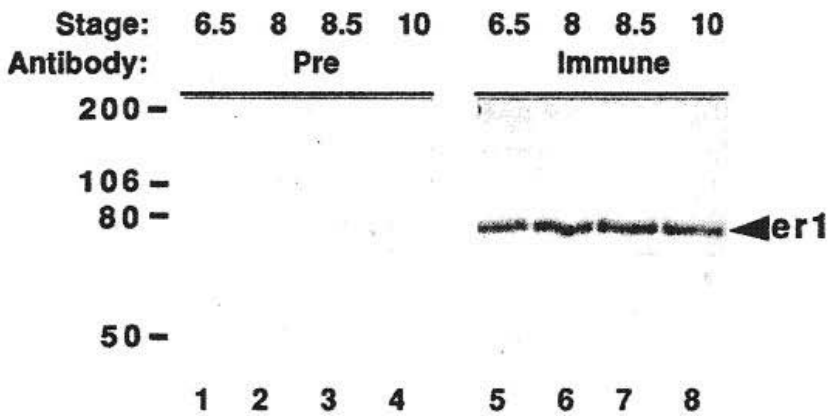


Figure 2.1 **ER1 protein is expressed during early development**
Embryo extracts from stages 6.5 (lanes 1 and 5), 8 (lanes 2 and 6), 8.5 (lanes 3 and 7) and 10 (lanes 4 and 8) were subjected to SDS-PAGE, blotted and stained with anti-ER1 (lanes 5 and 8). The blot was stripped and re-stained with pre-immune serum (lanes 1-4). The position of ER1 is indicated on the right and the molecular weight standards are on the left.

2.3.2 ER1 is differentially localized to the nucleus during early embryonic development

In embryo whole-mounts and sections stained with anti-ER1 antiserum, the first detectable staining is observed in the nucleus of marginal zone cells (presumptive mesoderm) of stage 8 blastula (Figs. 2.2 and 2.3), even though equivalent levels of ER1 protein are present at earlier stages (stage 6.5, Fig. 2.1). Thus, ER1 protein is present in the cells of the early stage embryo but does not become concentrated in the nucleus until mid-blastula stage.

No staining was observed at stage 6.5 using preimmune or anti-ER1 serum, (Fig. 2A and B, respectively). As development proceeds, more nuclei become stained and by late blastula (stage 8.5-9), virtually all nuclei in the animal hemisphere are stained (Fig. 2.1E,F). At this stage, the nuclei in the vegetal hemisphere begin to stain and by early gastrula (stage 10) ubiquitous nuclear staining is observed (Fig. 2.4). The same trend was observed for neurula stages.

During tailbud stages, endodermal and mesodermal tissues retain their nuclear staining (Fig. 2.5B, E, F); however, in ectodermally-derived tissues, such as the brain and epidermis, nuclear staining begins to disappear (Fig 2.5C, D). This pattern of decreasing concentration of ER1 in the nucleus of various tissues continues throughout late development and by tadpole stage; nuclear staining is observed only in some endodermal nuclei (Fig 2.6A, B). At tadpole stage of development, nuclear staining is no longer detected in any ectodermally or mesodermally-derived tissue (Fig. 2.6B-D), however, cytoplasmic staining is

observed in some mesodermal tissues (Fig. 2.6B-D). Neural tissue is not stained except for weak cytoplasmic staining in the eye (Fig. 2.6B, C).

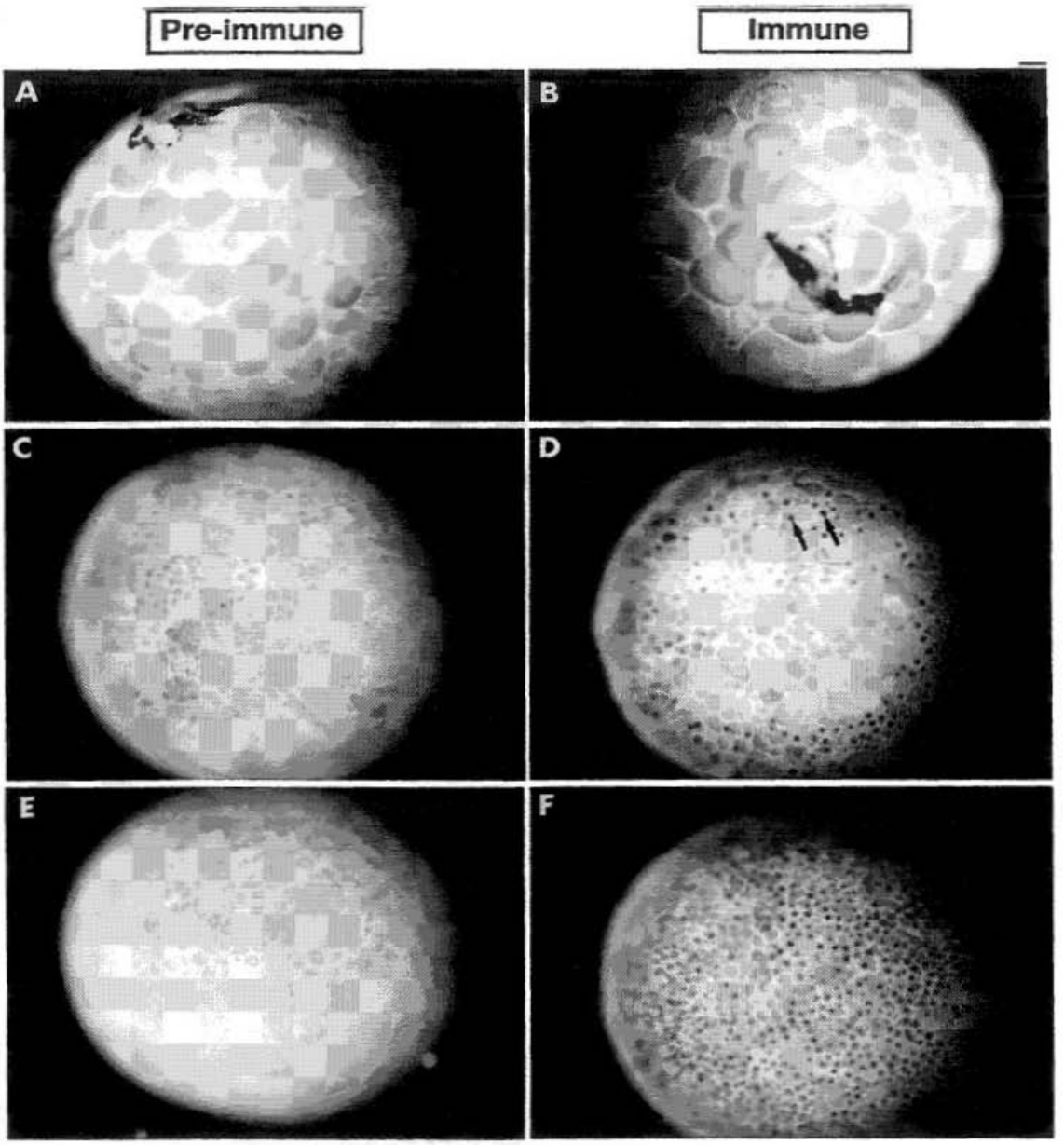


Figure 2.2 Localization of ER1 to the nucleus begins during blastula stages. Albino embryos were fixed at stages 6.5 (A,B), 8 (C,D) or 8.5 (E,F) and stained with either pre-immune serum (A, C, E) or anti-ER1 (B, D, F). Nuclear staining (see arrows in D) first appears in the marginal zone cells (presumptive mesoderm) of stage 8 blastulae; by stage 8.5 (one additional cell division), virtually all nuclei in the animal hemisphere are stained (F). The animal hemisphere of the embryo is shown facing up. Bar = 0.1 mm.

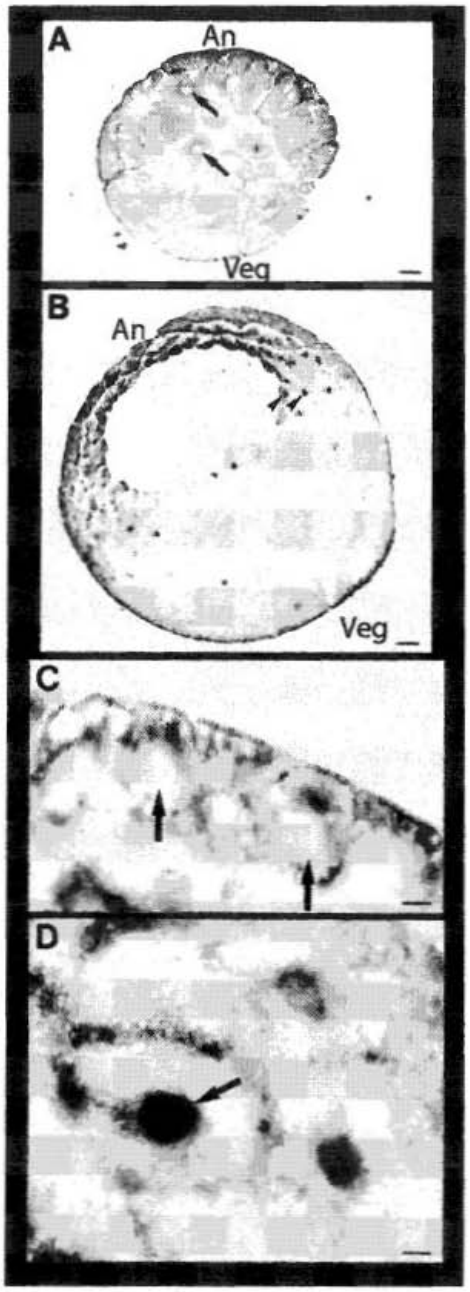


Figure 2.3 ER1 is concentrated in the nucleus of marginal zone cells in stage 8 blastulae.
Embryos were fixed at stages 6.5 (A) or 8 (B-D), sectioned and stained with anti-ER1. (A) The nuclei (arrows) remained unstained in early cleavage stages. (B-D) At stage 8, the nuclei (arrowheads in B and arrow in D) in the marginal zone begin to stain for ER1 while nuclei in the endoderm (B) as well as nuclei (arrows in C) in the rest of the animal hemisphere remained unstained. An = animal hemisphere. Veg = vegetal hemisphere. Bars = 0.1 mm in A, B and 0.02 mm in C, D.

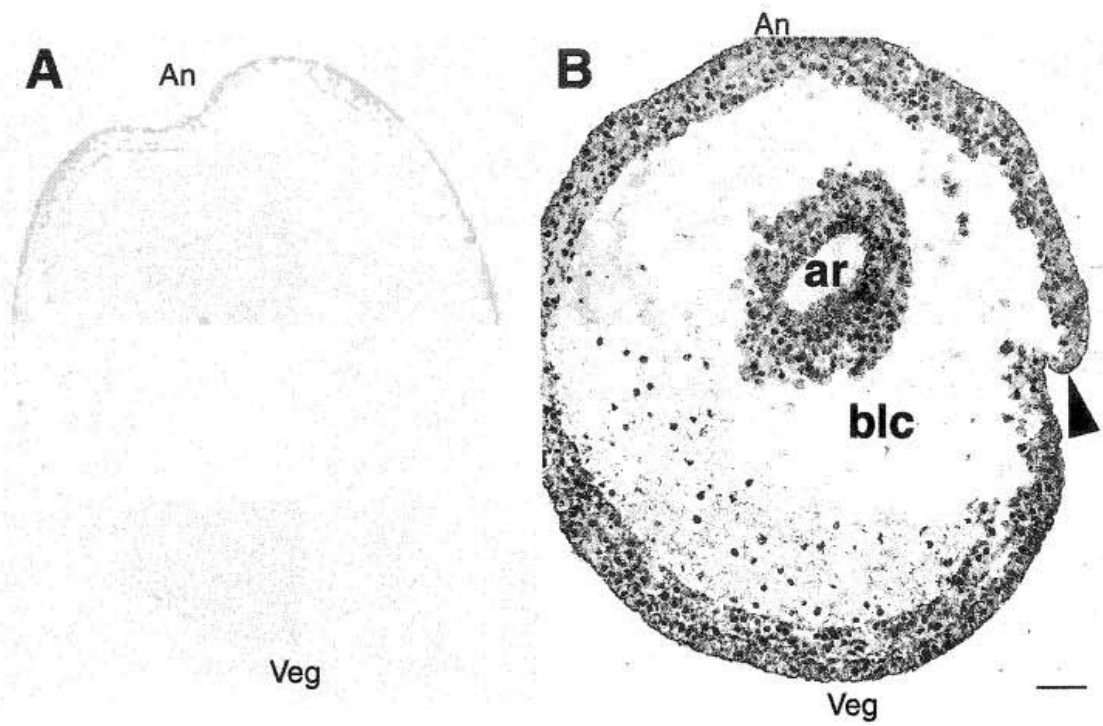


Figure 2.4 ER1 is concentrated in the nucleus of all cells in stage 10 gastrulae. Embryos were fixed at stage 10, sectioned and stained with either pre-immune (A) or anti-ER1 (B). at stage 10, ER1 is concentrated in the nucleus in virtually all cells of the three germ layers; the arrowhead indicates the involuting lip; ar, archenteron; blc, blastocoel. An = animal hemisphere, Veg = vegetal hemisphere Bars = 0.1 mm

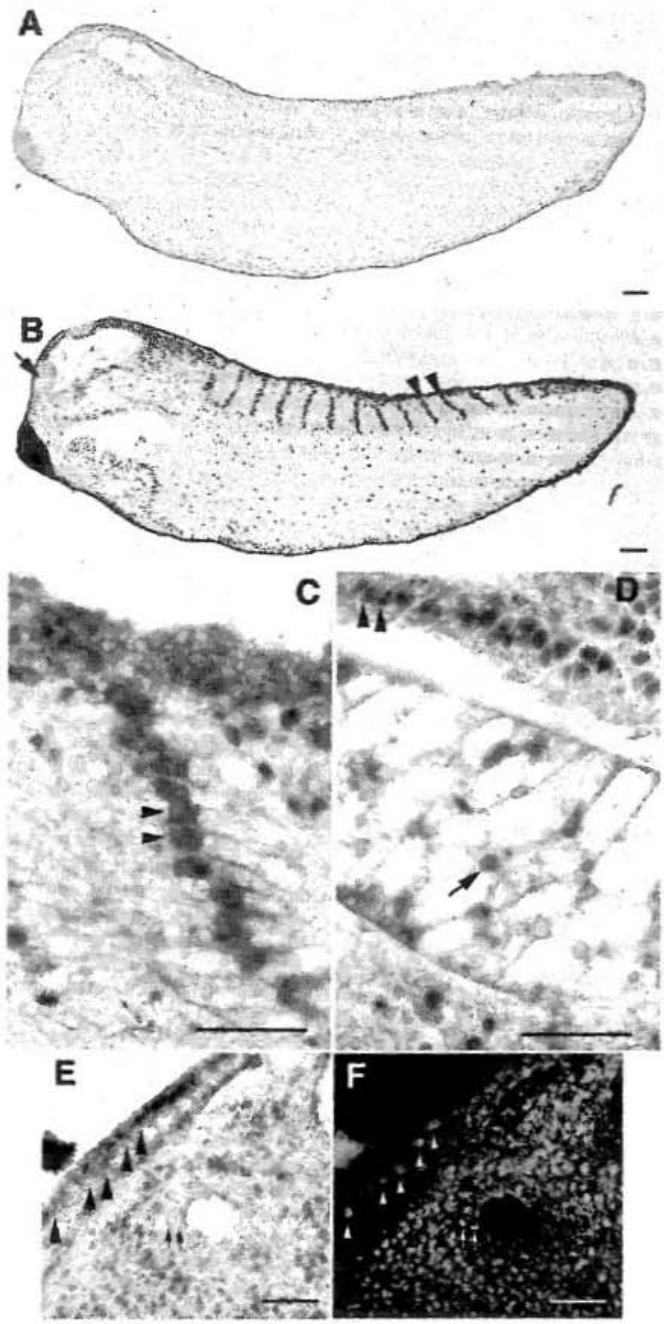


Fig 2.5 ER1 begins to disappear from the nucleus in the epidermis and brain during tailbud stages.

Embryos were fixed at stage 27, sectioned and stained with either preimmune (A) or anti-ER1 (B-F). At stage 27, nuclei are stained in the endoderm (B), somites (arrows in B and E), notochord (arrows in F), as well as in most of the spinal cord (tailed arrows in F). Many of the nuclei in the brain (tailed arrows in B-D) and epidermis (arrows in C, D) are no longer stained, as illustrated by comparing the anti-ER1 stained epidermis and brain in (C) with the same section incubated with a fluorescent nuclear stain (D). The black arrows in (C) mark the position of the nuclei identified by white arrows in D. Bars = 0.1 mm.

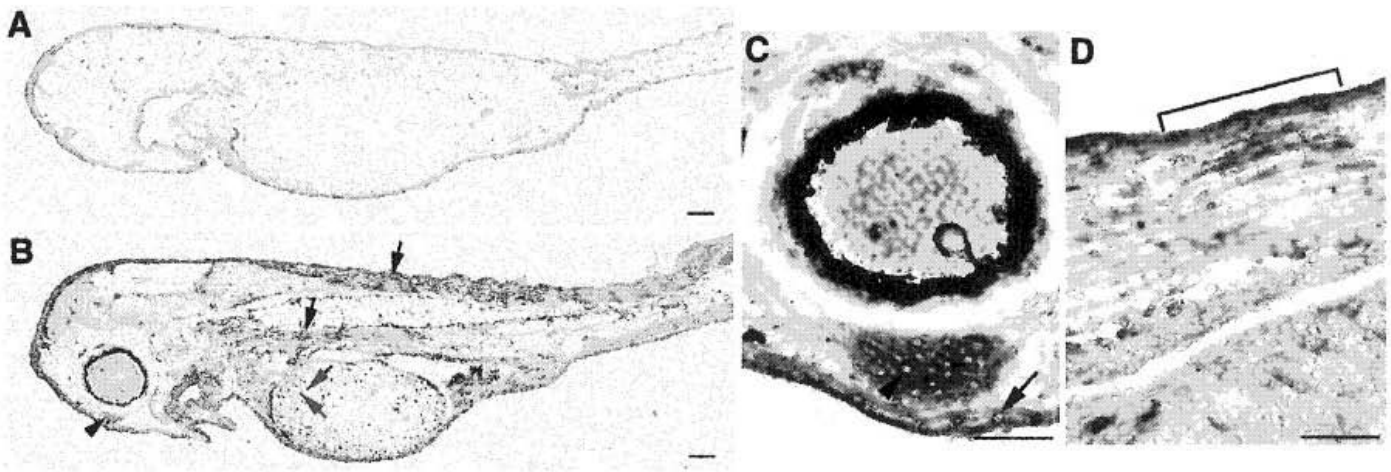


Figure 2.6 ER1 is no longer concentrated in the nucleus in stage 41 tadpoles
Embryos were fixed at stage 41, sectioned and stained with either preimmune (A) or anti-ER1 (B-D). At stage 41, staining is absent from neural tissue (B) except for weak cytoplasmic staining in the eye (C). Staining in mesodermal tissues is exclusively cytoplasmic and is observed in somites (tailed arrows in B and C). Nuclear staining is also absent in the epidermis (tailed black arrows in C) but is still observed in some of the endodermal cells (tailed arrows in B). Bars = 0.1 mm.

2.4 DISCUSSION

Analysis of the protein expression levels of ER1 during early developmental stages showed that, unlike the mRNA levels, the expression level of ER1 protein remained constant for all stages examined (Figure 2.1). ER1 protein is expressed at constant levels at stages when the mRNA levels go up (Figure 2.1 lanes 6 & 7, blastula stages) or are very low or undetectable (Figure 2.1 lane 8, gastrula; neurula and tailbud stages not shown). Although it was not possible to determine whether the ER1 protein detected on the Western blot was the product of maternal mRNA translated during early development and/or was maternal protein already present in the egg, it was clear that ER1 protein is present in the embryo from early cleavage stages at detectable and constant levels.

There are several mechanisms, which could be potentially involved in maintaining constant ER1 expression levels throughout the developmental stages examined: 1. The protein may be longer-lived than the maternal mRNA, since the protein is detectable at stages when the mRNA levels decrease; 2. The protein may be very efficiently translated from low levels of mRNA during the later developmental stages 3. The embryo may have a store of maternal ER1 protein as well as *er1* mRNA, and the protein detected on the Western blot could be from either or both sources.

There are examples of other proteins with similar protein expression patterns to that of ER1. An example is OCT1/POU, a maternally derived transcriptional activator in *Xenopus*, where the protein is at a constant level during early development and persists until neurula stages after downregulation of the mRNA (Veenstra et al., 1995; Veenstra et al., 1999). As discussed previously in this thesis, mRNA expression and localization during early development do not always give an accurate picture of the expression and distribution of the protein.

Many studies use RNA *in situ* hybridization techniques to visualize the tissues in which a gene is being actively transcribed. In the case of Xenopus embryos, maternal RNA is transcribed during oogenesis and localized to different parts of the egg, where it is in a stable form and is translated at different stages after fertilization. Localization of RNA during oogenesis is a means of regulating the translation of the protein to a specific embryo region and hence spatially regulating expression of the protein. However, mRNA is not always translationally active in regions where it is localized (Seydoux, 1996). For example, Xwnt-11 mRNA is symmetrically distributed in the vegetal region of the early Xenopus embryo, but Xwnt-11 protein is asymmetrically distributed along the dorso-ventral axis of the embryo, where it probably acts as a dorso-ventral determinant during establishment of the dorso-ventral axis (Schroeder et al., 1999).

Therefore, ER1 protein whole-mount antibody staining was used to visualize the spatio-temporal localization pattern during early cleavage and later developmental stages. During early blastula stages, ER1 is found in the cytoplasm (as shown by Western blotting, Figure 2.1) and is gradually localized to nuclei at mid- and late-blastula stages. The staining in the cytoplasm at early blastula stages is weak (almost similar to pre-immune staining). However, at these stages, the cytoplasm/nuclei ratio in the embryos is quite high, since the cells are very large. The staining is much darker at later stages when ER1 is localized to nuclei, as the protein spread over a large cell becomes confined to a smaller cytoplasm and a comparatively small nucleus. Although ER1 protein is present during early cleavage stages, nuclear localization only starts at mid-blastula stages. The spatio-temporal localization pattern of ER1 indicates that nuclear translocation of the protein is regulated during early development.

Appropriate subcellular localization is crucial for the proper functioning of proteins. Cytoplasmic localization is a way of regulating the functions of proteins including DNA replication, transcription and nuclear transport. There are several examples of proteins whose functions are regulated by spatio-temporal localization in *Xenopus*. For example, the Xnf-7 protein product, a maternally expressed putative transcription factor, is cytoplasmic during early cleavage stages in the *Xenopus* embryo and enters embryonic nuclei at mid-blastula transition (Dreyer and Hausen, 1983; Miller *et al.*, 1989; Kloc *et al.*, 1989; Reddy et *al.*, 1991b). Ultimately Xnf-7 becomes enriched in the nuclei of the cells of the

central nervous system at larval stages of development (Miller et al., 1989). OCT-1, a transcription activator in *Xenopus*, is also cytoplasmic until mid-blastula stages after which it gradually translocates to nuclei and is highly abundant in the nuclei of cells of ectodermal lineages during gastrulation (Veenstra et al., 1995). CCAAT, a maternal factor, which activates GATA-2 transcription in *Xenopus*, is localized within the cytoplasm of the embryo, until the beginning of gastrulation, when it becomes specifically translocated to the nucleus as zygotic GATA-2 transcription begins (Brewer et al., 1995). CRM1/XPO1, a protein involved in the specific export of proteins and RNA from the nucleus, is initially present in the cytoplasm of embryos in an inactive form, and becomes functional during the gastrula-neurula transition after which it becomes localized to the nuclear membrane (Kudo *et al.*, 1997).

The study of processes involved in the regulation of the localization of ER1 to the nucleus will be described in chapter 3.

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SECTION II ER1 IS DIFFERENTIALLY LOCALIZED DURING EARLY DEVELOPMENT IN XENOPUS EMBRYOS

CHAPTER 3 INVESTIGATION OF THE MECHANISM/S REGULATING NUCLEAR LOCALIZATION OF ER1 DURING EMBRYONIC DEVELOPMENT IN XENOPUS

Note: Part of this chapter has been submitted for publication.

3.1 INTRODUCTION

Numerous proteins have been identified in *Xenopus* whose subcellular localization is developmentally regulated (Dreyer, 1987). The germinal vesicle (GV) accumulates a reserve of maternal RNA and proteins, which are required in the early developmental stages of the embryo such as enzymes and precursors for DNA synthesis, chromatin assembly, nuclear formation and transcription (Almouzni and Wolffe, 1993;von Dassow et al., 1993). During oocyte maturation, the GV disintegrates and its proteins become distributed in the cytoplasm. Some maternal proteins quickly re-accumulate into nuclei during early cleavage stages

while others do not re-enter nuclei until blastula and gastrula stages (Dreyer, 1987). Examples of these are: nucleoplasmin, which rapidly migrates to the nuclei of the cleaving embryo after fertilization (Dreyer, 1987) and small nuclear ribonucleo proteins (snRNPs), which enter the nuclei only during late blastula and early gastrula stages (Zeller *et al.*, 1983). Xnf7 and Oct-1 are transcription factors that are also retained in the cytoplasm until mid-blastula to gastrula stages (Miller *et al.*, 1991; Veenstra *et al.*, 1999).

Different mechanisms in various cells have been identified that regulate retention of proteins in the cytoplasm. For example, members of the Rel/NF-κB family of transcription factors are retained in the cytoplasm through interaction with an inhibitor that is differentially phosphorylated (reviewed in Karin *et al.*, 1999). NF-κB heterodimers usually exist in the cytoplasm as a complex with the inhibitor IκB. Extracellular stimuli activate signalling pathways leading to phosphorylation and subsequent proteolysis of IκB, thereby releasing and enabling translocation to the nucleus of NF-κB heterodimers. In the case of Xnf-7, a 22-amino acid cytoplasmic retention domain (CRD) functions cooperatively with two phosphorylation sites within the Xnf-7 molecule to retain the protein in the cytoplasm (Li et al., 1994)

The developmentally regulated nuclear translocation of ER1 indicates that there is a tight control on nuclear localization of ER1 during early development. Regulated nuclear localization is often a means of controlling the function of

proteins such as transcription factors. Therefore, the controlled nuclear localization of ER1 during early *Xenopus* development may be an important mechanism in regulating its activity. In this chapter, different regulatory mechanisms that might control translocation of ER1 protein to the nucleus during early development in *Xenopus laevis* were investigated.

3.2 MATERIALS AND METHODS

3.2.1 In vitro transcription of RNAs

*Xenopus er*1, *Xenopus* fibroblast growth factor receptor (FGFR1) and dominant-negative FGFR (XFD, Amaya *et al.*, 1991) cRNA were prepared from cDNA templates that had been previously subcloned in the sp64T vector using the SP6 Ribomax large scale RNA production system (Promega Corporation) and cap analogue (New England Biolabs, Inc.). The cap analogue is a 7-methyl guanine residue, which is added onto the 5' terminus of the RNA to increase its stability and translation efficiency in *Xenopus*. The *er*1 and FGFR1 templates were linearized with the restriction endonuclease Xba1 (Amersham Pharmacia Biotech) and the XFD template with EcoR1 (Amersham Pharmacia Biotech). The following reagents were combined in a 1.5ml eppendorf tube: 10μl SP6 5xTranscription buffer, 2.5μl each 100mM ATP, CTP and UTP, 1.5μl 100mM GTP, 10μl 30mM cap analogue (New England Biolabs, Inc.), 10μg of linearized cDNA template, 5μl SP6 RNA polymerase enzyme mix and nuclease-free H_2O to

a final volume of 100µl. The mixture was gently pipetted up and down, briefly centrifuged and incubated in a 37^{0}C water bath for two hours. After incubation, 5U (5µl) of RQ1 RNAse free DNAse (Promega Corporation) was added to remove the cDNA template, and the reaction mixture was further incubated at 37^{0}C for 20 minutes. Then 200 µl of nuclease-free H_2O was added and phenol/chloroform extraction was sequentially performed by addition of equal volumes of buffer-saturated phenol and chloroform. The top aqueous layer was removed and precipitated overnight at –20^{0}C with one tenth the initial volume of 3M sodium acetate (Fisher Scientific), pH 5.2, and 2.5 volumes of 100% ethanol. The cRNA was pelleted by centrifuging at 10,000*g* for 20 minutes. The pellet was washed three times with 70% ethanol and briefly vacuum dried. The cRNA pellet was resuspended in 50µl DEPC-treated H_2O. To verify the presence of intact cRNA of the right size and the absence of impurities such as cDNA, 1µl of the each of the cRNA samples was run on a 0.8% agarose gel. Absorbance was measured at 260 and 280nm to determine concentration, purity and integrity of the cRNA. The cRNA samples were stored at –70^{0}C.

3.2.2 RNA injections

The cRNA was injected into embryos with a Drummond "Nanoinject" Microinjector using 31/2" Drummond glass capillary tubes (Fisher Scientific). The

needles were previously pulled vertically using a Narishige Model PB-7 micropipette puller and the tips bevelled at a 20^0 angle with a Narishige EG-40 grinder. For the overexpression studies, albino embryos were injected with 1-20ng *er1* cRNA in the animal pole at two-cell stage. Albino embryos were injected at two-cell stage in both cells with either 1ng *er1* cRNA alone or co-injected with 10ng FGFR1 or 10ng XFD, for experiments involving disruption of the FGF signalling pathway. All experiments were repeated 3-5 times and 80-100 embryos/experiment were injected. Injection volumes were 4.6nl.

3.2.3 Whole-mount antibody staining

Albino embryos were collected at stage 6.5-7, 8.5 and 9 and whole-mount antibody staining was performed as previously described (Luchman et al., 1999). A 1:400 dilution of anti-ER1 antiserum (Paterno *et. al.*, 1997) and a 1:400 dilution of alkaline-phosphatase-linked goat-anti-rabbit antibody (Invitrogen Life Technologies, Inc.) was used for staining. At each stage, 10-15 embryos were stained per experiment, which were repeated 3-5 times.

3.2.4 α-amanitin treatment and reverse transcription/polymerase chain reaction (RT/PCR)

*er*1 cRNA was prepared using the Ribomax large scale RNA production system (Promega Corporation) as described above. Embryos were injected with DEPC-treated H_2O or *er*1 cRNA (4 ng/embryo) plus or minus ∝-amanitin (Sigma-Aldrich) (50µg/embryo; Newport and Kirshner, 1982 & Sible *et al.*, 1997). Embryos were processed for whole-mount staining as described above at stage 7, 8 and 9. RNA was extracted from stage 8.5 and 10.5 embryos and processed for RT/PCR as described below.

3.2.4.1 RNA extraction

Ten whole embryos were transferred to 1.5ml eppendorf tubes containing 1ml of Tri-Reagent (Invitrogen Life Technologies, Inc.) and homogenized with a micropipette. RNA extraction was performed as instructed by the manufacturers. After the reaction, total RNA was re-suspended in 39µl of DEPC-treated dH_2O. Remaining traces of DNA were removed by incubating for 20-30 minutes in a 37° C water bath with 0.1U (1µl) of RNAguard RNase inhibitor (Amersham Pharmacia Biotech), 5µl 10X transcription buffer (400mM Tris-HCL, pH 7.5, 60mM $MgCl_2$, 20mM spermidine (Invitrogen Life Technologies Inc.) and 5U (5µl) RQ1 RNAse free DNase (Promega Corporation). After incubation, the final

volume in each tube was adjusted to 100µl with DEPC-treated dH_2O and phenol/chloroform extracted with equal volumes of buffer-saturated phenol and water-saturated chloroform. The remaining aqueous layer was precipitated with 2.5 volumes of 100% ethanol and one tenth volume of 3M sodium acetate, pH 5.2 at –20° C overnight then centrifuged at 4° C for 25 minutes at 10,000g. The supernatants were removed and the pellets were washed with 70% ethanol. The pellets were briefly vacuum dried, re-suspended in DEPC-treated dH_2O and stored at –70° C. The concentration and integrity of the RNA was examined by absorbance and by gel electrophoresis.

3.2.4.2 Reverse transcription of isolated RNA

About 200ng of each extracted RNA sample was diluted to a final volume of 15µl with DEPC-treated dH_2O in 0.5ml Eppendorf tubes. 2µl of 100ng/ml random hexanucleotide primers (Boeringher Manheim) was added to each tube, after which the RNA was denatured at 70° C for 10 minutes and placed immediately in an ice bath to prevent internal pairing of RNA strands and allow annealing of the primers to the RNA. To each tube, a reverse transcription mixture consisting of 8µl first strand buffer (Invitrogen Life Technologies Inc.), 8µl of 2.5mM deoxyribonucleotide triphosphates (dNTPs, Amersham Pharmacia Biotech), 4µl 100mM DTT (Invitrogen Life Technologies Inc.), 1 µl RNAguard RNAse inhibitor (Amersham Pharmacia Biotech) and 2µl M-MLV reverse

transcriptase (Invitrogen Life Technologies Inc.) was added. The reaction mixtures were incubated at 37° C for one hour after which the resulting cDNA samples were kept frozen at –20° C.

3.2.4.2 PCR of reverse transcribed products

A premix was prepared for PCR amplification reactions (10mg/ml 10xPCR buffer (Invitrogen Life Technologies Inc.), 1.5mM $MgCl_2$ (Invitrogen Life Technologies Inc.), 0.8mM dNTPs (Amersham Phamacia Biotech), 2ng/ml each primer of 1 set of specific primers (Oligos Etc.), 0.02 U/μl platinum Taq DNA polymerase (Invitrogen Life Technologies Inc.), and 50 μCuries/ml [^{32}P]ATP (Amersham Pharmacia Biotech) in dH_2O). Histone H4, was used as an internal control with 5'-CGGGATAACATTCAGGGTATCACT-3' and 5'-ATCCATGGCGGTAACTGTCTTCCT-3' as forward and reverse primers. EF1-∝ was amplified using 5'-CCTGAATCACCCAGGCCAGATTGGTG-3' and 5'-GAGGGTAGTCTGAGAAGCTCTCCACG-3'. In 0.5ml thin-walled eppendorf tubes (Fisher Scientific), 48μl of the master mix was added to 2μl of reversed transcribed cDNA. The reaction mixtures were vortexed briefly and 50μl of light mineral oil (Fisher Scientific) was added to the top of the mixtures to prevent evaporation during thermocycling. A Perkin Elmer Thermal Cycler was used for the thermocycling reactions. The [^{32}P]ATP-labelled PCR products were analyzed in the linear range for amplification, using the following program:

1 cycle: 94^{0}C, 5 minutes for activation of enzyme

'x' cycles: 55^{0}C, 1 minute for annealing of primers

72^{0}C, 1 minute for primer extension

94^{0}C, 1 minute for denaturation

1cycle: 55^{0}C, 1 minute for annealing of primers

72^{0}C, 7 minutes for primer extension.

'x' was empirically determined to be 22 cycles for Histone and 24 cycles for EF1-α. The PCR products were examined by a 6% polyacrylamide/6M urea gel electrophoresis and autoradiography.

3.2.5 Immunoprecipitation and Western Blotting

Extracts from embryos at different stages were prepared for Western blotting as described in Chapter 2, except 50 mM sodium pyrophosphate, 50 mM sodium fluoride, and 1mM sodium vanadate were added to the extraction buffer to prevent dephosphorylation. Extracts were immunoprecipitated with a 1:50 dilution of anti-ER1 antiserum (Paterno *et. al*, 1997) overnight at 4^{0}C, 50 μl of Protein-A-Sepharose (Amersham Pharmacia Biotech) was added and the mixture was rotated at 4 °C for one hour. The Sepharose protein was washed

three times with 1ml extraction buffer plus phosphatase inhibitors and twice with 1ml 150mM NaCl. To each sample, 35 µl of sample buffer (50mM Tris pH 6.8, 2% sodium dodecyl sulfate (SDS) (Fischer Scientific) was added and the samples were boiled for 4 minutes. The protein was resolved on an 8% acrylamide gel and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech). The membranes were stained with a 1:1000 dilution of biotin-conjugated anti-phosphotyrosine (Upstate Biotechnology, Inc.), anti-phosphoserine (Sigma-Aldrich) or anti-phosphothreonine (Sigma-Aldrich). Detection of ER1 protein was performed by loading 150 µg of total embryo extract for uninjected embryos or 7.5 µg total embryo extract for *er*1-injected embryos and staining with a 1:5000 dilution of ER1 antiserum. The Femtolucent system (Chemicon International, Inc.) was used for chemiluminescence detection. To compare expression levels of ER1 in H_2O-injected and *er*1 cRNA injected embryos, densitometric analysis was performed using a Canberra-Packard ChemiImager.

3.3 RESULTS

Several possible mechanisms were investigated in order to characterize the localization of ER1 to the nucleus.

3.3.1 ER1 is localization to the nucleus does not require zygotic transcription

The localization of ER1 to the nucleus appear to coincide with the start of zygotic transcription at mid-blastula stages. The possibility that targeting of ER1 to the nucleus was dependent on protein(s) translated from zygotic message(s) was therefore examined. Zygotic transcription was inhibited to determine if nuclear translocation is dependent on newly transcribed gene products.

Xenopus embryos were injected with or without α-amanitin, an inhibitor of RNA polymerases II and III, and/or with *er*1 cRNA. Co-injection was provide high concentrations of ER1 and α-amanitin at the same site. Subcellular localization of ER1 antibody staining was examined at several stages in whole-mount antibody preparations (Fig. 3.1). In both *er*1-injected and *er*1/α-amanitin injected embryos, ER1 remained cytoplasmic at stage 7 (Fig. 3.1A & B), was first found in the nuclei (see arrowheads in Fig. 3.1C & D) at stage 8 and became nuclear at stage 9 (Fig. 3.1E & F). Thus, no difference was detected in the subcellular localization pattern of ER1 in *er*1-injected compared to *er*1/α-amanitin

co-injected embryos, and both patterns were the same as that of endogenous ER1, previously described (Luchman *et al.* 1999).

To verify that α-amanitin was effective in blocking zygotic transcription, the expression levels of elongation factor 1-∝ (EF1-∝) was examined by ^{32}P-radiolabelling using RT-PCR (Fig. 3.2). EF1-∝ is a maternal gene whose expression level increases dramatically through zygotic transcription (Krieg *et al.*, 1989). Our results show that the expression level of EF1-∝ increases significantly in post-MBT embryos (Fig. 3.2, lanes 1 and 4; 2 and 5), but not in α-amanitin-injected embryos (Fig. 3.2, lanes 3 and 6).

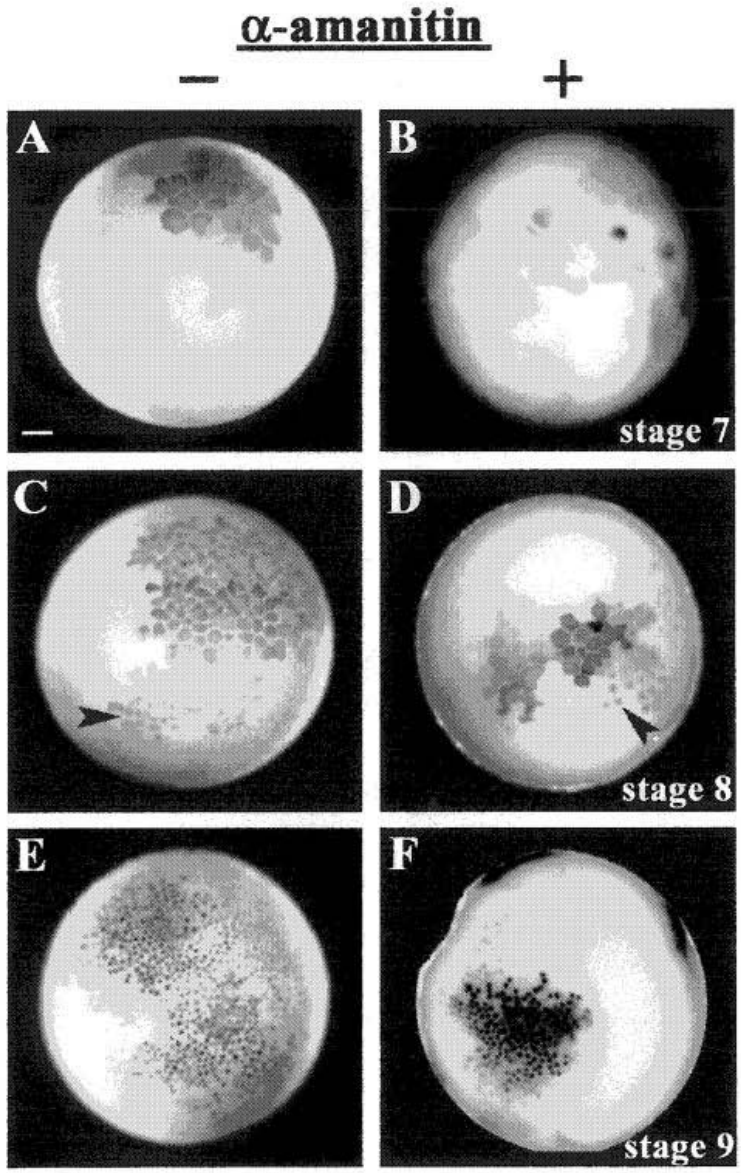

Figure 3.1 Translocation of ER1 to the nucleus is not dependent on zygotic transcription.

Fertilized embryos were injected with 4ng *er1* cRNA plus (+) or minus (-) α-amanitin as descibed under "Materials and Methods" and cultured until they reached stage 6.5-7, 8 or 9. Whole mount staining was performed as described under "Materials and Methods". Nuclear staining first appears at stage 8 (red arrowheads B and D). A total of 10-15 embryos per stage were stained for each experiment and the experiment was repeated three times. Embryos were photographed with the animal hemisphere facing up. Scale bar, 0.1mm.

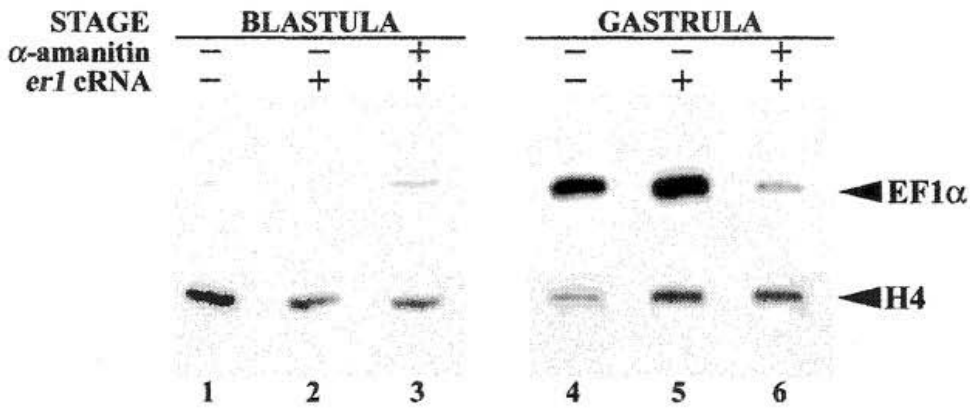

Figure 3.2 The expression of zygotic EF1-α is blocked by α-amanitin. Total RNA (8 embryos per condition) was extracted at blastula (lanes 1-3) or gastrula stages (lanes 4-6) from DEPC-H_2O injected (lanes 1 and 4), *er1* cRNA injected (lanes 2 and 5) or *er1* plus α–amanitin injected (lanes 3 and 6) embryos and analyzed by ^{32}P-labelled RT-PCR as described under "Materials and Methods". The positions of EF1-α and Histone H4 PCR products are indicated on the right.

3.3.2 Overexpressed ER1 is localized in the nucleus

The second possibility investigated was that ER1 is retained in the cytoplasm through its association with an inhibitor or anchor protein, as is the case for NF-κB (reviewed in Karin, 1999) and Xnf-7 (Li *et al.*, 1994), respectively. Inhibitor proteins mask the nuclear localization signals and prevent nuclear localization; for example, IκB associates with NFκB and prevents it from localizing to the nucleus (reviewed in Karin, 1999). Other proteins such as Xnf-7 have a cytoplasmic retention domain through which it associates with a cytoplasmic protein, and is anchored and retained in the cytoplasm (Li *et al.*, 1997). ER1 protein was overexpressed to titrate any possible protein causing retention of ER1 in the cytoplasm.

Embryos were injected with 1-20ng of *er*1 cRNA and ER1 protein was visualized by whole-mount antibody staining at stage 6.5, 8 and 9 (Figure 3.1A, C, and E, shows staining pattern of embryos injected with 4ng of *er*1 cRNA). The staining pattern in the overexpressed embryos (Figure 3.1A, C, and E) was the same as for endogenous ER1 (Fig. 2.2, B, D and F). ER1 remained cytoplasmic at stage 7, (Fig. 3.1A), was first found in the nuclei, (arrowheads in Fig. 3.1C), at stage 8, and became nuclear at stage 9, (Fig. 3.1E). This pattern was also observed when higher concentrations were injected. As can be seen in Fig. 3.3A(II), injection of as much as 20ng of *er*1 cRNA did not lead to premature translocation of ER1 to the nucleus at stage 6.5, a stage where endogenous ER1

is exclusively cytoplasmic (Luchman *et al.*, 1999). Injection of 20ng of er1 cRNA did not affect localization of ER1 to the nucleus at stages 8 and 9, the pattern of nuclear localization was the same as for endogenous ER1. That ER1 was indeed overexpressed in these embryos was verified by Western blot (Fig. 3.3B). Using densitometric analysis, the level of ER1 in injected embryos was determined to be in vast excess over endogenous ER1, at approximately 100-fold.

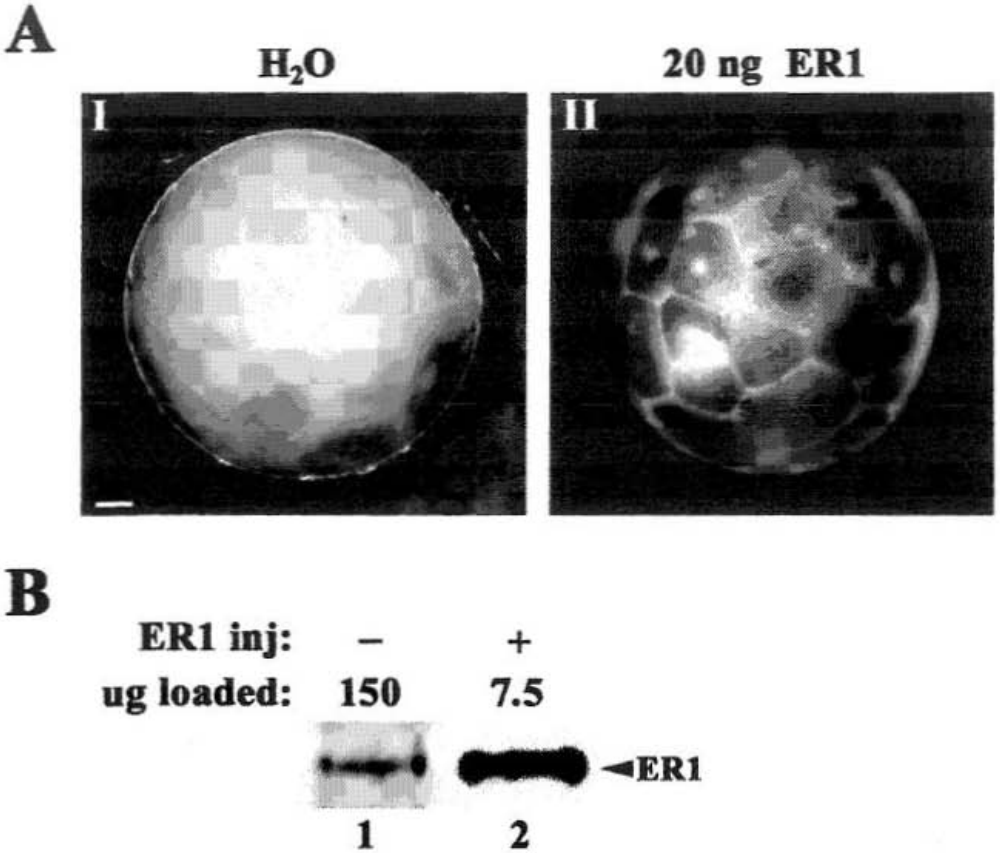

Figure 3.3 Overexpression of ER1 does not lead to premature targeting to the nucleus

(A) Fertilized embryos were injected with either DEPC H_2O (I) or 20 ng *er1* cRNA (II), fixed at stage 6.5 and stained by whole mount staining as described under " Materials and Methods". (B) ER1 protein was collected from DEPC-H_2O injected (-) and *er1* cRNA injected (+) embryos; Western blotting with anti-ER1 was performed as described (Luchman *et al.*, 1999). Due to the high levels of ER1 protein in the *er1*-injected embryos, only 7.5 µg of total embryo extract was loaded (lane 2), while 150 µg of extract from DEPC-H_2O injected embryos was loaded (lane1). Scale bar, 0.1 mm.

3.3.4 XFD injections disrupts nuclear localization of ER1

Given that *er1* transcription is activated in response to FGF (Paterno et al., 1997), a possible connection between nuclear localization of ER1 and the FGF signalling was investigated. The FGF signalling pathway was disrupted by co-expressing a dominant-negative FGF receptor, XFD, with ER1 in order to determine if nuclear translocation of ER1 was dependent on the FGF signalling.

Embryos were injected with 1ng of *er1* cRNA alone or co-injected with either, 1-10ng of wild-type *Xenopus* FGFR1 cRNA, or 1-10ng of the dominant-negative receptor, XFD, cRNA. Wholemount staining for ER1 was performed at stage 9 since we had previously shown that, in the animal hemisphere, ER1 was exclusively nuclear at this stage (Luchman *et al.*, 1999). Embryos were also scored morphologically. Embryos injected with XFD cRNA had truncations in the trunk and posterior region as previously reported by Amaya *et al.*, (1991). In embryos expressing both ER1 and XFD, a significant proportion of the cells around the injection site exhibited cytoplasmic staining at all concentrations of co-injected XFD. Fig. 3.4A shows the pattern obtained when the highest concentration of XFD, 10ng, is co-injected with *er1*. In cells co-expressing wild-type FGFR1 and ER1, on the other hand, staining was exclusively nuclear, even when 10ng of wild-type FGFR1 was co-injected with *er1* (Fig. 3.4B); the latter was similar to the staining pattern of cells expressing ER1 alone (Fig. 3.4C) as well as to the pattern of endogenous ER1 staining previously described

(Luchman et al., 1999). These results suggest that, nuclear translocation of ER1 is dependent on events triggered by FGF signalling.

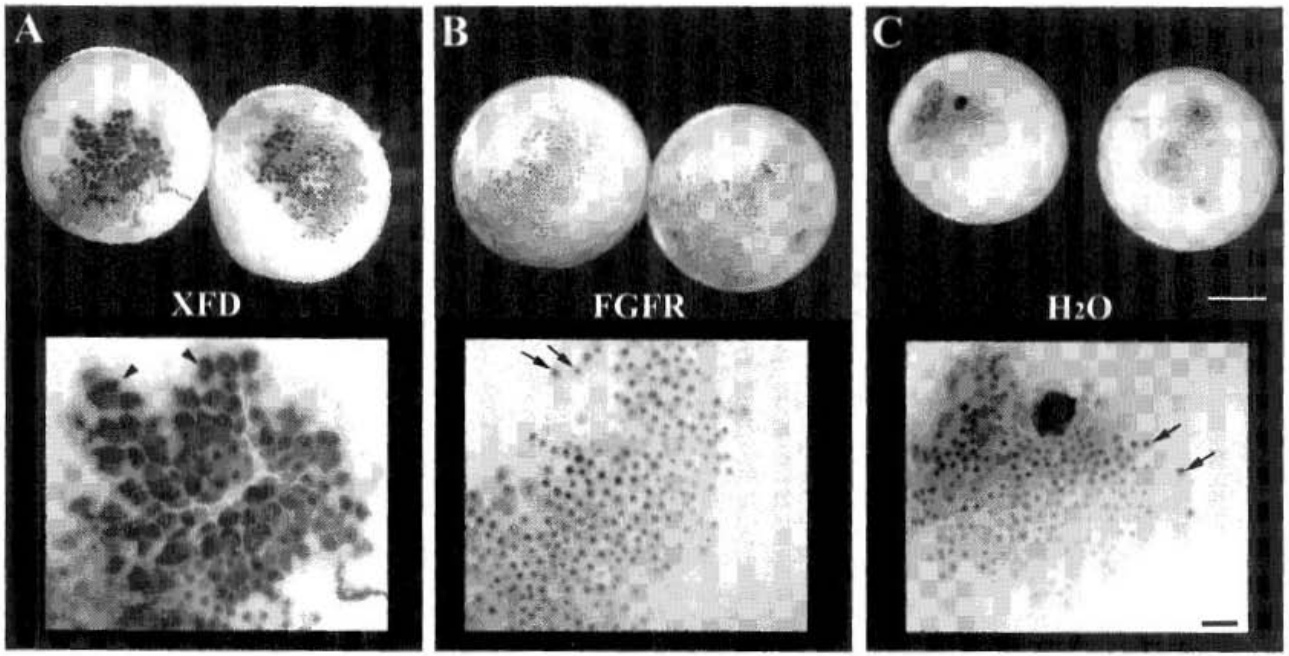

Figure 3.4 Nuclear translocation of ER1 is dependent on FGF signalling
Fertilized embryos were injected with 1ng *er1* cRNA alone (C) or with 10ng XFD (A) or FGFR (B) as described under "Materials and Methods" and cultured until they reached stage 9. Whole mount staining was performed as described under "Materials and Methods". At the bottom of each panel, a magnified view of the injection site in the embryo on the left is shown. Examples of cytoplasmic staining are indicated by arrowheads and nuclear staining by arrows. A total of 10-15 embryos per stage were stained for each experiment and the experiment was repeated five times. Embryos were photographed with the animal hemisphere facing up. Scale bar, 0.5 mm (white) 0.2 mm (black).

3.3.5 Different phosphorylated proteins are associated with cytoplasmic and nuclear ER1 \

Signal transduction cascades initiate events such as protein-protein interactions, protein phosphorylation and protein dephosphorylation. Furthermore, retention of nuclear proteins in the cytoplasm is often regulated by phosphorylation (Dreyer, 1987). Differential phosphorylation was therefore examined as a potential mechanism for the regulation of ER1 targeting to the nucleus.

Embryo extracts were immunoprecipitated with anti-ER1 antibody at stages when ER1 is either predominantly cytoplasmic (Reynolds et al., 1996) or predominantly nuclear (Ryan and Gillespie, 1994). Western blots were stained with anti-phosphotyrosine, anti-phosphothreonine, anti-phosphoserine or anti-ER1. Staining with anti-ER1 resulted in detection of a single band, with no evidence of additional slower migrating bands that might represent phosphorylated forms (Fig. 3.5, lane 3). This is consistent with our previous findings that ER1 was present as a single band at all developmental stages examined (Luchman *et al.*, 1999). In addition, ER1 did not co-migrate with any of the observed phosphoprotein bands (Fig. 3.5, lanes 1-6). Taken together, these results indicate that ER1 is not itself phosphorylated.

Staining with anti-phosphothreonine did not reveal any reproducible bands (results not shown), while staining with anti-phosphotyrosine and anti-phosphoserine revealed several bands co-precipitating with ER1 (Fig. 3.5, lanes

1-2, 4-5). Of the two phosphotyrosine bands detected, neither exhibited differences in intensity between stage 7 and 9 (Fig. 3.5, lanes 1 and 2).

Four phosphoserine bands (Fig. 3.5, arrowheads) of calculated molecular weight 226, 118, 76 and 38 kDa were detected and a significant decrease in the level of the latter three were detected at stage 9 when compared to stage 7 (Fig. 3.5, lanes 4-5). The 226 kDa band, on the other hand displayed little difference in intensity between the two stages. The identity of these phosphoserylproteins is presently unknown; therefore, I cannot measure their expression level to determine whether the differences in band intensity are due to changes in the amount of associated protein or due to changes in the level of phosphorylation. Interestingly, stage 9 embryos injected with XFD did not display these changes (Fig. 3.5, lane 6), but rather retained the phosphoserine pattern seen at stage 7, in which ER1 is retained in the cytoplasm (Fig. 3.5, compare lanes 6 and 4 respectively).

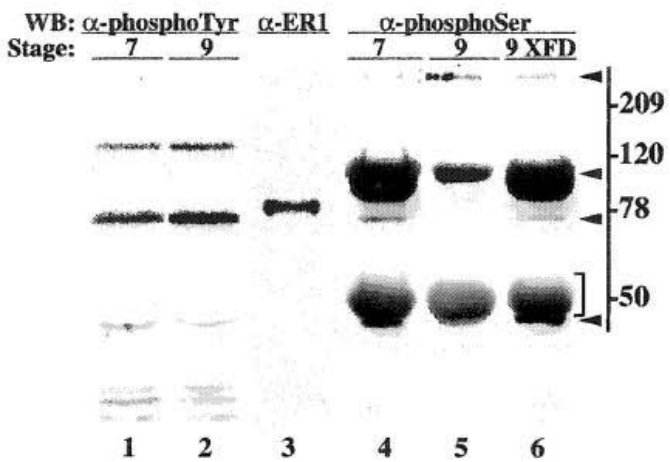

Figure 3.5 Changes in ER1-associated phophoserylproteins during early development are correlated with ER1nuclear translocation.
Embryo extracts (150 embryos/sample) were obtained from stage 7 (lanes 1 and 4) and stage 9 (lanes 2 and 5) embryos and from stage 9 embryos injected with XFD cRNA (lane 6). Immunoprecipitation and Western blotting were performed as described in "Materials and Methods". The Western blot was stained with anti-phosphotyrosine (lanes 1,2) and antiphosphoserine (lanes 4-6). For comparison, total embryo extract was loaded in lane 3 and stained with anti-ER1. The arrowheads indicate the four phosphoserine bands while the square bracket indicates the heavy chain of IgG. The positions of the molecular size markers is indicated on the right.

3.4 DISCUSSION

ER1 translocates to the nucleus in NIH 3T3 transfected cells (Paterno *et al.*, 1997; Post *et al.*, 2001) however, in *Xenopus* embryos the timing of nuclear translocation is precisely regulated. Therefore, there must be intrinsic mechanisms present in the embryo, regulating nuclear translocation of ER1 during early development. This may be an important mechanism for regulating ER1 activity during early development. Elucidation of these regulatory mechanisms would, therefore, provide clues to the function of ER1 in the embryo.

Recently, an NLS, ^{463}RPIKRQRMD^{472}, similar to the core NLS directing the human c-MYC protein to the nucleus (Makkerh *et al.*, 1996) was identified near the C-terminus of ER1. This sequence was found to be necessary and sufficient for ER1 targeting to the nucleus in NIH 3T3 cells (Post *et al.*, 2001). The presence of a functional NLS in the ER1 sequence confirms that the importin transport machinery has the ability to mediate the localization of ER1 to the nucleus. However, the presence of an NLS on a protein is not always sufficient to direct its nuclear import. For example, the NLS may be modified or masked so that it is no longer recognized by the nuclear transport machinery. Masking the NLS may be achieved by post-translational modifications such as phosphorylation or intra- or inter-molecular interactions. The protein is then sequestered in the cytoplasm until the NLS is unmasked, as is the case for the

proteins of the NF-κB family. NF-κB heterodimers usually exist in the cytoplasm as a complex with the inhibitor IκB. Extracellular stimuli activate signalling pathways leading to phosphorylation and subsequent proteolysis of IκBα, thereby releasing NF-κB heterodimers and enabling translocation to the nucleus (reviewed in Karin *et al.*, 1999).

Furthermore, as discussed earlier, an NLS-containing protein might be anchored in the cytoplasm by binding another protein as seen for Xnf-7 (Li *et al.*, 1994). Cadherins act as anchors for proteins such as β-catenin in the cytoplasm (reviewed in Ben-Ze'ev, 1999). My overexpression study attempted to titrate out any such factor.

Overexpression of ER1 did not result in its nuclear translocation. Although the possibility that a regulatory factor(s) is present at high enough concentration to prevent complete titration cannot be ruled out, the 100-fold overexpression of ER1 in injected embryos over uninjected embryos makes it unlikely that at least a partial titration would not have been observed. These considerations suggest that translocation of ER1 to the nucleus is not regulated by binding to an inhibitor or anchor protein and it is hypothesized that nuclear translocation of ER1 is regulated by another mechanism such as differential phosphorylation or interaction with a positive regulatory protein. This event/s may be triggered by a signalling pathway such as the FGF signalling pathway, which has previously been shown to activate *er1* transcription in *Xenopus* (Paterno *et al.*, 1997)

Nuclear translocation of ER1 does seem to be dependent on events triggered by the FGF signalling cascade since ER1 remained cytoplasmic in embryos injected with XFD, a dominant-negative FGFR, which shuts down the FGF signalling pathway. The pattern of nuclear translocation of ER1 is consistent with the same spatio-temporal pattern as the FGF signalling pathway during early development. FGF triggers several signal transduction cascades in the embryo; both the PI3' kinase and the ERK (MAPK) pathways are involved in mesoderm formation (Carballada *et al.*, 2001). Although some FGFs are expressed maternally, FGF signalling through MAPK is initiated at blastula stages (Christen & Slack, 1999). ERK activity is first detected at stage 8 in the embryo and at gastrula stages ERK is expressed in a ring around the newly formed mesoderm region in Xenopus embryos (Christen & Slack, 1999). ER1 is also first seen in nuclei of mesodermal cells at blastula stages (Luchman *et al.*, 1999).

Signal transduction cascades triggered by mitogens often result in events such as phosphorylation, dephosphorylation or protein-protein interaction and in turn may initiate nuclear translocation of certain proteins. For example, STATs are transcription factors that are involved in eliciting transcriptional effects in response to cytokine signalling. Nuclear translocation of STATs is dependent on receptor signalling by molecules such as EGF, PDGF or CSF-1, subsequent to which members of the Janus kinase (JAK) family are recruited to the receptor. The JAKs phosphorylate the cytoplasmic domains of the tyrosine kinase

receptors, which in turn recruit STAT members to the receptor via their SH2 domains. Once the STATs are receptor associated, they are phosphorylated by the JAKs on their essential tyrosine residues. Following phosphorylation, the STATs homo- or heterodimerize, and are translocated to the nucleus, where they can activate transcription (reviewed in Cartwright and Helin, 2000).

Several other proteins have been shown to localize to the nucleus following stimulation by mitogens. For example, in PC12 cells, the localization of the transcription factor FnIL-6 (which binds to the serum-response element (SRE) in the c-fos promoter in response to cAMP) changes following stimulation of cells with forskolin, which raises intracellular cAMP levels. FNIL-6 is normally located in the cytoplasm of untreated cells but becomes predominantly nuclear after treatment with forskolin. This re-distribution correlates with an increased phosphorylation of FNIL-6 (reviewed in Vandromme *et al.*, 1996).

Localization of four proteins involved in gene expression (c-fos and c-myc) or replication (DNA polymerase α and proliferating cell nuclear antigen (PCNA)) are subject to significant variation during cell cycle progression. In serum-starved NIH 3T3 cells, the proteins accumulate in the cytoplasm, whereas serum stimulation triggers nuclear translocation of c-fos and c-myc, followed by PCNA and DNA polymerase α (Vriz *et al.*, 1992). c-fos nuclear translocation is dependent on continuous stimulation of the cell with mitogens. It was shown that stimulation of quiescent cells with agents that raise intracellular cAMP levels

restored the nuclear translocation of c-fos in serum-starved cells (Roux *et al.*, 1990).

The phosphorylation results for ER1 demonstrate a link between nuclear translocation and changes in ER1-associated phosphoserylproteins and suggest that these changes are triggered by FGF signalling.

However, athough Phospho-antibodies provide a relatively simple and fast way to analyze phosphorylation *in vivo*, there are some inherent problems which have to be considered when using these antibodies. Phosphospecific antibodies do not always detect phosphorylation of certain proteins due to steric hindrance of the recognition sites. This is specially true for phosphothreonine and phosphoserine antibodies Kauffmann *et al.*, 2001). Furthermore, antibodies against phospho-serine and phospho-threonine are generally not specific enough to detect one phosphorylated serine or threonine side chain although they are specific for sequence motifs containing phospho-serine or phospho-threonine. This is because the epitope presented by a single phosphorylated serine or threonine side chain is too small (reviewed in Sickmann & Meyer, 2001; Sun *et al.*, 2001). The use of phospho-specific antibodies can also result in the isolation of non-phosphorylated species along with the phosphorylated species of interest (Conrads *et al.*, 2002). Detection of phosphorylation of ER1 or associated proteins may also have been limited by the relative abundance of the phosphorylated protein species; low levels of phosphorylated protein may not have been detected by the antibodies. Therefore, it is possible that, for the

reasons stated above, the phosphorylation profile of ER1 and proteins associated with it during nuclear localization has not yet been fully resolved.

Furthermore, the possibility that the critical event triggered by FGF signalling is interaction with a non-phosphoprotein that would thus have not been detected by the antibodies used cannot be ruled out. The results with ∝-amanitin suggest that these phosphoserylproteins are not zygotic proteins, but either maternal proteins or proteins translated from maternal message

The results presented in this chapter suggest that nuclear translocation of ER1 is regulated by changes in ER1-associated phosphoserylproteins, triggered by the FGF signalling pathway in *Xenopus* embryos.

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SECTION III INVESTIGATION OF THE FUNCTION OF ER1

CHAPTER 4 ANALYSIS OF PUTATIVE TRANSACTIVATION FUNCTION DOMAINS IN ER1 PROTEIN

Note: Part of this chapter was previously published in Paterno *et al.* (1997). The results presented here represent solely the thesis author's contribution to the manuscript.

4.1 INTRODUCTION

The N-terminus of ER1 includes several highly acidic stretches (Fig. 1) characteristic of the acidic activation domains of many transcription factors (Ptashne, 1988). ER1 also contains a proline-rich sequence near the C-terminus which corresponds to the PXXP motif found in all high affinity SH3-domain binding ligands (Cohen et al., 1995). As previously discussed in this thesis, a nuclear localization signal (NLS), ^{463}RPIKRQRMD^{472}, found to be necessary and sufficient for targeting to the nucleus, was identified near the carboxy terminus of ER1 (Post et al., 2001). These domains are highly characteristic of transcription factors.

Transcription factors or regulators fall into two classes, those that activate transcription (activators) and those that inhibit it (repressors). However, it has

become increasingly clear that there are several factors, that are capable of performing both these functions (Roberts and Green, 1995). Transcriptional activation domains, which are better characterized than transcription repression domains, have been loosely classed according to their amino acid composition (Hampsey, 1998; Orphanides *et al.*, 1996; Ptashne & Gann, 1997): regions rich in acidic amino acids, glutamine, or proline regions and bulky hydrophobic residues. Other that these general characteristics, activation domains do not show apparent sequence conservation (reviewed in Melcher, 2000). Typical examples of acidic activation domains are the potent activation domains of the herpes virus VP16 protein and yeast GAL4 protein (Cress & Triezenberg, 1990). Repression domains are less well characterized than activation domains (Johnson, 1995; Gashler *et al.*, 1993). A few of the known repression domains are rich in alanine (Licht et al., 1990), basic amino acids (Baniahmad et al., 1992) and prolines (Han & Manley, 1993; Ostling *et al.*, 1996; Madden *et al.*, 1991).

Transcription factors are synthesized in the cytoplasm and must translocate to the nucleus at some stage in order to potentially activate transcription. ER1 was shown to localize to the nuclei of *Xeno*pus embryos during certain developmental stages (chapter 2; Luchman et al., 1999). In this chapter, the ability of nuclear ER1 to intrinsically regulate transcription during *Xenopus* embryo development was investigated.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

NIH 3T3 cells (American type Culture Collection) were grown in tissue culture medium (13 g/L (w/v) Dulbecco's Modified Eagle Medium (DMEM, Invitrogen), 3.7 g/l (w/v) $NaHCO_3$, 10% (v/v) calf serum, 50 U/L Penicillin (Invitrogen Life Technologies Inc.), and 50µg/ml Streptomycin (Invitrogen Life Technologies Inc.) in dH_2O) at 37 °C, 10% CO_2. Cells were trypsinized (0.025% (v/v) trypsin (Invitrogen Life Technologies Inc.) in PBS/EDTA (137 mM NaCl (BDH), 2.7 mM KCl (Fisher Scientific), 1.4 mM KH_2PO_4 (Fisher Scientific), 8.1 mM $Na_2HPO_4 \cdot 7H_2O$ (Fisher Scientific), and 1mM EDTA (Fisher Scientific) in dH_2O)), passaged at a 1:5 dilution every three days, and plated into fresh 100 mm tissue culture dishes. Stocks of cells were frozen at -70°C in calf serum plus 10% (v/v) DMSO.

4.2.2 Plasmid construction

The expression vectors used were engineered to contain various regions of ER1 fused to the GAL4 BD (DNA binding domain) of the pM plasmid (CLONTECH) and are named according to the amino acids of ER1 that each encodes. Specific primers incorporating 5' and 3' *Bgl*II sites (ER 1-493 and ER 176-493) or a 5'*EcoR*1 and a 3' *Bam*H1 site (ER 1-175 and ER 1-25) were used

to amplify PCR fragments encoding the appropriate amino acids. The digested PCR fragments were inserted into the complementary sites of the pM plasmid, and all plasmids were sequenced to verify the insertion junctions, the *er1* sequence and the proper reading frame. ER 1-98 and ER 1-57 were generated by digesting the ER 1-175 construct with *Pst*I or *Pvu*II respectively, and re-ligating the largest fragments.

4.2.2 Transient transactivation assays

0.5 μg of a chloramphenicol acetyl transferase (CAT) reporter plasmid (pG5CAT, CLONTECH) was co-transfected into 3 x 10^5 cells with 1.0 μg of either the pM vector alone or one of the pM-*er1* fusion constructs using 12μl LipofectAmine reagent (Invitrogen Life Technologies Inc.) according to manufacturer's directions. After 48 h, cell extracts were prepared and assayed for CAT enzyme activity using a CAT enzyme-linked immunosorbent assay kit (Boehringer Manheim) according to the manufacturer's directions. The amount of CAT was normalized with the protein concentration.

4.2.3 Immunocytochemistry

For ER1 immunocytochemistry, NIH 3T3 cells were cultured and transfected with either 1μg of pcDNA3 vector (Invitrogen Life Technologies Inc.) or with 1μg *er1*-pcDNA3 using LipofectAmine as described above. Following 24h

transfection, cells were trypsinized with 0.025% trypsin in PBS/EDTA as described above and plated on chamber slides (Labtek, Nalge Nunc International). At 48h after transfection, the cells were fixed with 4% paraformaldehyde (Fisher Scientific) in PBS, pH7.2, and processed for immunocytochemistry using an anti-rabbit antibody detection system (Santa Cruz Biotechnology ABC staining system) as described below. The chamber slides were sequentially washed in PBS, incubated for 10 min in 0.2% Triton X-100 (Fisher Scientific) in PBS, incubated for 20 min in 1.5% normal goat serum in PBS, transferred to a 1:50 dilution of anti-ER1 antibody (Paterno *et al.*, 1997) in normal blocking serum, incubated at RT for two hours, washed in 0.2% Triton X-100 in PBS twice for 10 min each. The slides were incubated for 30 minutes at RT in normal blocking serum containing a 1:200 dilution of biotinylated goat-anti rabbit IgG secondary antibody, washed once with 0.1% Triton X-100 in PBS for 3 min and once with PBS for 3 min. The avidin-biotin-HRP complex from the detection kit was added to the slides for 30 min, followed by washing in PBS for 3 min. Detection was performed by DAB staining (Sigma-Aldrich) for 4-7 min or until a brown colour developed. The gasket was then removed from the slides and the slides were mounted in a 10% glycerol in PBS solution and viewed under light compound microscope.

4.2.4 Protein analysis

For Western blotting, transiently transfected cells were lysed in the CAT lysis buffer (Boeringher Manheim) on ice for 30 minutes. The protein extracts were centrifuged at 4^0C to pellet the insoluble proteins. The soluble proteins in the supernatant were precipitated with 100% acetone on ice for 25 minutes followed by centrifugation at 10,000*g* for 20 minutes. Protein pellets were washed with 70% acetone, dried under vacuum and prepared for SDS-polyacrylamide gel electrophoresis as described Chapter 2. Protein levels were standardized with the Bio-Rad assay kit as per the manufacturer's directions and subjected to electrophoresis and equal amounts of total protein (200μg) was loaded in each well in the gels. Western blots were stained with a 1:1000 dilution of anti-ER1 antiserum (Paterno *et al.*, 1997) or a 1:500 dilution of anti-pGAL4 antibody (UBI) as described in Chapter 2 and Western blots were analyzed using the Chemicon detection system (Chemicon International Inc.).

4.3 RESULTS

Subcellular localization of the ER1 protein was verified using a polyclonal anti-ER1 antibody to stain transfected NIH 3T3 cells. To test whether ER1 contains transactivation activity, ER1 protein was fused to the DNA-binding domain of yeast GAL-4 (GAL-4 BD) and the effect of the fusion on CAT reporter activity was examined. Furthermore, to identify domains in ER1 that may

mediate its transcriptional effect, deletion mutations of full length ER1 cDNA were constructed and fused to GAL-4 DNA binding domain.

4.3.1 ER1 localizes to the nucleus in transiently transfected cells

Anti-ER1 antibody, directed against a synthetic C-terminal peptide, recognizes full-length ER1 protein synthesized *in vitro* (Paterno *et al.*, 1997) and specifically stains the nuclei of cells expressing ER1 (Fig. 4.1 B). Cells transfected with the pcDNA3 vector alone (Fig. 4.1 A) as well as pcDNA3-*er1* transfected cells stained with pre-immune serum (data not shown) gave similar patterns and no specific nuclear staining.

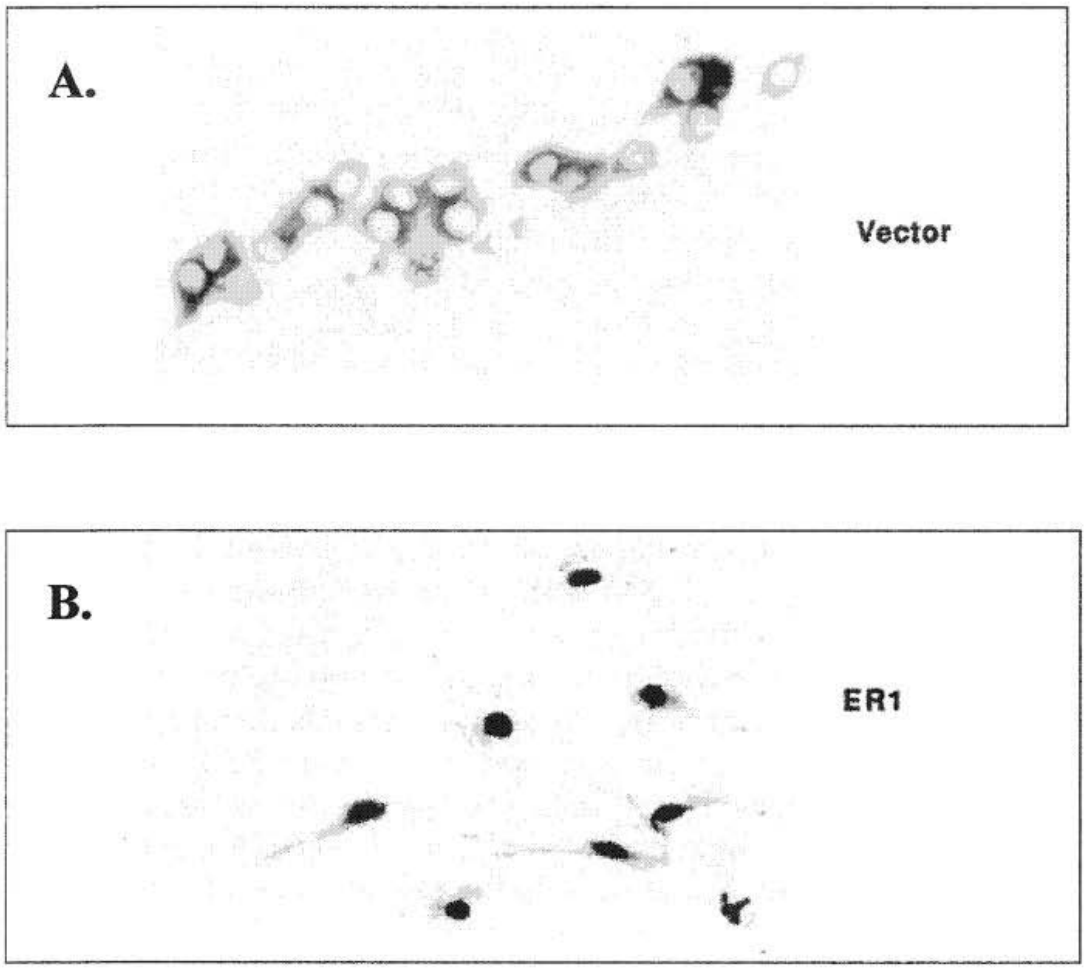

Figure 4.1 Nuclear localization of ER1 in transiently transfected NIH 3T3 cells.

NIH 3T3 cells were transfected with either the pcDNA3 vector alone (A) or *er*1-pcDNA3 (B). After 48 h, cells were fixed and stained with anti-ER1 as described in the "Materials and Methods" section. ER1 is localized within the nucleus in transfected NIH 3T3 cells (B).

4.3.2 ER1 has transactivation activity.

The fact that ER1 is targeted to the nucleus and that its N-terminus contains stretches of acidic residues characteristic of acidic activation domains (Paterno *et al.*, 1997), suggests that ER1 may function as a transcription factor. This hypothesis was investigated by testing the transactivation potential of various regions of the ER1 protein. Constructs, containing different regions of *er1* fused to the GAL4 DNA binding domain, were used along with a CAT reporter plasmid in transient transfections. Assays of CAT enzyme levels revealed that, although full-length ER1 did not activate transcription, the N-terminal region (ER 175), containing all four acidic amino acid regions stimulated transcription 10-fold (Fig. 4.2). The complementary C-terminal portion, ER 176-493, on the other hand, had no transactivational activity. Interestingly, deletion of the fourth acidic stretch to produce a construct containing only the first three acidic stretches (ER 1-98), resulted in a much more potent transactivator that stimulated transcription 80-fold (Fig. 4.3). These results suggest the presence of a negative regulatory region in amino acids 99-175. Further truncation of the N-terminus to generate ER 1-57 and ER 1-25 completely abolished transactivation. These results demonstrate that the N-terminus of the ER1 protein contains regions with transcription transactivating activity and that ER1 has the potential to function as a transcription factor.

In addition to the previously published results, (Paterno *et al.*, 1997) described in section 4.3.2, transient transfections and CAT assays were performed with a deletion construct lacking the fourth acidic stretch (ERΔ99-175). This

construct did not give any detectable CAT activity, although it lacked the putative negative regulatory region (amino acids 99-175). This additional result suggests that there may be additional negatively acting domain/s in the C-terminal portion of ER1. This idea is also supported by the fact that full-length ER1 does not activate transcription.

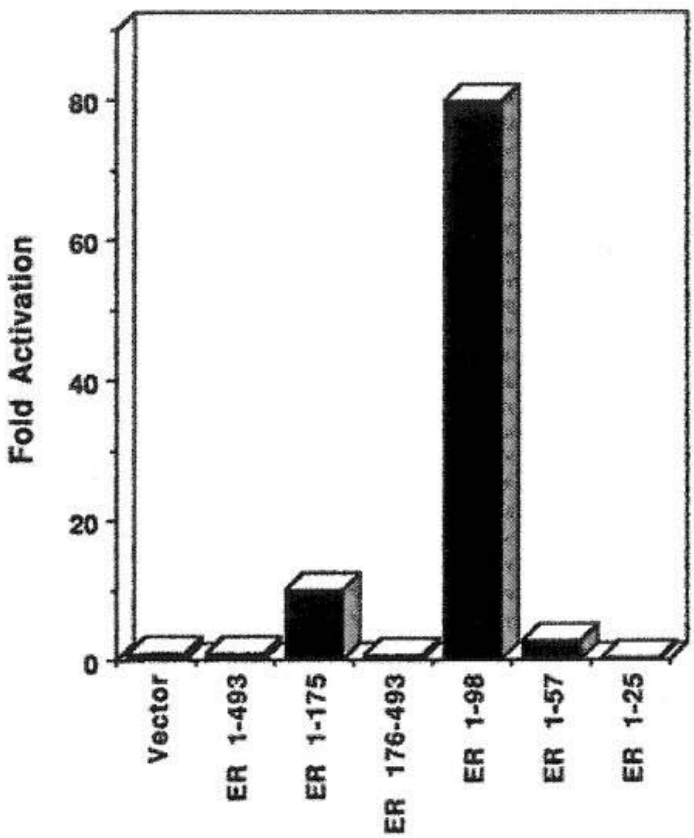

Figure 4.2 The N terminus of ER1 functions as a transcriptional activator. NIH 3T3 cells were transiently transfected with various GAL4-ER1 fusion constructs along with a CAT reporter plasmid. After 48 h, CAT enzyme levels were measured as described in the "Materials and Methods" section. Vector denotes the control pM plasmid, containing only the GAL4 DNA binding domain, whereas the numbers indicate the amino acids of ER1 encoded by each construct. The value for each construct represents the fold activation relative to the pM plasmid, averaged from 3 to 12 independent transfections.

In order to verify that the differences in fold transactivation of the different constructs were not due to unequal translation of the different constructs, equal amounts of total protein from NIH 3T3 cells transfected with the different constructs were resolved on an SDS-PAGE gel, transferred to nitrocellulose membrane and stained with anti-pGAL4 antibody. The Western blot shows that the different constructs yield varying amounts of fusion protein (Figure 4.3). The highest level of protein detected was for the full-length protein (ER 1-493), which does not have any transactivation activity in the CAT assay. Less protein was detected for ER 1-175, which activates transcription 10-fold, than for the full-length protein. Surprisingly, no protein was detected for ER 1-98 (lane 6), which has the highest transactivation activity in the CAT assay (Figure 4.2). In contrast, protein was detected for both ER 176-493 (lane 3) and ERΔ99-175 (lane 7), neither of which activates transcription. No protein was detected for ER 1-25 (lane 4), ER 1-57 (lane 5), or for the control GAL4 vector (lane 8). Although these results do show unequal expression of the different fusion constructs, it was not possible to determine whether the difference was due to unequal translation of the different constructs or to an detection problem since the fusion construct that gave the highest transactivation activity (ER 1-98) was not detected on the blot.

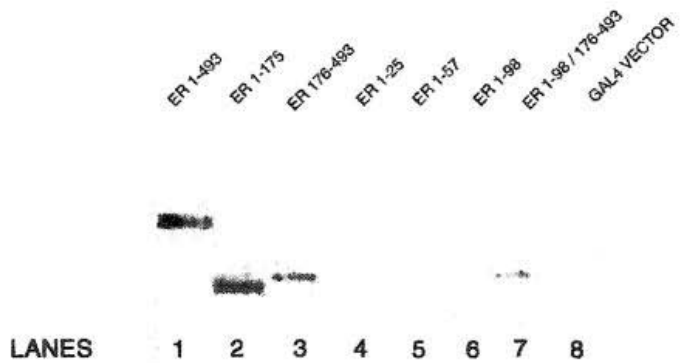

Figure 4.3 **Protein expression levels of transiently transfected ER1 constructs**
NIH 3T3 cells were transiently transfected with various GAL4-ER1 fusion constructs. After 48 h, total protein was extracted and prepared for Western blotting as described in the "Materials and Methods" section. The Western blot was stained with anti-pGAL4 (lanes 1-8). The protein loaded in each lane is indicated on the top.

4.4 DISCUSSION

The GAL4 DNA binding fusion protein has been used extensively to define the transcriptional activation domains of numerous proteins (Tolnay *et* al., 2000; Weintraub *et al.*, 1991; Nielsen *et al.*, 1992; Sadowski *et al.*, 1988; Sadowski & Ptashne, 1989). Since no mammalian protein binds to the GAL-4 recognition sites in the reporter plasmid, determination of the potential transactivating activity of the fused protein being tested is possible.

Several studies have used pGAL4 BD fusion constructs in combination with the CAT assay to define transactivation activity. Activity is measured as the fold-activation of the CAT enzyme compared with that of the empty pGAL4 vector. For example, the activation domain of thyroid receptor β1 activates transcription 40-fold (Wilkinson & Towles, 1997); Smad4, which mediates the transcriptional activation of target genes of the TGF-β signalling pathway, activates transcription of CAT 25-fold (Shioda *et al.*, 1998); the activation domain of hEZF activates transcription 50-fold (Yet et al., 1998). Other studies (Yet *et al.*, 1998) have reported much higher fold-activation, in the 200 to 300-fold range, but these numbers were obtained after normalization for transfection efficiency and protein expression. From the CAT assay kit, the HBx trans-activating factor, which stimulates the transcription of several cellular and viral genes, was tested as a positive control for detection of transactivation. The CAT assay kit provides

a four- to nine-fold increase in the steady state level of CAT protein was detected in the presence of HBx.

Full-length ER1 protein did not up-regulate transcription but the N-terminal portion of ER1 (ER 1-175) consisting of all four acidic stretches up-regulated transcription 10-fold and the first three acidic (ER 1-98) stretches 80-fold, showing the presence of an transactivation domain in ER1. These results also suggest that a negatively acting domain is located between amino acids 99-176. It is unclear why full-length ER1 was unable to stimulate transcription, but one possible explanation is that fusion of ER1 to GAL4 may alter the tertiary structure of the ER1 protein, affecting its activity. A similar observation was made with the ETS transcription factor ER81 fused to the GAL-4 BD, which lost its ability to activate transcription (Janknecht, 1996) Other proteins have also been identified for which deletion mutants encoding the activation domains have higher activity than the full-length proteins. For example, N-terminal deletion mutants of the NS5A protein have strong transcriptional activity when fused to the GAL-4 BD, whereas full length NS5A does not (Tanimoto et al., 1997). Although full-length ATF-2 protein fused to the GAL-4 BD is inactive, a chimeric protein containing the ATF-2 N-terminus fused to GAL-4 BD can stimulate the expression of a GAL-4 dependent reporter (Livingstone et al., 1995). These results are probably due to the masking of the activation domains in full-length proteins by inhibitory domains.

Another possibility is that ER1 may need to interact with other protein/s to become transcriptionally active or to expose the activation domains. This has been shown to be the case for NK-4, for which NK-4 dependent transactivation is augmented after physical interaction with the p300 co-activator (Choi et al., 1999).

Furthermore, transcription factors often have inhibitory regions masking their activation domains. Activation and inhibitory domains were identified in the C-terminal region of PEBP2αB1. The inhibitory domain was contained within a 40 amino acid region and was located next to the activation domain, keeping the full transactivation potential of the full length protein below its optimal level, probably through intramolecular masking of the activation domain (Yagi et al., 1999). IRF-1 has both transactivating domains and inhibitory domains. In addition to inhibiting transactivation activity by the adjacent activation domain in the IRF-1 protein, one of the inhibitory domains, when fused to the strong activator VP-16, reduced the transcriptional activity of the latter by 60%. The authors suggest several models through which inhibitory domains could potentially act: 1. Inhibitory domains can come in physical contact with the activator domain, thereby blocking its activity, 2. The inhibitory domain could also be interacting with the target molecules of the activation domains such as TAFs or other components of the transcription machinery and 3. The inhibitory domain may modify the enzymatic activity of stimulators involved in the transcription

activation process. *In vivo*, inhibitory activity is probably blocked by processes such as phosphorylation or protein-protein interaction.

ER1-98 activates transcription 80-fold, but ER1-175 only 10-fold. Therefore, the region containing the putative inhibitory domain (99-176) was deleted to test for transcriptional activity in the absence of the inhibitory region. However, a deletion construct (ERΔ99-175) fused to the GAL4 BD provided no transactivational activity. These results may indicate the presence of further negative regulatory regions in the C-terminal portion of ER1 or once again may be due to conformational changes created by fusion to GAL BD.

It is important to note that the use of truncated proteins to identify functionally important regions always involves uncertainty about the proper folding and conformation of the truncated protein. Removal of one part of a protein may affect the thermodynamic stability of the protein or alter the conformation of more distant regions (Tolnay et al., 2000).

Furthermore, deletion constructs are often not translated at the same levels or the protein products are not equally stable. Western blotting was therefore used to analyze the levels of protein expressed by the different constructs (Fig. 4.3).

Protein was detected for the N-terminal portion encoding all four acidic stretches of ER1 (1-176) and which activates transactivation 10-fold. The Western blot detected protein for the GAL-4 BD fusions with full length ER1 (1-493), the C-terminal portion (176-493) and ERΔ99-175, none of which have transactivation

activity. The Western blot (Fig. 4.3) failed to detect protein for the assays involving ER 1-25, ER 1-78 and ER 1-98, although the latter gave the highest activity in the transactivation assay.

It is probable that the CAT reporter assay detects activity by proteins that are present at too low levels to be detected by Western blotting. Full-length coding regions of Id proteins (Id1, Id2, Id3 and Id4) all activate transcription of a GAL-4-responsive reporter gene when fused to GAL-4 BD (Bounpheng *et al.*, 1999). However, Western blot analysis failed to detect GAL-4-Id3 protein even though it had strong transactivation activity. In the case of ER1, however, since the Western blot also failed to detect the control GAL4 protein, detection limitations cannot be excluded. At the time these experiments were done, the only anti-ER1 antibody available was unable to detect most of the fusion constructs. Most of the deletion constructs lacked the C-terminus portion of the ER1 protein and the antibody was made against a synthetic peptide encoding part of the C-terminal region of ER1 (Paterno *et al.*, 1997). Recently, a new anti-ER1 antibody that was prepared against the full protein has become available. It will be interesting to stain the Western blot with this new antibody and determine whether the staining patterns are repeated.

In view of the current Western blotting results, it is possible that the two fusion constructs for which protein was not detected, ER 1-25 and ER 1-57, also have transactivation activity and that activity was not detected because protein was

not produced or the proteins were not adequately stable and are therefore unable to activate transcription of the reporter gene.

It has been found that acidic activation domains can act synergistically, such as for the transcription activation domain of RelA, which contains several acidic modules (Blair et al., 1994). The first acidic module of RelA fused to the DNA-binding region is inactive by itself but a GAL4 fusion protein bearing two or more of the acidic modules is a very efficient activator of the reporter gene, even though all the fusion constructs express protein at the same levels (Blair et al., 1994). No consensus sequences other than clusters of acidic residues giving a net negative charge have been found to be common for acidic activation domains. However, it has been shown that bulky hydrophobic amino acids, such as phenylalanine, play a critical role in increasing the efficiency of transactivation by the activation domains of transcriptions factors such as VP16, GAL4, GCN4, Jun, Fos, and RelA (Cress & Triezenberg, 1991; Regier *et al.*, 1993; Hope *et al.*, 1988; Ma & Ptashne, 1987; Sutherland *et al.*, 1992; Blair *et al.*, 1994). In the case of ER1, the second acidic domain contains a phenylalanine residue bracketed by acidic amino acids (EFDDEOTLEEEEMLEGE). Therefore, it is possible that the first three acidic domains of ER1 or even all four (since the fusion encoding all four domains does activate transactivation 10-fold) are all required for transactivation activity.

As discussed above, the activity of inhibitory regions can be modulated through phosphorylation or protein-protein interactions. In the native protein, folding in the proper configuration would ensure that the activation domains would

act in a synergistic fashion and activate transcription. To further define the specific amino acids that are sufficient for the activation domain, further deletions will have to be constructed and analyzed. This could prove to be difficult, as activation domains can be partially deleted and have only a gradual loss of activity (Hope *et al.*, 1988; Triezenberg *et al.*, 1988).

It is important to note that the pGAL4 system can give false positives and that it is an artificial system. Non-transcriptional proteins can function as transcriptional activators when fused to a DNA-binding domain as baits in two-hybrid screens (Bartel *et al.*, 1993; Warbrick, 1997) and about 0.1-1% of random *Escherichia coli* peptides or random synthetic peptides (Ma & Ptashne, 1987) can substitute as activation domains when tethered to DNA. The ability of ER1 to function as a transcription factor is therefore being confirmed through other projects in the laboratory, listed below.

The experiments described in this chapter have shown that ER1 localizes to the nucleus and have provided support for ER1 functioning as a transcription factor. Other ongoing projects in the lab are investigating DNA-binding ability and protein-protein interactions in ER1, other key characteristics of transcription factors. ER1 has been shown to have consensus sequences for protein-protein interactions (Paterno *et al.*, 1997). Results demonstrating DNA-binding activity and/or interaction with proteins associated with the transcription machinery will help confirm transcriptional activity and determine which genes are transactivated by ER1.

The activation domain identified in ER1 (ER 1-98) stimulated high levels of transactivation activity in the CAT assay (80-fold) and coupled with intrinsic translocation to the nucleus of the full protein, it is highly probable that ER1 functions as a transcription factor *in vivo*. The investigation of the function of ER1 during early development in *Xenopus* embryos, will be described and discussed in Chapter 5.

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CHAPTER 5 FUNCTIONAL CHARACTERIZATION OF *er1* BY ITS OVEREXPRESSION XENOPUS EMBRYOS

5.1 INTRODUCTION

A common technique used to elucidate the function of genes and gene products in developmental and other studies is to over- or under-express them *in vivo*. The most effective technique developed to date for manipulating gene expression in *Xenopus* is the injection of cRNA synthesized from the cDNA of cloned genes. In over-expression studies, cRNA is injected shortly following fertization of embryos and its effect on development is followed to detect morphological abnormalities. The function of a protein in particular pathways can be analysed by looking examining for changes in the expression of molecular markers representative of particular tissues. Alternatively, the markers maybe expressed at specific times. By misregulating a gene and studying the effects, the functions of the gene and its product and the signalling pathways in which the latter is involved can be defined.

5.2 MATERIALS AND METHODS

5.2.1 RNA injection

er1 and XFD cRNA was prepared using the Ribomax large scale RNA production system (Promega Corporation), as described in Section 3.2.2 and dissolved in DEPC-treated H_2O. Embryos were injected with DEPC-treated dH_2O or 10ng, 5ng, 2ng, 1ng and 0.5ng *er1* cRNA in the marginal zone region at two-cell stage adjacent to the cleavage furrow. Each experimental condition included 40-60 embryos and experiments were repeated at least five times. Morphology differences between control and experimental conditions were scored and recorded by photographs using a RS Photometrics digital camera,attached to a SZ PT Olympus dissecting microscope. Embryos were staged (Nieuwkoop, 1994) and deviations from normal development were scored. Student T-tests were used to anlyze the numbers.

5.2.2 Total RNA extraction and reverse transcription

Embryos were injected at the two-cell stage, as described above, with DEPC-treated H_2O, 5ng *er1* cRNA or 5ng XFD cRNA respectively. Five whole embryos were transferred to 1.5ml Eppendorf tubes containing 1ml of Tri-Reagent (Life Technologies, Inc.) and homogenized with a micropipette. Embryos were collected at stage 10.5, 11.5 and 15. At stage 10.5, since there

were no morphology differences in the embryos in the three experimental groups and embryos were randomly selected for total RNA extraction. At stages 11.5 and 15, *er1* and XFD-injected embryos with gastrulation defects were used for RNA extraction. RNA extraction was performed as described in section 3.2.4.1. The concentration of the RNA was assayed before use by a combination of absorbance readings at 260nm (OD_{260}) and agarose gel electrophoresis. RT of the RNA samples was performed as described in Chapter 3.

5.2.3 RT-PCR

Table 5.1 lists forward and reverse primers used for molecular marker analysis. The number of amplification cycles used for each primer set is also shown in Table 5.1. PCR reactions for each primer pair were optimized so that amplifications cycle numbers were in the linear range. Reactions were performed with increasing numbers of cycles and resolved on electrophoresis gels. The gels were exposed to autoradiography films, which were examined by computer scanning densitometry. The cycle numbers were plotted against the densitometry values to determine the linear and plateau phases.

Table 5.1 List of primer pairs used for PCR reactions

Primer	Primer Sequence	Expected Size of Product	Number of PCR cycles	Reference
BMP-4F BMP-4R	5'GCATGTAAGGATAAGTCGATC3' 5'GATCTCAGACTCAACGGCAC3'	500 bp	26	Koster *et al.*, 1991
H4-F H4-R	5'CGGGATAACATTCAGGGTATCACT3' 5'ATCCATGGCGGTAACTGTCTTCCT3'	191 bp	22	Niehrs *et al.*, 1994
HoxB9-F HoxB9-R	5'GAGGCCACAGTGTAATGTTGG3' 5'ATTCCGCTCTGCGCAATTCCC3'	269 bp	26	Wright *et al.*, 1990
GSC-F GSC-R	5'GAGCAAAGTGGAGGAGGCAG3' 5'CCCACATCGTGGCACTGCTG3'	207 bp	30	Cho *et al.*, 1991
Xbra-F Xbra-R	5'CAAGGATCGTTATCACCTCTG3' 5'TGTGTAGTCTGTAGCAGCAG-3'	187 bp	26	Smith *et al.*, 1991
Xpo-F Xpo-R	5'CACTTAGGGATTGGTCTCAGGAGTC3' 5'TGAGGGAGGGCTATGGTCTAGG3'	500 bp	24	Sato *et al.*, 1991

F, Forward; R, Reverse

5.3 RESULTS

5.3.1 Over-expression of ER1 leads to posterior truncations in the embryo.

Injecting H_2O provides a control for the injection procedure and provides background levels for abnormalities arising from injection wounds. Injecting RNA controls is important to monitor morphological abnormalities that may be a factor when injecting non-physiological amounts of RNA. These samples require controls for contaminants, which may be present after the *in vitro* synthesis of the RNA. An appropriate RNA control was not available for *er1* when these experiments were done. A control *er1* RNA with a mutated start ATG codon was subsequently generated; injection of this RNA, at doses of 10ng or lower, generated only morphological abnormalities similar to those observed upon injecting DEPC-treated H_2O.

Further dose/response experiments are required to account for abnormalities due to injecting large amounts of RNA. In cases in which the phenotype is weak, hundreds of surviving embryos need to be examined in order to obtain an accurate representation of the injected embryo phenotype (Vize *et al.*, 1991).

er1 cRNA was microinjected into fertilized eggs at two-cell stage in the marginal zone region and the embryos were compared with control DEPC-treated H_2O injected embryos for their ability to develop into normal tadpoles. In the injection experiments, 0.5-10ng of RNA was used, and the injection series was

repeated at least 5 times. A total of 40-60 embryos were injected per condition in each experiment. These measures ensured that the phenotype observed upon injecting *er1* was due to the RNA rather than a non-specific effect.

Injection of RNA at high concentrations (10 ng) resulted in death of 90% of RNA-injected embryos at late gastrula stages. Embryos injected with RNA at concentrations of 0.5ng and lower did not show any significant abnormalities over control embryos. Figure 5.1 shows the percentage of abnormal embryos in the experimental series injected with DEPC-treated H_2O, 1 and 2ng *er1* respectively. 5% of DEPC-treated H_2O, 64% of embryos injected with 2ng *er1* and, 32% of embryos injected with 1ng *er1* developed abnormally. Student T-tests showed that these values were different from each other at 1% significance level (Table 5.2).

Embryos developed normally during early cleavage stages. Abnormalities in morphology were first observed at gastrulation stages 10-12h post-injection; gastrulation was incomplete, leaving an enlarged open blastopore and protruding yolk plugs. Embryos were scored at stage 36, three days after fertilization. Abnormalities were seen in 59% of embryos which were primarily posterior truncations resulting in normal heads but little or no tails (Figure 5.3). The remaining 5% of the embryos that developed abnormally had no truncations but displayed abnormalities such as split tails and bodies, enlarged heads, elongated shapes and poorly defined organs and tissues. In the control DEPC-treated H_2O injected, group, 5% of the embryos developed abnormally. Half of these abnormal

embryos failed to gastrulate normally and developed minor posterior abnormalities, such as kinked tails and/or spines, and mild posterior truncations. The remainder of the abnormal embryos in the control group had enlarged or poorly defined heads. The fact that the abnormal phenotype of the embryos injected with different doses of *er1* cRNA gave primarily one type of phenotype, posterior truncations as opposed to many different abnormalities seen in the controls, indicates that the phenotype is *er1*-specific.

In the injection experiments described above, the phenotype of the *er1* cRNA injected embryos was observed to be similar to the phenotype of embryos injected with a dominant negative FGFR, XFD cRNA, previously described in the literature (Amaya et al., 1991). Furthermore, since the levels of *er1* RNA had previously been found to increase in response to FGF treatment (Paterno et al., 1997), indicating the ER1 is on the FGF signalling pathway. Therefore, it was highly interesting to investigate this similarity further and determine the role that ER1 might play in the FGF signalling pathway. To follow this interest, XFD and *er1* RNA were injected in embryos for comparison purposes at both the morphological and molecular levels.

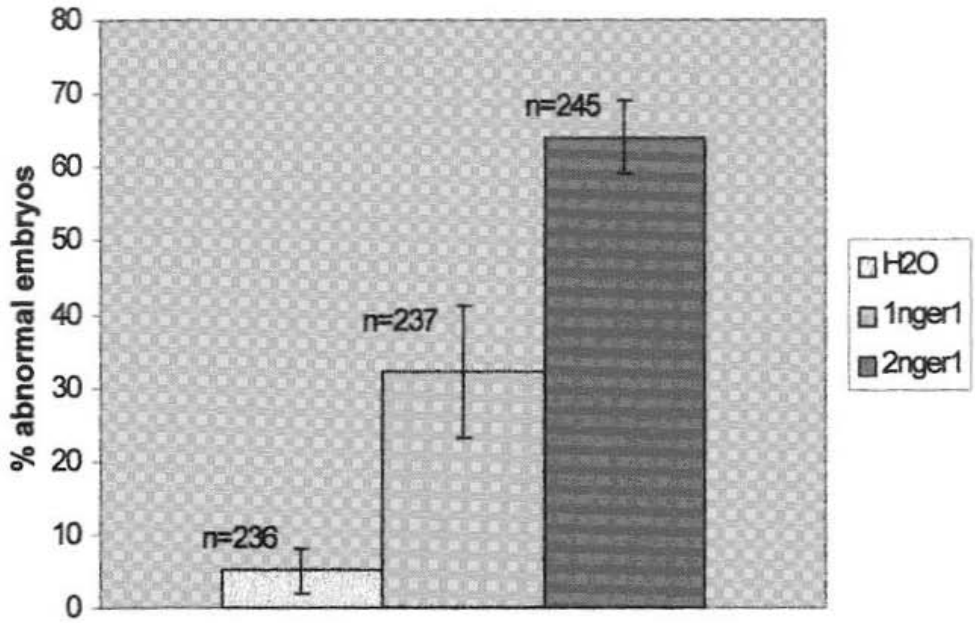

Figure 5.1 Over-expression of *er1* cRNA results in abnormal embryos

Embryos were injected with DEPC-treated dH_2O (control) or *er1* cRNA (2ng or 1ng) in the marginal zone region at two-cell stage as described in the "Materials and Methods" section. After 72h at room temperature, embryos were scored for abnormal phenotype; the percentage is based on total number (n) of embryos injected. Abnormal embryos in the *er1*-injected groups failed to complete gastrulation normally and as a result had heads but little or no tails (posterior truncations). The total number of embryos injected and standard deviation values (error bars) of six individual experiments are shown.

Table 5.2 Statistical analysis methods for results from *er1*-injected embryos.

The Student T-test was performed at the 1% significance level (Critical T-value = -4.604) and the following algorithm was used:

H0 : Mean of Test1 = Mean of Test2
H1 : Mean of Test1 ≠ Mean of Test2

Injections	Mean values	T-value	Interpretations
DEPC-treated H_2O	0.05 (Test 1)	-6.97	Hypothesis is rejected at the1% significance level.
1ng *er1*	0.32 (Test 2)		Mean of Test 1 is significantly different from mean of Test 2
DEPC-treated H_2O	0.05 (Test 1)	-24.78	Hypothesis is rejected at the1% significance level.
2ng *er1*	0.64 (Test 2)		Mean of Test 1 is significantly different from mean of Test 2
1ng *er1*	0.32 (Test 1)	-7.61	Hypothesis is rejected at the1% significance level.
2ng *er1*	0.64 (Test 2)		Mean of Test 1 is significantly different from mean of Test 2

Embryos were injected with either 2ng *er1* or 2ng XFD cRNA and the phenotypes of embryos were then scored. The abnormal embryos in both the *er1*- and XFD-injected groups failed to gastrulate properly, which resulted mainly in posterior truncations. The abnormal phenotypes from injecting either were highly similar (Figure 5.3). The percentage abnormal embryos in each group were compared. 59% of the embryos injected with *er1* cRNA and 62% of the embryos injected with XFD had posterior truncations. These values were not significantly different at the 1% significance level (critical T-value = -4.604; Test T-value = -0.47 at $\alpha = 0.01$).

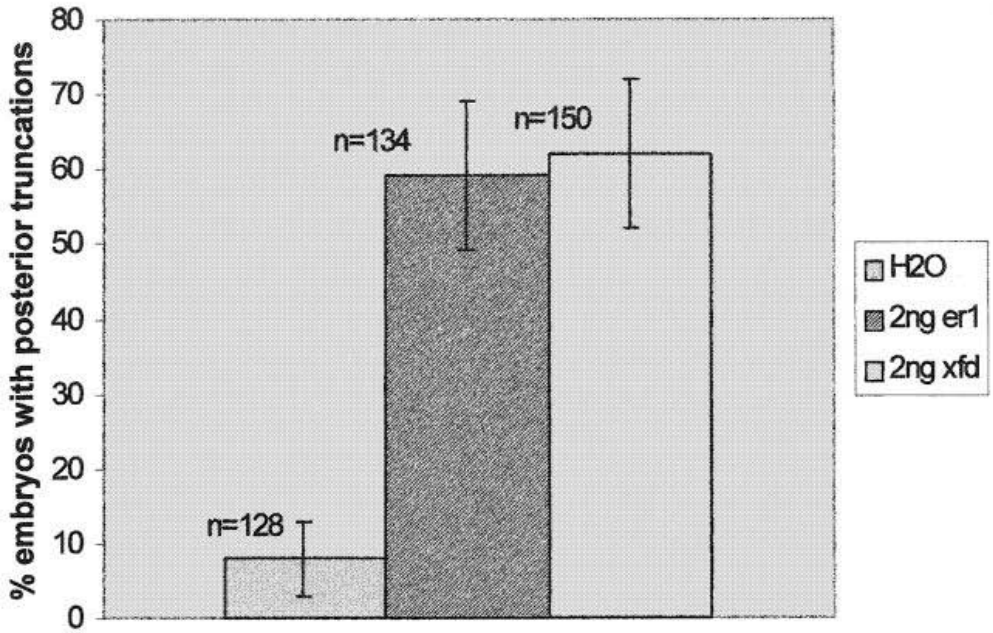

Figure 5.2 Overexpression of *er1* and XFD cRNAs result in similar percentages of embryos with posterior truncations.

Embryos were injected with DEPC-treated dH_2O (control), 2ng *er1* cRNA or 2ng XFD cRNA in the marginal zone region at two-cell stage as described in the "Materials and Methods" section. Embryos were left to develop for 72h at room temperature and scored for abnormalities. Both *er1*- and XFD-injected embryos failed to gastrulate properly resulting in posterior truncations (normal heads but little or no tails). Values were determined as the percentage of number of embryos injected. Error bars represent the mean (+/-) standard deviation (error bars) of four individual experiments.

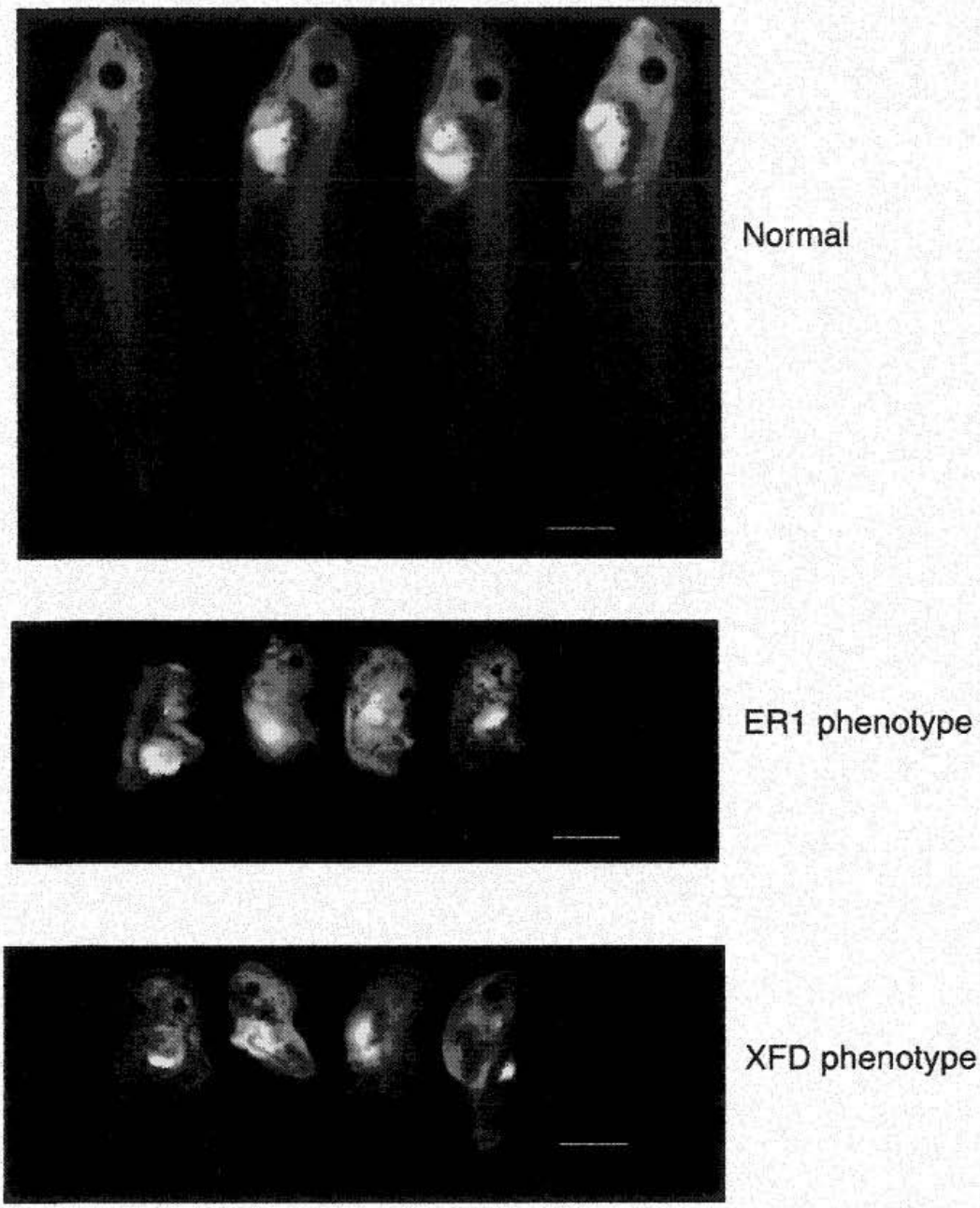

Figure 5.3 Effects of ER1 over-expression on embryonic development
Embryos were injected with 2ng *er1* cRNA or 2ng XFD cRNA, fixed in MEMFA after 72h and photographed. Examples are shown of embryos, which develop normally (normal) and of the posterior truncations observed in *er1*-injected embryos (ER1 phenotype) a nd XFD-injected embryos (XFD phenotype). Scale bar represents 0.1 mm.

5.3.2 Over-expression of ER1 during *Xenopus* development causes changes in the expression levels of early molecular markers.

Molecular markers were chosen to compare the effect of ER1 to the effects of XFD, which has been previously characterized (Amaya et al., 1993) on representative posterior mesodermal, general mesodermal and anterior mesodermal markers during early development. Embryos were injected with 5ng of RNA to ensure that there was an abnormal phenotype in the majority of the embryos injected since injections of 2ng of RNA resulted in abnormal phenotype in only approximately 60% of injected embryos (Figure 5.2).

The results presented here are preliminary results and the number of molecular markers examined was small. However, the effect of XFD overexpression on the chosen molecular markers has been well characterized and the primary objective of these experiments was to establish whether there was a similarity at the molecular level between ER1 and XFD, since similar phenotypes were observed upon overexpression of both ER1 and XFD. These experiments were used to establish a working model for further experiments examining the relationship between ER1 and FGF signal transduction.

The expression levels of the general mesodermal marker *Xbra*, posterior markers BMP-4, *Xpo,* and *HoxB9*, and the early anterior marker *goosecoid* in *er1* and XFD-injected embryos were examined. The effect of XFD on these markers has been previously described in the literature (Amaya *et al.*, 1993; Isaacs *et al.*,

1994; Pownall *et al.*,1996; Holawacz and Sokol, 1999)and was therefore useful for comparisons to the effects of *er1*. Figure 5.4 shows the expression patterns of *Xbra*, BMP-4, *HoxB9*, *Xpo*, and *Goosecoid* in embryos injected with DEPC-treated H_2O, *er1* or XFD cRNAs during subsequent development at stages 10.5, 11.5 and 15. *Histone*, H4, was used as an internal and gel loading control.

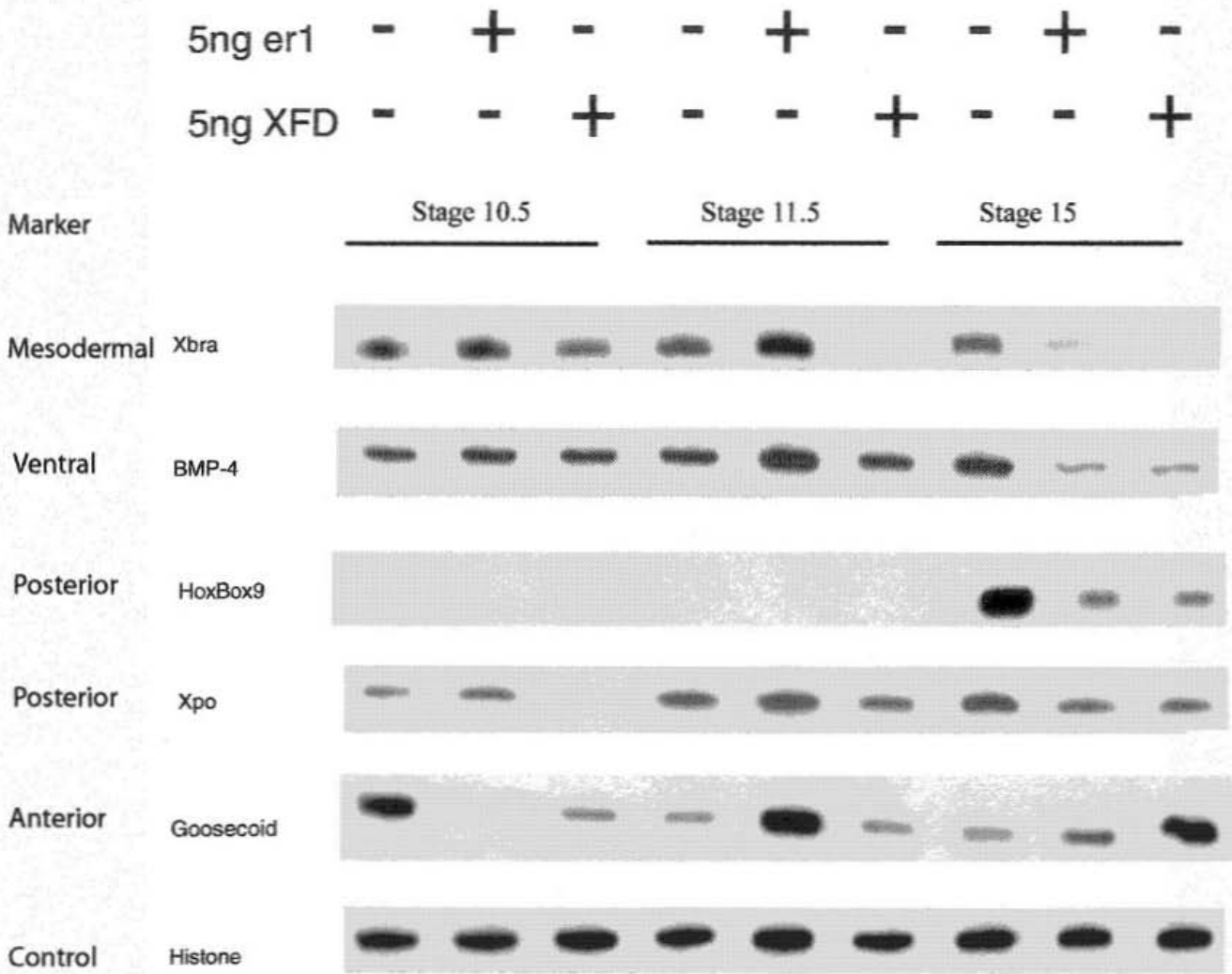

Figure 5.4 **Effects of overexpressing *er1*** and XFD on molecular markers Embryos were injected with DEPC-treated H_2O, 5ng *er1* (cRNA or 5ng XFD cRNA and RT-PCR was performed on RNAs extracted at stages 10.5, 11.5 and 14.

5.3.3.1 *Er1* down-regulates the expression of the mesodermal marker Xbra

The *Xenopus* brachyury gene (*Xbra*) is expressed throughout the marginal zone and serves as a general mesodermal marker (Smith et al., 1991). *Xbra* transcripts first appear in *Xenopus* embryos at the mid-blastula transition. The highest level of *Xbra* expression is at gastrula stages in the presumptive mesodermal cells around the blastopore lip. The level of expression starts to decline at the end of gastrulation but *Xbra* expression persists in the notochord during neurula stages.

Xbra is induced by either activin A or FGF-2 in animal caps in a cycloheximide-independent manner, suggesting that it is a general early mesodermal response gene for mesodermal induction (Smith et al., 1991). It has been found that the expression of *Xbra* is regulated by eFGF during development in *Xenopus* embryos (Isaacs et al., 1994) and *Xbra* levels were up-regulated in eFGF-injected embryos (Pownall et al., 1996). *In situ* hybridization techniques showed that embryos displayed a complete circle of *Xbra* expression throughout the marginal zone whereas XFD embryos lacked *Xbra* expression in a quarter to half of the marginal zone (Amaya et al., 1993). The RT-PCR results presented here (Fig. 5.4) are consistent with results reported for XFD in the literature; *Xbra* expression was lower than for control embryos in XFD-injected embryos at stage 10.5 and there was a complete lack of *Xbra* expression at stage 11.5 and 15 in

XFD-injected embryos. Relative to histone control, *Xbra* expression was similar to control H_2O-injected embryos in *er1*-injected embryos at stage 10.5 and 11.5 but was much lower than in control embryos at stage 15. Therefore, both XFD and ER1 over-expression were found to result in the down-regulation of *Xbra* expression. Previous experiments have shown that *Xbra* is necessary for the formation of posterior mesoderm (Conlon *et al.*, 1996; Cuncliffe and Smith, 1992). The RT-PCR results described here suggest that ER1 is inhibiting *Xbra* expression or is causing a premature decrease in *Xbra* expression level and thus acts as a negative regulator of the FGF signalling pathway during mesoderm induction.

5.3.3.2 *Er1* down-regulates the expression of the ventral marker BMP-4.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) family, which play diverse roles in embryonic development (reviewed in Dale & Jones, 1999). BMPs have been shown to be involved in both mesoderm induction and dorsoventral patterning (reviewed in Dale & Jones, 1999). BMP-4 transcripts are localized in the ventral and lateral regions of the gastrula stage embryo, and are absent from the dorsal side (Fainsod et al., 1994;Schmidt et al., 1995). Studies in *Xenopus* have identified a number of inhibitory proteins encoded by genes such as *chordin* and *noggin,* which bind to BMP-4, establishing a morphogen gradient of BMP-4 activity, which

specifies different dorsoventral fates in early gastrulae (reviewed in Dale & Jones, 1999; Thesis Introduction). Hence high BMP-4 expression in the ventral side of the embryo results in more ventrally derived tissues whereas inhibition on the dorsal side of the embryo gives dorsal derivatives. Genetic studies in mice have shown that BMP-4 plays an important role in mesodermal patterning; mice with a null mutation in the *bmp-4* gene typically die prior to gastrulation, but the small number that survive typically exhibit disorganised mesoderm and strongly truncated posterior structures (Winnier et al., 1995). This phenotype is similar to that obtained with the over-expression of XFD and *er1* in *Xenopus* embryos (Figure 5.3). The RT-PCR expression asays showed that the level of BMP-4 was similar to control level in both *er1* and XFD-injected embryos at stage 10.5 and 11.5, but lower than control for *er1*- and XFD-injected embryos at stage 15 (Figure 5.4).

Northrop *et al.* (1995) showed that BMP-4 rescues the expression of *Xbra* and *Xcad3*, which are also regulated by FGF, in XFD-injected embryos. The authors suggest that BMP-4 is involved in the ventral regulation of these genes. Our results suggest that, by down-regulating the expression of BMP-4, both XFD and ER1 inhibit the formation of ventrally derived tissues, a process which could result in posterior truncations.

5.3.3 *Er1* down-regulates the expression of HoxB9, a posterior marker.

Hox genes are widely accepted to be regulators of anteroposterior specification in animal groups ranging from *Drosophila* to vertebrates (Holland and Garcia-Fernandez, 1996;Pownall et al., 1996;Slack, 1993). The expression of some Hox genes has been shown to be regulated by FGF signalling during gastrula and neurula stages, after the period of mesoderm induction (Pownall et al., 1996). *HoxB9* expression starts at neurula stages and the marker is expressed at stage 14 in the posterior neural plate from the blastopore to approximately the middle of the antero-posterior axis in the embryo and is later expressed in the spinal cord until tailbud stages (Godsave et al., 1994;Pownall et al., 1996). *HoxB9* expression was upregulated in eFGF-injected embryos (Pownall et al., 1996b). Pownall *et al.*, (1998) showed that the activation of Hox genes during early neurula stages absolutely requires FGF signalling and transcriptional activation by Xcad3, while the maintenance of Hox gene expression in the trunk and tail during later development is independent of both FGF and Xcad. Since both XFD and *er1*-injected embryos have severe posterior truncations (Figure 5.3), it was expected that the expression level of a posterior marker such as *HoxB9* would be much lower than that of control in these embryos. The RT-PCR results showed that *HoxB9* expression can be first detected in control embryos at stage 15 and is considerably and comparably lower in both *er1*- and XFD-injected embryos (Fig. 5.4). The down-regulation of

HoxB9 by ER1 suggests that the FGF signalling pathway, critical to posterior patterning, is blocked in *er1*-injected embryos.

5.3.3.4 Expression levels of the posterior marker, Xpo, are unaffected by over-expression of *er1* as opposed to XFD.

Xpo is a posterior mesodermal marker. Xpo expression starts at or shortly after MBT. The RNA accumulates to a relatively low level, which remains constant until gastrulation then rapidly and transiently increases in posterior ectoderm and mesoderm (Sato and Sargent, 1991). Amaya *et al.*, (1993) found that *Xpo* was expressed strongly at the mid-gastrula stage in the lateral and ventral marginal zones. The authors report high expression levels in posterior structures at stage 13. Furthermore, *Xpo* gene expression is induced in animal caps by either activin A or FGF-2 (Sato and Sargent, 1991). *Xpo* expression was found to be up-regulated in eFGF-injected embryos (Pownall et al., 1996). At stage 11, *Xpo* expression was partially inhibited in XFD-injected embryos (Amaya et al., 1993). It was therefore expected that *Xpo* levels would be lower than control in both XFD and *er1*-injected embryos. RT-PCR analysis showed that the expression level of Xpo is initially lower than control levels in XFD-injected embryos at stage 10.5, and is only slightly lower than in control embryo levels at later stages (Figure 5.4). Amaya *et al.*, (1993) showed that the level of *Xpo* expression was down-regulated at stage 11, but there were no reports whether this effect was sustained past stage 11. Our results show that, similar to the results of Amaya *et al.*, (1993), there is a decrease in the level of *Xpo* expression in XFD injected embryos but that this effect is transient. In contrast, there were no detectable differences between *Xpo* levels in *er1*-injected and

control embryos at stage 10.5 and 11.5 and were only slightly lower in *er1*-injected than in control embryos at stage 15.

5.3.3.5 *Er1* overexpression delays the upregulation of the anterior marker goosecoid at *Xenopus* late gastrula stages.

Goosecoid is an anteriorly expressed marker, which encodes a homeobox containing transcription factor. *Goosecoid* is expressed early in the dorsal lip of the blastopore and the RNA levels decrease during neurulation (Cho et al., 1991;Pownall et al., 1996).

In XFD injected embryos, g*oosecoid* levels were lower than that of control embryos at stage 10.5, similar to controls at stage 11.5, and much higher than for controls at stage 15 (Fig. 5.4). Expression level of *goosecoid* was lower in *er1*-injected embryos than for controls at stage 10.5, higher than for controls at stage 11.5 and returned to the same level as for control embryos at stage 15 (Fig. 5.4).

Interestingly, *goosecoid* levels are lower for both *er1* and XFD-injected embryos at stage 10.5. The highest expression of *goosecoid* is seen at stage 11.5 for *er1*-injected embryos and 15 for XFD-injected embryos. Amaya *et al.*, (1993) found that *goosecoid* expression is not affected by XFD at stage 10, but did not investigate the effects of XFD on *goosecoid* expression at later stages. Pownall *et al.* (1996) showed that the expression of *goosecoid* is down-regulated by eFGF only during stage 12 and 15. No differences were seen between

control and eFGF-injected embryos at earlier or later stages. My results suggest that both ER1 and XFD delay the upregulation in the expression of *goosecoid*.

The up-regulation of *goosecoid* by ER1 further supports the hypothesis that ER1 may be acting as a negative regulator of the FGF signalling pathway during early *Xenopus* development. However, the lower expression of *goosecoid* in embryos injected with *er1* and XFD at stage 10.5 will need to be investigated further by RNase protection assays and *in situ* hybridization in order to determine whether *er1* and XFD repress or delay *goosecoid* expression at early gastrula stages. There is a delicate balance in the expression of patterning molecules during early development in *Xenopus*. As a negative regulator of the FGF signalling pathway, ER1 may be shifting the balance in favour of anteriorly expressed genes such as *goosecoid*.

5.4 DISCUSSION

The gross morphological abnormalities observed from overexpressing *er1* were very similar to the phenotype observed by Amaya *et al.*, (1991) upon injecting a mutant FGFR, XFD, which contains only an extracellular and a transmembrane domain and inhibits FGF signalling. When XFD is injected in embryos, they fail to complete gastrulation. There are major neurula stage deficiencies in lateral and posterior mesoderm, which later result in extreme trunk deficiencies and little or no tails but XFD embryos develop normal heads (Amaya et al., 1991).

Truncated posterior phenotype was observed in 64% of the embryos injected with 2ng of *er1*, the remaining embryos had normal phenotypes or minor defects (Figure 5.1). The percentage of abnormal embryos was only 32% when embryos are injected with 1ng *er1* cRNA (Figure 5.1) and no significant abnormalities are observed when lower amounts of cRNA are injected. The ER1 phenotype (Fig 5.3 B), is very similar to the XFD phenotype for which Amaya et al. (1991) reported severe posterior truncations in up to 60% of embryos injected with 4ng to 8ng of XFD cRNA whereas 29% of the injected embryos developed normally. The authors observed that when XFD is injected in embryos, normal embryos and less extreme phenotypes were present in the experimental group. The authors attribute this variation in severity of the phenotype to a non-uniform distribution of the injected RNA in some embryos and/or a low level of translation

of the injected cRNA. These factors may also account for the difference in severity of phenotype in in the 2ng *er1*-injected embryos, in which 36% of the embryos develop normally.

There are several lines of evidence indicating that FGF is a potent posteriorizing factor (Amaya et al., 1993; Cox and Hemmati-Brivanlou, 1995; Holowacz and Sokol, 1999; Isaacs et al., 1994;Lamb and Harland, 1995; Pownall et al., 1996). It has been shown that FGFs are secreted in the posterior of gastrula and neurula stage embryos (Isaacs *et al.*, 1992; Tanahill *et al.*, 1992; Isaacs *et al.*, 1995) and play a role in the posterior development of *Xenopus* embryos (Altaba and Melton, 1989, Isaacs *et al.*, 1994). Over-expression of eFGF in embryos during the gastrula stages produces a characteristic phenotype of reduction of the head and enlargement of the proctodeum (Isaacs et al., 1994). This observation of a similar phenotype is an indication that *er1*, like XFD, may be involved in blocking the FGF signalling pathway, and that ER1 may be a natural regulator or inhibitor of the FGF signalling pathway during early developmental processes in the *Xenopus* embryo. Thus, ER1 may function to turn off the FGF signalling pathway *in vivo*. However, the similarity in phenotype between ER1 and XFD overexpression has only been established by scoring gross morphological defects. In order to further characterize the effects of ER1 on specific tissues and similarity to XFD-injected embryos, it will be necessary to examine embryos histologically and use a larger array of molecular markers.

The abnormal phenotype obtained upon over-expressing ER1 is very similar to the XFD phenotype. Therefore, a molecular profile was established for ER1 by comparing the effect of over-expression on the expression of representative mesodermal, posterior and anterior markers on which the effects of XFD had been previously documented (Amaya et al., 1993).

Molecular biologists utilize many techniques in gene expression studies. Some of the most common techniques are Northern blot analysis, RNAse protection assays and *in situ* hybridizations. RT-PCR remains a relatively new technique and the technology is continually being optimized. It has proven to be a more sensitive and discriminating procedure than those mentioned above, as it permits the analysis of very small amounts of RNA. Furthermore, RT-PCR is a fairly rapid and simple procedure, in which simultaneous analysis of several transcripts from total RNA and quantitation can be achieved. One major shortcoming in using RT-PCR is the high degree of variability associated with the procedure. In addition to greatly amplifying the target, any errors or contaminations present are also amplified, thus affecting the accuracy and reliability of the result. However, with proper experimental design, it can be a useful technique.

Although the RT-PCR procedure followed here did not allow for quantitative analysis between the different developmental marker primer sets, the use of histone to standardize the amount of cDNA used in the PCR amplification with each primer set did allow comparison of samples. The methodology used

here allowed qualitative comparison of the effects of XFD and ER1 on embryos. Further analysis of these markers using *in situ* hybridization and RNAse protection assays would complement the RT-PCR results and help give a better indication of how the expression of these molecules are being quantitatively, spatially and temporally affected by *er1* over-expression.

Pownall *et al.* (1996) have previously shown that eFGF up-regulates the expression of posterior genes such as *XhoxC6 (Xlhbox1), HoxA7 (Xhox36), HoxB9 (Xlhbox6), Xcad*3 and *Xpo*. These authors also showed that eFGF up-regulates the expression of *Xbra,* a mesodermal marker and down-regulates the expression of anterior markers such as *goosecoid* and *otx*2. Conversely, XFD has been shown to down-regulate the expression of posterior molecular markers such as *Xpo*, *Xcad3,* and *HoxB9* and the mesodermal marker *Xbra* (Amaya *et al.*, 1993; Pownall *et al.*, 1996; Holawacz and Sokol, 1999). Our RT-PCR results with ER1 over-expression follow a similar pattern to that of XFD for the mesodermal marker *Xbra*, the ventral marker BMP-4 and the posterior marker *HoxB9* and the anterior marker *goosecoid*.

It is possible that XFD and ER1 are blocking the FGF signalling pathway at different steps and/or to different extents. Furthermore, XFD shuts down all signalling cascades triggered by FGF whereas a downstream regulator like ER1 may be affecting only specific pathways triggered by FGF. Hence the different temporal profiles are seen. It is also of interest to examine the effects of ER1 overexpression on these molecular markers at stages later than stage 15 in order

to determine whether the effect of ER1 on the expression levels is transient or sustained, since the expression of the molecular markers examined, such as *HoxB9*, only starts at neurula stages. RT-PCR was used in this study, whereas previous studies of factors other than *er1* had used *in situ* hybridization and RNAse protection assays. In the future, it is therefore of interest to confirm the *er1* results presented using such techniques.

When XFD is overexpressed in embryos, there is a reduction in mesoderm formation, abnormalities arising from the inhibition of normal gastrulation movements and there are defects in the formation of posterior parts (Isaacs *et al.*, 1994). It is believed that the mesoderm formation and cell movement defects are due to loss of *Xbra* expression and that the posterior defects are due to a lack of posterior Hox activity (Isaacs *et al.*, 1994; Pownall *et al.*, 1996). Our results show that *er1* over-expression results in gross morphological defects that are very similar to XFD. With the limited number of molecular markers used in this study and the different temporal profiles of these markers in response to ER1 and XFD, it may be too early to establish a definite similarity between the effects of ER1 and XFD at the molecular marker level. However, the molecular profile established for ER1 suggests that ER1 is a regulatory molecule, which is up-regulated by FGF pathway (Paterno et al., 1997), and negatively regulates the FGF pathway when developmental processes triggered by FGF signalling, such as mesodermal and posterior patterning, have been established.

In the future a larger spectrum of molecular markers, especially dorsal markers, such as *Chordin, Noggin* (inhibitory to the ventral patterning molecules) as well as more specific markers for tissue patterning, *Xnot* (notochord) and *β-actin* and MyoD (muscle), will confirm whether *er1* is truly a negative regulator of specific FGF signalling pathways and characterize the pathways involved.

Isaacs *et al.*, (1994) used XFD to investigate the role of eFGF in mesoderm during gastrula stages. When 2-cell stage embryos are injected with eFGF and animal caps are removed at stage 9 and cultured for three days, the animal caps develop fluid-filled mesoderm indicating the formation of mesodermal derivatives. Similarly, incubation of animal caps with other FGFs such as FGF-2 leads to differentiation of the animal caps into mesoderm of a ventro-lateral nature. Co-injection of eFGF with XFD leads to a marked reduction in the auto-induction activity of eFGF (Isaacs *et al.*, 1994). Repeating these types of experiments with *er1* and eFGF co-injection or incubating animal caps injected with *er1* with FGF-2 would demonstrate whether ER1 can suppress the mesoderm-inducing activity of FGFs and act in an inhibitory fashion similar to XFD by shutting off FGF signalling.

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SECTION IV

CHAPTER 6: GENERAL DISCUSSION, FUTURE DIRECTIONS AND CONCLUSION

6.1 GENERAL DISCUSSION

One of the main objectives of modern developmental biology has been and continues to be the identification of the molecular nature of inducing signals in early development. It is now known that key developmental pathways are triggered by members of the TGF-β and FGF families (see section 1.4-1.8). FGF has multiple functions in early development, including mesoderm formation, gastrulation movements and anteroposterior patterning (Slack, 1994). *Er1* was identified as a novel gene, whose expression is upregulated by FGF during mesoderm induction in *Xenopus,* (Paterno *et al.*, 1997). The objectives of this thesis were to characterize the expression pattern of ER1 protein, identify mechanisms regulating its expression and characterize and understand its function.

It was shown that the spatio-temporal nuclear translocation of ER1 is tightly regulated during early development (Chapters 2 and 3). Nuclear localization of ER1 is possibly a response to the FGF signalling pathway as suggested by the inhibition of nuclear localization of ER1 in XFD-injected

embryos (Chapter 3). Transcriptional activity was established for deletion constructs of ER1 protein, suggesting that ER1 may function as a transcription factor *in vivo* (Chapter 4). The results in Chapter 5 suggest that ER1 may function as a negative regulator of the FGF signalling pathway, since overexpression of ER1 gives a phenotype that is the converse of that of FGF overexpression and is similar to that of XFD overexpression. Furthermore, ER1 overexpression results in downregulation in the expression of molecular markers such as *Xbra* and *HoxBox9*, whose expression are activated, and in the case of *Xbra,* maintained, by FGF.

ER1 is initially translocated to the cell nuclei of the marginal zone and animal hemisphere of the early blastula stages embryo, regions that have been shown to express the three maternal forms of FGF: FGF-2, FGF-9 and eFGF(Song and Slack, 1994; reviewed in Isaacs, 1997). At gastrula and neurula stages, ER1 is ubiquitously present in all nuclei (Chapter 2). Gastrula and neurula stages are also stages where maternally expressed and zygotically expressed FGFs overlap in the embryo. Zygotic eFGF is expressed in the blastopore region at gastrulation (reviewed in Isaacs, 1997). At gastrula and neurula stages, FGF expression spans both the animal and vegetal regions of the embryo. At tailbud stages ER1 can be found in the nuclei of the notochord, somites, and spinal cord, but starts to disappear from the nuclei of the brain and epidermis. FGF-2 is zygotically expressed in the CNS and somitic tissue (Song and Slack, 1994) and eFGF is expressed in the notochord as well as in the

posterior region of the embryo (reviewed in Isaacs, 1997). At these stages, I found that ER1 is also nuclear in endodermal and mesodermal tissues and not in ectodermally derived tissues. The expression pattern of ER1 protein along with the inhibition of its nuclear localization by XFD suggests that ER1 may be a downstream effector of the FGF signalling pathway whose nuclear localization is analogous to the expression of various FGFs during early development.

The major role of the FGF pathway during early development is the formation of mesoderm and posterior structures in the *Xenopus* embryo. The current model for the role of FGFs during early amphibian development proposes that the maternal pool of FGFs is required to provide sub-threshold stimulation of the tyrosine kinase signal transduction pathway in the animal hemisphere (reviewed in Isaacs, 1997). The FGFs act as competence factors to vegetally localized inducing signals from activin-like factors (Hemmati-Brinvanlou and Melton, 1992; Thomsen and Melton, 1993). It has been proposed that mesoderm forms in the marginal zone because it is a region where FGF factors and activin-like factors overlap (Cornell and Kimelman, 1994). It has been shown that FGF is involved downstream of early mesoderm inductive signals in the maintenance of the *Xbra* expression in mesoderm precursors (Smith *et al.*, 1997). FGF signals also regulate the expression of the caudal gene family member *Xcad3* (Northrop and Kimelman, 1994; Pownall *et al.*, 1996), which has a critical function in the regulation of posterior hox genes (Isaacs *et al.*, 1998).

The results presented in Chapter 5 suggest that ER1 is functioning as a negative regulator of the FGF pathway. However, the expression of FGFs and the nuclear localization pattern of ER1 in the embryo show that ER1 is nuclear in regions where FGFs are active such as the marginal zone in the early embryo and later in the blastopore region, notochord, somites, and CNS. As a transcription factor, ER1 may be either 1) enhancing the transcription of genes whose products act to shut down the FGF pathway or 2) repressing the transcription of genes required for the FGF response. One possible scenario is that the supression of the FGF pathway is happening at varying levels in the embryo. Inductive events are the result of finely balanced positive and negative interactions. Therefore, the ability of ER1 to shut down the FGF pathway may be dependent on several factors such as the level of expression of the FGFs in different regions of the embryo as well as the expression of other spatially specific factors, which interact with the FGF pathway.

It is interesting to note that ER1 may be remaining cytoplasmic until the FGF pathway is itself activated as illustrated by the expression of activated MAPK, detected for the first time in the embryo at stage 8 (LaBonne and Whitman, 1997; Christen and Slack, 1999). ER1 also first localizes to the nuclei of marginal zone cells at stage 8. MAPK is initially present in the region of the future blastopore at stage 8 and around the blastoporal ring at gastrula stages. The staining pattern of activated MAPK has been fully described and is indicative of the regions in which FGF signals are being transduced through FGFR1

(LaBonne and Whitman 1997; Christen and Slack 1999; Umbhauer *et al.*, 2000; section 1.7.3). ER1 may be regulating the FGF pathway at a different threshold in the regions where MAPK is fully active, such as in mesodermal derivatives, than in tissues such as the ectoderm, where activated MAPK does not appear to be expressed (Christen and Slack, 1999). First, ER1 becomes undetectable in the nuclei of ectodermal derivatives, where FGF may no longer be active, and remains nuclear in tissues that retain FGF expression such as somites and notochord.

Although nuclear localization of ER1 coincides with the activation of the MAPK signalling cascade by FGF at blastula stages, it has not yet been determined whether the MAPK cascade is required for nuclear translocation of ER1. Preliminary results, not presented in this thesis, have shown that overexpression of a dominant-negative MAPK kinase (MEK-DN), which blocks MAPK signalling, does not prevent nuclear localization of ER1. It is therefore possible that nuclear localization of ER1 is triggered by a different signalling pathway, such as the PLCγ1 or PI3'K pathway. Previous work in our laboratory has shown that both phosphorylated PLCγ1 and PI3'K associate with FGFR1 during early blastula stages in *Xenopus* (Gillespie *et al.*, 1992; Ryan & Gillespie, 1994; Ryan *et al.*, 1998). Similar to MAPK signalling, these two pathways are therefore also activated correct time for initiating ER1 nuclear localization. The results in Chapter 3 showed that XFD blocks nuclear localization of ER1. However, XFD blocks FGF signalling at the receptor level and abrogates all FGF

signalling. Further work is therefore required to determine which specific downstream FGF signalling cascade may lead to dephosphorylation of ER1 associated proteins and consequent nuclear localization of ER1.

The results presented in this thesis suggest a model whereby FGF signalling is modulated by a negative feedback mechanism through ER1. As illustrated in Figure 6.1, nuclear localization of ER1 is initiated by FGF signalling. Signalling initiated by FGF leads to the dephosphorylation of proteins associated with ER1, and the latter event is correlated with translocation of ER1 to the nucleus. Once in the nucleus, ER1 may be activating the transcription of genes whose products act to inhibit FGF signalling, and/or repress the transcription of genes required for the FGF-mediated response during mesoderm induction.

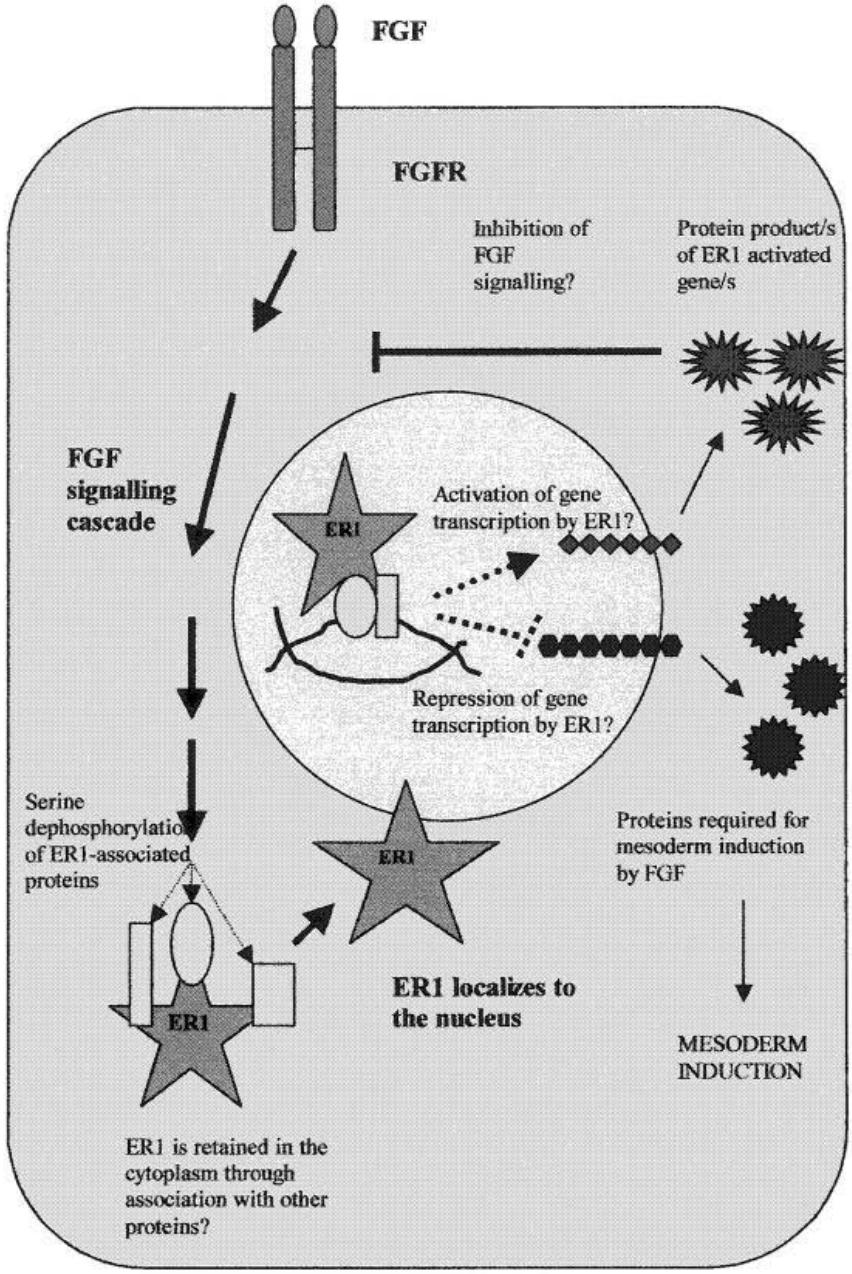

Figure 6.1 A model illustrating the putative mechanism underlying nuclear localization and function of ER1.
The above model proposes a negative feedback loop on FGF signalling by ER1. FGF binding to FGFRs triggers the FGF signalling cascade, which may lead to dephosphorylation events on ER1 associated proteins, followed by nuclear localization of ER1. In the nucleus, ER1 possibly activates transcription of gene/s whose products shut down FGF signalling and/or represses the transcription of gene/s required for mesoderm induction.

6.2 FUTURE DIRECTIONS

Future experiments of relevance to each of the chapters were discussed earlier. After considering all the information presented, there are several areas of immediate interest, which can be examined to confirm the results presented in this thesis.

6.2.1 Down-regulating ER1 expression or function.

Overexpression of ER1 in the embryo was useful in determining its function in the embryo. Another strategy often used in unravelling the function of proteins is the use of dominant-negative mutants. The dominant-negative FGF receptor, XFD, has been crucial in investigating some functions of FGFs in the embryo. Overexpression of a dominant-negative ER1 will provide further clues to the function of the protein. Hence, if ER1 truly functions as a regulator of the FGF pathway, overexpression of a dominant-negative form of the protein should give reverse effects to those observed here from overexpressing ER1 and putatively similar results to overexpression of FGFs. A protein, which does not exhibit transcriptional activity but blocks endogenous ER1 activity by interacting with normal ER1 partners, should act as a good dominant-negative. Other ongoing projects in the laboratory are attempting to characterize the ER1 domains ER1, that may be responsible for DNA-binding or protein-protein interaction.

Other strategies, which can be used to cause "loss of function", are RNA interference and the use of antisense oligonucleotides and morpholino oligonucleotides. RNA interference technology, using double stranded RNA corresponding to the gene of interest, has been found to be effective in downregulating gene expression in *Xenopus* (Nakano *et al.*, 2000). Antisense oligonucleotides lead to degradation of mRNA and morpholino oligonucleotides act by preventing the translation of specific mRNAs into protein, hence ablating the function of the gene of interest (Summerton & Weller, 1997).

6.2.2 Determining which FGF pathway is responsible for nuclear translocation of ER1

FGF signalling activates three major pathways, Ras/MAPK, PLCγ1 and PI3'K pathways (reviewed in Powers *et al.*, 2000). The work in this thesis has shown that nuclear localization of ER1 is tightly regulated during early developmental stages and is under the control of FGF signalling. Dominant-negative mutants (such as dominant negative MAPK) and chemical inhibitors (such as wortmannin, which inhibits PI3'K, and U713122, the synthetic inhibitor of PLCγ) or neutralizing antibodies to specific components of these pathways, will help determine which of the three pathways listed above is responsible for nuclear localization of ER1. Nuclear localization of ER1 in response to overexpressing of dominant-negative mutants or chemical inhibitors can be verified by whole-mount antibody staining.

Activated PLCγ and PI3'K both associate with FGFR during mesoderm induction (Ryan & Gillespie, 1994; Ryan *et al.*, 1998). Activated PKC, a component of the PLCγ pathway, has been detected in FGF-treated explants (Gillespie *et al.*, 1992). Simultaneous treatment of explants with TPA (an activator of PKC) and FGF results in significant inhibition of mesoderm induction by FGF, suggesting that activation of PKC could be part of a negative feedback mechanism on the FGF mesoderm induction pathway (Gillespie *et al.*, 1992). It is possible that nuclear localization of ER1 is triggered by either the PLCγ or the PI3'K pathway as part of a negative feedback mechanism to regulate mesoderm induction by FGF.

6.2.3 Further characterization of the role of ER1 during mesoderm induction

A low level of activity from maternal FGFs is required for the transcription of the mesodermal marker *Xbra* in late blastula marginal zone. Xbra then activates the zygotic expression of eFGF in the early mesoderm leading to a period of autocatalytic activity of eFGF and Xbra in the nascent mesoderm of the marginal zone. During gastrula and neurula stages, eFGF continues to regulate the expression of *Xbra* in the blastopore region and the notochord. The FGF-Xbra autocatalytic pathway is important for the establishment of the mesoderm. Different types of mesoderm are induced in animal cap explants at varying doses of FGFs (Slack *et al.*, 1987; Slack *et al.*, 1988). Mesodermal tissue of a ventral nature such as mesenchyme and mesothelium are induced at low doses, and of

a more lateral nature such as muscle at higher doses (reviewed in Isaacs, 1997). These induced caps express mesodermal molecular markers such as *Xbra*. Animals caps from embryos injected with *er1* should inhibit the ability of FGFs to induce mesoderm. This would be verified by visually scoring the explants, looking at the expression of molecular markers for mesoderm and by histological analysis for mesodermal derivatives. These experiments will confirm the ability of ER1 to regulate the mesoderm-inducing ability of FGF.

6.2.4 Investigating effect of ER1 on the MAPK pathway and rescue of the ER1 phenotype by activated MAPK.

Mesoderm and posterior signals transduce through FGFR1 by activation of the Ras/MAPK pathway (Umbhauer *et al.*, 2000). Transduction of FGF signals leading to *Xbra* maintenance involves Ras, Raf and the MAPK cascade (Gotoh *et al.*, 1995; LaBonne *et al.*, 1995; MacNicol *et al.*, 1993; Umbhauer *et al.*, 1995) and downstream the heterodimeric AP-1 transcription factor (Kim *et al.*, 1998). In *Xenopus*, the FGF family seems to be responsible for the full pattern of activated MAPK in early development since activation can be blocked completely by XFD until tailbud stages (Christen and Slack, 1999). The connection of ER1 to the FGF signalling pathway can be further investigated by looking at the effect of ER1 on the expression of activated MAPK. The level of activated MAPK in embryos overexpressing ER1 can be verified by whole mount staining or by Western blotting.

Since ER1 may be having a negative regulatory effect on FGF signalling during mesoderm induction and the main pathway activated during mesoderm induction by FGF is through MAPK, it will be interesting to determine whether expression of activated MAPK will rescue the phenotype seen in embryos overexpressing ER1.

6.3 CONCLUSION

This study has provided insight into the expression and function of ER1 in the *Xenopus* embryo. There is preliminary evidence that ER1 functions as a transcription factor. The protein has a tightly regulated spatio-temporal nuclear localization pattern in the embryo. Investigation of the mechanisms regulating this nuclear localization pattern suggests that nuclear translocation of ER1 is regulated by changes in ER1-associated phosphoserine proteins, triggered by the FGF signalling pathway in *Xenopus* embryos.

ER1 over-expression results in posterior truncations in the embryo and inhibits the expression of mesodermal and posterior markers, which are induced by FGF, suggesting that ER1 is a modulator of the FGF pathway. Further expression analysis of a larger array of markers will support this premise. In addition, analysis of the response of components specific to the FGF signalling pathway will determine the specificity of the ER1-FGF connection.

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