STUDIES ON THE EXPRESSION, EFFECTS AND REGULATION OF FIBROBLAST GROWTH FACTOR-2 BY GENE TRANSFER IN CARDIAC MYOCYTES AND GLIAL CELLS

By
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Dedicated to my parents

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ABSTRACT

Basic fibroblast growth factor (FGF-2) is a potent mitogen and exists in multiple forms because of alternate initiation from the same mRNA. FGF-2 mediates various biological responses by binding to cell surface receptors of the tyrosine kinase family. Inability of adult cardiac myocytes to regenerate following myocardial infarctions has been an acute problem in the clinical cardiology. Embryonic cardiac myocytes are proliferative and express predominantly high molecular weight forms of FGF-2. Adult cardiac myocytes lose their proliferative potential and this correlates with the reported loss of high molecular weight forms of FGF-2. FGF-2 is, therefore, an excellent candidate for the control of myocardial growth and development. FGF-2 also exerts potent multipotential trophic effects on neurons, endothelial cells and glia of the central nervous system. It is required for the origin and development of neuronal and glial cells as well as nerve regeneration. Glial cells produce high levels of FGF-2, stimulating autocrine growth as well as survival and functions of neurons in a paracrine manner. Recent studies indicate that altered levels of FGF-2 may be involved in the disease process of neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's diseases. Clearly, this potent mitogen must be tightly regulated to avoid uncontrolled proliferation of cells. Thus, the hypotheses of my doctoral studies were: (i) overexpression of high molecular weight forms of FGF-2 can increase proliferation and decrease differentiation of cardiac myocytes (ii) FGF-2 expression in glial cells can be controlled at the transcriptional level.

Modified rat FGF-2 cDNAs that can preferentially express high (22-21.5 kD) or low molecular weight forms (18 kD) of FGF-2 were generated and their expression products were characterized in cardiac and noncardiac cell types. Increases in DNA synthesis and proliferation were observed with both forms of FGF-2, however, overexpression of 22-21.5 kD but not 18 kD FGF-2 was associated with clumping of the DNA in embryonic as well as postnatal ventricular myocyte cultures. In addition, these studies provided evidence that the stimulation of mitosis by 22-21.5 or 18 kD FGF-2 likely represents a proximity-dependent paracrine effect. In contrast to the effects on hyperplastic growth which were similar for high and low molecular weight FGF-2, overexpression of high but not low molecular weight FGF-2 was associated with an increase in cardiac

myocyte binucleation in an intracrine manner. Further, overexpression of both high and low molecular weight FGF-2 in ventricular myocyte cultures resulted in disorganization of sarcomeric structure as well as significant decreases in the differentiation markers myosin and desmin but not α-sarcomeric actin. With a view to testing our hypothesis *in vivo* that FGF-2 is involved in postnatal cardiac growth, we generated transgenic mouse models for high and low molecular weight FGF-2 and these models are currently being characterized.

As a prerequisite for understanding transcriptional regulation of FGF-2 gene, the 5′-flanking sequence (~1.1 kb) of rat FGF-2 gene was cloned and sequenced. Transcriptional start sites of FGF-2 gene were mapped using primer extension analysis of rat brain and heart RNA. The organization of FGF-2 gene was studied by DNA (Southern) blotting. The 5′-flanking region of the FGF-2 gene was subcloned into a promoterless luciferase plasmid and several deletion constructs were generated. These hybrid genes were transfected into rat glioma C6 cells to examine the putative FGF-2 promoter activity and the results indicated that ~167 nucleotides of upstream sequences are sufficient to maintain the minimal promoter activity. This promoter was also found to be active in rat cardiac cell line H9c2 as well as human astrocytoma U87-MG. FGF-2 promoter activity was increased in response to mitogenic stimuli serum and a protein kinase C activator (phorbol myristate acetate or PMA) in C6 cells. A repressor activity was localized to -1058/-911 region of the rat FGF-2 gene in the absence of mitogenic stimuli and it was relieved completely in response to serum treatment but only in part by PMA. Further, deletion of -1058/-911 region was sufficient for the loss of phorbol ester responsiveness of the rat FGF-2 gene in glioma C6 cells. In addition, specific and differential DNA protein interactions were identified in -1058/-911 region of FGF-2 gene by gel mobility assay using nuclear extracts from C6 cells treated with or without mitogenic stimuli. These DNA protein interactions in FGF-2 gene were confirmed by nuclease protection assay.

In conclusion, my doctoral studies provided substantial evidence that FGF-2 can be used to stimulate the proliferation of postnatal cardiac myocytes and its levels can be regulated in glial cells at the transcriptional level and thus suggest a therapeutic potential for this growth factor in diseases associated with the cardiovascular and central nervous systems.

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ABBREVIATIONS

aka	also known as
ANF	atrial natriuretic factor
ATP	adenosine triphosphate
ß-gal	beta galactosidase
BSA	bovine serum albumin
bp	base pair
CAT	chloromphenicol acetyl transferase
°C	degrees centigrade
cDNA	complementary deoxyribonucleic acid
CMF	calcium and magnesium-free
CMV	cytomegalo virus
CNS	central nervous system
cpm	counts per minute
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
dpm	disintegrations per minute
DTT	dithiothreitol
ECM	extra cellular matrix
EDTA	ethylenediaminetetraacetic acid
eg.	example
EGF	epidermal growth factor
FBHE	fetal bovine heart endothelial cells
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Fig.	figure
GAPDH	glyceraldehyde phosphate dehydrogenase

GH	growth hormone
GR	glucocorticoid receptor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hnRNA	heterogeneous nuclear ribonucleic acid
HS	horse serum
Ig	immunoglobulin
IGF	insulin-like growth factor
kb	kilobase
Kd	dissociation constant
kD	kilodalton
LI	labelling index
luc	luciferase
LTR	long terminal repeat
M	molar
MEM	minimum essential medium
mg/μg/ng	milli/micro/nanogram
MAPK	mitogen-activated protein kinase
MHC	myosin heavy chain
min	minute
ml	millilitre
MLC	myosin light chain
mM/μM/nM	milli/micro/nanomolar
mRNA	messenger ribonucleic acid
nt	nucleotide
NTP	nucleotide triphosphate
p	promoter
PAGE	polyacrylamide gel electrophoresis
PAS	Periodic Acid Schiff staining
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction

PDGF	platelet-derived growth factor
PIPES	1,4-piperazine diethanesulfonic acid
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethlyl-sulfonic fluoride
PRL	prolactin
Rb	retinoblastoma
RNA	ribonucleic acid
rpm	revolutions per minute
RSV	Rous sarcoma virus
RT	reverse transcriptase
RTK	receptor tyrosine kinase
SEM	standard error of mean
SDS	sodium dodecyl sulphate
SV40	simian virus 40
TBE	Tris borate EDTA buffer
TE	Tris-EDTA buffer
TGF	transforming growth factor
TSH	thyroid stimulating hormone
v/v	volume per volume
w/v	weight per volume
%	percent

Chapter 1

Introduction

Cardiovascular and neurological disorders are two major problems of public health in North America. Heart disease remains the number one cause of death while neurodegenerative diseases pose a considerable burden on the affected individual, the family and the community. Inability of adult cardiac myocytes to regenerate following myocardial infarctions has been an acute problem in clinical cardiology. The damaged area is replaced by fibrotic scar tissue following myocardial infarction. This scar formation compromises the cardiac performance and thereby reduces the lifespan. Factors or mechanisms that promote the ability of damaged myocardium to stimulate regenerative growth of cardiac myocytes would reduce fibrotic scar and thus, prolong cardiac lifespan. By contrast, inability of central nervous system to protect neuronal cells from death during neurodegenerative diseases leads to major complications such as chorea, muscle spasms, paralysis and loss of speech or memory. Factors or mechanisms that promote the ability of central nervous system to enhance the neuronal survival during disease state would minimise neurological complications. However, there is scant information about the factors or mechanisms regulating cardiac myocyte proliferation, and neuronal survival/regeneration. This drawback has created a large interest in the areas of cardiovascular and neuroscience research to identify means or factors that can regulate the cellular growth/regeneration with a view to prevent/protect as well as treat the affected individuals.

Fibroblast growth factor-2 (FGF-2 or basic FGF) is a potent mitogen for tissues of mesodermal and neuroectodermal origin and plays a vital role in the growth and development of cardiovascular and central nervous systems. FGF-2 exists in high and low molecular weight forms due to alternate initiation of translation from the same mRNA, alternative splicing as well as proteolysis. High molecular weight forms of FGF-2 have been implicated in the regeneration of liver cells. Loss of proliferative potential in adult cardiac myocytes correlates with the reported loss of high molecular weight forms of FGF-2. It is possible that the proliferative potential of cardiac myocytes can be prolonged by

increasing the levels of high molecular weight forms of FGF-2. **However, this premise is yet to be tested due to the unavailability of purified high molecular weight forms of FGF-2 protein.** In addition, FGF-2 possesses both angiogenic and cardioprotective properties which are of benefit to cardiac function and/or recovery from infarction. Thus, FGF-2 is an excellent candidate to stimulate cardiac myocyte growth as well as cardioprotection during myocardial infarctions.

FGF-2 exerts potent multipotential trophic effects on neurons, endothelial cells and glia of the central nervous system. This growth factor has also been implicated in nerve cell regeneration and is required for the glial cell proliferation, neuronal survival, neurite outgrowth and synthesis of neurotransmitters. The glial cells express high levels of FGF-2 and its high affinity receptor (FGFR-1), and also serve as a reservoir for FGF-2 that can enhance the survival and differentiated functions of neurons during normal and disease conditions. **Clearly, this potent mitogen must be tightly regulated in order to avoid uncontrolled proliferation of cells, however, the mechanisms of its regulation are largely unknown.** Recent studies indicate that altered levels of FGF-2 may be involved in the disease process of neurodegenerative disorders such as Alzheimer's disease, Huntington's disease and Parkinson's disease. Therefore, regulation of endogenous FGF-2 levels may serve as a useful therapeutic tool to promote survival of neurons during neurodegenerative diseases.

Thus, the first of two hypotheses addressed by my doctoral work was that overexpression of high molecular weight forms of FGF-2 can increase proliferation and decrease differentiation of cardiac myocytes. Towards this, the objectives were (i) to generate FGF-2 cDNAs that can preferentially express high or low molecular weight forms of FGF-2 and (ii) to examine the effects of high and low molecular weight forms of FGF-2 on cardiac myocyte growth and differentiation, after gene transfer. **The second hypothesis was that FGF-2 expression in glial cells can be controlled at the transcriptional level.** To address this hypothesis, my objectives were (i) to clone the 5′-flanking region containing promoter and regulatory sequences of rat FGF-2 gene and (ii) to characterize the regulatory regions of FGF-2 gene that respond to various stimuli in glial cells. The outcome of this research work should provide answers to whether FGF-2 (i) can be used to stimulate the

growth of postnatal cardiac myocytes with a view to promoting cardiac regeneration/protection during ischemia or myocardial infarctions and (ii) can be regulated in glial cells at the transcriptional level with a view to promoting neuronal survival during neurodegenerative diseases.

Chapter 2

Review of the Literature

2.1 Cardiogenesis

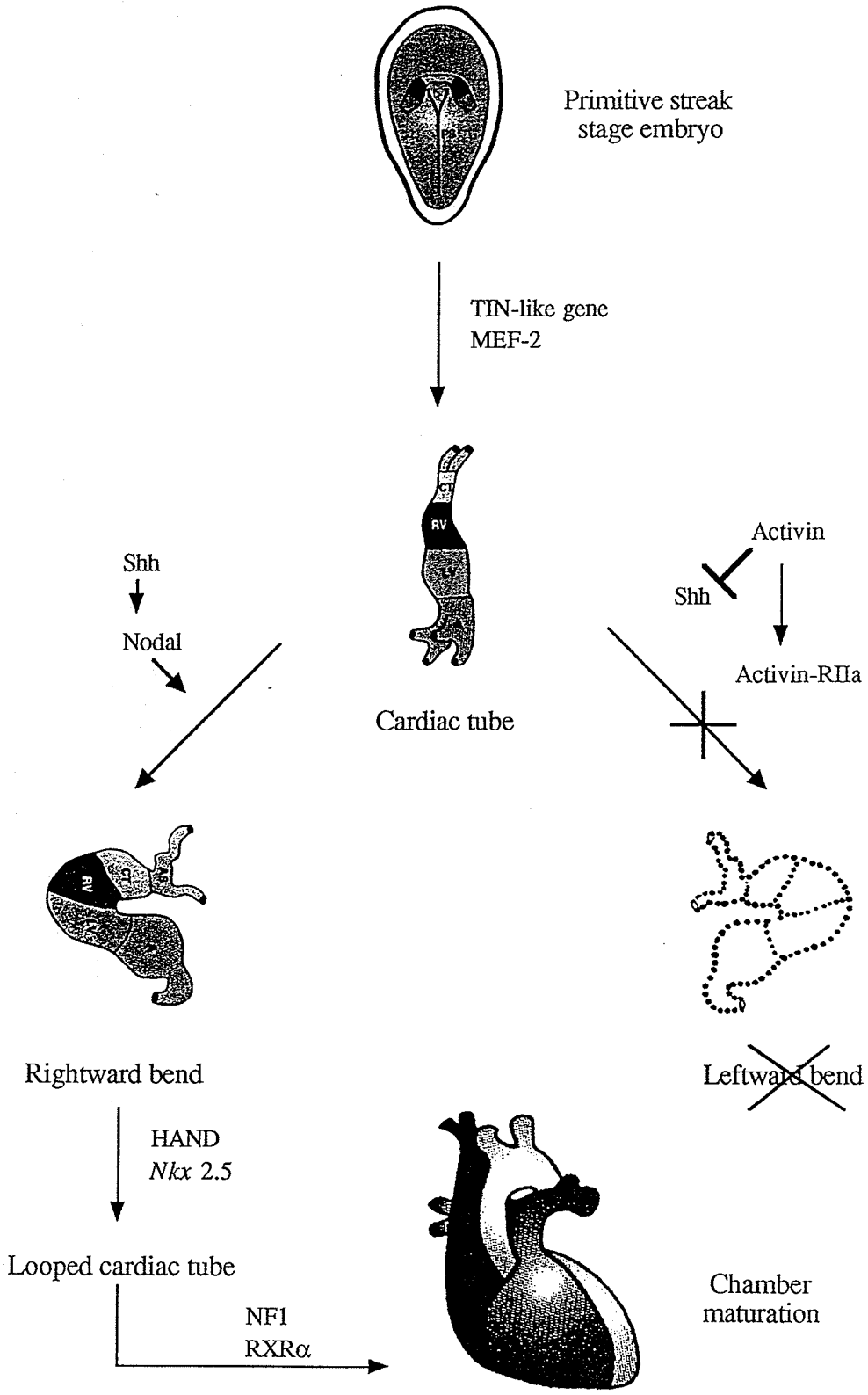
An understanding of the molecular events involved in the process of cardiogenesis and identification of genes involved in lineage determination, proliferation and differentiation of cardiac myocytes will greatly enable clinical efforts to regenerate heart muscle cells during myocardial damage.

2.1.1 Early cardiac development

Although early developmental events during the process of cardiogenesis are well defined in many species at anatomical and physiological levels, the genetic basis of these events is largely unknown. In humans, appearance of the cardiogenic area and pericardial cavity begins at the embryonic stage of ~2.5 weeks (Robbins *et al.,* 1992). Unknown mechanisms regulate the predetermination of mesodermal precursor cells in the cardiogenic area to become myocytes. However, this commitment of precursor cells to a cardiogenic fate in humans occurs by embryonic day 20 (Olson and Srivastava, 1996). Although definitive precontractile cardiac myocytes do not appear in the avian embryo until stage 9/9+, there is evidence that cells have become committed to a cardiogenic lineage as early as stage 4 in the pregastrulation embryo (Sanchez and Bader, 1990). Commitment to cardiac lineage is followed by formation of two parallel endothelial heart tubes, one on each side of the midsagittal plane, which subsequently fuse by day 22-23 in human (Fig. 1). The fused cardiac tube is comprised of an external myocardial layer (epicardium), internal endothelial tube (endocardium) and an intermediary layer of cardiac jelly. This tubular heart initiates rhythmic contractions by day 23 in humans and tends to loop rightwards due to extensive inductive interactions between cells of different layers. Looping of the heart to the right is an evolutionarily conserved mechanism in all vertebrate species and is normally directed by the asymmetric expression patterns of various morphogens. In the heart forming region of a chick embryo, expression of morphogen sonic hedgehog (Shh) induces chick nodal-related morphogen (cNR1) on the left side while expression of activin induces activin

Fig. 1

Fig. 1. Schematic diagram indicating the molecular signals and pathways that have been implicated in cardiac development. MEF-2 = myocyte enhancer binding factor-2; Shh = sonic hedgehog morphogen; Nodal = chick nodal-related morphogen (cNR1); Act-RIIa = activin receptor IIa; NF1 = neurofibromatosis gene; RXR = retinoid receptor. (Adapted from Olson and Srivastava, 1996).


Primitive streak
stage embryo
TIN-like gene
MEF-2
RV
Cardiac tube
Shh
Nodal
Activin
Shh
Activin-RIIa
Rightward bend
Leftward bend
HAND
Nkx 2.5
Looped cardiac tube
NF1
RXRα
Chamber
maturation

receptor IIa (Act-RIIa) but suppresses Shh levels on the right side, creating asymmetric expression patterns which ultimately lead to rightward looping (Levin *et al.*, 1995). Morphologically identifiable atrial and ventricular chambers appear after looping due to the development of anteroposterior (A-P) polarity, possibly under the influence of homeobox genes (Yutzey and Bader, 1995). Partition of the cardiac tube into the atria, ventricles, and outflow tract is accomplished by mesenchymal outgrowth or cardiac cushions that result from regional swellings of the cardiac jelly (Eisenberg and Markwald, 1995). However, there is scant information about the molecular cues that are responsible for the regional specification and determination of specialized cells within the heart.

2.1.2 Transcriptional regulation of cardiogenesis

In *Drosophila melanogaster*, the homeobox gene *tinman*, is expressed initially in all ventral mesoderm cells but later becomes restricted to the precardiac mesoderm. The product of the *tinman* gene, a homeodomain containing transcription factor, is required for subsequent mesoderm patterning and heart formation. Targetted disruption of the gene *tinman*, resulted in the complete absence of the heart, suggesting a vital role for this protein in the formation of cardioblasts (Azpiazu and Frasch, 1993; Bodmer, 1993). The vertebrate homologue of *tinman* is the homeobox gene *Nkx*2.5 or *Csx* (Komuro and Izumo, 1993; Lints *et al.*, 1993) and its inactivation affects heart morphogenesis but does not prevent formation of the primitive heart tube, cardiac muscle differentiation and muscle gene expression except for the ventricular specific myosin light chain gene (Buckingham, 1994). Another transcription factor that appears to play a role in cardiogenesis in both *Drosophila* and vertebrates is myocyte enhancer binding factor-2 (MEF-2), which activates transcription through a conserved A-T- rich DNA sequence in the regulatory regions of several muscle specific genes (Edmondson *et al.*, 1994). Targetted disruption of MEF-2 in *Drosophila* (*D-mef-2*) did not effect the expression of *tinman* and heart formation but resulted in loss of contractile protein gene expression (Lilly *et al.*, 1995). The consequences of MEF-2 mutations on vertebrate cardiogenesis are not yet known.

Although cardiac and skeletal muscle cells express similar muscle specific proteins, they appear to have distinct predetermination pathways. Members of the basic helix-loop-helix (bHLH) family of transcription factors, MyoD, myogenin, Myf5 and MRF4,

regulate skeletal myogenesis in addition to neurogenesis and hematopoiesis through interaction of their DNA binding domain with the E-box consensus sequence (CANNTG) in the control regions of target genes. These skeletal muscle specific bHLH proteins are not expressed in the heart, however, recent reports suggest that similar bHLH proteins may control cardiac gene expression (Srivastava *et al.*, 1995). A regulatory bHLH protein Id which is an inhibitor of cellular differentiation, is transiently expressed at high levels in the endocardial cushions of the fetal heart (Bilsen and Chein, 1993) and its role in a cardiac context is not completely understood. Two bHLH proteins, dHAND and eHAND regulate the process of cardiac looping and inhibition of their expression in chick embryos (stage 11-12) by antisense oligonucleotides resulted in the arrest of looping, followed by poor hemodynamic performance and embryonic death (Srivastava *et al.*, 1995). Cardiac looping is thought to occur as a result of cellular proliferation and specific interaction with extracellular matrix molecules (Yost, 1995). Thus, dHAND and eHAND may regulate one or more genes required for cardiac looping.

Members of the GATA transcription factor family also appear to control cardiogenesis. GATA-4 expression is observed as early as ~7.5 days postcoitum in mouse precardiac cells. It was shown to activate the transcription of several cardiac genes including atrial natriuretic peptide, B-type natriuretic peptide, α-myosin heavy chain and troponin C. Inhibition of GATA-4 expression with an antisense construct in pluripotent embryonal carcinoma P19 cells, specifically blocked cardiac muscle gene expression and inhibited the appearance of beating cardiac cells. These data suggest that GATA-4 is necessary for development and differentiation of cardiac myocytes (Grepin *et al.*, 1995). GATA-5 and GATA-6 bind the same DNA sequence as GATA-4 and their expression pattern is similar to GATA-4 in the cardiac lineage. It is possible that these factors are also involved in cardiogenesis (Laverriere *et al.*, 1994; Jiang and Evans, 1996).

2.1.3 Growth factors

Growth factors play a vital role in embryonic cardiogenesis. Treatment of cultured anterior lateral plate mesoderm from Hamburger-Hamilton (H-H) stage 6 chick embryos with antisense oligonucleotide complementary to the second exon of chicken fibroblast

growth factor-2 (FGF-2 or basic FGF) mRNA caused a significant inhibition of myocyte proliferation and contractility (Sugi *et al.*, 1993). It was suggested that the precardiac mesoderm was induced by a FGF-2 like protein present in the adjacent endoderm cells during avian heart development (Parlow *et al*, 1991). A dominant negative fibroblast growth factor receptor type 1 (FGFR-1) that can block the FGFR signalling pathway, was also shown to suppress myocyte proliferation and or survival in tubular stage hearts, during the first week of chicken embryonic development (Mima *et al.*, 1995). Treatment of cultured cardiac cushion tissue with antisense oligonucleotides complementary to transforming growth factor (TGF) ß-3 mRNA inhibited the epithelial-mesenchymal transition of valve precursor cells (Potts *et al.*, 1991). Although, the addition of TGF-ß to embryonic or neonatal rat cardiac myocyte cultures did not show any effect on the DNA synthesis, it was shown to inhibit the stimulatory effects of FGF-2 and insulin like growth factor (IGF) in those cultures (Kardami, 1990). In addition both IGF-I and IGF-II were shown to stimulate the hyperplastic growth of cardiac myocytes and were implicated in postnatal heart growth (Engelmann *et al.*, 1992; Kardami, 1990). It is possible that a direct or indirect cross-talk exists between these growth factors and bHLH transcription factors during cardiogenesis.

2.1.4 Genetic mutations causing hypoplasia of ventricular wall

Retinoic acid and related vitamin A derivatives modulate complex morphogenic events during vertebrate development by binding to retinoic acid receptors (RAR) and retinoid receptors (RXR) which belong to a nuclear receptor superfamily. Targetted disruption of the RXRα gene by homologous recombination in the mouse germ line was shown to cause embryonic lethality between E13.5 and E16.5 due to hypoplastic development of the ventricular chambers of the heart with concurrent defects in ventricular septation. (Sucov *et al.*, 1994). Embryonic vitamin A deficiency was also shown to cause severe defects in cardiac morphogenesis (Wilson *et al.*, 1949; 1953). Deficiencies of the cell adhesion molecules α4 integrin and VCAM (vascular cell adhesion molecule) resulted in epicardial dissolution and subsequent myocardial thinning. A similar phenotype was observed in mice carrying mutations in the N-*myc*, transcription enhancer factor-1 (TEF-

1), Wilms tumor (WT1) and neurofibromatosis (NF1) genes (Rossant, 1996).

2.2 Cardiac growth and development

Cardiac growth can be divided into three distinct developmental stages. During embryonic development, the heart increases in size due to proliferation of both cardiac myocytes as well as nonmuscle cells (endothelia cells, fibroblasts, smooth muscle cells) (Bogenmann and Eppenderber, 1980). Neonatal cardiac development represents a transitional stage between the embryonic and adult stages. The neonatal cardiac myocytes can undergo DNA synthesis and mitotic division during the first week but they fail to induce cell division and DNA synthesis at the end of first and second weeks respectively (Ueno *et al.*, 1988). The DNA synthesis that occurs after birth in the rat heart is part of the process during which the muscle cells become binucleated (Clubb and Bishop, 1984). Therefore DNA replication can be uncoupled from cell division soon after birth. During the postnatal development when cardiac myocytes cease to proliferate, the increased demand placed on the heart is met by physiological hypertrophy of myocytes (Rumayantsev, 1977). Rat cardiac myocytes are mononucleated during fetal and early neonatal development and about 85% of cardiac myocytes become binucleated by the third week after birth (Clubb and Bishop, 1984). Postnatal growth is also accompanied by certain dramatic changes in gene expression. For example, during the fetal stage, ß-myosin heavy chain (MHC) is the most abundant isoform in the heart, whereas the α-MHC isoform becomes predominant in adult rodents (Lompre *et al.*, 1984). In humans, there is only a transient increase in the α-MHC isoform following birth (Chizzonite *et al.*, 1984). The catalytic α subunit of Na/K-ATPase is also subjected to isoform switching (from α3 to α2) (Orlowski and Lingrel, 1990). Another remarkable feature of developing heart is the overall increase in metabolic rate accompanied by a shift from carbohydrate to fatty acid utilisation.

Although it is generally accepted that adult cardiac myocytes are terminally differentiated and indeed limited in their capacity to reenter the cell cycle, there are several exceptions to this paradigm. Ventricular trauma in newt directly leads to regeneration by ventricular myocyte proliferation (Oberpriller *et al.*, 1988). Unlike the newt heart, trauma in mammalian hearts does not lead to ventricular regeneration, however, it is followed by increases in tritiated (^{3}H) thymidine uptake (a sign of DNA synthesis) and proliferation of

atrial myocytes (Rumyantsev, 1974 and 1977). In other studies, limited DNA synthesis was observed in adult ventricular myocytes maintained in culture (Claycomb and Moses, 1988). Aging myocardium tends to lose myocytes progressively and this loss is replaced by myocyte cellular hyperplasia (Anversa *et al.*, 1990). Recently, a few ventricular myocytes undergoing mitosis were also observed in sections of the adult rat myocardium (Kajstura *et al.*, 1994). All these studies suggest that the terminal differentiation of adult cardiac myocytes may not be irreversible.

2.2.1 Cardiac hypertrophy

Cardiac hypertrophy occurs during chronic mechanical overload and is characterized by an additional increase in cell size which results due to increases in both RNA as well as protein levels. As the myocytes differentiate and lose their ability to proliferate soon after birth, they respond to an increased work load by an increase in cell size (hypertrophy), and not by an increase in cell number (hyperplasia). Chronic increase in wall tension leads to left ventricular hypertrophy and ventricular dysfunction (Lejemtel and Sonnenblick, 1993). The pressure overload hypertrophy can be mimicked *in vitro*, by the administration of a variety of stimuli, i.e., adrenergic agonists, endothelin-1, angiotensin II, FGF, and TGFß (Bilsen and Chein, 1993). IGFs are also known to be involved in the cardiac hypertrophy (Han *et al.*, 1987; Turner *et al.*, 1988). Both IGF-I and IGF-II were shown to stimulate cardiac myocyte specific protein synthesis leading to an increase in cell size (Adachi *et al.*, 1994; Florini and Ewton, 1992; Fuller *et al.*, 1992). In addition, mechanical stretch of myocytes is associated with the activation of a hypertrophic response (Sadoshima *et al.*, 1992; Schneider *et al.*, 1991). Several second messengers including $p21^{ras}$, MAP kinase (MAPK), phospholipase C (PLC) and protein kinase C (PKC) have been implicated in the process of hypertrophy (Rozengurt, 1991). Ventricular myocytes re-express a panel of fetal proteins or protein isoforms, which include atrial natriuretic factor (ANF), atrial myosin light chain-1 (MLC-1a) isoform (Hirzel *et al.*, 1985), beta myosin heavy chain (ß-MHC) isoform (Nagai *et al.*, 1987; Nagai *et al.*, 1989; Mercadier *et al.*, 1990), skeletal α-actin (Schwartz *et al.*, 1986), tropomyosin isoform ß (Izumo *et al.*, 1988) and Na^+/K^+ ATPase α2 isoform (Lattion *et al.*, 1986a) (see Table 1) during hypertrophy.

Table 1

Table 1. Phenotypic changes in the expression pattern of myocardial proteins during cardiac growth and hypertrophy. MHC = myosin heavy chain; MLC = myosin light chain; SR = sarcoplasmic reticulum. Presence (+) or absence (-) as well as relative abundance (+, ++, +++) of myocardial proteins during embryonic and adult stages are indicated. Levels of these proteins were reported to be up-regulated (↑) or down-regulated ( ) and in some cases remain unchanged (↔). Adapted from Parker and Schneider, 1991. Changes in the expression of MLC (Hirzel *et al.*, 1985), tropomyosin (Izumo *et al.*, 1988) and Na+/K+ ATPase (Lattion *et al.*, 1986a) isoforms are also included.

Muscle marker	Embryonic	Adult	Levels during hypertrophy
α-MHC	-	+ + +	↓
β-MHC	+ + +	–	↑
α-Actin (Cardiac)	++	+++	↔
α-Actin (Skeletal)	++	±	↑
α-Actin (Smooth)	++	–	↑
MLC-1a	+	–	↑
MLC-1v	+	+	↔
MLC-2v	+	+	↑
α-Tropomyosin	+	+	↔
β-Tropomyosin	+	–	↑
ANF	+++	–	↑
α1-Na^+/K^+ ATPase	+	+	+
α2-Na^+/K^+ ATPase	+	–	↑
SR Ca^{++} ATPase	+	+++	↓

Re-expression of these isoforms during hypertrophy is also accompanied by a loss of atrial/ventricular specificity. For example, atrial specific ANF (Lattion *et al.*, 1986a,b) and MLC-1a (Hirzel *et al.*, 1985; Sutch *et al.*, 1992) are re-expressed in ventricles in response to cardiac overload. All these changes are often referred to as "a return to a fetal gene programme" (Parker and Schneider, 1991).

Adaptive changes observed during cardiac hypertrophy may vary with the species studied and the etiology. In rats, pressure overload leads to a form of hypertrophy that is characterized by a reduction in sarcoplasmic reticular Ca-ATPase activity and a shift from α-MHC to ß-MHC isoform expression (Nagai *et al.*, 1987; Nagai *et al.*, 1989; Mercadier *et al.*, 1990). In contrast, administration of thyroid hormone leads to a different form of hypertrophy that is associated with increased Ca-ATPase activity (Nagai *et al.*, 1987; Nagai *et al.*, 1989; Mercadier *et al.*, 1990; Sayen *et al.*, 1992) and enhancement of α-MHC form levels (Scheuer and Buttrick, 1987). Similarly, FGF-1, FGF-2, TGFß1 and norepinephrine increase the levels of the ß-MHC isoform, while thyroid hormone decreases the levels of ß-MHC isoform in cardiac myocytes (Parker and Schneider, 1991). It is important to unravel the molecular events that initiate hyperplastic as well as hypertrophic growth of the myocardium with a view to prevent or protect the heart from failure.

2.2.2 Apoptosis in the heart

Although, hypertrophy is considered as a compensatory response to the overload of myocardium, beyond a certain period of compensation, cardiac failure ensues. There is scant information about the mechanisms associated with cellular deterioration and dysfunction of myocardium. Apoptosis is a tightly regulated process of cell death involving single cells which exhibit cytoplasmic and nuclear condensation followed by activation of nuclear endonucleases that can cleave chromatin at internucleosomal sites (Wyllie, 1987). By contrast to the nuclear changes in apoptotic cells, necrotic cell death is characterized by loss of plasma membrane integrity and severe changes in the cytoplasmic components (Arends *et al.*, 1990). Recent studies have suggested that apoptosis may serve as a major contributor to the loss of myocytes during myocardial infarctions, while necrotic myocyte cell death follows apoptosis and contributes to the progressive loss of cells with time after infarction (Kajstura *et al.*, 1996). The enhanced levels of Fas (involved in the promotion of cell death; Oltavi *et al.*, 1993; Tanaka *et al.*, 1994) and Bcl-2 (involved in the protection of cells against apoptosis; Reed, 1994) were found in the areas of myocardial infarctions (Kajstura *et al.*, 1996). Apoptosis has also been linked to the control of

the primitive myocardial cell overgrowth associated with cardiac rhabdomyoma and removal of damaged cardiac myocytes after reperfusion injury (Gottileb *et al.*, 1994; Medioni *et al.*, 1994).

2.2.3 *In vitro* and *in vivo* models of cardiac growth and development

Inability of adult cardiac myocytes to regenerate following myocardial infarctions has been an acute problem in clinical cardiology. It is possible that adult cardiac myocytes cease to proliferate due to a block in the cell cycle pathway. The proliferating cell nuclear antigen (PCNA) is essential for DNA replication and expressed at the G1/S phase boundary of the cell cycle. PCNA is normally associated with proliferating cells and its mRNA is present even in adult cardiac myocytes that no longer divide. However PCNA protein is found only in embryonic and neonatal rat cardiac myocytes but not in adult rat cardiac muscle cells, suggesting that this protein is regulated at the posttranscriptional level in adult cardiac myocytes (Marino *et al.*, 1991).

Retinoblastoma protein, the product of the tumor suppressor gene (Rb) is involved in the regulation of cell cycle as well as the transcriptional apparatus of the cell. This protein is unphosphorylated in resting cells and G1, but is phosphorylated during DNA replication. The unphosphorylated form exerts its growth suppressive effect by binding to transcription factors E2F, DRTF1 and the *myc*-oncogene product. When Rb is phosphorylated by the cell cycle kinases (*cdc2*), it releases the transcription factors, allowing them to activate gene transcription necessary for progression through the cell cycle (Marx, 1991). Oncoproteins of certain animal tumor viruses (eg. E1A protein of adenovirus, SV40 large T antigen) work by binding to the Rb protein and preventing it from exerting its growth supressive action. Several other tumor suppressor gene products exist in different cell types (eg. p53, p107, Lee *et al.*, 1995).

It is hypothesized that tumor suppressor products in adult cardiac myocytes (Claycomb, 1992) are constitutively unphosphorylated due to a block or decreased levels of cell cycle kinases. This in turn leads to unavailability of transcription factors that are necessary for the cell to traverse through the cell cycle. If this is the case, overexpression of the proteins that can bind unphosphorylated tumor supressor genes, should lead to reactivation of the cell cycle in adult cardiac myocytes. Adenovirus mediated expression of SV40 large T antigen in neonatal rat ventricular myocytes induced cell proliferation without the loss of expression of differentiation proteins (Sen *et al.*, 1988). However, the infected myocytes divided very slowly and it was often difficult to passage them. It was speculated that the T antigen can not induce proliferation in cells already exited from the cycle (Claycomb, 1992). Transgenic mice expressing an atrial natriuretic factor-SV40 T antigen fusion gene developed unilateral right atrial tumors composed of

differentiated and dividing cardiac myocytes and cardiac arrhythmias (Field, 1988). Later, a fusion gene comprised of the rat α-cardiac myosin heavy chain promoter and T antigen was used to target expression to the myocardium with a view to inducing ventricular myocyte proliferation. The oncogene expression was associated with hyperplasia of both atrial and ventricular cardiac myocytes in adult transgenic animals and these cells can undergo a limited number of passages while retaining the differentiated phenotype (Katz *et al.*, 1992).

Proto-oncogenes such as c-*myc* play a pivotal role in the developmental program of many cells. Increased expression of c-*myc* is reported to prevent differentiation in chicken embryo fibroblasts, hematopoietic and skeletal muscle cells (Jackson *et al.*, 1990). In the heart, transition of cardiac myocytes from proliferative to a terminally differentiated phenotype is synchronous with a decrease in c-*myc* mRNA levels (Schneider *et al.*, 1986). Transgenic mice overexpressing the c-*myc* oncogene in cardiac myocytes, under the control of Rous sarcoma viral (RSV) long terminal repeats (LTR) revealed both atrial and ventricular enlargement compared to their nontransgenic littermates. This increase in cardiac mass was due to additional hyperplastic growth of myocyte during fetal development. Although, there was constitutive expression of c-*myc* in addition to cardiac enlargement in the adult transgenic mice, cardiac myocyte proliferation ceased during postnatal development. Since the interaction of normal cells with v-*myc* transformed cells can suppress the transformed phenotype (LaRocca *et al.*, 1989), it was speculated that non myocytic cells in the hearts of c-*myc* transgenic mice could perform a similar function (Jackson *et al.*, 1990). Indeed, epicardial mesothelial cells (nonmuscle) were shown to induce changes in phenotype and function of adult rat ventricular myocytes in coculture conditions (Eid *et al.*, 1992).

Therapeutically, cardiac injury can be corrected either by stimulation of myocyte proliferation or by grafting with a view to substitute damaged myocytes. Intracardiac grafting may serve this purpose, provided that the grafted cells can contribute to myocardial function. Fetal cardiac myocytes from transgenic mice expressing an α-cardiac MHC-ß-galactosidase fusion gene, when grafted into the myocardium of a syngenic host, formed stable grafts and were viable as long as two months. There were no negative effects of graft formation on the host myocardium and electron microscopic studies revealed the presence of nascent intercalated disks connecting the engrafted fetal cardiomyocytes and the host myocardium (Soonpa *et al.*, 1994). Transplantation of whole heart is another promising way to alleviate severe cardiac damage. Xenotransplantation from swine to humans is increasingly viewed as the best alternative as

there is an acute shortage of organs available for transplantation (McCurry *et al.*, 1995). However, the xenografts are susceptible for hyper acute rejection by the recipient due to failure of complement regulatory proteins (CRPs) present in the graft endothelium to control activation of the recipient's complement system. Hearts from transgenic swine expressing the human CRPs, when transplanted into baboons revealed markedly less vascular injury and functioned for prolonged periods compared to hearts from nontransgenic swine (McCurry *et al.*, 1995).

2.3 Development of the central nervous system (CNS)

The brain and spinal cord of the CNS provide a rapid communication between widely separated parts of the body. Development of CNS begins with neural induction during gastrulation in the mammalian embryo. During gastrulation, the mesoderm triggers an overlying region of ectoderm to become neuroectoderm. At this stage, all of the ectoderm is competent to receive the trigger from mesoderm (McKay,1989). The neuroectoderm (also called neural plate) is first distinguishable soon after 7.5 days post coitus (p.c.) in mouse, then rolls up along the length of the embryo and pinches off to form the neural tube at about 8.0-8.5 days p.c. A variety of motive forces are implicated in folding the simple sheet of neuroepithelium and causing it to roll up into a tube. These forces include microfilaments, microtubules, cell division, convergent extension of cell movements and expansion of the extracellular matrix underlying the neural plate (Schoenwolf and Smith, 1990). Closure of the anterior neuropore is completed by the 15- to 20- somite stage (9.0 days p.c.), whereas the posterior neuropore remains open until the 32- somite stage (10.0-10.5 p.c.). These events are followed by cell division of neuroepithelium lining the neural tube which then leads to regional differentiation along the anteroposterior axis to generate morphologically distinct retina, forebrain, midbrain, hindbrain and spinal cord domains (Deutsch and Gruss, 1991). There is scant information about the mechanisms or factors controlling the regional differentiation of neuroepithelium. The neural crest is formed from a group of cells that originates in the dorsal part of the neural tube at the junction between neuroepithelium and surface ectoderm. Crest cells migrate extensively from the neural tube to ventral and dorsolateral locations where they differentiate into a wide variety of cell types which include adrenomedullary cells, bone,

cartilage, melanocytes, glial, Schwann cells and several other kinds of neurons. Regional differentiation and dorsoventral cellular patterning are determined by the differential expression of genes such as Hox, Wnt, Pax, Pou, homeodomain, helix-loop-helix, Pax, Lim and others, in restricted domains (Deutsch and Gruss 1991).

2.3.1 Origin and diversification of glial cells

Both central and peripheral neural tissues consist of two major classes of cells, neurons and glial cells that are derived from the neuroepithelial cells of the early mammalian embryo. The neurons are electrically excitable and their fundamental task is to receive, conduct and transmit signals in the body. The glial cells are not electrically excitable and surround neurons to support them. Moreover, neurons cannot divide after they have differentiated, while glial cells remain capable of dividing throughout life. Furthermore, glial cells are diversified into oligodendrocytes, astrocytes, microglia and ependymal cells in the CNS and Schwann cells in the peripheral nervous system. The oligodendrocytes as well as Schwann cells extend processes that wrap concentrically around the axons to form an insulating myelin sheath (McKinnon *et al.*, 1991). The functions of astrocytes are largely unknown, although it is known that they extend processes to the surface of nerves, to blood vessels and to nodes of Ranvier. These astrocytes serve as a main source of FGF-2 which was shown to be an essential neurotrophic factor for the survival of differentiated neurons in the CNS (Morrison, 1991). Ependymal cells line the internal cavities of the brain and spinal cord, while microglia are functionally akin to macrophages and surround the nerve. Microglia play a vital role in development, immune response and wound healing in the CNS (Giulian, 1987; Suzumura *et al.*, 1987). These cells produce interleukin-1 and FGF-2 to promote the proliferation of astrocytes (Giulian, 1987; Giulian and Lachman, 1985; Hetier *et al.*, 1988) and neuronal survival (Shimojo *et al.*, 1991) respectively. With the exception of microglia which originate from hemopoietic tissue, all the glial cells share a common embryonic origin with the neurons with which they are associated (Raff, 1989).

The best studied model of glial cell diversification is rat optic nerve, because it contains several types of glial cells but no intrinsic neurons (Raff, 1989). Cell suspensions from the optic nerve during different stages of rat development, contained mainly three types of glial cells and it was found that type-1 astrocytes first appear on embryonic day

16, oligodendrocytes appear on the day of birth and type-2 astrocytes appear at the beginning of the second week after birth (Miller *et al.*, 1985). Type-1 astrocytes extend processes that terminate on blood vessels (Miller *et al.*, 1985), where they induce the underlying endothelial cells to form the blood-brain barrier (Janzer and Raff, 1987). Type-2 astrocytes and oligodendrocytes seem to extend their processes to nodes of Ranvier (Miller *et al.*, 1985) and myelin sheath (Raff, 1989), respectively. Oligodendrocytes and type-2 astrocytes develop from a common, bipotential O-2A progenitor cell (Raff *et al*, 1983), whereas type-1 astrocytes develop from a different neuroepithelial precursor cell (Raff *et al.*, 1984).

2.3.2 Differentiation of O-2A progenitor cells into oligodendrocytes and astrocytes

In the rodent CNS, O-2A progenitor cells originate in the germinal neuroepithelium surrounding the ventricles during late embryonic development, migrate into the destined regions, then differentiate into oligodendrocytes and type-2 astrocytes shortly after birth (LeVine and Goldman, 1988; Reynolds and Wilkin, 1988). The timing of these events vary slightly in different regions of the CNS but the sequence of events is similar. When O-2A cells are cultured *in vitro* in the presence of 10% fetal bovine serum (FBS) they become type-2 astrocytes that express transcripts for glial fibrillary acidic protein (GFAP), whereas when they are cultured in the absence of FBS they become oligodendrocytes that express transcripts for basic myelin protein (BMP) (Raff *et al.*, 1983; Stallcup and Beasley, 1987). This suggests that oligodendrocyte development is the constitutive pathway of O-2A progenitor cell development in the absence of any extracellular signals *in vivo*, while type-2 astrocyte differentiation is an induced pathway in the presence of FBS.

Type-1 astrocytes were shown to secrete growth factors that keep O-2A cells proliferating and prevent their premature differentiation (Noble and Murray, 1984; Noble *et al.*, 1988). Both platelet derived growth factor (PDGF) as well as FGF-2 were shown to inhibit the differentiation of O-2A cells into oligodendrocytes. FGF-2 localized in the subependymal zone of CNS, was shown to prevent progenitor cell terminal differentiation and maintain a high level of PDGF receptor (McKinnon *et al.*, 1990). PDGF produced by type-1 astrocytes, was shown to act as a chemoattractant to direct the migration of these

progenitor cells into regions of the CNS destined to become white matter. Migration of progenitor cells away from their source of FGF-2 would release the block to differentiation, placing the cells under the control of PDGF. However, PDGF unlike FGF-2, promotes only a limited number of cell divisions and thus drives a molecular clock that controls the extent of proliferation during migration as well as timing of progenitor cell differentiation into oligodendrocytes (McKinnon *et al.*, 1991). Progenitor cells can be induced to differentiate prematurely into type-2 astrocytes, if they are cultured in 10% FBS. The optic nerve extracts from a 3-week old rat were found to have ~50 times higher GFAP inducing activity compared to similar extracts from a 1-week old animal, when added to progenitor cells (Hughes and Raff, 1987). Later, it was shown that addition of purified ciliary neurotrophic factor (CNTF) induces O-2A progenitor cells to express GFAP in culture (Hughes *et al.*, 1988; Lillien *et al.*, 1988). Type-1 astrocytes were shown to release CNTF like molecules when the cultures were injured by scratching or passaging cells (Lillien *et al.*, 1988). Thus, type-1 astrocytes play a critical role in the timing of both oligodendrocyte and type-2 astrocyte differentiation. However, the effect of CNTF on O-2A cells appeared to be transient as the induced cells were shown to lose GFAP and become oligodendrocytes (Raff, 1989). This suggests that CNTF can initiate type-2 astrocyte differentiation, but other factors are required to complete the process.

2.4 The fibroblast growth factor (FGF) family

The growing family of fibroblast growth factors (FGF) currently consists of ten members *viz.*, FGF-1 to FGF-10 (see Table 2). FGF was first identified as an activity in the bovine brain (Trowell *et al.*, 1939) and pituitary extracts (Hoffman, 1940) that stimulated the proliferation of BALB-C 3T3 fibroblasts. Later, FGF-2 was identified by its ability to cause the proliferation and phenotypic transformation of BALB-C 3T3 fibroblasts (Gospadarowicz, 1974), whereas FGF-1 was identified by its ability to cause the proliferation and delayed differentiation of myoblasts (Gospadarowicz *et al*, 1975). The prototypic members of the FGF family, FGF-1 (*aka* acidic FGF) and FGF-2 (*aka* basic FGF) were purified to homogeneity based on their ability to bind to heparin (Gospadarowicz *et al.*, 1987). The highly purified preparations of FGFs were subjected for amino acid sequencing and this ultimately led to the cloning of cDNAs for both FGF-1

Table 2

Table 2. Members of the fibroblast growth factor (FGF) family. Adapted from Baird and Klagsbrun, 1991. The number of amino acids (Baird, 1994) and additional references are included for further information.

Member of FGF family	Historical names	Acronyms	Amino acid number	Additional references
FGF-1	Acidic fibroblast growth factor/Heparin binding growth factor-1	Acidic FGF/HBGF-1	155	Gospadarowicz et al, 1987
FGF-2	Basic fibroblast growth factor/Heparin biniding growth factor-2	Basic FGF/HBGF-2	155/196/ 201/210	Baird and Walicke, 1989
FGF-3	int-2	int-2	239/268	Goldfarb, 1990
FGF-4	Kaposi sarcoma FGF/Human stomach cancer transforming gene-1	K-FGF/hst-1	194	Herbert et al., 1990
FGF-5	Fibroblast growth factor-5	hst-1/HSTF-1	267	Goldfarb et al., 1991
FGF-6	hst-1 related gene	hst-2	198	Coulier et al., 1991
FGF-7	Keratinocyte growth factor	KGF	194	Aaronson et al., 1991
FGF-8	Androgen-induced growth factor	AIGF	215	Ohuchi et al., 1994
FGF-9	Glia-activating factor	GAF	208	Miyamoto et al., 1993
FGF-10	FGF-10	FGF-10	215	Yamasaki et al., 1996

(Jaye *et al.*, 1986) and FGF-2 (Abraham *et al.*, 1986a) and determination of their genomic organization (Abraham *et al.*, 1986b,c). Thereafter, new members of the FGF family were identified and their amino acid and nucleotide sequences were determined (Baird and Bohlen, 1990). All the members are structurally related, believed to derive from a common ancestral gene and share a homology of 33-65% at the amino acid level (Jaye *et al.*, 1992). FGFs are proteins with a molecular mass of 16-30 kD and were shown to be potent mitogens *in vitro* for cells of mesodermal and neuroectodermal origin. More recently, FGFs have been implicated in the mitogenesis of other ectodermal cell types and also for endodermal derivatives (Mason, 1994). Their *in vitro* actions also include the modulation of cell motility, differentiation, extension of neurites and survival. They are also involved in normal physiological processes such as embryonic and fetal development, neovascularization and responses to wounding. Some of them are implicated in pathological processes due to their involvement in oncogenesis (eg. FGF-3, FGF-4, FGF-5, FGF-8) or cellular transformation (eg. FGF-1, FGF-2, FGF-6) (Coulier *et al.*, 1991; Baird and Walicke, 1989; Lorenzi *et al.*, 1995).

The expression of FGF-1 is detected in brain, retina, heart, bone matrix and osteosarcoma but predominantly seen in the nervous system. By contrast, FGF-2 is found in all organs, solid tissues, tumours and cultured cells examined so far (Gospadarowicz *et al*, 1987). The expression patterns of FGF-3 to FGF-6 suggest that they are mainly restricted to the embryonic stage of development. FGF-3 gene is actively transcribed throughout embryogenesis but not in adult tissues (Goldfarb, 1990). The expression of FGF-4 gene is restricted to pre-gastrula stage embryo (Herbert *et al.*, 1990). The FGF-5 gene is expressed through most phases of embryogenesis and is also expressed weakly and exclusively in the adult central nervous system (Goldfarb *et al.*, 1991). In adult tissues FGF-6 gene is expressed in skeletal muscle, heart and testis (Coulier *et al.*, 1991). The FGF-7 transcript is detected in several stromal fibroblast lines derived from epithelial tissues of embryonic, neonatal and adult sources, normal adult tissues including kidney and gastrointestinal tract but not brain or lung (Aaronson *et al.*, 1991). Intriguingly, FGF-7 is expressed in the proliferating embryonal carcinoma cells and turned off during differentiation (Curatola and Basilico, 1990), while FGF-3 is turned on in these cells after differentiation (Jakobovits *et al.*, 1986). The temporal and spatial expression patterns of

FGF-8 suggest its involvement in gastrulation, regionalization of the brain and organogenesis of the limb and face (Ohuchi *et al.*, 1994). However, the FGF-8 transcript is exclusively detected at a low level in poly (A)+ RNA isolated from adult mouse testis (Lorenzi *et al.*, 1995). The FGF-9 transcript is detected at low levels in kidney and brain but not in other tissues (Miyamoto *et al.*, 1993). The cellular localization of FGF-9 mRNA suggests that it is preferentially expressed in neurons (Tagashira *et al.*, 1995). Recently, the tenth member of FGF family (FGF-10) is identified and its cDNA has been isolated from rat embryos by homology-based polymerase chain reaction (Yamasaki *et al.*, 1996). The FGF-10 mRNA is expressed in several discrete regions of the embryo and in adult tissues, it is preferentially expressed in lung (Yamasaki *et al.*, 1996). Thus, the expression patterns of members of FGF family appear to be regulated in a temporal and spatial manner.

2.4.1 Multiple forms of FGFs

FGFs exist in multiple forms due to initiation of translation from alternative codons in the same mRNA (Florkiewicz and Sommer, 1989), alternative splicing of RNA (Borja *et al.*, 1993), as well as posttranslational modifications (Doble *et al.*, 1991; Klagsbrun *et al.*, 1987; Mason, 1994). However, the biological significance of these multiple forms is not clear. Translational initiation at a classical methionine (AUG) codon results in low molecular weight forms, while high molecular weight forms can also result by translation from upstream leucine (CUG) codons in the same mRNA (eg. FGF-2 and FGF-3; Acland *et al.*, 1990; Powell and Klagsbrun, 1991). A novel FGF-2 isoform with a unique N-terminal extension in chicken (Borja *et al.*, 1993) as well as multiple isoforms of murine FGF-8 (Tanaka *et al.*, 1992) appear due to alternative splicing of mRNA. Further heterogeneity in the FGF family is also created by proteolysis of high molecular weight forms and other posttranslational modifications. FGF-2 was originally purified as a 16.5 kiloDalton (kD) form from bovine pituitary (Esch *et al.*, 1985) and was subsequently shown to arise by proteolysis of an 18 kD species (Klagsbrun *et al.*, 1987). Secreted forms of Xenopus FGF-3 are subjected to proteolytic cleavage at a consensus cleavage site for plasmin to generate multiple forms (Kiefer *et al.*, 1993). Glycosylation, methylation, phosphorylation, ribosylation and nucleotidylation of FGF-2 isoforms have been reported (Mason, 1994) and these modifications might differentially regulate their intracellular

activities. Nucleotidylation was also reported for FGF-1, FGF-6 and FGF-7 (Mason, 1994).

2.4.2 Fibroblast growth factor receptor (FGFR) family

FGFs mediate various biological responses including mitogenesis, angiogenesis and repair of tissue injury by binding to two classes of cell surface receptors (Jaye *et al.*, 1992). The high affinity, low capacity receptors (FGFR) bind FGFs with a K_d of 20-600 pM and belong to the tyrosine kinase family (Partanen *et al.*, 1992). Mammalian cells express four types of FGF receptors (FGFR-1-FGFR4; see Table 3) which consist of extracellular, transmembrane and intracellular domains (Coughlin *et al.*, 1988; Fig. 2). The intracellular domain has an intrinsic tyrosine kinase activity. In addition, a nontyrosine kinase cysteine-rich transmembrane protein has also been identified as a high affinity receptor for FGFs, although its function remains unknown (Burrus *et al.*, 1992). The low affinity, high capacity receptors are cell surface proteoglycans containing heparan sulphate side chains and bind FGFs with a K_d of 2-20 nM (Burgess and Maciag, 1989). Low affinity receptors for FGFs include the extracellular matrix proteoglycan perlecan, transmembrane molecules such as syndecan, betaglycan, certain isoforms of CD44, glypican and cerebroglycan (Hardingham and Fosang, 1992). The cytoplasmic domain of syndecan is associated with the cellular actin cytoskeleton and thus the binding of FGFs to this low affinity receptor may regulate cell morphology and behaviour (Jaye *et al.*, 1992). However, FGFs seem to transduce signals to the cytoplasm mainly through their high affinity receptors (Johnson and Williams, 1993), while the low affinity receptors have been implicated in FGF storage and stabilization of FGF-FGFR interaction (Saksela *et al.*, 1988).

High affinity FGF receptor tyrosine kinases (FGFRTK) show a high degree of similarity particularly in their tyrosine kinase domain, to other members of the tyrosine kinase receptor family which include receptors for platelet derived growth factor (PDGF), epidermal growth factor (EGF) and insulin (Aaronson, 1991). The members of the FGFRTK family (FGFR-1 to FGFR-4) share a high degree of homology at the amino acid level (60-70%) and have a similar structure (Partanen *et al.*, 1992). FGFRTKs exhibit an enormous repertoire of variants each possessing different ligand binding properties, which

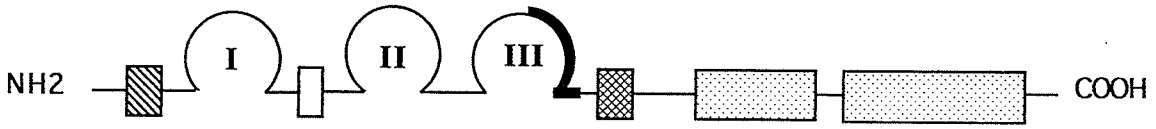
Table 3

Fig. 2

Table 3. Members of the fibroblast growth factor receptor (FGFR) family. Adapted from Partanen *et al.*, 1992 and Johnson and Williams, 1993.

Fig. 2. Schematic representation of the structure of high affinity receptor for fibroblast growth factor (FGFR). The striped box indicates a hydrophobic leader sequence, the three loops (I-III) indicate the immunoglobulin-like domains in the extracellular portion of the receptor, the open and cross-hatched boxes indicate the acid box and transmembrane domains, respectively, and the stippled boxes represent kinase domains (Johnson and Williams, 1993). The location of exon III in the third immunoglobulin-like domain is indicated by a broad black line.

Member of FGFR family	Acronyms	Exon III splice variants	FGF binding
FGFR-1	flg, cek-1	III a III b III c	FGF-2>FGF-1 FGF-1>FGF-2 FGF-1=FGF-2
FGFR-2	bek, cek-3, K-sam, KGFR	III b III c	FGF-1=FGF-7>FGF-2 FGF-1=FGF-2
FGFR-3	Cek-2	III c	FGF-1, FGF-2
FGFR-4	—	III c	FGF-1



result from alternative splicing or alternative polyadenylation of the transcripts of the FGFR genes. All members of FGFR family exist as short or long isoforms due to alternate splicing which can result in the presence of two (short) or three (long) immunoglobulin like domains (*aka* Ig loops) in the extracellular region (Jin *et al.*, 1994). The short form of FGFR lacks the most distal Ig loop I and still can be activated by FGFs and hence loop I appears to be dispensable for ligand mediated signalling. Although loop I is not required for ligand binding, its presence may alter the binding affinity of FGF as well as heparin and its physiological importance is not fully understood (Mason, 1994). However, loops II and III have been implicated in ligand binding and exhibit striking specificity in their interactions with different FGFs. For example FGF-1 binds to loop II at high affinity but does not interact detectably with loop III, while FGF-7 shows high affinity binding to loop III but not loop II. The binding specificities of these ligands were confirmed using short peptides of individual Ig loops, which were tested for their ability to neutralize mitogenic responses (Cheon *et al*, 1994). Although FGFRTK can bind to more than one type of FGF, their affinities vary from ligand to ligand. This is achieved by the alternative splicing of exons encoding the C-terminal region of Ig loop III to generate IIIa, IIIb and IIIc isoforms of FGFR. These exon III isoforms exhibit varying affinities to different ligands (see Table 3; Partanen *et al.*, 1992; Johnson and Williams, 1993). For example FGFR-1-IIIc can bind to both FGF-1 and FGF-2 with comparable affinities, while FGFR-1-IIIb can bind to FGF-1 with a higher affinity than FGF-2 (Johnson and Williams, 1993). Differential expression of FGFR isoforms has been observed in mouse tissues during development. The IIIc exon was expressed in all tissues examined with the exception of liver. The IIIa exon was expressed in brain, skeletal muscle and skin, while the IIIb exon was expressed predominantly in skin and at lower levels in brain, kidney, muscle and placenta (Werner *et al.*, 1992). Thus the existence of these receptor variants makes it possible for the multiple members of FGF family to exert their biological effects with a high degree of specificity in a temporal and spatial manner during development.

2.4.3 FGFR signalling at the cell surface

There is a growing interest in the area of intracellular signalling pathways involved in FGFR activation. Binding of FGF to its high affinity receptor induces receptor

dimerization which in turn leads to their autophosphorylation. Both homodimeric and heterodimeric receptor species can be formed between members of the FGFR family (Ueno *et al.*, 1992). However, dimerization of a mutant high affinity FGFR that lacked the tyrosine kinase domain with the wild type FGFR led to the inhibition of signal transduction (Amaya *et al.*, 1991). Receptor dimerization induces phosphorylation of tyrosine residues in their counterparts and as a result, various intracellular signalling molecules such as phospholipase C-γ (PLC-γ) that contain SH2 domains, are recruited to the activated receptors. Interaction of FGFR with signalling molecules leads to a cascade of cellular events which include stimulation of calcium mobilization, activation of protein kinase C, hydrolysis of phosphoinositides, increased phosphorylation of cellular proteins and increased transcription of a subset of cellular genes including c-*myc* and c-*fos* (Jaye *et al.*, 1992).

Depending on the cell type, FGFs trigger proliferation, differentiation and inhibition or maintenance of differentiated phenotype via a receptor mediated pathway. The specific signalling mechanisms that can give rise to these manifestations are unknown. Phosphorylation of tyrosine (Tyr) 766 residue in the C-terminal of FGFR creates a binding site for the SH2 domain of PLC-γ1 and mutation of this site into phenylalanine (Phe) blocks PLC-γ1 action without affecting the mitogenic pathway (Mohammadi *et al.*, 1992; Peters *et al.*, 1992a). Thus, PLC-γ1 may not be important for pathways leading to FGF-induced mitogenesis. The mutant receptor (Tyr 766 to Phe) does, however, autophosphorylate and mediate increased tyrosine phosphorylataion of other cellular proteins (Mohammadi *et al.*, 1992; Peters *et al.*, 1992a). The kinase deficient forms of FGFR-1 lacking an intracellular kinase domain and tyrosine autophosphorylation sites at amino acids 653 and 766, were shown to act as dominant negative inhibitors of FGFR-1 and suppress both proliferative responses and phosphorylation of Tyr 653 (Mason, 1994). It is possible that the FGF-induced mitogenesis requires phosphorylation of either Tyr 653 alone or both Tyr 653 and Tyr 766 residues. However, the kinase deficient forms could not supress phosphorylation of Tyr 766 or the consequent activation of PLC-γ of the wild type FGFR-1 containing kinase domain. This suggests that phosphorylation of Tyr 766 occurs in *cis* whereas phosphorylation of Tyr 653 requires a *trans* intermolecular mechanism (Hou

et al., 1993; Shi *et al.*, 1993). Stimulation of cells with FGF was also reported to lead to increased phosphorylation of *Raf*-1 (Morrison *et al.*, 1988) and mitogen-activated protein (MAP) kinase (Creuzet *et al.*, 1995; Liu *et al.*, 1995). In addition, FGF-2 mediated MAP kinase activation was shown to be regulated by a MAP kinase phosphatase in MM14 skeletal myoblast cells (Campbell *et al.*, 1995). Currently, the intracellular signalling molecules that bind to FGFRTK are unknown other than PLC-γ. Also, there is scant information about other components involved in the signal transduction pathway of FGFRTK.

2.4.4 Mode of FGF release

FGFs fall into two distinct categories with respect to their mode of release from cells. Several members of the FGF family possess "classical" leader sequences found in most exported proteins and are released from the cells. FGF-1, FGF-2 and FGF9 lack the characteristic hydrophobic signal peptide sequences and their mode of secretion has yet to be resolved. It is possible that these factors are released from cells when the integrity of the plasma membrane is compromised during tissue injury and cell death (Gajdusek and Carbon, 1989). However, recent reports indicate that these molecules are released from cells by novel secretory mechanisms. FGF-1 appears to be released from cells in response to heat shock (Jackson *et al.*, 1992). Further it is released as a dimer that is inactive in mitogenic assays and cannot bind to heparin (Jackson *et al.*, 1992). FGF-2 can be release from cells via matrix associated sites by heparin, heparan sulphate and heparinases (D'Amore, 1990), exocytosis independent of the endoplasmic reticulum-Golgi pathway (Mignatti *et al*, 1992), plasminogen activator mediated proteolysis (Saksela and Rifkin, 1990), injuries resulting from complement-mediated reactions (Floege *et al.*, 1992), contraction mediated sarcolemmal injury (Clarke *et al.*, 1995) and gentle mechanical wounding (Kardami *et al.*, 1991a). Recently, it has been shown that FGF-2 expressed in COS-1 cells can be exported into the culture medium in a time dependent fashion and this release can be blocked in the presence of cardioglycosides oubain, digitoxin and digoxin (Florkiewicz *et al.*, 1996). As the cardiacglycosides specifically inhibit the electrochemical gradients generated by cell membrane associated Na^+/K^+ ATPase, it has been proposed that

FGF-2 may be released from the cells through an active transport mechanism via Na^+/K^+ ATPase. Further, endogenous ouabain-like compounds may regulate the natural release of FGF-2 during injury repair, inflammation and angiogenesis (Florkiewicz *et al.*, 1996). Although, FGF-9 lacks a typical N-terminal signal sequence, it is constitutively secreted from transfected COS cells (Miyamoto *et al.*, 1993). Despite entering the "classical" secretory pathway and undergoing primary glycosylation, FGF-3 is shown to accumulate in an immature form in the Golgi complex from where it is released very inefficiently compared to FGF-4 and FGF-5. However, FGF-3 is released very efficiently from certain fibroblast lines. Further studies indicated that the sequences for retention in Golgi are located toward the N-terminus of FGF-3. This suggests a novel mechanism of regulation for release of FGF-3 between cell types (Kiefer *et al.*, 1993).

2.5 Fibroblast growth factor-2 (FGF-2 or basic fibroblast growth factor)

FGF-2 (Basic fibroblast growth factor) is a potent mitogen for tissues of mesodermal and neuroendodermal origin and exerts its biological activities by binding to the cell surface receptors of tyrosine kinase family (FGFR-TK; Gospadarowicz *et al.*, 1987). This growth factor was originally purified as a 16.5 kiloDalton (kD) form from bovine pituitary (Esch *et al.*, 1985), 17.5 kD form from human placenta (Sommer *et al.*, 1987) and was subsequently shown to arise by proteolysis of an 18 kD species (Klagsbrun *et al.*, 1987). More recently, high molecular weight forms of FGF-2 (21-25 kD) were identified (Rifkin and Moscatelli, 1989) which result from the use of alternate translation initiation codons (Florkiewicz and Sommer, 1989) or alternative splicing of mRNA (Borja *et al.*, 1993). FGF-2 is a basic protein (pI>9.0) and shares about 55% sequence homology at the amino acid level with FGF-1 (pI 5.6) and about 43% with other members of the FGF family (Rifkin and Moscatelli, 1989). Both FGF-2 and FGF-1 possess the ability to bind to heparin sulphate proteoglycans (HSPG) in the extra cellular matrix (ECM) and can be eluted from heparin-sepharose columns with 1.5 M and 1.0 M NaCl, respectively (Esch *et al.*, 1985). The heparin-FGF-2 complex protects FGF-2 from proteolytic degradation and association of FGF-2 with HSPG in the ECM offers a mechanism for its distribution and storage *in vivo* (Gospadarowicz and Cheng, 1986; Rifkin and Moscatelli, 1989). Structure-function analysis of FGF-2 revealed two regions that are involved in high affinity receptor

binding (residues 32-76 and 114-123) and low affinity heparin binding (42 C-terminal residues) (Basilico and Moscatelli 1992). Phosphorylation of the Thr 120 residue by protein kinase A was shown to increase the affinity of FGF-2 for its receptor (Basilico and Moscatelli 1992). FGF-2 contains four cysteine residues. Two of them are conserved among all members of the FGF family (Yoshida *et al.*, 1987) and are important in determining the tertiary structure of FGF-2 through intramolecular disulfide bonds, while the remaining two residues are not essential for biological activity (Fox *et al.*, 1988).

FGF-2 is found in all organs, solid tissues, tumours and cultured cells examined so far (Rifkin and Moscatelli, 1989). It is predominantly an intracellular molecule due to the absence a signal sequence for its secretion and postulated to act in an intracrine manner (Logan, 1990). However, recent reports indicate that it is also found in serum and the levels may vary between 190 to 200 ng/L (Kurobe *et al.*, 1992). It is possible that FGF-2 is released from cells when the integrity of the plasma membrane is compromised during tissue injury and cell death (Gajdusek and Carbon, 1989). FGF-2 may also be released from cells via matrix associated sites by heparin, heparan sulphate and heparinases (D'Amore, 1990), exocytosis independent of the endoplasmic reticulum-Golgi pathway (Mignatti *et al.*, 1992), plasminogen activator mediated proteolysis (Saksela and Rifkin, 1990), injuries resulting from complement-mediated reactions (Floege *et al.*, 1992), contraction mediated sarcolemmal injury (Clarke *et al.*, 1995), gentle mechanical wounding (Kardami *et al.*, 1991a) and via Na^+/K^+ ATPase mediated active transport (Florkiewicz *et al.*, 1996).

2.5.1 FGF-2 gene structure

The human FGF-2 gene spans over 40 kb on chromosome 4 and is reported to exist as a single copy gene in the genome (Abraham *et al.*, 1986b,c; Mergia *et al.*, 1986). The gene contains three exons interrupted by two introns (Abraham *et al.*, 1986b). The first intron is at least 16 kb long and separates codons 60 and 61, while the second intron is 16 kb long and separates codons 94 and 95 (Abraham *et al.*, 1986b; Shibata *et al.*, 1991). FGF-2 cDNAs were cloned from human (Abraham *et al.*, 1986b; Kurokawa *et al.*, 1987), bovine (Abraham *et al.*, 1986a), rat (Shimasaki *et al.*, 1988), mouse (Hebert *et al.*, 1990) and chicken (Borja *et al.*, 1993) sources and their coding sequences have been highly

conserved (84 to 98%) during evolution (Borja *et al.*, 1993). For example human FGF-2 shares about 98.7% of sequence homology with bovine FGF-2 (Abraham et al., 1986a,b) and about 87% with rat FGF-2 (Kurokawa *et al.*, 1987 and 1988). In addition, the FGF-2 gene shares a high degree of homology with the other members of the FGF family and boundaries for exon 1, 2, and 3 of all the known genes of the FGF family (FGF-2, FGF-3, FGF-4 and FGF-5) align perfectly except that the exon 1 boundary for FGF-2 is shifted by three nucleotides. This suggests that their genes are derived from a single ancestral gene through processes of duplication and evolutionary divergence (Baird and Bohlen 1990; Gospadarowicz *et al.*, 1987). Transcription of the human FGF-2 gene appeared to rely on GC rich sequences in stead of a canonical TATA box (Shibata *et al.*, 1991). RNA blot analysis of FGF-2 transcripts in several tissues and cell lines indicated the presence of multiple species of mRNA ranging from 1-7 kb in size, which hybridize with FGF-2 cDNA specific probes (Abraham *et al.*, 1986; Powell and Klagsbrun, 1993; Murphy *et al.*, 1988a,b). These size differences may result due to transcriptional termination followed by multiple poly A signals (Kurokawa *et al.*, 1987) or varying lengths of 5′- and 3′-untranslated sequences (Prats *et al.*, 1989), alternative splicing (Borja *et al.*, 1993; el-Husseini *et al.*, 1992) as well as RNA degradation (Abraham *et al.*, 1986b, el-Husseini *et al.*, 1992). Although the major transcript coding for FGF-2 is about 6 kb in several rat tissues, a shorter 1.8 kb transcript was found to be predominant in the embryonic rat brain (Powell *et al.*, 1991). Transcription of antisense RNA from the opposite strand of FGF-2 gene has also been reported in Xenopus (Kimelman and Kirschner, 1989) as well as human (Murphy and Knee, 1994).

2.5.2 High and low molecular weight forms of FGF-2

FGF-2 exists in multiple forms due to initiation of translation from alternative codons (leucine versus methionine) in the same mRNA (Florkiewicz and Sommer, 1989), alternative splicing of RNA (Borja *et al.*, 1993) as well as proteolysis of high molecular weight forms (Doble *et al.*, 1991; Klagsbrun *et al.*, 1987). The 18 kD low molecular weight form of FGF-2 is initiated at a classical methionine (AUG) codon, and high molecular weight forms of FGF-2 can also result by translation from upstream leucine (CUG) codons in the same mRNA (Florkiewicz and Sommer, 1989). The 18 kD species is

highly conserved and there is about 90% sequence homology between proteins from human, bovine, rat, mouse and chicken (Abraham *et al.*, 1986a,b; Borja *et al.*, 1993; Hebert *et al.*, 1990; Shimasaki *et al.*, 1988). The high molecular weight forms also appear to be conserved between species and contain higher levels (~29%) of arginine (Arg) in their amino terminal extensions. This may confer unique properties to these forms (Rifkin and Moscatelli, 1989). However, high molecular weight forms of FGF-2 differ in their sizes depending on the species. For example, 22 and 21.5 kD forms in the rat (Powell and Klagsbrun, 1991), 20.3 and 19 kD forms in the chicken (Borja *et al.*, 1993) and 24, 23 and 22.5 kD forms in the human (Florkiewicz and Sommer, 1989), are translated from upstream CUG sites. There is scant information about the functional significance of high molecular weight forms of FGF-2 due to the unavailability of purified proteins.

Immunolocalization studies indicate that high and low molecular weight forms of FGF-2 are not equally distributed within the subcellular compartments. The low molecular weight form of FGF-2 is primarily cytoplasmic, whereas the other forms are predominantly nuclear (Bugler *et al.*, 1991). This has raised the possibility of specific nuclear signalling function for these molecules that is parallel to the classical tyrosine kinase transduction pathway from the cell surface. FGF-2 is normally detected in the nuclei of astrocytes and neurons of the rodent brain and appears in the cytoplasm following central nervous system lesion (Logan *et al.*, 1992). Exogenously added FGF-2 is translocated to the nuclei of various cell types *in vitro* during the transition from G_0 to G_1 phase of the cell cycle (Bouche *et al.*, 1987; Baldin *et al.*, 1990). Stimulation of cells by extracellular FGF-1 leads to redistribution of FGFR-1 to a perinuclear location followed by phosphorylation of a number of proteins including p85 which shares homology with the cytoskeleton associated protein cortactin. Thus it is proposed that both the cytoskeleton and high affinity receptor are involved in translocating exogenously added FGF to the nucleus (Zhan *et al.*, 1993). FGF-2 was shown to bind to casein kinase II in the nuclei of adult bovine aortic endothelial cells (ABAE), increase phosphorylation of its substrate nucleolin and thereby increase the transcription of ribsomal genes (Amalric *et al.* 1994). In addition, nuclear forms of FGF-2 have been implicated in the transcription of phosphoglycerate kinase genes (Nakanishi *et al.*, 1992) and also in DNA synthesis (Baldin *et al.*, 1990; Bugler *et al.*, 1991; Cattini *et al.*, 1991; Florkiewicz *et al.*, 1991; Renko *et al.*, 1990). In adult bovine aortic endothelial

cells infected by recombinant retroviruses expressing high or low molecular weight forms of FGF-2, constitutive expression of the low molecular weight form led to cell transformation whereas expression of high molecular weight forms resulted in cell immortalization (Couderec *et al.*, 1991).

2.5.3 Biological activities of FGF-2

Biological functions of FGF-2 reported so far, can be largely attributed to 18 kD FGF-2, since a recombinant form of this protein is widely used for *in vitro* studies. There is scant information about the importance of high molecular weight forms of FGF-2 resulting from the upstream CUG sites due to the unavailability of purified proteins. High molecular weight FGF-2 binds to the same cell membrane receptors and is mitogenic similar to 18 kD FGF-2 (Moscatelli *et al.*, 1987; Amalric *et al.*, 1991). A role for the high molecular weight (25 kD) FGF-2 species in liver regeneration has been suggested (Presta *et al.*, 1989). FGF-2 exerts a wide variety of activities in many cell types, including mitogenesis, induction of plasminogen activator, interstitial and type IV collagenase and increased cell migration *in vitro* (Mignatti *et al.*, 1989; Moscatelli *et al.*, 1986; Presta *et al.*, 1986). It can stimulate neovascularization/angiogenesis in cornea, cheek pouch, chorioallantoic membrane, kidney capsule and skin and participate in wound repair *in vivo* (Baird and Walicke, 1989; Rifkin and Moscatelli, 1989). In addition, it can also stimulate endothelial cell proliferation both *in vitro* and *in vivo* (Folkman and Klagsbrun, 1987). FGF-2 is present in high quantities in all vascularized tissues, including tumours and thus, appears to be involved in both physiological and pathological regulation of new blood vessel growth (Baird and Bohlen, 1990). In addition to its ability to promote cellular proliferation, FGF-2 also influences the differentiation of a variety of cell types. It can induce the neurite outgrowth of hippocampal neurons (Walicke *et al.*, 1986), cerebral cortical neurons (Morrsion *et al.*, 1986) and support the survival of cholinergic neurons *in vivo* (Anderson *et al.*, 1988). Addition of FGF-2 to skeletal muscle myoblasts was shown to inhibit differentiation into myotubes (Clegg *et al.*, 1987). The ability of FGF-2 to influence the differentiation of a variety of cell types suggests that it may play important roles during development. Application of purified FGF-2 to ectodermal explants of early Xenopus embryos led to mesoderm induction (Slack *et al.*, 1987). It was also shown to

enhance the synthesis and release of prolactin (PRL) from lactotropes and thyrotropin (TSH) from thyrotropes (Baird *et al.*, 1985). FGF-2 is not a potent transforming factor unless its coding sequence is fused to a signal sequence for secretion from either immunoglobulin or growth hormone (Blam *et al.*, 1988; Rogelj *et al.*, 1988). However, aberrant production of FGF-2 or dysregulation of its signal transduction pathway may contribute to pathological conditions. FGF-2 is a potent mitogen for vascular smooth muscle cells and it has been implicated in the development of atherosclerosis (Baird and Walicke, 1989). High levels of FGF-2 were associated with diabetic complications which include retinopathy, nephropathy and neuropathy (Karpen *et al.*, 1992).

2.5.4 Regulation of FGF-2

FGF-2 is widely distributed and known to exert potent multipotential trophic effects on several cell types. However, the bioavailability of this potent mitogen must be restricted in all the tissues to avoid uncontrolled proliferation of cells. A high affinity of FGF-2 for heparin suggests that heparin sulphate proteoglycans in the ECM can serve as a storage depot as well as a local source of growth factor *in vivo*. FGF-2 bound to the ECM can be made bioavailable by either increased expression of high affinity receptors on the target cells which can remove the growth factor from low affinity binding sites or by damage of the matrix during injury and tissue remodelling. The ability of basement membrane to regulate endogenous levels of FGF-2 could be compromised in diseases associated with changes in ECM (eg. diabetes) resulting in characteristic complications of cell proliferation (Baird and Walicke, 1989).

In addition to its regulation of bioavailability by ECM, FGF-2 expression is also controlled at transcriptional and translational levels. The stability of its mRNA may be regulated by natural antisense transcripts in human astrocytoma U87MG cells (Murphy and Knee, 1994) and Xenopus oocytes (Kimelman and Kirschner, 1989). Also, there is a high degree of conservation of AT rich motifs in the 3′ untranslated region of FGF-2 mRNA (el-Husseini *et al.*, 1992), which have been shown to play a major role in mRNA stability of many growth regulatory genes (Akashi *et al.*, 1991; Brawerman, 1987; Reeves *et al.*, 1987). The levels of FGF-2 mRNA were increased rapidly in rat glioma C6 cells (Powell and Klagsbrun, 1993) and human astrocytoma U87MG cells (Murphy *et al.*, 1988a,b) after

treating them with serum, phorbol esters and other mitogenic stimuli suggesting that the transcriptional activation of the FGF-2 gene plays an important role during the early events of a mitogenic response. Similarly, direct stimulation of adenylate cyclase with forskolin or protein kinase C with phorbol ester treatment was also shown to increase FGF-2 mRNA levels in bovine adrenal medullary cells (Stachowiak *et al.*, 1994). It was also demonstrated that FGF-2 can induce its own mRNA expression but not FGF-1 mRNA expression in serum starved rat aortic smooth muscle cells (Alberts *et al.*, 1994). A recent report indicated that the human FGF-2 promoter can be repressed by the tumor suppressor gene product p53 and activated by a mutant p53 (Ueba *et al.*, 1994).

FGF-2 exists in multiple forms due to the initiation of translation from alternate codons (AUG versus CUG) in the same mRNA (Florckiewicz and Sommer, 1989; Renko *et al.*, 1990) as well as proteolysis of high molecular weight forms (Klagsbrun *et al.*, 1987). These forms have distinct intracellular localization and can modify cell phenotypes differently (Bikfalvi *et al.*, 1995). It was suggested that global or alternative translation from FGF-2 mRNA can be modulated by *cis*-acting elements corresponding to secondary or tertiary RNA structures, which could be the targets of cell-specific *trans*-acting factors (Prats *et al.*, 1992). Also, alternative translation from human FGF-2 mRNA was shown to be controlled by internal ribosomal entry sequences (IRES) located in between nucleotides 154 and 318 of the 5′ untranslated region in a cap-independent manner (Vagner *et al.*, 1995). In addition, multiple isoforms of FGF-2 exist due to glycosylation, methylation, phosphorylation, ribosylation and nucleotidylation (Mason, 1994) and these modifications might differentially regulate their intracellular activities. The factors or mechanisms that can regulate the relative levels of these forms during development are largely unknown.

2.6 FGF-2 in the heart

The mechanisms involved in the cellular growth and differentiation of cardiac myocytes during physiological as well as pathological growth of the mammalian heart are poorly understood. A fundamental constraint to the development of therapeutic approaches for the treatment of heart disease, is the inability of adult cardiac myocytes to divide. This has stimulated an enormous amount of interest in the area of cardiovascular research to identify factors that would be able to communicate growth messages from their receptors

on the sarcolemmal cell surface to DNA, in order to initiate cellular growth, regulate synthesis of various muscle proteins and increase the proliferative potential of cardiac myocytes. The multifunctional polypeptide growth factors have been implicated in the normal growth and differentiation of cells from a variety of tissues and could fulfil the above roles as possible regulators of myocardial growth and differentiation. Several growth factors [eg. fibroblast growth factor (FGF), transforming growth factor (TGF) ß, insulin like growth factor (IGF), platelet-derived growth factor (PDGF), heparin binding-epidermal growth factor (EGF) and myotrophin] have been linked to the myocyte growth (Cummins, 1993; Engelmann *et al.*, 1992; Whitman and Melton, 1989; Abraham *et al.*, 1993, Mukherjee *et al.*, 1993). Recent studies show that FGF-1 and FGF-2, and their receptors play critical roles in the growth and development of the heart and vasculature and are involved in angiogenesis, vasculogenesis and cardiomyogenesis (Engelmann *et al.*, 1993; Flamme and Risau, 1992; Hughes and Hall, 1993; Liu and Nicoll, 1988). This section will focus mainly on the role of FGF-2 in cardiac growth and development.

2.6.1 Early cardiac development

A role for FGF-2 has been implicated in the induction of mesoderm in cultured animal caps of the Xenopus blastula (Slack *et al.*, 1987; Kimelman *et al.*, 1988). This is consistent with the presence of FGF-2 mRNA and protein in early embryos (Kimelman *et al.*, 1988; Slack and Isaacs, 1989). Formation of vertebrate heart requires commitment of mesodermal precursor cells to the cardiac lineage. It was suggested that the precardiac mesoderm was induced by a FGF-2 like protein present in the adjacent endoderm cells during avian heart development (Parlow *et al.*, 1991). Thus, it is possible that FGF-2 is a key player in early cardiac development. Indeed, its involvement has been elegantly shown by treating precardiac lateral plate mesoderm from chick embryos (H-H stage 6) with antisense oligonucleotide complementary to the second exon of FGF-2 mRNA which resulted in a significant inhibition of myocyte proliferation and contractility (Sugi *et al.*, 1993). Disruption of the FGFR signalling pathway by retroviral mediated expression of a dominant negative FGFR-1 was also shown to suppress myocyte proliferation and or survival in tubular stage hearts, during the first week of chicken embryogenesis (Mima *et al.*, 1995). However, expression of dominant negative FGFR-1 after the second week of

embryogenesis, did not block the proliferation of myocytes (Mima *et al.*, 1995). Although dominant negative FGFR-1 used in this study could block all types of FGFR mediated signalling pathways (Amaya *et al.*, 1991), it would not be able to block signalling by non-tyrosine kinase cysteine rich FGFR, whose function in embryonic development is largely unknown (Olwin *et al.*, 1991). Taken together, these reports indicate that FGF-2 regulates *in vivo* mitogenesis and or survival of embryonic myocytes early in cardiogenesis.

2.6.2 Distribution

During development, FGF-2 staining in the embryonic chicken heart (Joseph-Silverstein *et al.*, 1989) first appeared as punctate sarcoplasmic inclusions at a stage (H-H stage 9) which precedes the onset of contraction (Parlow *et al.*, 1991). This growth factor is widely distributed in several tissues including myocytes, valves, smooth muscle of aorta, coronary arteries and other vessels (Cummins, 1993). In adult bovine cardiac sections, it was shown to be associated with the nuclei, intercalated discs and endomysium of both atrial and ventricular fibres. Higher levels of FGF-2 staining as well as protein were found in the atria compared to ventricles, suggesting a correlation with a higher regenerative potential of atrial myocytes. Also blood vessels, nonmuscle cells and connective tissue cells were shown to react strongly with FGF-2 specific antibodies (Kardami and Fandrich, 1989). FGF-2 was also localised to endothelial cells and cardiac myocytes in the human ventricles (Casscells *et al.*, 1990). However, similar patterns of distribution for FGF-1, FGF-2 and TGFß were shown during embryonic and neonatal development of the rat heart from 11-20 days of gestation and up to five weeks after birth (Spirito *et al.*, 1991). Recently, FGF-2 has also been localized to cardiac gap junctions (Kardami *et al.*, 1991) and intense staining was also reported at the areas of myocardial infarction/injury (Padua and Kardami, 1993). In isolated neonatal (Kardami *et al.*, 1993) and adult rat cardiac myocytes (Speir *et al.*, 1992), FGF-2 staining appeared to be mainly perinuclear and diffuse in the cytoplasm.

2.6.3 High affinity receptors

FGF-2 is known to exert its biological effects by binding to the cell surface receptors of tyrosine kinase family. FGFR-1 is the only high affinity receptor expressed in

both embryonic and adult heart (Engelman *et al.*, 1993). The levels of FGFR-1 were reported to be higher in fetal heart and decline after birth, suggesting that the reduction of FGFR-1 levels may play a role in the transition of heart from a hyperplastic to hypertrophic phenotype (Engelman *et al.*, 1993; Liu *et al.*, 1995). Perfusion of adult rat heart with FGF-2 was associated with increased levels of tyrosine phosphorylation *in situ.* Similarly, treatment of adult cardiac myocytes with FGF-2 resulted in the activation of MAP kinase *in vitro* and myocytes were also able to internalize the ligand via high affinity receptors (Liu *et al.*, 1995). The majority of FGFR-1 transcripts in the embryo as well as adult heart contained exon IIIc which is associated with isoforms that display the highest affinity for FGF-2 compared to FGF-1 (Jin *et al.*, 1994; Pasumarthi *et al.*, 1995). FGFR-1 exists as long or short isoforms in the heart due to alternative splicing of mRNA (Jin *et al.*, 1994; Pasumarthi *et al.*, 1995; Liu *et al.*, 1995). Both long and short FGFR-1 isoform cDNAs were cloned from the embryonic mouse heart, which generated 102 and 86 kD proteins respectively, following *in vitro* translation (Jin *et al.*, 1994). Although both long and short isoforms appear to bind to the same FGF ligands, it is largely unknown whether these isoforms are linked to different signal transduction pathways (Jaye *et al.*, 1992). However, their expression patterns varied during cardiac development, long isoform was more abundant in the embryo while short isoform predominated in the adult mouse heart (Jin *et al.*, 1994; Pasumarthi *et al.*, 1995). Cardiac myocytes were also shown to express *N*-syndecan, the low affinity receptor for FGF-2 (Ross and Hale, 1990).

2.6.4 Cardiac myocyte proliferation and differentiation

Exogenously added FGF-2 was shown to stimulate the proliferation of both embryonic and neonatal cardiac myocytes in culture (Kardami, 1990). Immunolocalization studies revealed close association of FGF-2 with chromosomes during various phases of cell cycle in cardiac myocytes (Liu, 1994). The FGF-2 mediated increase in DNA synthesis and proliferation of cardiac myocytes was truncated by TGFß as well as thyroid hormone (Kardami, 1990). Although it exists in high and low molecular weight forms, the effects of FGF-2 on cardiac myocytes reported so far can be attributed to the low molecular weight form due to its commercial availability. The physiological significance of high molecular weight forms in the context of cardiac myocyte growth is largely unknown. However,

there appears to be a switch in the pattern of FGF-2 expression during cardiac development. High molecular weight forms of FGF-2 were abundant in the neonatal heart which has proliferative potential, while low molecular weight forms of FGF-2 were predominant in the differentiated adult heart (Liu *et al.*, 1993). Thus a positive correlation was suggested between the presence of high molecular weight forms of FGF-2 and proliferative potential of cardiac myocytes (Liu *et al.*, 1993). It was also shown that thyroid hormone exerts a negative regulatory effect on the accumulation of high molecular weight forms of FGF-2 in the heart *in vivo* as well as in cardiac myocytes *in vitro*. In addition to its ability to stimulate proliferation of embryonic and neonatal cardiac myocytes, FGF-2 was also shown to stimulate DNA synthesis in adult ventricular myocyte cultures (Claycomb and Moses, 1988; Speir *et al.*, 1992). Higher concentrations of FGF-2 were found in the atria compared to the ventricles which was correlated with the increased capacity for atrial myocytes to synthesize DNA in adult hood (Kardami and Fandrich, 1989). This growth factor was also shown to alter the programme of differentiated gene expression in cardiac myocytes. Treatment of cardiac myocytes with FGF-2 resulted in increases in the levels of ß-myosin heavy chain (ß-MHC), α-skeletal actin, α-smooth muscle actin and a decrease in the levels of α-myosin heavy chain (α-MHC) with no changes in α-sarcomeric actin (Parker *et al.*, 1990).

2.6.5 Angiogenesis

FGF-2 induces new capillary blood vessel growth or neovascularization in the avascular areas of various animal models (Esch *et al.*, 1985; Gospadarowicz *et al.*, 1985). Due to this property, FGF-2 is an attractive candidate to stimulate the formation of collateral vessels during ischemia. In deed, FGF-2, when infused onto the arterial wall, was able to stimulate vascularization in the vasa vasorum of rat carotid artery. (Baird and Ling, 1987). It was suggested that endogenous FGF-2 may be activated during ischemic injury by processes such as hypoxia and local changes in pH and thus increase the capillary bed that vascularizes the wounded tissue (Brick, 1959). Later, it was found that myocardial infarct tissue contains higher levels of FGF-2 than noninfarct tissue (Yanagisawa-Miwa *et al.*, 1992; Padua and Kardami, 1993). In a canine experimental infarct model, intracoronary injection of FGF-2 was shown to increase the number of arterioles and capillaries due to its

potent anigiogenic action (Yanagisawa-Miwa *et al.*, 1992).

2.6.6 Cardioprotection

Ventricular dysfunction following reperfusion of ischemic myocardium is associated with ultrastructural damage to mitochondria. This damage may be in part due to an increase in free oxygen radical generation resulting from a decrease in the levels of catalase antioxidant enzyme. Treatment of confluent cultures of neonatal rat ventricular myocyte cultures with H_2O_2 led to lethal sarcolemmal disruption *in vitro* (Janero *et al.*, 1991). Similarly, oxidative stress induced by free radicals was shown to be the major cause of myocardial injury (Slezak *et al.*, 1995) and it is of clinical interest to explore the means that can increase the levels of endogenous anti-oxidants during ischemia. Addition of FGF-2 to neonatal rat ventricular myocyte cultures treated with H_2O_2 or starved from serum resulted in improved cardiac myocyte survival, as assessed by lactate dehydrogenase levels, nuclear morphology and myofibrillar integrity (Kardami *et al.*, 1993). Further, it was suggested that FGF-2 which possesses four thiol groups in the reduced state (Thomson, 1992), may act as an anti-oxidant and offer protection to the differentiated myocardium during injury (Kardami *et al.*, 1993).

Cardioprotective effects of FGF-2 *in vitro*, were also found to be physiologically important *in vivo*. Perfusion of a rat heart Langendorff preparation with FGF-2 and subsequent exposure to 60 minutes of global ischemia resulted in ~95% recovery of preischemic contractile force compared to ~60% in the absence of FGF-2 (Padua and Kardami, 1993). Immunolocalization in the perfused rat heart revealed association of added FGF-2 with cardiac myocytes and cells of the circulatory system supporting the notion that FGF-2 may contribute to vasodilation (Rosenblatt *et al.*, 1994) and offer cardioprotection via improved tissue perfusion (Padua and Kardami, 1993). In addition, interaction of exogenous FGF-2 with cardiac myocytes was associated with increased levels of tyrosine phosphorylation *in situ* (Liu *et al.*, 1995). In a canine experimental infarct model, intracoronary injection of FGF-2 was shown to increase the number of arterioles and capillaries in the infarct, improve cardiac systolic function and reduce the size of the infarct (Yanagisawa-Miwa *et al.*, 1992). Similarly, in porcine infarction/ischemia models, administration of FGF-2 was shown to promote both angiogenesis as well as cardiac

function (Battler *et al.*, 1993; Harada *et al.*, 1994). It is considered beneficial to the heart to increase the coronary collaterals in salvaging the myocardium from ischemia in experimental animal models of coronary stenosis or obstruction (Schaper *et al.*, 1988) and in ischemic heart disease (Schaper *et al.*, 1988; Sabri *et al.*, 1991). Thus, the application of FGF-2 may bring a therapeutic modality for the salvage of the infarcted myocardium.

2.7 FGF-2 in the central nervous system (CNS)

The mechanisms regulating the growth signals in the cells of CNS during physiological as well as pathological events, are largely unknown. Dysregulation of these growth signals in the CNS often results in degeneration of neuronal cells and impaired functions of CNS some of which include loss of speech or memory, chorea, muscle spasms and paralysis. Neuronal death is the characteristic of neurodegenerative diseases which pose a considerable burden on the affected individual, the family and the community. This has stimulated an enormous amount of interest in the area of neurobiology to identify factors that would be able to promote the survival and or regeneration of neurons. Several growth factors [eg. nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and fibroblast growth factor (FGF)] have been implicated in the normal growth and development of the CNS (Baird, 1994; Lindsay *et al.*, 1994). Recent studies showed that altered levels of FGF-2 may be involved in the disease process of neurodegenerative disorders such as Alzheimer's disease (Cotman and Gomez-Pinilla, 1991) Huntington's disease (Tooyama *et al.*, 1993a) and Parkinson's disease (Tooyama *et al.*, 1993b). In this context, administration of FGF-2 was shown to prevent neuronal death *in vivo* (Riva and Mocchetti, 1991) and this suggests that the increased availability of FGF-2 may serve as a specific treatment regimen to slow or regress the progression of neurodegenerative diseases. This section will focus mainly on the role of FGF-2 in the growth and development of the CNS.

2.7.1 CNS development

The neural crest (NC) is a population of cells that arise during neurulation in vertebrate embryos. These cells migrate from the dorsal neural tube and differentiate into a

variety of cell types which include melanocytes of the integument and iris, Schwann cells, sensory and autonomic neurons, various endocrine and paracrine cells and mesenchymal cells of the head and face (Sherman *et al.*, 1991). The mechanisms controlling the diversity of NC cells are largely unknown. It is possible that the NC cells are multipotent and their commitment is influenced regionally by specific environmental cues. Peripheral nerve cultures from avian embryos, which consist largely of Schwann cell precursors but no melanocytes, were shown to undergo pigmentation in response to phorbol ester 12-*O*-tetradecanolyl phorbol-13-actetate (TPA) treatment (Ciment *et al.*, 1986). Phorbol esters are known to upregulate the mRNA levels of FGF-2 through a PKC-dependent mechanism in several cell types of the CNS including glioma C6 (Powell and Klagsbrun, 1993) and astrocytoma U87-MG cells (Murphy *et al.*, 1988b). There was an induction (~63%) in the pigmentation of peripheral explant cultures when they were treated with FGF-2 but not with other growth factors (FGF-1, NGF, EGF, PDGF, TGF-α, and TGF-ß1) indicating that this growth factor can reverse the developmental restriction of melanogenesis (Sherman *et al.*, 1991). Dorsal root ganglion cultures from 7 day old embryos (E7) can normally undergo pigmentation. These cultures could not undergo pigmentation when they were maintained in the presence of neutralizing antibodies for FGF-2 and this inhibition could be overcome by adding excess recombinant human FGF-2 (Stocker *et al.*, 1991). Similarly, FGF-2 was shown to inhibit the differentiation of O-2A progenitor cells of the CNS into oligodendrocytes (McKinnon *et al.*, 1990).

2.7.2 Distribution in the CNS

Although, the mammalian brain is one of the richest sources of FGF-2, its cellular location is still controversial. It was reported that FGF-2 is exclusively localized in neurons in the normal brain (Pettman *et al.*, 1986). However, it was found that only a restricted number of neuronal populations showed immunoreactivity to FGF-2 in the adult brain (Cotman and Gomez-Pinilla, 1991; Woodward *et al.*, 1992). Both RNA and immunoblot analyses have revealed that FGF-2 is expressed in cultured astrocytes (Ferrara *et al.*, 1988) and microglia (Shimojo *et al.*, 1991). FGF-2 was found in the cytoplasm or nucleus depending on the area of the brain examined, the antibody used and the fixation procedure employed (Hanneken and Baird, 1992). This growth factor has been localized to specific

foci which include cingulate cortex and CA2 hippocampus of the CNS (Emoto *et al.*, 1989). Neurons in CA2 showed strong FGF-2 immunoreactivity around the perinuclear area and weak staining in the cytoplasm and processes proximal to soma. In the brain stem there was strong FGF-2 immunoreactivity within the facial nerve nucleus and the motor and spinal components of the trigeminal nucleus (Cotman and Gomez-Pinilla, 1991). It is also expressed in Purkinje cells of the cerebellum (Matsuda *et al.*, 1992), a subpopulation of sensory neurons (Weise *et al.*, 1992) and in several other areas of the CNS (Matsuyama *et al.*, 1992).

2.7.3 Spatial and temporal expression in the CNS

There is scant information about the expression of the FGF-2 gene during the development of CNS and the mechanisms regulating its production. There was a marked increase (~13 fold) in the mitogenic activity of brain extracts from the embryonic stage to the third postnatal week, when assayed using Balb/c 3T3 cells. Most of this mitogenic activity was shown to be associated with both FGF-1 and FGF-2 using heparin-affinity chromatography and immunoblotting. However, the levels of FGF-1 were negligible at postnatal day (P) 10 and markedly increased by P40. By contrast, substantial levels of FGF-2 were already present at P10, increasing by only 3-fold at P40. This suggests that both FGF-1 and FGF-2 are differentially regulated in the CNS to serve different functions during development (Caday *et al.*, 1990). Embryonic brain (E13-E21) was shown to express predominantly a FGF-2 transcript of 1.8 kb, while the adult brain contained multiple FGF-2 mRNA species of 6.0, 3.7, 2.5, 1.8, 1.6 and 1.0 kb. Similarly, embryonic hypothalamus was found to contain a 1.8 kb transcript, whereas the 6.0 kb transcript was abundant in the adult hypothalamus. Adult pituitary and cortex contained the 1.8 kb and other lower molecular weight FGF-2 mRNAs but not the 6.0 kb mRNA (Powell *et al.*, 1991). In adults, FGF-2 mRNA is distributed throughout the brain, the highest levels being observed in cerebral cortex, hippocampus and spinal cord. The levels of FGF-2 mRNA in the brain were reported to be lower during the neonatal stage, and increase thereafter to reach peak expression around postnatal day 21 (Riva and Mocchetti, 1991). It appears that FGF-2 mRNA levels vary even during the first month of postnatal CNS development. The mRNA levels were (i) steadily increased in the inferior colliculus

and occipital cortex, (ii) constant in pons medulla and (iii) decreased in cerebellum (el-Husseini *et al.*, 1994).

The expression of individual forms of FGF-2 is regulated in the CNS with regard to both developmental stage and location. Embryonic rat brain extracts contained the 18 and 21 kD FGF-2 protein isoforms, but lacked the 22 kD form. Expression of the 22 kD form was first detected in the neonate and then steadily increased up to one month of age. All three forms were identified in adult rat brain extracts. In spinal cord and cortex, the predominant FGF-2 forms in embryonic and adult tissues were the same as those observed in whole brain extracts. Adult spinal cord and cortex expressed higher levels of the 21 and 22 kD forms and lower levels of the 18 kD form. By contrast, the cerebellum exhibited a different pattern of expression. The embryonic cerebellum expressed predominantly 18 and 21 kD forms. However, during the postnatal maturation of the cerebellum the levels of the 18 kD form predominate due to a reduction in the 21 kD form in addition to the weak expression of the 22 kD form. These data suggest that different forms of FGF-2 may have distinct roles during the maturation and organization of the nervous system (Giordano *et al.*, 1992).

2.7.4 High affinity receptors

In the developing chick nervous system, high affinity FGF receptor mRNA initially appears in the germinal neuroepithelium (Heuer *et al.*, 1990). Although FGFR-1, FGFR-2, and FGFR-3 are expressed in the germinal epithelium of the neural tube during murine development, their expression patterns were reported to dissociate in the adult brain. FGFR-1 was preferentially expressed by neuronal cells while FGFR-2 expression was associated predominantly with glial cells (Asai *et al.*, 1993; Peters *et al.*, 1992b). In addition, regional and temporal differences in the expression patterns of FGFR were also reported. The relative amounts of FGFR-2 mRNA in the occipital cortex and inferior colliculus increased with age during the first month of postnatal development while no change was detected in the cerebellum. With regard to the expression of FGFR-1 mRNA, there was a steady state decrease in the occipital cortex and cerebellum with age, while no change was detected in the inferior colliculus. Increased levels of FGFR-2 in the CNS during the first month of postnatal development, a period of active astroglial proliferation,

suggest that the FGF-2 may be involved in glial cell development by acting through FGFR-2 (el-Husseini *et al.*, 1994).

2.7.5 Activities in the CNS

FGF-2 elicits a broad repertoire of effects on the cells of mesodermal and neuroectodermal origin. Despite its abundance in neural tissue, a precise cellular localization for FGF-2 synthesis has not been unequivocally determined. Although FGF-2 immunoreactivity has been localized to astroglial cells and specialized neurons, astrocytes may represent a potential source of FGF-2 expression in the CNS under appropriate circumstances (Morrison, 1991). It promotes the proliferation of glial cells such as oligodendrocytes (Saneto and DeVellis, 1985; Eccleston and Silverberg 1985), astrocytes (Morrison and DeVellis 1981; Pettmann *et al.*, 1985) and Schwann cells (Pruss *et al.*, 1981). Transformed glial cells were shown to express elevated levels of FGF-2 and their growth was suppressed in the presence of FGF-2 specific antisense oligonucleotides indicating that this growth factor is involved in the regulation of glioma growth and invasion in an autocrine pathway (Morrison, 1991). Also, FGF-2 was shown to act in both paracrine and intracrine manners. A paracrine mode of action was proposed for this growth factor in case of baby hamster kidney cells which will not initiate neuronal differentiation of neural crest progenitors unless they have been transfected with FGF-2 cDNA (Brill *et al.*, 1992). An intracellular action of FGF-2 was proposed to cause the trans-differentiation of neural crest-derived Schwann cell precursors into melanocytes (Logan, 1990). It also modulates the expression of astrocyte-specific proteins such as glial fibrillary acidic protein (GFAP), glutamine synthetase and S100 protein (Morrison *et al.*, 1985; Weibel *et al.*, 1985), influences the morphological maturation of astrocytes (Weibel *et al.*, 1985) as well as changes the membrane structure (Wolburg *et al.*, 1986) and thus is responsible for the differentiated function of astrocytes. In addition, FGF-2 stimulates the nonmitogenic functions of glial cells such as the migration of astrocytes (Senior *et al.*, 1986) and the release of plasminogen activators by astroglial cells (Rogister *et al.*, 1988). Furthermore, FGF-2 from glial cells was shown to enhance the survival of cerebral and hippocampal neuronal cells (Morrison *et al.*, 1986; Walicke *et al.*, 1986).

FGF-2 is a mitogen for neuroblasts from young embryos, enhances survival of

post-mitotic differentiated neurons and promote the extension of neurite outgrowth (Morrison *et al.*, 1986; Unsicker *et al.*, 1987; Walicke, 1988). It is known to stimulate the differentiation of neuroepithelial progenitor cells into "mature" neuronal cells and glia (Murphy *et al.*, 1990; Sherman *et al.*, 1991; Vescovi *et al.*, 1993) and is essential for the development and mature function of brain neurons (Baird, 1994). Both FGF-1 and FGF-2 were shown to regulate the expression of cholinergic and noradrenergic neurotransmitter expression independently in sympathetic neurons *in vitro*, without affecting the survival of these cells (Zurn, 1992). FGF-2 regulates the cholinergic, GABAnergic (Yokoyama *et al.*, 1994) and dopaminergic (Takayama *et al.*, 1995) function of neurons. Both FGF-1 and FGF-2 were shown to regulate the synthesis and secretion of NGF by fibroblasts and astroctyes in concert with other cytokines (Yosida and Gage, 1992). Other *in vitro* effects of FGF-2 on neurons include promoting the survival of hypothalamic neurons (Ishikawa *et al.*, 1992), development of cholinoceptive neurons from fetal cerebrum in culture (Kushima *et al.*, 1992) as well as protection of striatal neurons from NMDA receptor mediated cytotoxicity (Freese *et al.*, 1992). It is not clear whether these effects are mediated through FGF-2 produced from either neuronal cells or non-neuronal cells.

2.7.6 Neuronal survival and regeneration

FGF-2 is neuroprotective against excitotoxic damage produced *in vivo* as well as *in vitro* (Riva *et al.*, 1995). It was shown to prevent or minimize the cellular damage following lesions of specific neuronal pathways or after excitotoxic insults (Anderson *et al.*, 1988; Mattson *et al.*, 1989). The levels of FGF-2 and its high affinity receptor FGFR-1 were shown to be elevated during brain injury (Logan *et al.*, 1992). This growth factor prevents glutamate-induced degeneration of hippocampal neurons *in vitro* (Cheng and Mattson, 1991; Mattson *et al.*, 1989), while *in vivo* it is effective against the degeneration of specific neuronal populations after lesion or ischemic insult (Anderson *et al.*, 1988; Otto *et al.*, 1989; Yamada *et al.*, 1991). It can also protect hippocampal neurons against ischemia (Nakata *et al.*, 1993), spinal cord neurons following lesion (Blottner and Baumgarten, 1992) and photoreceptors from light damage (LaVail *et al.*, 1992; Faktorovich *et al.*, 1992). The potential significance of this growth factor in retinal regeneration was also well documented (Park and Hollenberg 1993). FGF-2 can also induce motor neuron

sprouting similar to CNTF (Gurney *et al.*, 1992). The exogenous administration of FGF-2 to both CNS and the peripheral nervous system was found to be beneficial by many groups. Infusion of FGF-2 into the ventricle prevented the death of cholinergic septal neurons after lesions to the hippocampus (Anderson *et al.*, 1988). Similarly administration of FGF-2 to the severed sciatic nerve (Baird and Walicke, 1989) or optic nerve (Sievers *et al.*, 1987) enhanced the rate of neuronal survival and nerve regeneration.

2.7.7 Diseases of the CNS

Overexpression of FGF-2 is associated with the autocrine growth of a number of tumor cell types including gliomas, astrocytomas and meningiomas (Maxwell *et al.*, 1991; Murphy *et al.*, 1988a,b; Takahashi *et al.*, 1991, 1992). However, it is not known whether the expression of FGF-2 in these tumors is associated with the onset of the disease. Altered expression of FGF-2 has been implicated in the disease process of several neurodegenerative disorders. Increased levels of FGF-2 were found in the astroglial cells of severely affected areas in Alzheimer's disease (Cotman and Gomez-Pinilla, 1991), Huntington's disease (Tooyama *et al.*, 1993a) and decreased levels of FGF-2 were found in the dopaminergic neurons of the midbrain in Parkinson's disease (Tooyama *et al.*, 1993b). Alzheimer's disease specifically causes neuronal degeneration in selected brain areas including the entorhinal cortex, the main input into the hippocampus. During the early course of disease, when cells are lost, the remaining cells undergo sprouting in a compensatory fashion to maintain the continuity of neural circuits. This process is associated with an induction of FGF-2 levels in both neurons and astrocytes (Cotman and Gomez-Pinilla, 1991). In the later course, the senile plaques, extracellular deposits of amyloid protein, appear to sequester FGF-2 to decrease its local availability and thereby promote neuronal degeneration (Baird, 1994). In this context, administration of FGF-2 was shown to prevent neuronal death *in vivo* (Riva and Mocchetti, 1991) and this suggests that the increased availability of FGF-2 may serve as a specific treatment regimen to slow or regress the progression of neurodegenerative diseases.

Parkinson's disease is characterized by a loss of dopamine-containing neurons within the substantia nigra. Decreased levels of FGF-2 were found in the dopaminergic neurons of the midbrain in Parkinson's disease (Tooyama *et al.*, 1993b). Fetal dopamine-

containing neurons transplanted into the brain of adult rats with experimental Parkinson's disease, reverse some motor impairments of the grafted animals (Perlow *et al.*, 1979). However, the viability of such grafts was poor. It was shown that cografting of fetal dopamine neurons with fibroblasts genetically engineered to express FGF-2 into rats with experimental Parkinson's disease improved the survival, growth and functional efficacy of transplanted neurons (Takayama *et al.*, 1995). In Huntington's disease, FGF-2 levels are upregulated following reactive gliosis and the increase is reported to be proportional to the extent of disease (Tooyama *et al.*, 1993a). In addition to the dysregulation of FGF-2 during neurodegenerative diseases, FGF receptor deficiency was also found to be associated with dystrophic retinal-pigmented epithelium (Malecaze *et al.*, 1993).

Thus, a survey of the literature indicates that FGF-2 plays an important role in the growth and development of both cardiovascular and central nervous systems. As a result, my doctoral studies were focused on (i) overexpression of FGF-2 in cardiac myocytes with a view to stimulate postnatal cardiac growth as a step toward cardiac regeneration (chapters 4 to 7) and (ii) transcriptional regulation of FGF-2 in glial cells with a view to understand the control of FGF-2 production as a step toward increasing neuronal survival (chapters 8 and 9).

Chapter 3

Materials and Methods

3.1 Cells and Tissues

African green monkey kidney COS-1 cells, fetal bovine heart endothelial (FBHE), rat C6 glioma, heart myoblast H9c2(2-1) and human astrocytoma U87-MG cells were obtained from the American Type Culture Collection and grown in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in the presence of 5% CO_2.

Embryonic cardiac ventricular myocytes were isolated from embryonic chicken hearts (6-7 days) by enzymatic disaggregation with trypsin, collagenase I and deoxyribonuclease (Sigma, St. Louis, MO) as described previously (Kardami, 1990). Cells were counted using a Coulter counter (Coulter Electronics of Canada Ltd., Surrey, Vancouver, B.C) and plated on collagen-coated dishes in 50% (v/v) Eagles minimal essential medium and 50% (v/v) Ham's F12 medium (MEM/F12), 2% (v/v) fetal bovine serum (FBS) and 50 pg/ml insulin (2% FBS-MEM/F12).

Postnatal cardiac ventricular myocytes were isolated from newborn Sprague-Dawley rat (1 day) hearts by enzymatic disaggregation with 0.1% (w/v) trypsin (Sigma, St. Louis, MO) using a temperature regulated (35 °C) spinner flask, with or without subsequent fractionation on a Percoll gradient (Iwaki *et al.*, 1990). In the absence of Percoll fractionation, cells were preplated for 1.5 hours without collagen to allow non muscle cells to attach. The remaining unattached cardiac myocytes were obtained by low speed centrifugation and used for our experiments. Cells were counted using a hemocytometer and plated on collagen-coated dishes in Ham's F10 medium (F10) containing 10% (v/v) FBS, 10% (v/v) horse serum (HS) and 140 μg/ml (w/v) calcium chloride (F10 growth medium). For collagen coating, 0.1% (w/v) collagen stock solution was made by dissolving 100 mg of type 1 collagen (Upstate Biotechnology Inc., Lake Placid, NY) in 100 ml of autoclaved distilled water. Culture dishes with or without glass coverslips (22 mm in diameter) were coated using 0.8 ml of 0.1% collagen/60 mm dish, dried under an ultraviolet light in a hood for ~4 hours and rinsed with fresh medium before

plating cells. Collagen-coated dishes were used within 24 hours after their preparation.

Plating densities of cells used in this study were as follows: COS-1 cells and rat C6 glioma cells: 0.5 x 10^6 cells per 100 mm dish or 1.0 x 10^6 cells per 150 mm dish; rat heart myoblast H9c2(2-1) and human astrocytoma U87-MG cells: 0.8 x 10^6 cells per 100 mm dish; chicken cardiac myocytes: 3.5 x 10^6 per 100 mm dish or 1.2 x 10^6 per 60 mm dish or 0.4 x 10^6 per 35 mm dish and rat cardiac myocytes: 3.5 x 10^6 per 100 mm dish or 1.5-2.0 x 10^6 per 60 mm dish or 0.7 x 10^6 per 35 mm dish.

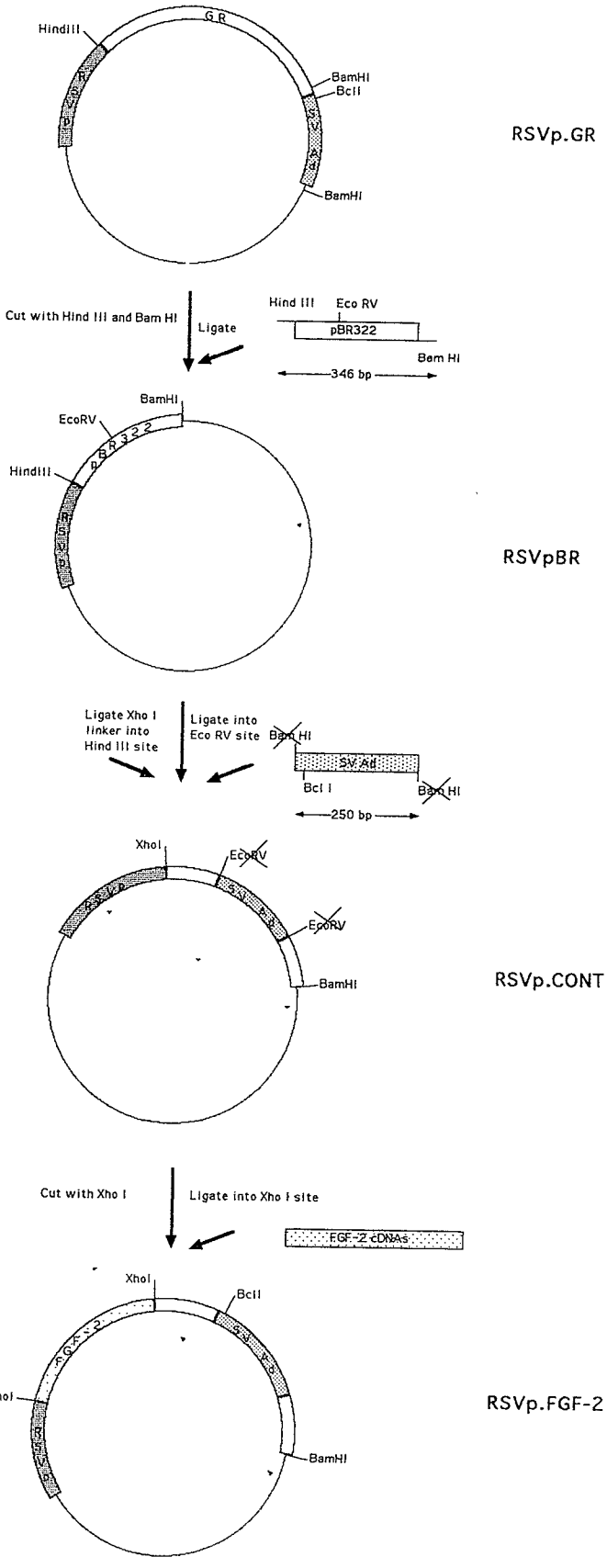
Adult rat tissues, brain, heart, ovary and liver were dissected aseptically from 6 months old Sprague Dawley rats. All the procedures involving experimental animals were performed according to the protocols authorized by the animal care committee at University of Manitoba.

3.2 Generation of expression vectors containing wild type and modified FGF-2 cDNAs

In order to generate an expression vector (containing Rous sarcoma viral (RSV) promoter (p) and simian virus 40 (SV40) polyadenylation signal) for subcloning FGF-2 cDNAs, the plasmid RSVp.GR (Miesfeld *et al.*, 1986, Fig. 3) was digested with *Hin*dIII and *Bam*H1 to remove the glucocorticoid receptor cDNA and ligated with a 246 bp *Bam*H1/*Hin*dIII fragment from pBR 322 (Bolivar, 1978). The resulting plasmid (RSVpBR; Fig. 3) was further modified by inserting a single *Xho*1 linker (5′-CCTCGAGG -3′; Pharmacia Canada Inc., Quebec) at the blunted *Hin*dIII site as well as a 250 bp blunt *Bam*H1 fragment (containing SV40 polyadenylation signal from RSVp.GR) at *Eco*RV site to generate RSVp. The rat FGF-2 cDNA obtained as a 1.0 kilobase (kb) *Xho* I fragment (Shimasaki *et al.*, 1988) was inserted at *Xho*1 site in the plasmid RSVp. to generate RSVp.FGF. The *Nco* I site containing the ATG sequence in RSVp.FGF, was cut, blunted (Maniatis *et al.*, 1982) using mung bean nuclease (Pharmacia Canada Inc., Quebec) and finally modified by introduction of a *Hind* III linker (5′-CCAAGCTTGG- 3′; Pharmacia Canada Inc., Quebec). Blunting and addition of a *Hind* III linker eliminated the methionine codon but maintained the translational reading frame in the resulting plasmid RSVp.ΔmetFGF. The *Sma*1 fragment (~1 kb) from RSVp.FGF was gel purified and

Fig. 3

Fig. 3. Progenitors of the expression vector (RSVp.CONT), which was used to generate wild type and modified FGF-2 hybrid genes. RSV promoter and the SV40 polyadenylation signal were obtained from RSVp.GR (Miesfeld *et al.*, 1986) and the wild type rat FGF-2 cDNA was obtained as an 1 kb *Xho* 1 fragment from Shimasaki *et al.*, 1988.


HindIII
G.R
BamHI
BclI
BamHI
RSVp.GR
Cut with Hind III and Bam HI
Ligate
Hind III
Eco RV
pBR322
Bam HI
346 bp
BamHI
EcoRV
HindIII
pBR322
RSVpBR
Ligate Xho I linker into Hind III site
Ligate into Eco RV site
Bam HI
SV Ad
Bcl I
Bam HI
250 bp
XhoI
EcoRV
EcoRV
BamHI
RSVp.CONT
Cut with Xho I
Ligate into Xho I site
FGF-2 cDNAs
XhoI
BclI
XhoI
BamHI
RSVp.FGF-2

inserted into the blunted *Xho*1 site of RSVp. to generate RSVp.metFGF. Two *Xho*1 sites flanking the insert were recreated in RSVp.metFGF after the fusion of *Sma*1 and blunt *Xho*1 sites. To generate the RSVp.FGF/CAT fusion gene, RSVp.ΔmetFGF was digested with *Hin*dIII and *Bam*H1 and ligated with a 1.6 kb *Hin*dIII/*Bam*H1 fragment from SVp.CAT (containing the coding region of CAT gene including the methionine codon; Promega Corp., Wisconsin, U.S.A.), after the removal of the FGF-2 cDNA coding region. RSVp.FGF/CAT was digested with *Hin*dIII, blunted and religated to maintain the leucine codons in the upstream FGF-2 cDNA seqeunce, in frame with the methionine codon of CAT gene. Bacterial genes coding for chloramphenicol acetyl transferase (CAT) and ß-galactosidase (ß-gal) (Walker *et al.*, 1983) were also fused to the RSV promoter to obtain RSVp.CAT and RSVp.β–gal, respectively.

3.3 Gene transfer

Cells were transfected by the calcium phosphate/DNA precipitation method (Cattini and Eberhardt, 1987). Briefly, calcium phosphate/DNA precipitate for triplicate dishes was made by adding 0.5 ml of DNA mix buffer (30-60 μg of test plasmid DNA and 6 μg of RSVp.*cat* in 252 mM calcium chloride) to 0.5 ml of 2X HEBS buffer (280 mM sodium chloride, 50 mM Hepes and 1.5 mM sodium phosphate dibasic, pH 7.1) while bubbling with a sterile air line. Precipitate was allowed to form at room temperature for 30 minutes, vortexed gently and 325 μl was added to each dish containing freshly applied 10% FBS-DMEM unless stated otherwise. After 24 hours, cells were refed with growth medium (2% FBS-MEM/F12 for embryonic ventricular myocytes, F10 growth medium for postnatal ventricular myocytes and 10% FBS-DMEM for all other cell types) and maintained for a further 48-72 hours before processing unless stated otherwise.

For experiments involving serum stimulation of rat C6 glioma cells, cells were transfected in 10% FBS-DMEM for 24 hours, refed with either 10% FBS-DMEM or serum free DMEM and maintained for a further 48 hours before processing. For experiments with phorbol esters, rat C6 glioma cells were transfected in 10% FBS-DMEM for 24 hours, refed with serum free DMEM for 24 hours and treated with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) in serum free conditions for a further 24 hours. PMA was prepared as a 10 μM stock solution in 0.001% (v/v) dimethyl sulphoxide

(DMSO) (Sigma chemicals, St. Louis, MO) and stored as 50 µl aliquots at -70 °C for one month.

3.4 Enzyme assays for the reporter genes

For β-galactosidase activity, individual plates of transfected cardiac myocyte cultures were rinsed with calcium and magnesium-free (CMF) phosphate buffered saline (PBS), then lifted with 1.5 ml of 0.25% (w/v) trypsin and 1 mM ethelynediaminetetraacetic acid (EDTA) (Gibco-BRL, Burlington, ON) and added to 2.0 ml of 2% FBS-DMEM. The cells were pelleted at 1,250 x g for 2 minutes and resuspended in 1.0 ml of CMF-PBS on ice. An aliquot (0.25 ml) was removed and the cells pelleted at 2,000 x g for 1 minute and resuspended in 0.5 ml of X-gal solution containing 1 mM magnesium chloride, 3.3 mM potassium ferrocyanide, 3.3 mM potassium ferricyanide, 0.15 M sodium chloride, 10 mM sodium phosphate buffer pH 7.0 and 0.2% (w/v) X-gal (Xu *et al.*, 1992), and incubated for 18 hours at 37 °C. Cells were assessed using a hemocytometer and the percentage of stained cells was determined.

For chloramphenicol acetyl transferase (CAT) and luciferase assays, cells were rinsed with CMF-PBS, lifted and lysed in 300-500 µl of 100 mM Tris-HCl, pH 7.8, containing 0.1% trition X 100. The lysates were spun at 13,000 x g for 15 min at 4 °C, supernatants were collected and protein concentrations were measured (Bradford, 1976). CAT activity was measured using a modification of the two-phase fluor diffusion assay (Nickel *et al.* 1990a). Quantitative values for CAT activity were determined by regression analysis to give cpm/µg of cell lysate protein. Luciferase activity per mg of lysate protein was determined (Jin *et al.*, 1995) by using the "Luciferase Assay System" (Promega Corp., Wisconsin, U.S.A.) and a luminometer (ILA911 Luminometer, Tropix Inc., Bedford, MA, U.S.A.) according to manufacturers instructions.

3.5 Cell extracts and immunoblotting

Transfected cultures were rinsed with PBS twice and lysed in 1 M sodium chloride (NaCl) solution (1.0 ml/100 mm plate) containing 5 µg/ml leupeptin, 5 µg/ml pepstatin, 5 µg/ml aprotinin and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma chemicals, St. Louis, MO). The lysates were sonicated for 30 seconds and centrifuged at 30,000 rpm for

30 minutes, using Ti 75 rotor in an ultracentrifuge (Beckman Instruments Canada Inc., Mississauga, ON). Supernatants which represent total cell lysates were saved and protein concentrations of all the samples were determined using the Bradford protein assay (Bradford, 1976). Total cell lysates were fractionated using heparin-Sepharose beads to evaluate levels of different forms of FGF-2 and starting concentrations (~2 mg of total cellular lysate) were normalized in all the treatments prior to the fractionation. For heparin-Sepharose adsorption, the salt concentrations of the lysates were adjusted to 10 mM Tris-HCl (pH 7.0) and 500 mM NaCl, mixed with 100 µl of heparin-Sepharose beads (Pharmacia Canada Inc., Quebec) and rocked at room temperature for 1 hour. The beads were collected after centrifugation at 50 x g (800 rpm) for 1 minute and washed (i) twice with 0.6 M NaCl and 10 mM Tris-HCl (pH 7.0), (ii) once with 1.1 M NaCl and 10 mM Tris-HCl (pH 7.0) and (iii) twice with 0.11 M NaCl and 10 mM Tris-HCl (pH 7.0). After the last wash, the remaining buffer was aspirated from the beads using a tuberculine syringe. The beads were resuspended in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [1% (w/v) SDS, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue in 50 mM Tris-HCl (pH 6.8)], boiled for 5 minutes and chilled on ice. Heparin-Sepharose bound protein was resolved in a 12.5% SDS-PAGE gel [containing acrylamide and N,N´-methylene-bis-acrylamide at a ratio of 30:0.8 (w/v)], and transferred onto Immobilon P membrane (Millipore, ON, Canada). Blots were blocked with 1% (w/v) gelatin in CMF-PBS for 30 minutes at room temperature and probed with rabbit polyclonal anti-FGF-2 antibodies (Kardami *et al.*, 1991b) at a dilution of 1:5,000 for 18 hours at 4 °C. FGF-2 was visualized by incubation with 15 µl of [^{125}I] iodinated-protein A (75 µCi/ml, Amersham Corp, IL) in 15 ml of Tris buffer saline [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% tween-20] followed by autoradiography and the levels were quantitated by densitometry (Ultrascan XL, LKB, Broma, Sweden). Samples of recombinant human FGF-2 (10 ng) (Upstate Biotechnology Inc., Lake Placid, NY, USA) as well as prestained SDS-PAGE standards (10 µg) (low range; BioRad, ON, Canada) were used as molecular weight markers.

For nuclear fractionation, COS-1 cells were lifted in 0.2 % (w/v) EDTA-PBS, dounce homogenized in 5 ml of breaking buffer (250 mM sucrose, 100 mM KCl, 15 mM Hepes (pH 7.5), 1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml pepstatin, 10 µg/ml

aprotinin; Powell and Klagsbrun, 1991), centrifuged at 1000 x g (2500 rpm) in JA 21 rotor (Beckman Instruments Canada Inc.) for 5 minutes. The nuclear pellet was resuspended in 2 ml of breaking buffer and the purity of nuclei was confirmed by direct microscopic examination. Nuclear extracts were further fractionated by using heparin-Sepharose beads as described above to evaluate levels of different forms of FGF-2 in nuclei. Protein conentrations of all the extracts were determined using the Bradford protein assay (Bradford, 1976) and thus starting concentrations (2 mg of total cellular lysate) were normalized in all the treatments prior to heparin-Sepharose adsorption. Heparin bound protein was resolved in a 12.5% gel by SDS-PAGE, transferred onto Immobilon P membrane (Millipore, ON, Canada) and probed with rabbit FGF-2 antibodies (at a dilution of 1:10,000) (Kardami *et al.*, 1991b). Antigen-antibody complexes were visualized by incubation with ^{125}I-protein A (Amersham Corp., IL) and autoradiography.

3.6 Tritiated thymidine incorporation

Ventricular myocytes were plated in 35 mm collagen-coated dishes and triplicates (per experiment) were transfected with each construct (10 µg of plasmid DNA/35 mm dish) in 2% FBS-DMEM for 24 hours. Cells were refed with 2% FBS-MEM/F12 and maintained for a further 43 hours. Cells were rinsed with PBS and pulsed with tritiated thymidine (5 µCi/ml) for 5 hours. Radioactive medium was aspirated and cells were rinsed with PBS and then lysed in 1 ml of 10 mM Tris-HCl, pH7.4, 1 mM EDTA, 100 mM NaCl and 0.5 % SDS. Lysates were transferred to glass tubes and precipitated with equal volumes of 20 % trichloroacetic acid (TCA), vortexed and incubated on ice for 10 minutes. The suspension was filtered onto glass microfibre disks (GF/A; Whatman). Disks were washed with equal volumes of 5 % TCA (5 times) and 95 % ethanol. Disks were dried at room temperature (18 hours), transferred to scintillation vials containing 4 ml of cytoscint and radioactivity was measured in a scintillation counter.

3.7 Labelling Index (LI)

Ventricular myocytes in 60 mm dish (collagen-coated) containing 2 square collagen-coated coverslips (22 x 22 mm) were transfected for 24 hours in 10% FBS-DMEM, refed

with 10% FBS-F10 for 24 hours and then pulsed with [^{3}H]thymidine (10 μCi/ml) in fresh medium for a further 24 hours at 37 °C. Cells were rinsed with PBS and fixed with formyl-alcohol (9:1, 37% formaldehyde and 95% ethanol) at room temperature for 15 minutes. Myocytes on coverslips were identified by staining histochemically for glycogen using periodic acid Schiff (PAS; Sigma, St. Louis, MO, USA) stain. Subsequently, [^{3}H]thymidine uptake in myocyte cultures was visualized by autoradiography using Kodak NTB emulsion. Slides were developed 14 days later in D19 Kodak developer, fixed, dehydrated and mounted in Permount (Fisher Scientific, Ottawa, ON). A total of about 600-800 radiolabelled or unlabelled PAS positive (PAS+) cells were scored per each treatment (150-200 cells per coverslip, n=4). LI equals the proportion of radiolabelled PAS+ cells (LI = radiolabelled PAS+ cells/total number of PAS+ cells) and was expressed as a percentage (Kardami, 1990). Further, PAS+ cells were also scored in 12-16 random fields to estimate cardiac myocyte number.

3.8 Bromodeoxyuridine (BrdU) Labelling

For bromodeoxyuridine (BrdU) labelling, rat ventricular myocytes on collagen-coated coverslips, were transfected for 24 hours, maintained for 24 hours and incubated with 3 μg/ml (w/v) BrdU (Sigma, St. Louis, MO, USA) for a further 24 hours. Myocyte cultures were fixed with 1% paraformaldehyde for 15 minutes, then with 70% ethanol for 30 minutes at room temperature and permeabilized with 0.1% (v/v) Triton X100 in PBS for 15 minutes at 4 °C. Coverslips were then processed for immunofluorescence microscopy (see section 3.15) to visualize bromodeoxyuridine positive cells. For quantitation, about 2,500 cardiac myocytes from each culture transfected with RSVp.CONT, RSVp.FGF, RSVp.ΔmetFGF or RSVp.metFGF, were assessed from 11 randomly selected fields on 4 separate coverslips, representing 2 independent transfection experiments. The fraction of nuclei staining for BrdU was determined and the results are expressed as the fold difference relative to RSVp.CONT which was arbitrarily set to 1.0.

3.9 Cell number

Cells were rinsed with CMF-PBS and lifted with 0.25% trypsin and 1 mM EDTA

and pelleted at 1,250 x g for 2 minutes. For ventricular myocytes, COS-1, C6 glioma, H9c2(2-1) and U87-MG cells, pellets were resuspended in 2 ml of medium on ice and then cell number from each sample was assessed using a hemocytometer. FBHE cells were counted using a Coulter counter.

3.10 Mitotic Index (MI) and Assessment of Binucleation

The Mitotic Index, defined here as the fraction of cardiac myocytes in metaphase, anaphase or telophase, as well as the degree of binucleation were determined for cultures transfected with RSVp.CONT, RSVp.FGF, RSVp.ΔmetFGF or RSVp.metFGF. Cardiac myocytes in various stages of mitosis or containing 2 nuclei were identified by fluorescence microscopy and a combination of DNA (Hoechst dye 33342) and anti-myosin staining (see section 3.15). About 3,000-3,500 cardiac myocytes from each transfected culture were assessed from 25-35 randomly selected fields on 8 separate coverslips, representing 3 independent transfection experiments. The results are expressed as the fold difference relative to the levels seen with RSVp.CONT, which were arbitrarily set to 1.0. In addition, the effect of individual cardiac myocytes overexpressing FGF-2 (ΔmetFGF and metFGF) on mitosis in surrounding cells was assessed in the presence of either 10 μg/ml anti-bovine basic FGF, Type 1 (mouse monoclonal IgG1κ; Upstate Biotechnology Inc., Lake Placid, NY, USA) or 10 μg/ml normal mouse IgGs (NM Ab; Sigma Immunochemicals, St. Louis, MO, USA). The anti-bovine basic FGF, Type 1 preparation contains neutralizing FGF-2 antibodies which were used successfully to block a FGF-2 mediated growth response (Matsuzaki *et al.*, 1989). Following transfection, cells were refed growth medium containing FGF-2 antibodies or NM Ab for 48 hours. The fraction of mitotic cells in an area (~125 μm radius) surrounding or not containing a myocyte overexpressing FGF-2 was determined in the same culture (15 areas from 3 coverslips). Under the culture conditions employed, 125 μm corresponded to a 5 cell radius. "Background" values were also determined from cultures transfected with RSVp.CONT and maintained in the presence of antibodies to FGF-2 (15 areas from 3 coverslips) or NM Ab (20 areas from 3 coverslips). The effect of neutralizing FGF-2 antibodies on the degree of binucleation was also assessed (30 areas from 3 coverslips). In addition, the level of binucleation was determined in the population of cardiac myocytes overexpressing FGF-2. About 400

cardiac myocytes overexpressing FGF-2 were assessed from cultures transfected with RSVp.FGF, RSVp.ΔmetFGF or RSVp.metFGF and stained with Hoechst dye as well as antibodies to FGF-2 and myosin.

3.11 Growth assay using FBHE cells

During FGF-2 neutralizing antibody experiments, it was necessary to ensure that sufficient levels of antibodies were present in the medium of transfected ventricular myocyte cultures to neutralize the FGF-2 released from cells. Therefore, conditioned medium was removed at the end of ventricular myocyte transfection experiments and used to perform a growth assay on FBHE cells (which require FGF-2 for growth/survival; Gospadarowicz *et al.*, 1976). FBHE cells were plated at a density of 3 x 10^3 cells per 35 mm dish in DMEM with 10% FBS and 3 ng/ml human recombinant FGF-2 (Upstate Biotechnology Inc., Lake Placid, NY, USA). The conditioned medium from each experimental dish containing 10 μg/ml FGF-2 antibodies or 10 μg/ml NM Ab and 3 ng/ml human recombinant FGF-2 were added to each dish of FBHE cells. The FBHE cells were allowed to grow for 5 days before counting by a Coulter counter.

3.12 Reverse transcriptase-polymerase chain reaction (RT-PCR)

For cDNA synthesis, 1 μg of total RNA from postnatal ventricular myocytes (see RNA extraction procedure in section 3.20) was incubated for 2 hours at 37 °C in a reaction mixture (10 μl) containing 200 units of Moloney murine leukemia virus reverse transcriptase (RT; Gibco-BRL, Burlington, ON), 10 μM of random hexadeoxynucleotide primer (pdN6; Pharmacia Canada Inc., Baie d'Urfe, PQ), 16 units of RNA guard (Pharmacia Canada Inc., Baie d'Urfe, PQ), 10 mM dithiothreitol, 0.01% (w/v) bovine serum albumin and 5% (v/v) DMSO. The extra cellular region of rat FGF-2 high affinity receptor (FGFR-1) cDNA with (long isoform) or without (short isoform) the first Ig-like domain, was amplified by polymerase chain reaction (PCR) using sense (5′-GAGACCAAGCTTGGACCTGAACGGATCCCAGG-3′, nucleotides) and antisense (5′-AGTCATCGAATTCGTCATCGTCCTCCGAGGATGG-3′) primers including nucleotides 105-124 and 400-422 respectively, based on sequences reported earlier (Kim *et al.*, 1993). Primers were provided by the Regional DNA Synthesis Laboratory (Calgary, Alberta,

Canada). PCR was carried out in a mixture (50 µl) containing 1 µl of the above cDNA reaction (~100 ng), 1 µM each of sense and antisense primers and 2 units of Taq DNA polymerase (Gibco-BRL, Burlington, ON). PCR reactions were carried out in a thermocycler (PTC-100™, MJ Research Inc., Watertown, Massachusetts) for 30 cycles, each consisting of denaturation at 95 °C for 1 minute, annealing at 60 °C for 45 seconds and extension at 72 °C for 1.5 minute as described earlier (Liu *et al.*, 1995). As a control, samples were also processed routinely in the absence of any treatment with RT. The reaction products were separated in 1.5% agarose gels and visualized by ethidium bromide staining.

3.13 New protein synthesis

Ventricular myocytes were plated in 35 mm collagen-coated dishes and triplicates (per experiment) were transfected with each construct (10 µg of plasmid DNA/35 mm dish) in 2% FBS-DMEM for 24 hours. Cells were refed with 2% FBS-MEM/F12 and maintained for a further 43 hours. Cell were rinsed with PBS twice and incubated with 1.5 ml of short term labelling medium per 35 mm plate (2% FBS-methionine and glutamine free DMEM, 0.4 mM glutamine, 100 U/ml penstrep, and 0.37% sodium bicarbonate) for 15 minutes in 5% CO_2 to deplete intracellular pools of methionine. The cells were rinsed with PBS three times and then incubated with the short term labelling medium containing ^{35}S-methionine (25 µCi/1.5 ml/35 mm plate) at 37 °C for 7 hours in 5% CO_2. At the end of pulsing, radioactive medium was aspirated and cells were rinsed twice with PBS and scraped in 3 ml of PBS. Cells were pelleted at 2,000 x g for 5 minutes at 4 °C and the pellets were resuspended in 0.5 ml of PBS. Labelled cell suspension (50 µl) was added to 0.5 ml of PBS containing 0.1 mg/ml bovine serum albumin (BSA) in a glass tube and precipitated with 0.5 ml of ice cold 20% TCA. Tubes were incubated on ice for 30 minutes and the suspensions were filtered through a filtration apparatus under vacuum onto glass microfiber disks (GF/A; Whatman). Disks were washed twice with 5 ml of 10% TCA, twice with 5 ml of 95% ethanol and air dried at room temperature overnight. Disks were transferred to scintillation vials containing 4 ml of cytoscint (ICN Biomedicals Inc., Irvine, CA) and the radioactivity was measured in a scintillation counter.

3.14 Total myosin, desmin and alpha sarcomeric actin accumulation

Ventricular myocytes were plated on 35 mm collagen-coated dishes and triplicates (per experiment) were transfected with each construct (10 μg of plasmid DNA/35 mm dish). Embryonic chicken ventricular myocytes were lysed in 0.5 ml of lysis buffer (2% SDS, 50 mM Tris-HCl pH 8.8). The lysates were vortexed vigorously, boiled for 10 min, sonicated and spun 14,500 x g at 4 °C for 10 minutes. Supernatants were saved and protein concentrations were determined using the Bradford protein assay (Bradford, 1976). Total protein of 10 μg was fractionated on a 7.5% SDS-PAGE gel and transferred to immobilon P membrane (Millipore, ON, Canada). The membrane was blocked with 1% BSA and incubated with monoclonal antibodies (CCM 52) which react with all chicken striated muscle myosins (Clark *et al.*, 1982; Sweeney *et al.*, 1987; gift from Dr. R. Zak, University of Illinois, Chicago, IL) (1:5000) for 18 hours at 4 °C. Blots were subsequently incubated with anti-mouse rabbit immunoglobulin (Sigma Immunochemicals, St. Louis, MO), followed by ^{125}I labelled protein A. Immunoreactive proteins were visualized by autoradiography. The myosin band (200 kD) was assessed by densitometry (Ultrascan XL, LKB, Broma, Sweden).

Postnatal rat cardiac myocytes were also lysed in 0.5 ml of lysis buffer (2% SDS, 50 mM Tris-HCl pH 8.8) and protein concentrations were measured (Bradford, 1976). Total protein of 10 μg was fractionated on a 7.5% (for myosin) or 12.5% (for desmin and alpha sarcomeric actin) SDS-PAGE gel and transferred to immobilon P membrane (Millipore, ON, Canada). The membranes were blocked with 5% milk powder (Carnation) for about 30 minutes at room temperature and incubated with the primary antibodies for 15 hours at 4 °C. Myosin, desmin and cardiac alpha sarcomeric actin were detected using monoclonal antibodies (1:5,000) for striated myosin (gift from Dr. R. Zak, University of Illinois, Chicago, IL), polyclonal antibodies (Sigma, St. Louis, MO) for desmin (1:500) and sarcomeric actin (1:1500) respectively. Muscle specific proteins were visualized using ECL (Amersham) detection system according to the manufacturer's instructions. The intensities of the 200 kD myosin band, 55 kD desmin band and 45 kD cardiac alpha sarcomeric actin band were assessed by densitometry (Ultrascan XL, LKB, Broma, Sweden).

3.15 Immunofluorescence microscopy

Transfected cardiac myocytes on collagen-coated 60 mm dishes (containing 3 coverslips, 22 mm in diameter) were fixed 48 hours after transfections with 1% paraformaldehyde for 15 minutes and then permeabilized with 0.1% (v/v) Triton X100 in PBS for 15 minutes at 4 °C. Alternatively, cells were permeabilized with acetone and methanol (1:1) for 15 minutes, followed by acetone alone for a further 15 minutes. All fixation and permeabilization steps were done at 4 °C and produced similar results.

Coverslips were incubated with rabbit FGF-2 antiserum (1:1,000) in 1% (w/v) BSA in PBS, then with biotinylated anti-rabbit immunoglobulins (Ig, 1:20; from Amersham Corp, Arlington Heights, IL, USA) and followed by fluorescein conjugated to streptavidin (1:20; from Amersham Corp, IL, USA). Rabbit FGF-2 antibodies used in this study were raised against the amino terminal residues 1-24 of bovine FGF-2 (Esch *et al.*, 1985; Kardami *et al.*, 1991b). These antibodies are highly specific to FGF-2, and staining is competed with a synthetic peptide corresponding to residues 1-10 of FGF-2 (Cattini *et al.*, 1991; Kardami *et al.*, 1993). Labelling for myosin was done using monoclonal antibodies against striated myosin (1:2,000; a generous gift from Dr. R. Zak, University of Illinois, IL, USA) in 1% (w/v) BSA in PBS followed by visualization with Texas Red conjugated anti-mouse Ig (1:20; from Amersham, IL).

Simultaneous labelling for myosin and/or BrdU in ventricular myocytes was done using monoclonal antibodies against striated myosin (1:2,000) and BrdU (1:2, from Amersham, IL, USA and 1:7 from Becton Dickinson, San Jose, CA, USA) in 1% (w/v) BSA in PBS. For BrdU labelling, fixed coverslips were treated with 70 mmol/L sodium hydroxide for 2 minutes and then rinsed with PBS prior to the addition of primary antibodies. Both myosin and BrdU were visualized with Texas Red conjugated anti-mouse Ig (1:20; from Amersham, IL, USA). Cellular DNA was stained with Hoechst dye 33342 (Calbiochem-Behring, CA, USA) in PBS (10 μg/ml) for 30 seconds as described (Kardami and Fandrich, 1989). Coverslips were mounted, examined and then photographed with a Nikon Diaphot microscope equipped with epifluorescence optics. To test for non-specific fluorescence, preparations were routinely treated with non-immune serum at identical dilutions made for immune Ig.

3.16 Cloning of 5´-flanking region of rat FGF-2 gene

A Sprague Dawley rat testis genomic library in Lamda DASH™ II (Stratagene, La Jolla, CA, U.S.A.) was screened according to standard procedures (Maniatis *et al.*, 1982) using an intact 1 kilobase (kb) rat ovarian FGF-2 cDNA (Shimasaki *et al.*, 1988) as well as an upstream 350 base pair (bp) *Xho*1/*Sma*1 fragment of the FGF-2 cDNA as described in section 3.16.4.

3.16.1 Preparation of host bacterial cells

Host bacterial cell (SRB, Stratagene, La Jolla, CA, U.S.A.) glycerol stock with disc was transferred into 50 µl of LB broth (1% bactotryptone, 0.5% bactoyeast and 0.5% sodium chloride) containing 0.2% maltose and 10 mM magnesium sulphate (LBMM), vortexed and centrifuged at 13,000 x g for 5 seconds. 5 µl of broth was streaked on a LB glucose plate (1.5% agarose and 20% glucose in LB broth) and incubated for 18 hours at 37 °C to obtain colonies. A single colony was inoculated into 50 ml of LBMM and grown for 18 hours while shaking at 30 °C. Cells were centrifuged at 3,000 x g for 10 minutes and diluted to OD_{600}=0.5 with 10 mM magnesium sulphate.

3.16.2 Titering of the genomic library

The pre-made genomic library was thawed and initially diluted (1:10) in SM solution (0.6% sodium chloride, 0.2% magnesium sulphate, 0.01% gelatin and 50 mM Tris-HCl, pH 7.5) to reduce the concentration of DMSO to 0.7%. Further dilutions (10^{-2} to 10^{-7}) of the lambda phage were made in SM solution for titering and screening purposes. Diluted phage (10 µl) from each serial dilution was added separately to 100 µl of host bacterial cells (OD_{600}=0.5) per tube and incubated at 37 °C for 20 minutes. Inoculum was mixed with 3.5 ml of top agar (0.75% agar in LB broth) at 65 °C , plated on LB glucose plates (100 mm) and incubated for 15 hours at 37 °C to allow plaque formation. The number of plaques was counted to determine the concentration of genomic library (plaque forming units/ml or pfu/ml) based on dilutions.

3.16.3 Primary screening

Based on the titer of the library, 50,000 pfu per 100mm were plated in 20 LB glucose plates to screen ~ 1 x 10^6 plaques and incubated at at 37 °C for 15 hours as described above. Plates were refrigerated at 4 °C for at least two hours to prevent top agar from sticking to the nitrocellulose during the screening procedure. For "lifting" plaques, a nitrocellulose filter was overlaid on each plate carefully avoiding air bubbles and left for 2 minutes before processing further. For making duplicate filters, the second filter was overlaid on the same plate for 4 minutes. Filters were pricked with a needle dipped in india ink (through the membrane into the agar) for orientation. Each filter was denatured by submerging in 1.5 M sodium chloride and 0.5 M sodium hydroxide solution for 2 minutes, neutralized in 1.5 M sodium chloride and 0.5 M tris-HCl, pH 8.0 solution for 2 minutes, transferred to a fresh neutralization solution for an additional 2 minutes and finally rinsed in 6 x SSC for 3 minutes. All the filters were air dried, baked at 80 °C for 90 minutes under vacuum and hybridized to radiolabelled 1 kilobase (kb) rat ovarian FGF-2 cDNA (Shimasaki *et al.*, 1988) as described under the section 3.18. Positive signals were visualized by autoradiography.

3.16.4 Additional screenings

After aligning the signals on the autoradiogram from the primary screening with the nitrocellulose filter and then master plate (based on orientation marks), the strongest positive clones were selected. The area where a putative clone lined up with the autoradiogram signal was cut out from the master plate by using the broader end of a pasteur pipette and transferred to a 1.5 ml microfuge tube containing 1.0 ml of SM solution with 2.5% chloroform. The microfuge tube was vortexed vigorously, left at 4 °C for 18 hours to facilitate separation of phage particles from agar and this suspension was stored up to one month at 4 °C until further use. Serial dilutions (10^{-2} to 10^{-7}) of the lambda phage were made in SM solution for secondary screening, used to inoculate host bacterial cells and plated on LB glucose plates as described above. Plaques were "lifted" to nitrocellulose filters and were hybridized with radiolabelled rat ovarian FGF-2 cDNA. Positive clones obtained from secondary screening were re-screened with radiolabelled FGF-2 cDNA in

the same manner for tertiary screening to enrich the "putative" clones. Subsequently, positive clones from tertiary screening were re-screened a fourth time with an upstream 350 base pair (bp) *Xho*1/*Sma*1 fragment of the FGF-2 cDNA containing untranslated sequences to identify a "double-positive" clone that was most likely to contain the promoter and 5′-flanking region of rat FGF-2 gene.

3.17 Isolation of Lamda DASH™ II phage DNA

For phage genomic DNA preparation, an isolated, positive plaque was transferred from LB glucose plate into 100 µl of SM solution containing 25% (v/v) chloroform, vortexed and left at room temperature for 2-4 hours. About 20 µl of this phage suspension was used to inoculate 4 tubes containing 100 µl of host bacterial cells (OD_{600}=0.5) and incubated at 37 °C for 20 minutes. Inoculum in each tube was mixed with 3.5 ml of top agar at 65 °C, plated on LB glucose plates (100 mm) and incubated for 15 hours at 37 °C to allow plaque formation. Then the plates were overlaid with 5 ml of SM solution and rocked gently at 4 °C overnight. The supernatants were pooled and transferred into a centrifuge tube and chloroform was added to a final concentration of 0.25% (v/v). Bacterial debris was removed by centrifugation at 3000 x g for 20 minutes and the supernatant containing phage particles was transferred into a new tube. This supernatant was used to inoculate 100 ml of host bacterial cells grown in LBMM (OD_{600}=0.5) and incubated at 37 °C for 15 hours while shaking. Later, chloroform was added to a final concentration of 2% (v/v), incubated for a further 5-10 minutes and supernatant was collected by centrifuging at 3,000 x g for 20 minutes. Ribonuclease A (RNase A) and deoxyribonuclease (DNase I) were added to the supernatant at a final concentration of 1 µg/ml (w/v) each and incubated at 37 °C for 1 hour. Phage DNA was precipitated using an equal volume of 20% poly ethylene glycol (PEG) at 4 °C for at least 1 hour and pelleted by centrifuging at 3,000 x g at 4 °C for 30 minutes. The pellet was resuspended in 2 ml of SM solution, treated with 20 µg proteinase K and 0.1% SDS at 37 °C for 30 minutes and then with 5 mM EDTA at 65 °C for 15 minutes. DNA was extracted with phenol-chloroform-isoamyl alcohol three times, then once with chloroform-isoamyl alcohol and precipitated with an equal volume of isopropanol at room temperature for 30 minutes. The pellet was collected after centrifuging at 3000 x g for 30 minutes, resuspended in 0.5 ml of TE (10 mM Tris-HCl, pH 8.0 and 1

mM EDTA) containing 25 μg RNase and incubated at 37 °C for 30 minutes. Phage DNA was precipitated again and resuspended in 0.5 ml of TE and the concentration was determined by spectrophotometry.

3.18 DNA (Southern) blotting

Genomic DNA from the positive bacteriophage λrFGF-2-c4 (see section 3.17) as well as Sprague Dawley rat liver (Ausubel *et al.*, 1990; Gross-Bellard *et al.*, 1973) was isolated, digested with various restriction endonucleases and electrophoresed in 1.0 % or 1.7% (w/v) agarose gels. Genomic DNA from the primary chicken ventricular myocyte cultures was isolated according to the published protocol (Nickel *et al.*, 1990b). Plasmid DNA was isolated using a Qiagen Maxi Kit (Qiagen Inc., CA, U.S.A.) according to the manufacturer's instructions. Restriction digests of genomic DNA and plasmid DNA were blotted to nitrocellulose as described by Maniatis *et al.*, 1982. Probes were radiolabelled routinely to a specific activity of ~$1x10^9$ cpm/μg using alpha ^{32}P dATP by random priming (Promega Corp., Madison, U.S.A.). DNA blots were hybridized to radiolabelled probes at 42 °C in the presence of 50% formamide for 20-24 hours, washed three times for 15 minutes each time at 65 °C in 0.1X SSC (20X SSC: 3 M sodium chloride, 0.3 M sodium citrate) with 0.1% sodium dodecyl sulfate (SDS) and visualized by autoradiography.

3.19 Subcloning of the rat FGF-2 gene 5′-flanking region

Based on the results of DNA blotting, two fragments that were candidates to contain coding and 5′-flanking regions of rat FGF-2 gene, were identified in *Bam*H1 digests of phage genomic DNA. These fragments of 1.0 kb (B1) and 1.4 kb (B2) were subcloned into *Bam*H1 site of pUC 119 (Vieira and Messing, 1987). Several deletion constructs were also generated based on the partial restriction mapping for the purposes of sequencing. The B2 subclone was digested with *Xba*1 to release an insert of ~1.1 kb and religated to generate a 3′deletion plasmid B2Δxba (Fig. 4). B2Δxba was digested with *Bam*H1 and *Bgl*II to release an insert of ~0.1 kb and religated to generate a 5′deletion plasmid B2Δbbg (Fig. 4). The B2Δxba was also digested with *Bgl*II and *Xba*1 to release an insert of ~0.15 kb, blunted with the enzyme Klenow and religated to generate a

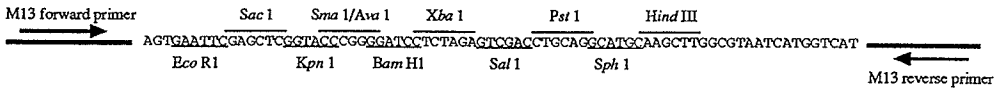
Fig. 4

Fig. 4. Schematic diagram showing the subclones used for sequence analysis of FGF-2 genomic fragments cloned from the rat genomic library.

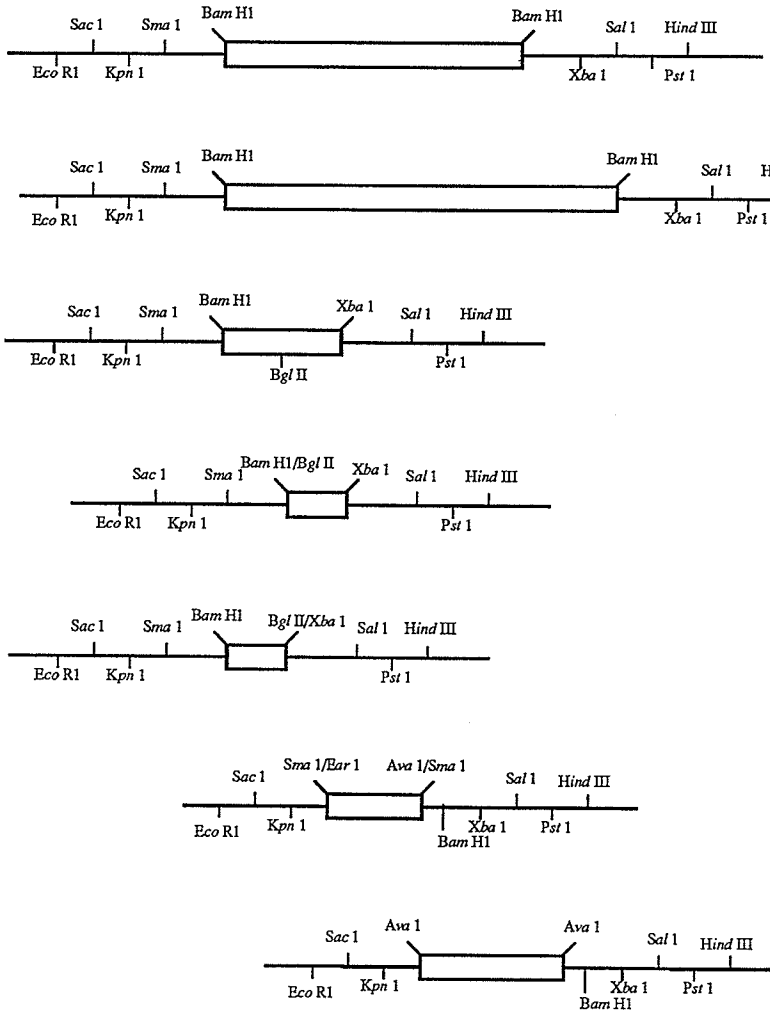
(a) Restriction enzyme sites and sequence of the polylinker region of pUC119 or pUC 19 plasmids used for subcloning of the rat FGF-2 genomic fragments. Relative locations of M13 forward and reverse primers used for DNA sequencing are indicated by arrows.

(b) Physical maps of subclones containing the 1 kb (B1) and 1.4 kb (B2) rat FGF-2 genomic fragments as well as the deletion clones (B2Δxba; B2Δbbg; B2Δbgx; B2ea and aspUC19) obtained from the B2 subclone.

(a)


M13 forward primer
Sac 1
Sma 1/Ava 1
Xba 1
Pst 1
Hind III
AGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCAT
Eco R1
Kpn 1
Bam H1
Sal 1
Sph 1
M13 reverse primer

(b)


Sac 1
Sma 1
Bam H1
Bam H1
Sal 1
Hind III
Eco R1
Kpn 1
Xba 1
Pst 1
B1
Sac 1
Sma 1
Bam H1
Bam H1
Sal 1
Hind III
Eco R1
Kpn 1
Xba 1
Pst 1
B2
Sac 1
Sma 1
Bam H1
Xba 1
Sal 1
Hind III
Eco R1
Kpn 1
Bgl II
Pst 1
B2XbaΔ
Sac 1
Sma 1
Bam H1/Bgl II
Xba 1
Sal 1
Hind III
Eco R1
Kpn 1
Pst 1
B2Δbbg
Sac 1
Sma 1
Bam H1
Bgl II/Xba 1
Sal 1
Hind III
Eco R1
Kpn 1
Pst 1
B2Δbgx
Sac 1
Sma 1/Ear 1
Ava 1/Sma 1
Sal 1
Hind III
Eco R1
Kpn 1
Xba 1
Pst 1
Bam H1
B2ea
Sac 1
Ava 1
Ava 1
Sal 1
Hind III
Eco R1
Kpn 1
Xba 1
Pst 1
Bam H1
aspUC19

3´deletion plasmid B2Δbgx (Fig. 4). The *Ear*1/*Ava*1 fragment (~0.25 kb) from B2 was gel purified and ligated into the *Sma*1 site of pUC 119 to generate B2ea (Fig. 4). The *Ava*1fragment (~0.4 kb) of B2 was subcloned into *Ava*1 site of pUC19 to generate aspUC19 (Fig. 4).

3.20 RNA (northern) blotting

Total RNA was isolated from primary rat ventricular myocytes, cell lines C6, H9c2 and U87-MG as well as rat tissues brain, heart, ovary and liver by the guanidinium isothiocyanate method (Chomczysi and Sacchi, 1987). RNA (100 µg/lane) from rat tissues as well as cell lines C6, H9c2 and U87-MG was electrophoresed in a denaturing formaldehyde-agarose gel [1.5% (w/v) agarose in 20 mM sodium salt of 3-(N-Morpholino) propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA and 2.2 M formaldehyde] and blotted to nitrocellulose (Maniatis *et al.*, 1982). The RNA blot was hybridized to radiolabelled fragments from the 5´-flanking region of FGF-2 gene and rat FGF-2 cDNA (Shimasaki *et al.*, 1988) as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH; kindly provided by Dr. I. Dixon, St. Boniface Hospital Research Centre, Winnipeg, MB, Canada). Probes were routinely labelled to a specific activity of ~1x10^9 cpm/µg of DNA using a random prime kit (Promega Corp., Madison, U.S.A.). Blots were hybridized to the radiolabelled probes at 42 °C in the presence of 50% formamide for 20-24 hours, washed three times for 15 minutes at 65 °C in 0.1X SSC with 0.1% SDS and assessed by autoradiography.

RNA (~15 µg/lane) from the transfected primary rat ventricular myocyte cultures was transferred to the nitrocellulose as described above and hybridized to radiolabelled oligonucleotides (kindly provided by Dr. I. Dixon, St. Boniface Hospital Research Centre, Winnipeg, MB, Canada; Epp *et al.*, 1993) specific for α-MHC (5´-AGACTCTGCGGCCCAG-3´) and ß-MHC (5´-TTTCCCTGCTGCTGCTCTCAG-3´) as well as cDNAs for ANF (a 600 bp *Pst* 1 insert was kindly provided by Dr. M. Nemer, Clinical Research Institute of Montreal, Canada) and 28S ribosomal RNA. About 100 ng of oligonucleotides was end labelled (~5x10^6 cpm) in a 20 µl reaction containing 70 mM Tris-HCl (pH7.6), 10 mM $MgCl_2$, 15 mM DTT, 200 µM spermidine, 5 µl of γ^{32}P-ATP, 10

units of T4 polynucleotide kinase (New England Biolabs, Ltd., ON, Canada) at 37 °C for 1 hour. RNA blots were hybridized with oligonucleotides at 40 °C in the presence of 28% formamide for 20 hours, washed three times for 15 minutes at room temperature in 2X SSC/0.1% SDS; 0.5X SSC/0.1% SDS and 0.1X SSC/ 0.1% SDS and assessed by autoradiography. ANF and 28S inserts were labelled by random priming and used for hybridizations as described above.

3.21 Primer extension analysis

Primer extension experiments were carried out using two antisense oligonucleotides primers: FGFLS3 (5´-CGAGAGACCGAGCCCCTGTGTGCTAC-3´), and B2LS1 (5´-CAGTTGTTAGATGTTTCTTGGAAAGAAC-3´) according to the method described (Ausubel *et al.*, 1990; McKnight and Kingsbury, 1982). Total RNA (50 µg) from rat heart, brain and C6 glioma cells was co-precipitated with the ^{32}P-end-labelled (~3x10^6 cpm; see section 3.20) oligonucleotide (~2.0 pmol), resuspended in 15 µl of 10 mM Tris-HCl, pH 8.3 and 50 mM magnesium chloride, denatured for 1 minute at 95 °C and hybridized for 2 hours at 65 °C. After hybridization, reverse transcription was done by adding 30 µl of buffer containing 30 mM Tris-HCl pH 8.3, 15 mM magnesium chloride, 8 mM dithiothreitol, 220 µM of each deoxyribonucleotide, 7 µg of actinomycin D and 10 units of AMV reverse transcriptase (Gibco BRL; Burlington, ON) to each tube and incubating for 90 minutes at 42 °C. The reaction was stopped by adding 105 µl of RNase mix containing RNase A (10 µg/ml; Sigma, St. Louis, MO) and salmon sperm DNA (0.1 mg/ml; Sigma, St. Louis, MO) and incubating for 30 minutes at 37 °C. Samples were extracted using phenol/chloroform/isoamyl alcohol followed by ethanol precipitation and resuspended in 5 µl of sequencing gel loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanole). Primer extended products were run in denaturing 8% polyacrylamide/8 M urea gels (7.6 g acrylamide (w/v), 0.4 g N,N´-methylene-bis-acrylamide (w/v), 50 g urea (w/v) and 10 ml (v/v) of 20x TBE (1 M Tris-HCl, pH8.3, 1 M boric acid and 20 mM EDTA) in a final volume of 100 ml) and visualized by autoradiography.

3.22 S1 nuclease protection

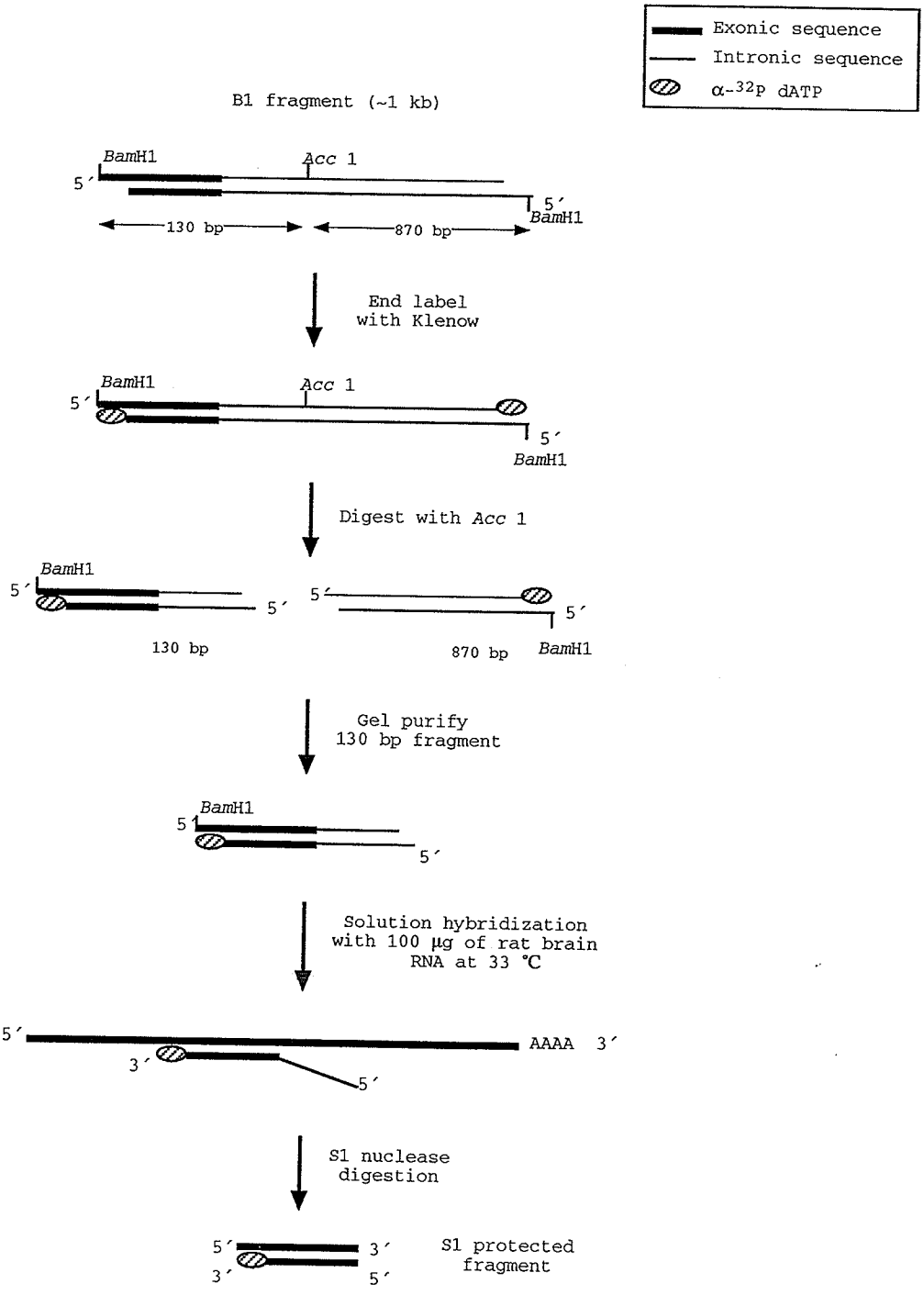
S1 nuclease protection was employed to locate the exon-intron boundary in the B1 subclone (Fig. 5). The 1 kb B1 fragment was radiolabelled with α-^{32}P dATP using klenow enzyme, digested with *Acc*1 and ~130 bp *Bam*H1/*Acc*1 fragment was gel isolated. Total RNA (100 µg) was coprecipitated with 250 fmol of radiolabelled (~5x10^5 cpm) *Bam*H1/*Acc*1 fragment, resuspended in 32 µl of deionized formamide and 8 µl of S1 hybridization buffer (80% formamide, 400 mM NaCl, 10 mM disodium salt of PIPES/piperazine-N,N´-bis[2-ethane-sulfonic acid]; 1,4-piperazine diethanesulfonic acid) and hybridized for 3 hours at 33 °C. Similarly, yeast tRNA (100 µg) was also hybridized with the probe as a control. After hybridization, samples were digested with 250 units of S1 nuclease (Pharmacia Canada Inc., Quebec) in 360 µl of S1 digestion buffer (200 mM NaCl, 30 mM sodium acetate, pH 5.2, 1 mM zinc sulphate) for 3 hours at 37 °C. Samples were extracted using phenol/chloroform/isoamyl alcohol followed by ethanol precipitation and resuspended in 5 µl of sequencing gel loading buffer. The products were resolved on denaturing 8% polyacrylamide/8 M urea gels (see section 3.21) and visualized by autoradiography.

3.23 Dideoxy sequencing (Sanger's method)

Nucleotide sequence of the 5´-flanking region of the rat FGF-2 gene was determined by the dideoxy method using a f-mol sequencing kit (Promega Corp., Madison, WN, U.S.A.). Several subclones (see Fig. 4) of the 5´-flanking region were sequenced using M13/pUC primers (forward: 5´-GTTTTCCCAGTCACGAC-3´; reverse: 5´-AGCGGATAACAATTTCACACAGGA-3´) as well as primers that can bind to the FGF-2 sequence (FGFLS3: 5´-CGAGAGACCGAGCCCCTGTGTGCTAC-3´; FGFLS4: 5´-CACGCCCGGCGCCTCCAAGTTGC-3´ and B2LS1: 5´-CAGTTGTTAGATGTTTCTTGGAAAGAAC-3´). All primers were obtained from UCDNA services, University of Calgary, Alberta, Canada. In the f-mol sequencing method, DNA template and primer are repeatedly annealed and enzymatically extended/terminated in a thermocycler using a thermo stable *Taq* DNA polymerase. Briefly, each sequencing reaction (G, A, T and C) consisted of 0.25 µg of plasmid DNA, 5 ng of

Fig. 5

Fig. 5. Strategy used for the identification of putative exon-intron boundary located in the 1 kb (B1) rat genomic fragment using S1 nuclease protection analysis.


Exonic sequence
Intronic sequence
α-32P dATP
B1 fragment (~1 kb)
BamH1
Acc 1
5′
5′
BamH1
130 bp
870 bp
End label
with Klenow
BamH1
Acc 1
5′
5′
BamH1
Digest with Acc 1
BamH1
5′
5′
5′
5′
130 bp
870 bp
BamH1
Gel purify
130 bp fragment
BamH1
5′
5′
Solution hybridization
with 100 µg of rat brain
RNA at 33 ℃
5′
AAAA 3′
3′
5′
S1 nuclease
digestion
5′
3′
3′
5′
S1 protected
fragment

primer, 0.25 µl of alpha ^{32}P dATP, 50 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 2 µl of respective nucleotide mix and 1.25 U *Taq* DNA polymerase in a total volume of 6 µl. All four nucleotide mixes (G, A, T and C) contained 20 µM each of 7-deaza dGTP, dATP, dTTP and dCTP but only one respective dideoxy (dd) nucleotide at varying concentrations. (ddGTP: 30 µM, ddATP: 350 µM; ddTTP: 600 µM and ddCTP: 200 µM). The sequencing reactions were carried out in a thermocycler for 30 cycles, each consisting of denaturation at 95 °C for 1 minute, annealing at 55 °C for 30 seconds and extension at 70 °C for 1 minute. The reactions were stopped by adding 3 µl of f-mol sequencing stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanole). The reaction products were seperated in 8 M urea and 8% acrylamide gels (see section 3.21). The gels were dried at 80 °C for 1 hour using a gel drier (Biorad, ON, Canada) and the sequence was visualized by autoradiography.

3.24 Chemical sequencing (Maxam and Gilbert's method)

Chemical sequencing of the radiolabelled fragments was performed (Maxam and Gilbert, 1977) in order to identify the boundaries of nuclease protected areas during DNase I protection assays. B2Δbgx plasmid was digested with *Eco*R1 or *Hin*dIII, end labelled with klenow fragment and redigested with *Hin*dIII or *Eco*R1 to release ~170 bp DNA fragments (Fig. 4) that were radiolabelled on either *Eco*R1 or *Hin*dIII ends. The radiolabelled fragments were gel purified and used for chemical sequencing. For "G" reaction, ~5 ng of end labelled DNA fragment (~1.0 x 10^6 cpm) in 7 µl of sterile distilled water was added to 7 µl of cacodylate buffer (50 mM sodium cacodylate, 1mM EDTA, pH 8.0) and 100 µl of fresh DMS (1% dimethyl suphate in cacodylate buffer) and incubated at 20 °C for 2 minutes. The reaction was stopped by adding 50 µl of DMS stop buffer (1.5 M sodium acetate, pH 7.0, 1.0 M ß-mercaptoethanol and 100 µg/ml tRNA), extracted with 750 µl of 95% ethanol on dry ice for 5 minutes, centrifuged for 10 minutes and the pellet was resuspended in 100 µl of water. The sample was mixed with 1 ml of n-butanol, vortexed vigorously, spun for 1 minute and pellet was resuspended in 100 µl of water. Extraction with n-butanol was repeated once more, the pellet was resuspended in 150 µl of 100 mM piperidine and stored at 4 °C until the "G+A" reactions were completed. For

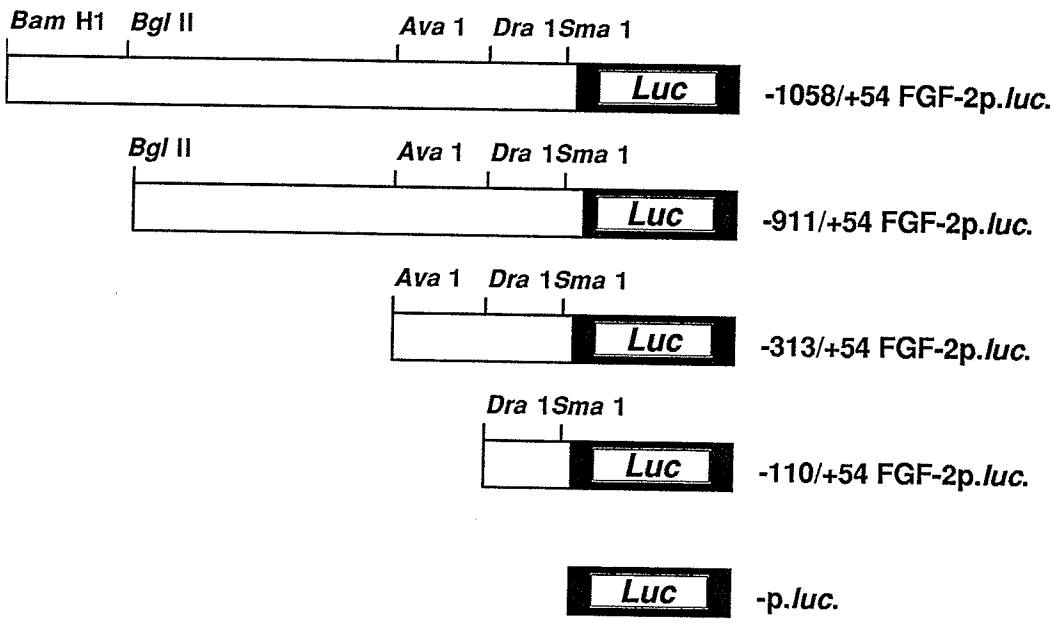
"G+A" reaction, 1 µl of 1M formic acid was added to ~5 ng of end labelled DNA fragment (~1.0 x 10^6 cpm) in 30 µl of sterile distilled water and incubated at 37 °C for 25 minutes. The reaction was stopped by adding 150 µl of 100 mM piperidine. Both "G and G+A" reactions were incubated at 90 °C for 30 minutes, allowed to cool down to room temperature, extracted twice with 1.2 ml of n-butanol and resuspended in 6 µl of sequencing gel loading buffer. The reaction products were seperated along with the DNase I protection assay samples (see section 3.28) in 8 M urea and 8% acrylamide gels (see section 3.21). The gels were dried at 80 °C for 1 hour using a gel drier (Biorad, ON, Canada) and the sequence was visualized by autoradiography.

3.25 Hybrid luciferase Plasmid constructions

Fragments of the rat FGF-2 gene were introduced upstream of the translation start site of the firefly luciferase reporter gene in pXP1 (Nordeen, 1988). The 1112 bp *Bam*H1/*Sma*1 fragment (Fig. 6) was introduced at the *Bam*H1 and *Hin*dIII (blunted) sites of the pXP1, to generate -1058/+54FGF-2p.*luc*; the minus and plus values indicate the number of bases included upstream and downstream of the transcription start site (nucleotide +1) of rat FGF-2 gene. The 1002 bp *Bam*H1/*Dra*1 fragment (Fig. 6) was introduced at the *Bam*H1 and *Hin*dIII (blunted) sites of the pXP1, to generate -1058/-111FGF-2p.*luc*. The 147 bp *Bam*H1/*Bgl*II fragment (Fig. 6) was removed from -1058/+54FGF-2p.*luc* by digestion with *Bam*H1 and *Bgl*II to generate -911/+54FGF-2p.*luc*. The FGF-2 *Ava*1/*Sma*1 fragment (Fig. 6) was released from aspUC19 (Fig. 4) by *BamH*1/*Sac*1 digestion and inserted into corresponding sites in pXP1 to yield -313/+54FGF-2p.*luc*. A 164 bp FGF-2 *Dra*1/*Sma*1 fragment (Fig. 6) was obtained from aspUC19 as a *Dra*1/*Sac*1 fragment and ligated into corresponding sites of the pXP1 to generate -110/+54FGF-2p.*luc*. All the recombinant luciferase plasmids were verified by sequencing. These hybrid genes were used to transiently transfect the rat glioma C6 cell line. A promoterless luciferase gene (pXP1/-p.*luc*) was used to transfect cells as a control for random transcription initiation. As a positive control, cells were also transfected with CMVp.*luc* (de Wet *et al.*, 1987) and transfection efficiency was normalized using RSVp.CAT .

Fig. 6

Fig. 6. Hybrid FGF-2p/luciferase plasmid constructions. The 1112 bp *Bam* H1/*Sma* 1 fragment containing 5´-flanking region of the rat FGF-2 gene was placed upstream of the luciferase coding sequence in the promoterless plasmid (-p.*luc*; Nordeen, 1988) to generate -1058/+54FGF-2p.*luc*. As indicated, restriction fragments containing various lengths of the rat FGF-2 gene were used to generate 5´ deletion constructs (-911/+54FGF-2p.*luc*.; -313/+54FGF-2p.*luc*. and -110/+54FGF-2p.*luc*.). These FGF-2p/luciferase hybrid genes were used to test the cloned fragments for promoter activity and or responsiveness to mitogenic stimuli in rat and human cells after gene transfer. Promoterless -p.*luc*. was used as a control for random transcription initiation. CMVp.*luc*. was used as a positive control for transfections. RSVp.CAT was used as a control for DNA uptake.


Bam H1
Bgl II
Ava 1
Dra 1
Sma 1
Luc
-1058/+54 FGF-2p.luc.
Bgl II
Ava 1
Dra 1
Sma 1
Luc
-911/+54 FGF-2p.luc.
Ava 1
Dra 1
Sma 1
Luc
-313/+54 FGF-2p.luc.
Dra 1
Sma 1
Luc
-110/+54 FGF-2p.luc.
Luc
-p.luc.

3.26 Nuclear extracts

For generating nuclear extracts, C6 glioma cells were plated in 150 mm culture dishes and maintained (i) in the absence of serum for 48 hours, (ii) in the presence of serum for 48 hours, or (iii) in the absence of serum for 48 hours but treated with phorbol ester (100 nM PMA) for the final 24 hours. Cells were lifted using 0.25% (w/v) trypsin and 1 mM EDTA (Gibco-BRL; Burlington, ON), pelleted at 3,000 x g for 2 minutes and pellets were frozen at -70 °C until further use. Nuclear extracts were prepared according to the previously described method (Schreiber *et al.*, 1989; Muller *et al.*, 1989) with modifications. Briefly, cells were thawed, resuspended in 1.5 ml of cold CMF-PBS using a 1 ml pipetteman and pelleted again. The pellets were resuspended in 0.4 ml of buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM magnesium chloride, 10 mM potassium chloride, 0.5 mM dithiothreiotol (DTT),1 mM PMSF and 2 µg/ml aprotinin) and allowed to swell for 15 min on ice. All the steps were carried out at 4 °C unless stated otherwise. After 15 min, cells were examined under a microscope to confirm whether the nuclei are completely released. The cell suspension was then treated with 25 µl of Nonidet P-40 (NP-40, Sigma, St. Louis, MO), vortexed for 10 seconds and centrifuged for 30 seconds. Supernatant was discarded and the pellets were resuspended in 0.1 ml of buffer C (20 mM HEPES-KOH pH 7.9, 25% (v/v) glycerol, 1.5 mM magnesium chloride, 420 mM sodium chloride, 0.2 mM EDTA, 0.5 mM DTT,1 mM PMSF and 2 µg/ml aprotinin), rocked for 20 min and centrifuged for 5 min at 13,000 xg. Supernatants were saved, aliquoted and stored at -70 °C until further use. Protein concentrations were assessed using the Bradford protein assay (Bradford, 1976).

3.27 Gel mobility shift assays

Gel mobility shift assays were employed (Baldwin, 1990) in order to detect interactions (if any) of proteins from C6 nuclear extract with the radiolabelled DNA fragments from the 5´-flanking region of rat FGF-2 gene. Binding reactions were performed in 20 µl reaction mixtures containing 4 to12 µg of nuclear extract and radiolabelled DNA fragment (10^4 cpm/0.5 ng) in the presence of 15 mM HEPES-KOH pH 7.9, 15% (v/v) glycerol, 2 µg calf thymus DNA (Sigma, St. Louis, MO), 6.25 mM magnesium chloride, 75 mM potassium chloride, 150 µM EDTA, 375 µM DTT and 750

μM PMSF. Binding was allowed to proceed for 20 min at 4 °C. The binding reactions were then analyzed by electrophoresis on a 4% polyacrylamide gel [4% acrylamide, 0.05% bis-acrylamide, 2.5% (v/v) glycerol in 0.5X TBE (20X TBE: 1 M Tris-HCl, pH8.3, 1 M boric acid and 20 mM EDTA)] buffer at 30 mA for ~2 hours.

3.28 DNase I protection assay

DNase I protection assays were carried out to identify the sites of DNA protein interactions indicated by the results of the gel mobility shift assays, according to published protocols (Allegretto *et al.*, 1990; Nachtigal *et al.*, 1993). Briefly, binding reactions in triplicates were performed in 20 μl reaction mixtures containing 15 μg of nuclear extracts and radiolabelled DNA fragment (10^4 cpm/0.5 ng) under the similar conditions described for gel mobility shift assay for 20 min at 4 °C and for an additional 5 min at room temperature. Later, 24 μl of nuclease mix [0.05 to 0.1 units of DNase I (RQ 1, Promega Corp., Wisconsin, U.S.A.) in 3.3 mM HEPES-KOH pH 7.9, 3.3% (v/v) glycerol, 16.5 mM potassium chloride, 4.17 mM calcium chloride, 8.33 mM magnesium chloride, 33 μM EDTA, 82 μM DTT and 165 μM PMSF] was added to each binding reaction and the triplicates were incubated at room temperature for 1 min, 1.5 min and 2 min respectively. DNase I digestion was stopped by adding 160 μl of stop buffer (120 mM tris-HCl, 16 mM EDTA, 188 mM NaCl, 1.3% SDS (w/v), 100 μg of proteinase K and 4 μg of tRNA) and incubating for 30 min at 37 °C. Samples were then extracted with phenol-chloroform-isoamyl alcohol, ethanol precipitated and resuspended in 6 μl of sequencing gel loading buffer. As a control, labelled DNA fragment was also digested under similar conditions but in the absence of nuclear extract. Nuclease protection sites were visualized by running the samples in a 8% polyacrylamide/8 M urea sequencing gel and subsequent autoradiography.

3.29 Statistical analysis

Data presented in the text and figures are mean plus or minus standard error of the mean. Statistical analysis (Bruning and Kintz, 1977) of the data related to embryonic chicken ventricular myocytes, was done using a paired or unpaired Students t-test. Statistical analysis of the data related to postnatal rat ventricular myocytes, was done by a one-way analysis of variance (ANOVA) and the Bonferroni multiple comparisons *post hoc*

test. The results were accepted if Bartlett's test for homogeneity of variances indicated that the difference between standard deviations from each test group was not significant. When this difference was shown to be significant (level of binucleation in population of cardiac myocytes overexpressing FGF-2), analysis was done using the Mann-Whitney test (non parametric). In all cases, a value was considered statistically significant if p was determined to be <0.05.

Chapter 4

Generation of FGF-2 cDNAs that can preferentially express high and or low molecular weight forms of FGF-2 and characterization of their expression products

4.1 Background

Basic fibroblast growth factor (FGF-2) is a potent mitogen for tissues of mesodermal and neuroendodermal origin (Baird *et al.*, 1986). FGF-2 exists in multiple forms due to the initiation of translation from alternative codons (leucine versus methionine) in the same mRNA (Florkiewicz and Sommer, 1989), alternative splicing of RNA (Borja *et al.*, 1993) as well as proteolysis of high molecular weight forms (Doble *et al.*, 1991; Klagsbrun *et al.*, 1987). This growth factor is predominantly an intracellular molecule due to the absence a signal sequence for its secretion. However, it has been implicated to exert its biological effects in both paracrine (cell surface receptor dependent pathway) and intracrine (cell surface receptor independent pathway) manners (Logan, 1990). Mitogenic function of FGF-2 reported so far, can be largely attributed to the 18 kD FGF-2, since a recombinant form of this protein is widely used for *in vitro* studies. There is scant information about the importance of high molecular weight forms of FGF-2 resulting from the upstream CUG (leucine) codons due to the unavailability of purified proteins. High molecular weight FGF-2 binds to the same cell membrane receptors and has similar mitogenic activity as 18 kD FGF-2 (Moscatelli *et al.*, 1987; Amalric *et al.*, 1991). A role for the high molecular weight FGF-2 (25 kD) has been implicated in the liver regeneration (Presta *et al.*, 1989). Further, an evidence for its intracellular effect was shown recently based on phenotypic changes occurring in mouse 3T3 cells overexpressing dominant negative FGFR-1 (Bikfalvi *et al.*, 1995). Thus, high and low molecular weight forms may have distinct functions in the context of cellular growth and differentiation.

4.2 Rationale

Although much is known about low molecular weight form of FGF-2, the

physiological significance of high molecular weight forms of FGF-2 is largely unknown due to the unavailability of purified proteins. Recent studies implicated high molecular weight forms of FGF-2 in liver cell regeneration (Presta *et al.*, 1989). The levels of these forms were also shown to be developmentally regulated in the central nervous system as well as cardiovascular system (Giordano *et al.*, 1992; Liu *et al.*, 1993). Therefore, this work was aimed at generating expression vectors containing wild type and modified rat FGF-2 cDNAs that could preferentially express high and or low molecular weight forms of FGF-2. Generation of these hybrid FGF-2 genes will enable us to study the effects of individual forms of FGF-2 in different cell types using gene transfer.

4.3 Hypothesis

Hybrid FGF-2 genes that are generated by the modification of leucine or methionine initiation codons in the wild type rat FGF-2 cDNA, can be used to preferentially overexpress either high or low molecular weight forms of FGF-2 in COS-1 cells after gene transfer.

4.4 Specific Aims

1. To generate expression vectors containing wild type and modified rat FGF-2 cDNAs in between the Rous sarcoma viral (RSV) promoter (p) and simian virus 40 (SV 40) poly adenylation signal.
2. To characterize the expression products of wild type and modified rat FGF-2 cDNAs and their subcellular distribution in COS-1 cells after gene transfer.

4.5 Results and Discussion

4.5.1 Generation of expression vectors containing wild type and modified FGF-2 cDNAs

The full length complementary DNA coding for rat ovarian FGF-2 was obtained as a 1.0 kilo base (kb) *Xho* I fragment (Shimasaki *et al.*, 1988). The rat FGF-2 cDNA was predicted to have three potential sites for initiation of translation based on *in vitro* translation studies (Powell and Klagsbrun, 1991). These included a classical methionine

(AUG) codon and two upstream leucine (CUG) codons (Powell and Klagsbrun, 1991). The methionine (ATG) codon of the wild type FGF-2 cDNA (Shimasaki *et al.*, 1988), responsible for initiating the 18 kD form, was modified by an insertional mutation such that the methionine codon was eliminated but the translational reading frame was maintained. As a result the amino acid sequence in the methionine region -Gly-Ala-Met-Ala-Ala- was changed to -Gly-Ala-Gln-Ala-Trp-Ala-Ala-. Thus, the modified cDNA (ΔmetFGF) had the potential to generate only high molecular weight FGF-2 species from the upstream CTG sites. The putative leucine (CTG) start sites were removed from the wild type FGF-2 cDNA to generate a truncated sequence (metFGF). In this case, the modified cDNA (metFGF) has the potential to generate only the low molecular weight FGF-2 species from the ATG site. Wild type (FGF) as well as modified FGF-2 sequences (ΔmetFGF and metFGF) were cloned in between the Rous sarcoma viral (RSV) promoter (p) and simian virus 40 (SV40) polyadenylation signal (see section 3.2; Fig. 4), to obtain RSVp.FGF, RSVp.ΔmetFGF and RSVp.metFGF (Fig. 7). The expression vector without any FGF-2 sequence (RSVp.CONT) was used as a control. Bacterial genes coding for chloramphenicol acetyl transferase (CAT) and ß-galactosidase (ß-gal) were also cloned in the identical expression vector to obtain RSVp.CAT and RSVp.β–gal, respectively and were used as a control for DNA uptake of the "test" genes. The upstream sequence from ΔmetFGF which contains leucine codons, was fused to the coding sequence of the CAT gene to generate RSVp.FGF/CAT hybrid gene (Fig. 7).

4.5.2 Preferential expression of different forms of FGF-2 from the wild type and modified FGF-2 hybrid genes in COS -1 cells

The wild type and modified FGF-2 cDNAs were introduced into COS-1 cells by gene transfer and their expression products were analysed by immunoblotting with antibodies to FGF-2. COS-1 cells were chosen to test the expression of these hybrid genes because they contain low levels of endogenous FGF-2 (Florkiewicz and Sommer, 1989). Heparin binding fractions of total lysates from COS-1 cells alone or cells transfected with the expression vector (RSVp.CONT) revealed endogenous forms (22 and 18 kD) of FGF-2 (Fig. 8, lanes a,b). Following transfections with RSVp.ΔmetFGF and RSVp.FGF, expression of 22 and 21.5 but not the 18 kD form of FGF-2 was detected above

Fig. 7

Fig. 7. Schematic representation of hybrid FGF-2 genes used in this study. Rat FGF-2 cDNA (Shimasaki *et al.*, 1988) was introduced in between the RSV promoter and SV40 polyadenylation signal to generate RSVp.FGF which contains two potential CTG initiation sites in addition to an ATG site. For RSVp.ΔmetFGF, the ATG codon was removed by inserting a *Hind* III linker at the *Nco* I site, leaving the potential CUG sites intact. For RSVp.metFGF, CTG sites were eliminated by *Sma* I digestion. The expression vector containing bacterial gene coding for either chloramphenicol acetyl transferase (RSVp.CAT) or ß-galactosidase (RSVp.ß-gal) was used for co-transfection. For RSVp.FGF/CAT, upstream sequences from the ΔmetFGF cDNA, containing two CTG codons were fused in frame with the methionine codon of CAT gene in place of FGF-2 coding sequence. Identical vector without any FGF-2 cDNA sequence (RSVp.CONT) was used as a control for transfections.

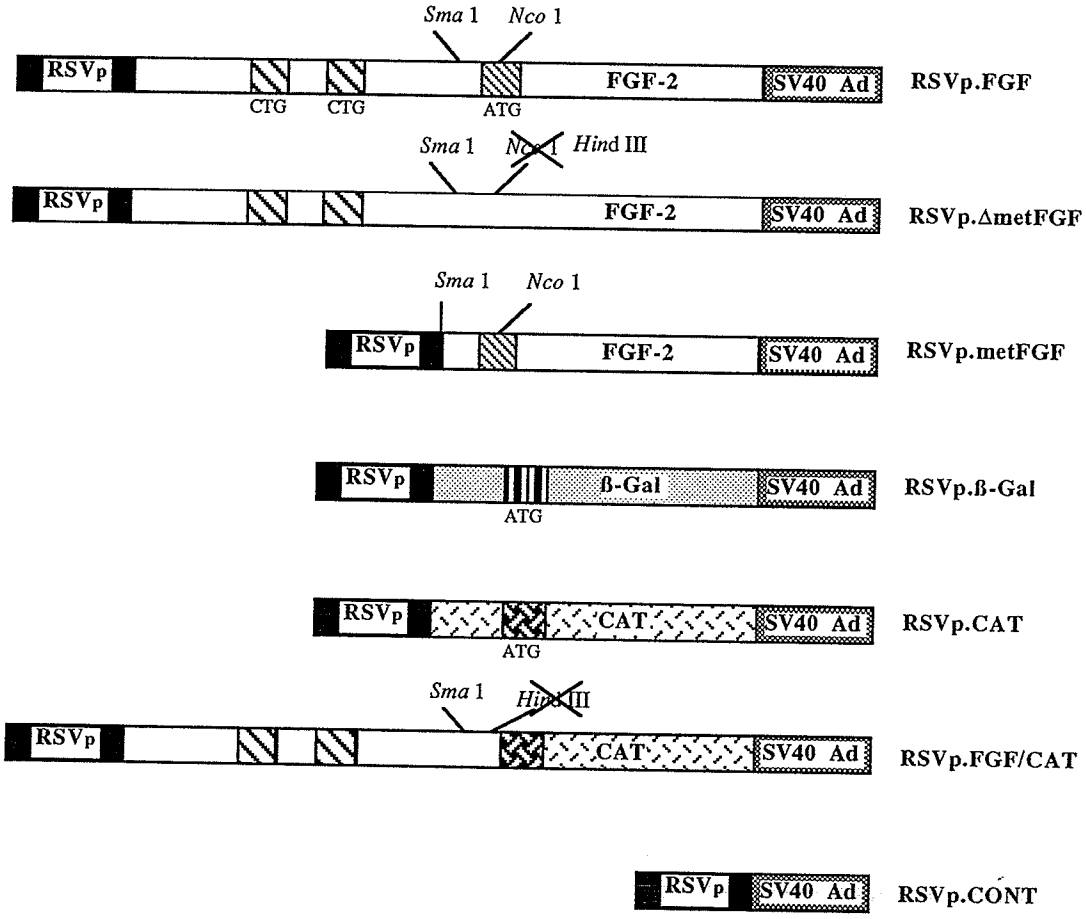
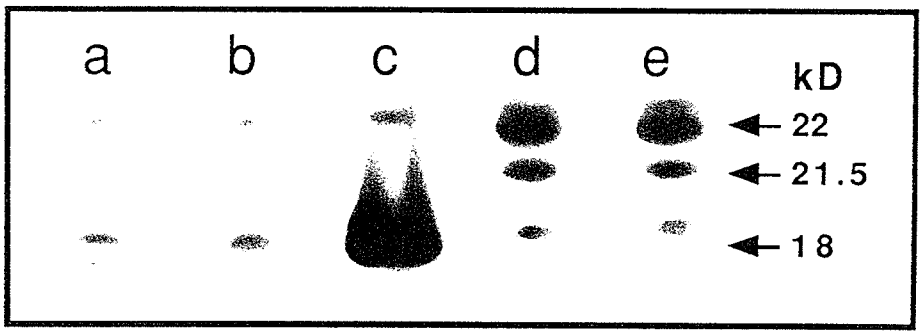

Sma 1
Nco 1
RSVp
CTG
CTG
ATG
FGF-2
SV40 Ad
RSVp.FGF
Sma 1
Nco 1
Hind III
RSVp
FGF-2
SV40 Ad
RSVp.ΔmetFGF
Sma 1
Nco 1
RSVp
FGF-2
SV40 Ad
RSVp.metFGF
RSVp
ß-Gal
SV40 Ad
ATG
RSVp.ß-Gal
RSVp
CAT
SV40 Ad
ATG
RSVp.CAT
Sma 1
Hind III
RSVp
CAT
SV40 Ad
RSVp.FGF/CAT
RSVp
SV40 Ad
RSVp.CONT

Fig. 8

Fig. 8. Expression pattern of wild type and modified hybrid FGF-2 genes in simian kidney COS-1 cells. Wild type (RSVp.FGF) and modified (RSVp.ΔmetFGF, RSVp.metFGF) hybrid FGF-2 genes, as well as the RSVp vector without any FGF-2 sequence (RSVp.CONT), were used to transiently transfect COS-1 cells. Heparin-sepharose purified COS-1 cell total lysates were fractionated in a 12.5% SDS-PAGE gel, immunoblotted, probed with rabbit FGF-2 antibodies, and visualized with ^{125}I-protein A. Lysate from: non transfected cultures, lane a; transfected with the expression vector (RSVp.CONT), lane b; RSVp.metFGF, lane c; RSVp.ΔmetFGF, lane d; and RSVp.FGF, lane e. The mobilities of 22, 21.5 and 18 kD FGF-2 are indicated.


a
b
c
d
e
kD
22
21.5
18

background levels (lanes d,e). The levels of 18 kD form observed were comparable to the amount of endogenous FGF-2 seen with the non transfected or RSVp.CONT (Fig. 8, lanes a,b). This suggests that 22 and 21.5 kD high molecular weight forms are the major products of the RSVp.ΔmetFGF and RSVp.FGF genes. Transfection of COS-1 cells with the RSVp.metFGF construct resulted in the expression of 18 kD FGF-2 but not the 22 or 21.5 kD species (Fig. 8, lane c). Removal of the upstream sequences containing CTG sites (Fig. 4; Fig. 7), resulted in high levels of 18 kD FGF-2 expression from the RSVp.metFGF gene. There was no evidence for the expression of high molecular weight 22 and 21.5 kD FGF-2 species from the transfected RSVp.metFGF gene as levels were comparable to background (Fig. 8). Further, the level of 18 kD FGF-2 following overexpression of metFGF was consistently 5 fold greater than 22 and 21.5 kD FGF-2 generated from ΔmetFGF and FGF cDNAs (determined by densitometry, n=8). This result can not be attributed to differences in DNA uptake by the cells, as there was no significant difference between the CAT activity measured resulting from co-transfection with the RSVp.CAT gene. Also, the difference in the levels of 18 kD FGF-2 expressed by RSVp.FGF versus RSVp.metFGF, suggests the presence of repressor sequences which impede the use of the methionine (AUG) site in the RSVp.FGF transcripts. These sequences would be deleted in the metFGF cDNA construct.

4.5.3 Effect of rat FGF-2 cDNA sequences located upstream of the AUG initiation codon on the expression of a hybrid FGF/CAT gene

In light of differences in the expression levels of high and low molecular weight forms of FGF-2 from the modified cDNAs, a FGF-2/CAT fusion gene was generated to test the repressor effect of upstream sequences of rat FGF-2 cDNA, on the translation from AUG codon of CAT gene. The upstream sequences containing leucine but not methionine codons of rat FGF-2 cDNA, were fused in frame with the methionine codon of CAT gene to generate RSVp.FGF/CAT (Fig. 7). RSVp.CAT which lacks the upstream rat FGF-2 cDNA sequences was used for comparison. RSVp.CONT was also used as a negative control. All genes were introduced into COS-1 cells by transient gene transfer and their expression was analysed in terms of CAT activity. The cells were also co-transfected with CMVp.*luc* for normalizing the transfection efficiency. The corrected CAT activity of

RSVp.CAT was significantly higher (~4 fold, $p<0.002$) compared to the activity of RSVp.FGF/CAT (Fig. 9). Thus, these results suggest that the upstream rat FGF-2 cDNA sequences exert a repressor effect on the translation from the downstream methionine codon. These repressor sequences could be (i) the CUG start sites or (ii) RNA upstream of the AUG codon that could possibly form a secondary structure. The latter possibility is supported by data from Prats *et al.*, (1992), who demonstrated that RNA structures surrounding the human FGF-2 AUG start codon can act negatively on its accessibility. Deletion of the entire 5′ untranslated region and alternate translation region, containing potential CUG codons, resulted in overexpression of the 18 kD FGF-2 form. This indicated that 5′ leader sequences are responsible for a strong inhibition of translation from the AUG codon. Deletion of intervening sequences (87 nucleotides) between the CUG and AUG sites also led to an increase in translation from the AUG start codon (as well as from some but not all CUG start sites). In contrast, conversion of CUG to AUG sites did not alter translation from the wild type or mutated AUG codon (Prats *et al.*, 1992). Thus, these data support the possibility that the 5′ sequences other than CUG sites repress translation from the wild type AUG site. Regulation of CUG- versus AUG- initiated forms of FGF-2 was also reported during the development of heart (Liu *et al.*, 1993) and brain (Giordano *et al.*, 1992). However, the factors or mechanisms that augment the selective translation of one form versus another are largely unknown.

4.5.4 Subcellular localization of the expression products of wild type and modified rat FGF-2 cDNAs in COS-1 cells

COS-1 cells transfected with wild type and modified FGF-2 sequences were assessed for the subcellular localization of FGF-2 by immunofluorescent staining with specific antibodies. Expression of FGF-2 above background levels (RSVp.CONT; Fig. 10d) was seen after transfection with the modified and wild type FGF-2 cDNAs (Fig. 10a-c). Staining of cells transfected with RSVp.ΔmetFGF and RSVp.FGF, which produce predominantly 22 and 21.5 kD FGF-2 forms (Fig. 8), was largely nuclear (Fig. 10a,b) although some cytoplasmic staining was observed. This could be due to the fact that RSVp.FGF contains wild type FGF-2 cDNA (Shimasaki *et al.*, 1988) which has the potential to produce both high and low molecular weight forms of FGF-2. In contrast,

Fig. 9

Fig. 9. Effect of upstream rat FGF-2 cDNA sequences containing CTG codons on the expression of a FGF/CAT fusion gene in COS-1 cells. A hybrid RSVp.FGF/CAT gene was used to test the effect of upstream FGF-2 cDNA sequences on the usage of the methionine codon in the CAT gene. COS-1 cells were transfected with RSVp.FGF/CAT, RSVp.CAT and RSVp.CONT. The transfection efficiency was normalized by co-transfecting with CMVp.*luc*. The CAT activities (CAT/luciferase) for RSVp.FGF/CAT or RSVp.CAT represent the mean from at least 4 determinations after subtraction of background levels (2.9×10^6) of CAT activity obtained from cells transfected with RSVp.CONT. There is a significant decrease (~4 fold, $p<0.002$) in corrected CAT activity of RSVp.FGF/CAT compared to the activity seen with RSVp.CAT. Error bars represent the standard error of the mean.

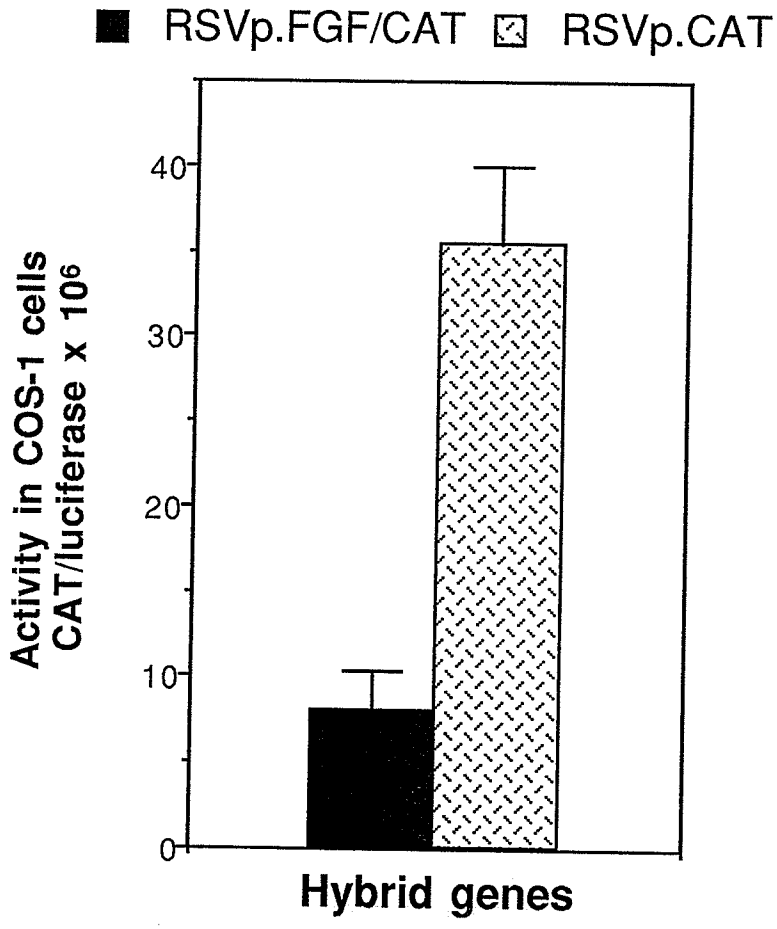
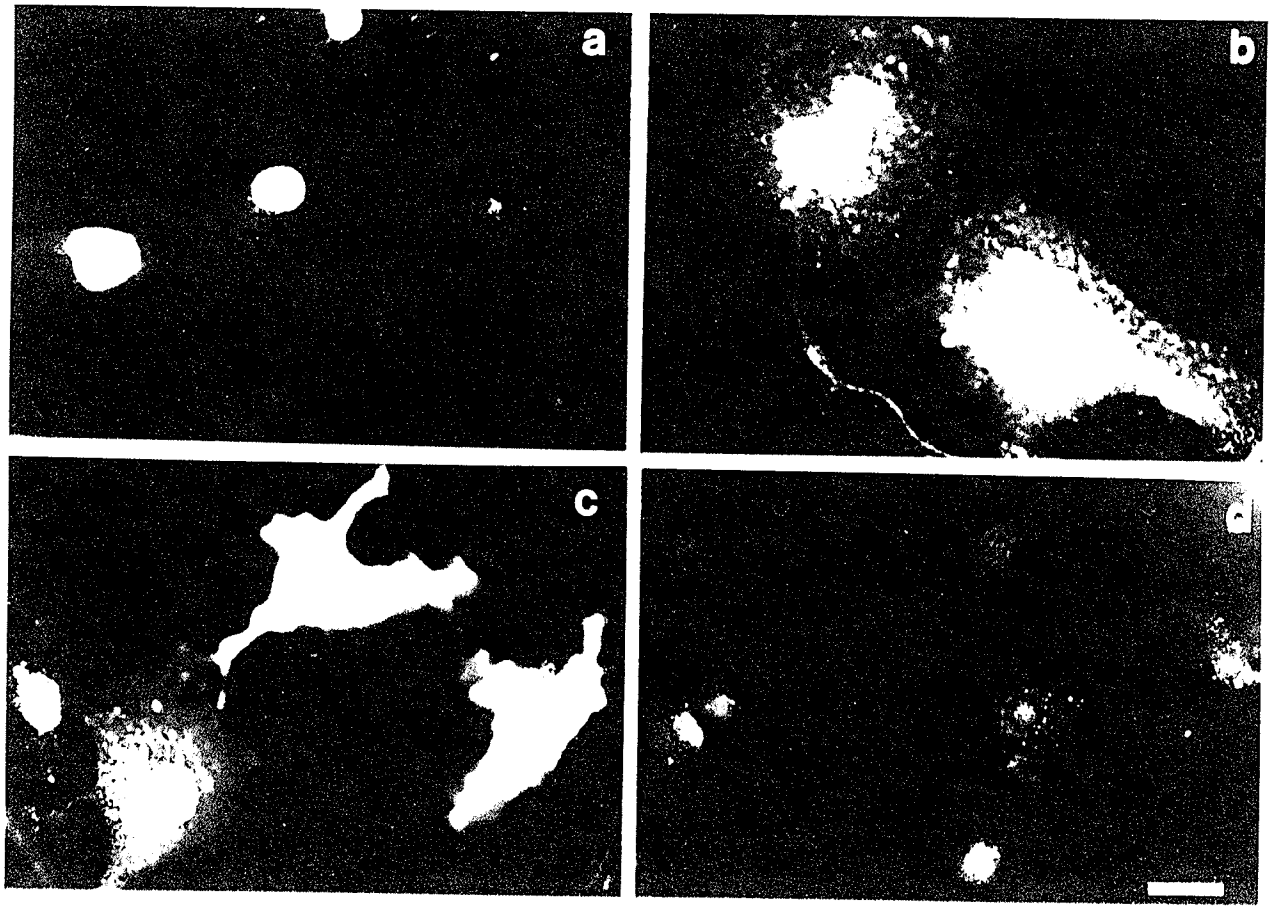

RSVp.FGF/CAT
RSVp.CAT
Activity in COS-1 cells
CAT/luciferase x 10⁶
40
30
20
10
0
Hybrid genes

Fig. 10

Fig. 10. Subcellular localization of FGF-2 in simian kidney COS-1 cells transfected with wild type and modified FGF-2 cDNAs. COS-1 cells were transfected with RSVp.ΔmetFGF (a), RSVp.FGF (b), RSVp.met FGF (c) and RSVp.CONT (d) and stained for FGF-2. Note the background levels of FGF-2 staining in cells transfected with RSVp.CONT. By contrast, intense nuclear FGF-2 staining was observed in cells transfected with either RSVp.ΔmetFGF (a) or RSVp.FGF (b). However, some cytoplasmic staining was also observed with RSVp.FGF (b). COS-1 cells transfected with RSVp.metFGF (c) revealed predominantly cytoplasmic and nuclear staining pattern in addition to exclusively cytoplasmic or nuclear staining patterns. Bar is equivalent to 20 μM.


a
b
c
d

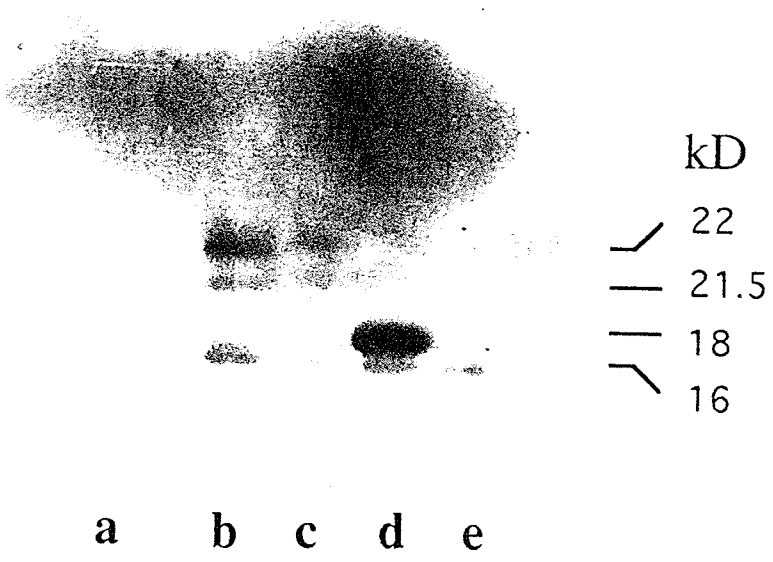
RSVp.metFGF which generates 18 kD FGF-2, revealed three different staining patterns: (i) both cytoplasmic and nuclear, (ii) cytoplasmic and (iii) nuclear (Fig. 10c). The majority of those cells (75%) displaying intense immunostaining (relative to background levels) after transfection with RSVp.metFGF had both nuclear as well as cytoplasmic localization.

4.5.5 Assessment of different forms of FGF-2 in the nuclear extracts of COS-1 cells transfected with wild type and modified rat FGF-2 cDNAs

In order to confirm whether the intense FGF-2 nuclear staining that was observed in the transfected cells is not merely a nuclear surface staining, the nuclear extracts from the transfected cells were analysed by immunoblotting. High molecular weight forms (22 and 21.5 kD) were detected with FGF-2 antibodies in the heparin sepharose fractionated nuclear extracts of COS-1 cells transfected with RSVp.FGF and RSVp.ΔmetFGF (Fig. 11, lanes b,c). In contrast, the nuclei from COS-1 cells transfected with RSVp.metFGF revealed high levels of 18 kD form, but not the 22 and 21.5 kD species (Fig. 11, lane d). Although a 16 kD band was detected in all nuclear samples, levels were identical to those seen in cells transfected with the RSVp.CONT (Fig. 11, lane e) and, thus, likely reflect an endogenous gene product. No significant difference was measured in the uptake of FGF-2 hybrid genes by comparing co-transfected RSVp.CAT gene activity. Thus, the immunoblotting results substantiate the immunofluorescence results and indicate that both high and low molecular weight forms of rat FGF-2 can localize to the cell nucleus. These results are in agreement with other studies on the expression of bovine and rat FGF-2 (Baldin *et al*, 1990 and Powell and Klagsbrun, 1991). In humans, however, high and low molecular weight forms of FGF-2 were exclusively localized to the nucleus and cytoplasm, respectively (Bugler *et al*, 1991) and this nuclear localization of high molecular weight forms was due to the presence of a nuclear localization signal in their amino terminal extensions (Bugler *et al*, 1991). This presumably, represents a species difference between rat and human as both high and low molecular weight forms of rat FGF-2 were found in the nucleus (Powell and Klagsbrun, 1991). There are at least three possible mechanisms by which 18 kD FGF-2 could enter the nucleus: (i) through the presence of a nuclear transport signal; (ii) during cell division; (iii) by binding to a nuclear transport protein; and (iv) by

Fig. 11

Fig. 11. Localization of high and low molecular weight forms of FGF-2 in the nuclear extracts of COS-1 cells transfected with wild type and modified FGF-2 cDNAs by immunoblotting. Nuclear extracts were generated from COS-1 cells transfected with RSVp.FGF, RSVp.ΔmetFGF and RSVp.metFGF as well as RSVp.CONT. Heparin-sepharose purified COS-1 nuclear extracts were fractionated in a 12.5% SDS-PAGE gel, immunoblotted, probed with rabbit FGF-2 antibodies, and visualized with ^{125}I-protein A. Recombinant human 18 kD bFGF marker (10 ng), lane a; Nuclear extract from COS-1 cells transfected with the RSVp.FGF, lane b; RSVp.ΔmetFGF, lane c; RSVp.metFGF, lane d; expression vector (RSVp.CONT), lane e. The mobilities of 22, 21.5, 18 and 16 kD FGF-2 are indicated.


kD
22
21.5
18
16
a
b
c
d
e

proteolytic cleavage of preexisting high molecular weight forms. A region of 27 to 32 amino acids downstream of the AUG start site was suggested as a putative nuclear transport signal (Isacchi *et al.*, 1991) and, of course, this signal would also be present in the 22 and 21.5 kD forms. It is also possible that 18 kD FGF-2 could be trapped in the nucleus following breakdown and re-formation of the nuclear membrane as a part of the cell cycle (Baldin *et al.*, 1990; Bugler *et al.*, 1991). Finally, proteolytic cleavage of FGF-2 was also described (Baldin *et al.*, 1990; Doble *et al.*, 1991; Powell and Klagsbrun, 1991).

4.6 Concluding Remarks

In the present study, hybrid genes containing wild type and modified FGF-2 cDNAs were generated and tested for their expression in COS-1 cells. The results indicate that (i) the hybrid FGF-2 genes can preferentially express high or low molecular weight forms of FGF-2 in COS-1 cells after gene transfer, (ii) expression of the low molecular weight form of FGF-2 is repressed by the sequences located upstream of the AUG codon in the rat FGF-2 cDNA and (iii) low molecular weight FGF-2 localizes to the cytoplasm as well as nucleus whereas high molecular weight FGF-2 is found predominantly in the nucleus of transfected COS-1 cells.

Chapter 5

Effects of overexpression of high and low molecular weight forms of FGF-2 on the growth and differentiation of embryonic ventricular myocytes

5.1 Background

Basic fibroblast growth factor (FGF-2) and other growth factors play a vital role in cardiogenesis (Sugi *et al.*, 1993) as well as proliferation and differentiation of cardiac myocytes (Claycomb and Moses, 1988; Kardami, 1990). FGF-2 exists in high and low molecular weight forms because of alternate translation initiation from the same mRNA (Florkiewicz and Sommer, 1989). Both high and low molecular weight forms and their high affinity receptor FGFR-1 are present in cardiac myocytes (Hughes and Hall, 1993; Kardami *et al.*, 1995). DNA synthesis and cell division in embryonic cardiac myocyte cultures are stimulated by the exogenous addition of low molecular weight form of FGF-2 (Kardami, 1990). However, there is scant information about the importance of high molecular weight forms of FGF-2 particularly in the cardiac context, due to the unavailability of purified protein. High molecular weight forms were implicated in DNA synthesis (Baldin *et al.*, 1990; Bugler *et al.*, 1991; Cattini *et al.*, 1991; Florkiewicz *et al.*, 1991; Renko *et al.*, 1990) and transcription of specific genes (Nakanishi *et al.*, 1992). Also these forms were shown to be developmentally regulated in the heart (Liu *et al.*, 1993) and brain (Giordano *et al.*, 1992). In addition, FGF-2 possesses both angiogenic and cardioprotective properties which are of benefit to cardiac function and/or recovery from infarction (Padua and Kardami, 1993; Yanagisawa-Miwa *et al.*, 1992). This growth factor has also been implicated in neuronal (Baird, 1994), retinal (Sievers *et al.*, 1987) and liver (Presta *et al.*, 1989) cell regeneration.

5.2 Rationale

Inability of adult cardiac myocytes to divide following myocardial infarctions has been an acute problem in clinical cardiology. As a result, the damaged area is replaced by

scar tissue. Further, the scar formation compromises cardiac performance and thereby reduces its lifespan. This drawback has created a large interest in the area of cardiovascular research to explore the means or mechanisms that can increase the proliferative potential of cardiac myocytes during disease conditions with a view to prolonging the cardiac lifespan. FGF-2 has been implicated in cardiogenesis, growth of cardiac myocytes, angiogenesis, cardioprotection as well as tissue regeneration (Kardami *et al.*, 1993) and is, therefore, an excellent candidate for the control of myocardial growth during disease conditions. However, FGF-2 exists in high and low molecular weight forms and the physiological significance of high molecular weight forms is largely unknown due to the unavailability of purified protein. Furthermore, the high molecular weight forms were shown to be developmentally regulated in the heart (Liu *et al.*, 1993). Therefore, the present study utilized the previously characterized wild type and modified rat FGF-2 hybrid genes (see chapter 4) to examine the effects of high and low molecular weight forms of FGF-2 on the growth of embryonic cardiac myocytes after gene transfer. Embryonic cardiac myocytes were chosen initially because (i) it was easier to culture these cells; (ii) they were more proliferative and thus likely to have a higher level of transfection efficiency compared to postnatal cardiac myocytes; (iii) the requirement for FGF-2 in the heart was the most compelling based on data pertaining to chicken heart development (Sugi *et al.*, 1993); and (iv) it was essential to establish whether different forms of FGF-2 can increase proliferative potential of dividing myocytes through gene transfer approach, before attempting to examine their effects on the growth of postnatal cardiac myocytes.

5.3 Hypothesis

Overexpression of high and low molecular weight forms of FGF-2 can affect the growth of embryonic ventricular myocytes in a differential manner.

5.4 Specific Aims

1. To express wild type and modified rat FGF-2 hybrid genes in embryonic ventricular myocytes by gene transfer.
2. To examine the effects of overexpression of high and low molecular weight forms

of FGF-2 on the proliferation of embryonic ventricular myocyte cultures.

3. To examine the effects of overexpression of high and low molecular weight forms of FGF-2 on the differentiation of embryonic ventricular myocyte cultures.

4. To examine the subcellular distribution of high and low molecular weight forms of FGF-2 in embryonic ventricular myocytes transfected with wild type and modified FGF-2 cDNAs.

5.5 Results and Discussion

5.5.1 Expression of wild type and modified FGF-2 cDNAs in embryonic ventricular myocytes

Embryonic ventricular myocyte cultures derived from 6-7 day old chick embryo hearts were used to test the expression of the wild type and modified FGF-2 cDNAs after gene transfer. Heparin binding fractions of total lysates from myocyte cultures transfected with wild type or modified FGF-2 cDNAs were analyzed by immunoblotting with antibodies that detect chicken as well as rat high and low molecular weight forms of FGF-2 (Borja *et al.*, 1993; Kardami and Fandrich, 1989; Kardami *et al.*, 1991b). Both high and low molecular weight forms of FGF-2 were observed in non transfected cultures as well as cultures transfected with RSVp.CONT. The mobilities of the high molecular weight species suggested sizes closer to 22 and 20 kD (Fig. 12, lanes a and b) than the 20.3 and 19 kD previously reported on the basis of amino acid composition (Borja *et al.*, 1993; see Table 4). The slight discrepancy between deduced molecular weights and those estimated by SDS-PAGE also extends to rat FGF-2 (Table 4). Further, the expression pattern of FGF-2 hybrid genes in the myocyte cultures was identical to the pattern observed in noncardaic COS-1 cells (Fig. 8, see section 4.5.2).

Following transfection with RSVp.ΔmetFGF, expression of 22 and 21.5 but not the 18 kD form of FGF-2 was detected above background levels. An increase (~5 fold) in the levels of 22 kD FGF-2 as well as the appearance of a 21.5 kD form of FGF-2 were observed following transfection with RSVp.ΔmetFGF (lane d). The levels of 20 and 18 kD FGF-2 species were comparable to the amount of endogenous FGF-2 seen with the RSVp.CONT (compare lanes b and d). This is consistent with the removal of the ATG site responsible for 18 kD FGF-2 in the ΔmetFGF gene construct. An increase in 22 kD FGF-2

Fig. 12

Fig. 12. High and low molecular weight forms of FGF-2 are expressed in embryonic chicken cardiac cells transfected with modified FGF-2 cDNAs. Heparin-sepharose purified cardiac cell lysates were fractionated in a 12.5% SDS-PAGE gel, immunoblotted, probed with rabbit FGF-2 antibodies, and visualized with ^{125}I-protein A. Lysate from: non transfected cultures, lane a; transfected with the expression vector (RSVp.CONT), lane b; RSVp.metFGF, lane c; RSVp.ΔmetFGF, lane d; and RSVp.FGF, lane e. The mobilities of 22, 21.5, 20 and 18 kD bands are indicated. Note the presence of 21.5 kD band generated by transfection with RSVp.ΔmetFGF or RSVp.FGF in lane d and lane e, respectively.

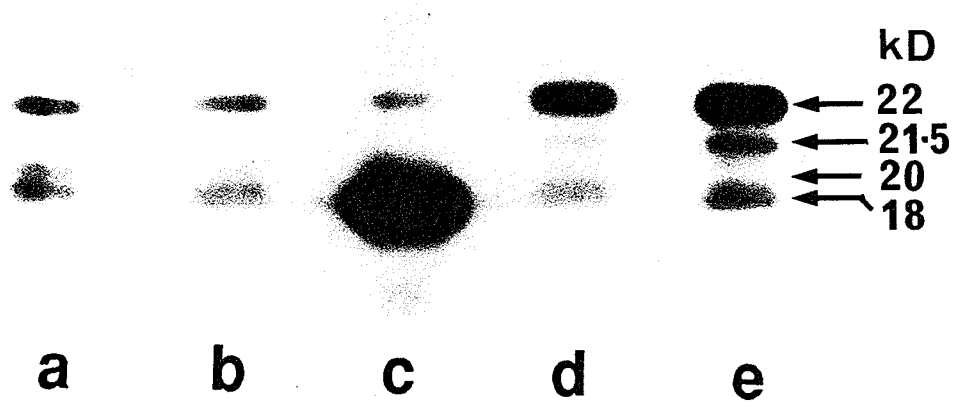

kD
22
21·5
20
18
a
b
c
d
e

Table 4

Table 4. Comparison of the molecular weights of rat and chicken FGF-2 based on deduced amino acid composition and SDS-PAGE. Sequences reported for rat (Shimasaki *et al.*, 1988) and chicken FGF-2 (Borja *et al.*, 1993), as well as mobility data for rat FGF-2 (Liu *et al.*, 1993) were used for comparison.

	RAT	CHICKEN
Initiation codons in mRNA (position)	CUG (-34), CUG (-26), AUG (+1)	CUG (-31), CUG (-19), AUG (+1)
Amino acids (AA) in the translated protein	188, 180, 154	189, 177, 158
Molecular weights based on AA sequence (kD)	20.2, 19.5, 17.1	20.3, 19.0, 17.5
Molecular weights based on SDS-PAGE (kD)	22.0, 21.5, 18.0	22.0, 20.0, 18.0

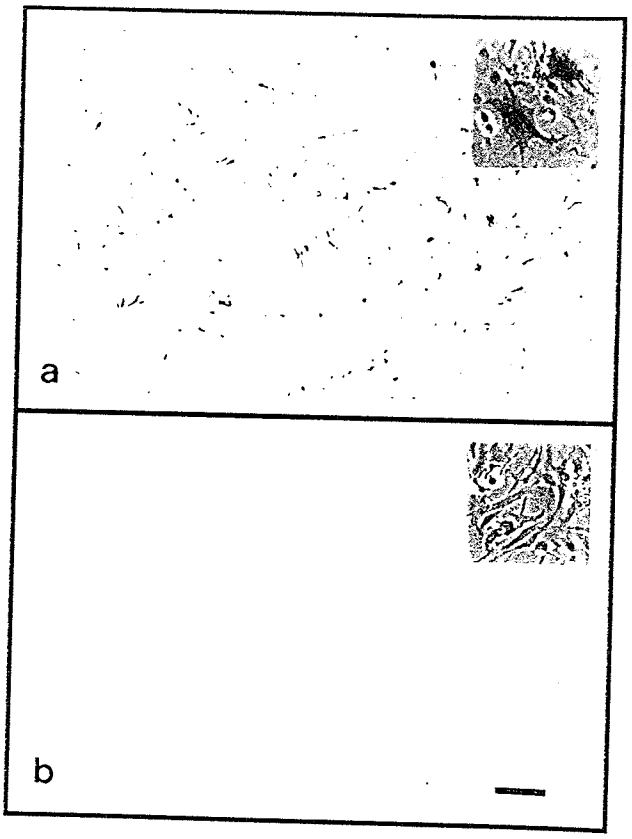
levels (>5-fold) as well as the expression of 21.5 kD FGF-2 were also seen in cultures transfected with RSVp.FGF (lane e) compared to the control (lane b). In contrast to RSVp.ΔmetFGF, however, an increase (~2-fold) in the levels of 18 but not 20 kD FGF-2 was also observed after transfection with RSVp.FGF (lane e). These results are in agreement with the fact that the RSVp.FGF gene has the potential to synthesize both high and low molecular weight forms of FGF-2. The mobility of the endogenous 20 kD form (open arrow) is distinct from the 21.5 kD band (closed arrow) generated by the transfected rat FGF-2 cDNA (lanes d and e). The 5-fold increase in levels of the 22 kD band and the presence of the 21.5 kD band (lanes d and e), a unique product of transfection, suggest that these high molecular weight forms are the major products of the RSVp.FGF and RSVp.ΔmetFGF genes. Transfection of myocyte cultures with the RSVp.metFGF construct resulted in the overexpression of 18 kD FGF-2 (lane c). The level of 18 kD FGF-2 expression was higher (~20 fold) than the levels of 22 and 21.5 kD forms generated using an identical (RSV) promoter (Fig. 12). This result cannot be attributed to differences in DNA uptake by the cells, as there was no significant difference between the CAT activity measured resulting from co-transfection with the RSVp.CAT gene. Further, the levels of 22 or 20 kD FGF-2 species seen in extracts from cultures transfected with RSVp.metFGF (lane c) were comparable to endogenous levels (lanes a and b). The difference in levels of 18 kD FGF-2 expressed by RSVp.FGF versus RSVp.metFGF, suggests the presence of repressor sequences which impede the use of the methionine (AUG) site in the RSVp.FGF transcripts. This repressor effect on the translation of low molecular weight FGF-2 in the presence of upstream sequences, was also observed in noncardiac COS-1 cells (see sections 4.5.2 and 4.5.3) and it could be due to the presence of repressor sequences as described for human FGF-2 RNA (Prats *et al.*, 1992).

5.5.2 Efficiency of transfection in embryonic cardiac myocytes

Transfection efficiency for the myocyte cultures was determined initially by expression and detection of ß-galactosidase following transfection with RSVp.ß-gal (Fig. 13). The ß-gal staining was not detected in cultures transfected with RSVp.CONT (Fig. 13). Staining was detected in 17.5±5.4% (n=3) cells of RSVp.ß-gal transfected cultures. However, this value must be considered an underestimate. Transfection efficiency reflects

Fig. 13

Fig. 13. Expression of ß-galactosidase in embryonic chicken ventricular myocytes. Cultures of embryonic chicken ventricular myocytes were transfected with (a) RSVp.ß-gal or (b) expression vector (RSVp.CONT). Cells were assessed for ß-galactosidase activity 48 hours later. The insets show the presence (a) or absence (b) of myocytes overexpressing ß-gal under phase contrast microscopy. Bar is equivalent to 150 μm for the light microscopic field and 50 μM for the insets.


a
b

the number of cells in cultures transfected with RSVp.ß-gal in which overexpression was detected by the ß-galactosidase assay. It is likely that cells which are expressing the transfected gene but not at a sufficient level for the detection of a definitive blue colour in the ß-galactosidase assay were present. Similarly, this would also be expected for the transfected FGF-2 cDNAs. As a result the level of transfection and overall production of FGF-2 in the cultures will be greater than suggested by the ~18% figure.

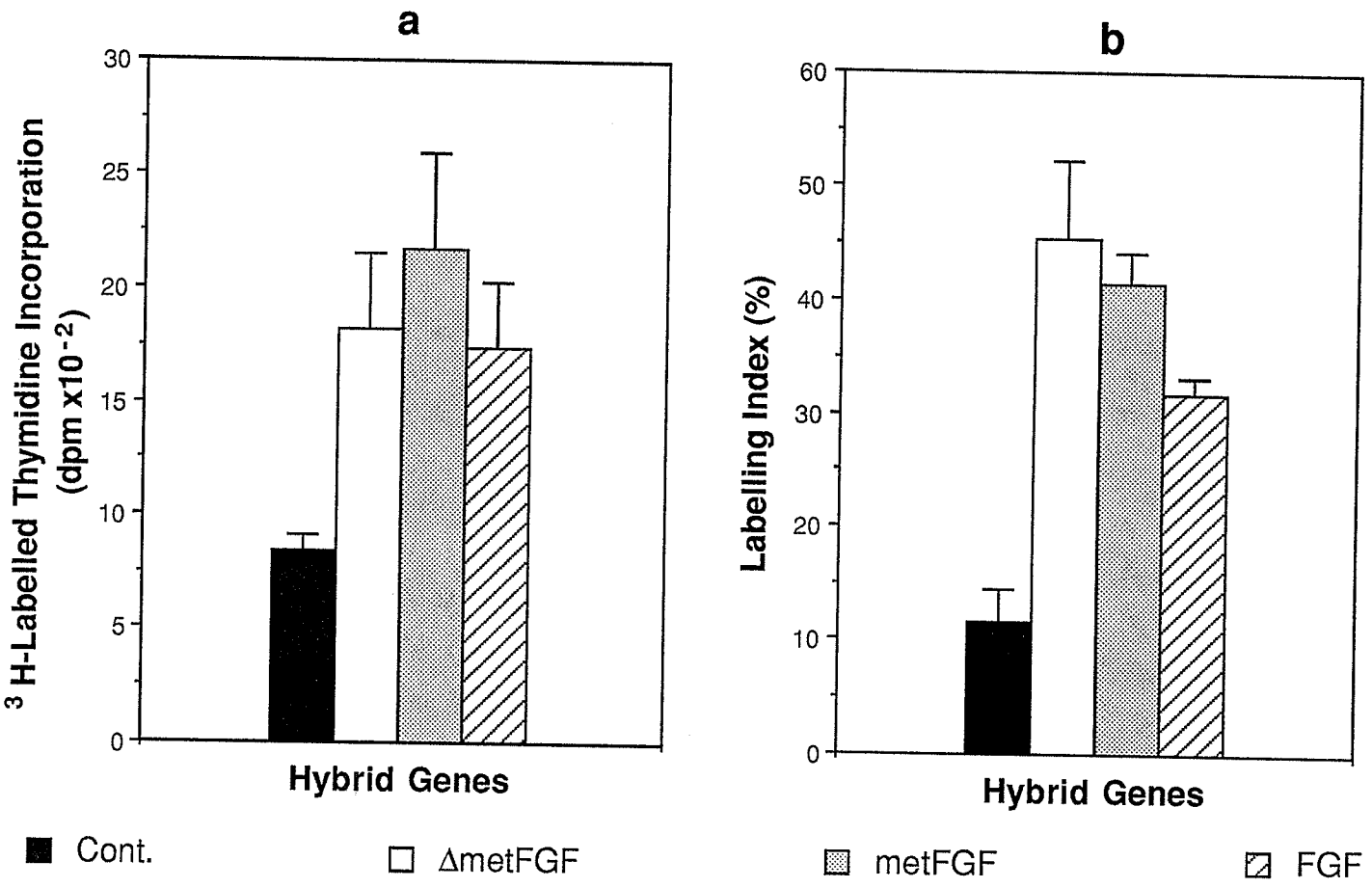
5.5.3 Effect of wild type and modified FGF-2 cDNA expression on DNA synthesis

Embryonic ventricular myocyte cultures transfected with or without FGF-2 sequences, were pulsed for 5 hours with tritiated thymidine. DNA synthesis was assessed by measuring the incorporation of tritiated thymidine into the cultures. The results from 2 experiments (n=6) are shown in Fig. 14a. A significant increase (~2.6 fold) in thymidine incorporation, compared to cells transfected with the RSVp.CONT, was observed in cultures transfected with RSVp.ΔmetFGF (3.2 fold, $p<0.02$), RSVp.metFGF (2.5 fold, $p<0.02$) and RSVp.FGF (2.2 fold, $p<0.02$). There was no significant difference between the effect of ΔmetFGF versus metFGF overexpression.

A combination of *in situ* autoradiography and histochemistry was also used to obtain a Labelling Index (LI) as a more accurate assessment of DNA synthesis in cardiac myocytes following transfection with or without FGF-2 cDNAs. The LI is the proportion of PAS positive/glycogen-containing cells (myocytes) that also show the presence of tritiated thymidine. The results from 2 experiments (n=4) are shown in Fig. 14b. A significant increase (~3.5 fold) in LI was observed in cultures transfected with RSVp.ΔmetFGF (4.0 fold, $p<0.005$), RSVp.metFGF (3.6 fold, $p<0.0005$) and RSVp.FGF (2.8 fold, $p<0.0001$) compared to cells transfected with the RSVp.CONT. There was no significant difference between the effect of ΔmetFGF versus metFGF overexpression. It is possible that the stimulation of cardiomyocyte DNA synthesis in the cultures overexpressing high or low molecular weight FGF-2 (Fig. 12) is mediated by a paracrine/autocrine or intracrine mechanism of action for these proteins. Because of different levels of expression, however, these data may be masking a difference in potency between high and low molecular weight forms. This problem can be resolved by using

Fig. 14

Fig. 14. Incorporation of ^{3}H-thymidine in embryonic cardiac myocyte cultures transfected with modified FGF-2 cDNAs. (a) Total tritiated thymidine uptake was determined in cardiac myocyte cultures after transfection with the hybrid genes by pulsing with ^{3}H-thymidine (5 μCi/ml) for 5 hours. The results (dpm x 10^{-2}) are expressed as the mean plus or minus the standard error of the mean from 6 determinations. Using an unpaired t-test, there is a significant (~2.6 fold) increase in thymidine incorporation in cultures transfected with RSVp.ΔmetFGF, RSVp.metFGF and RSVp.FGF, compared to cells transfected with the RSVp.CONT. There is no significant difference between the effect of ΔmetFGF versus metFGF overexpression. (b) Labelling index (LI=radiolabelled PAS+ cells/total number of PAS+ cells) was determined in cardiac myocyte cultures after transfection with hybrid genes by pulsing with ^{3}H-thymidine (10 μCi/ml) for 24 hours and by using a combination of *in situ* autoradiography and histochemistry. The results (%) are expressed as the mean plus or minus the standard error of the mean from 4 determinations. Using an unpaired t-test, there is a significant (~3.5 fold) increase in labelling index in cultures transfected with RSVp.ΔmetFGF, RSVp.metFGF and RSVp.FGF, compared to cells transfected with the RSVp.CONT. There is no significant difference between the effect of ΔmetFGF versus metFGF overexpression.


a
b
^{3}H-Labelled Thymidine Incorporation (dpm x10^{-2})
Labelling Index (%)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
Hybrid Genes
Hybrid Genes
Cont.
ΔmetFGF
metFGF
FGF

more efficient gene transfer methodologies which include viral transfections and transgenics.

5.5.4 Effect of wild type and modified FGF-2 cDNA expression on cell division

To determine whether the increase in DNA synthesis observed was also reflected by an increase in cell number, embryonic myocyte cultures were maintained for 48 hours after transfection with the RSV expression vector with or without FGF-2 sequences and then cell number was assessed using a hemocytometer. The results from 2 experiments (n=6) are shown in Fig. 15a. A significant increase (~2.6 fold) in cell number, compared to cells transfected with the RSVp.CONT, was observed in cultures transfected with RSVp.ΔmetFGF (3.2 fold, $p<0.001$), RSVp.metFGF (2.5 fold, $p<0.005$) and RSVp.FGF (2.2 fold, $p<0.0001$). Although there was an apparent increase in cell number in cultures expressing ΔmetFGF versus metFGF, this was not statistically significant.

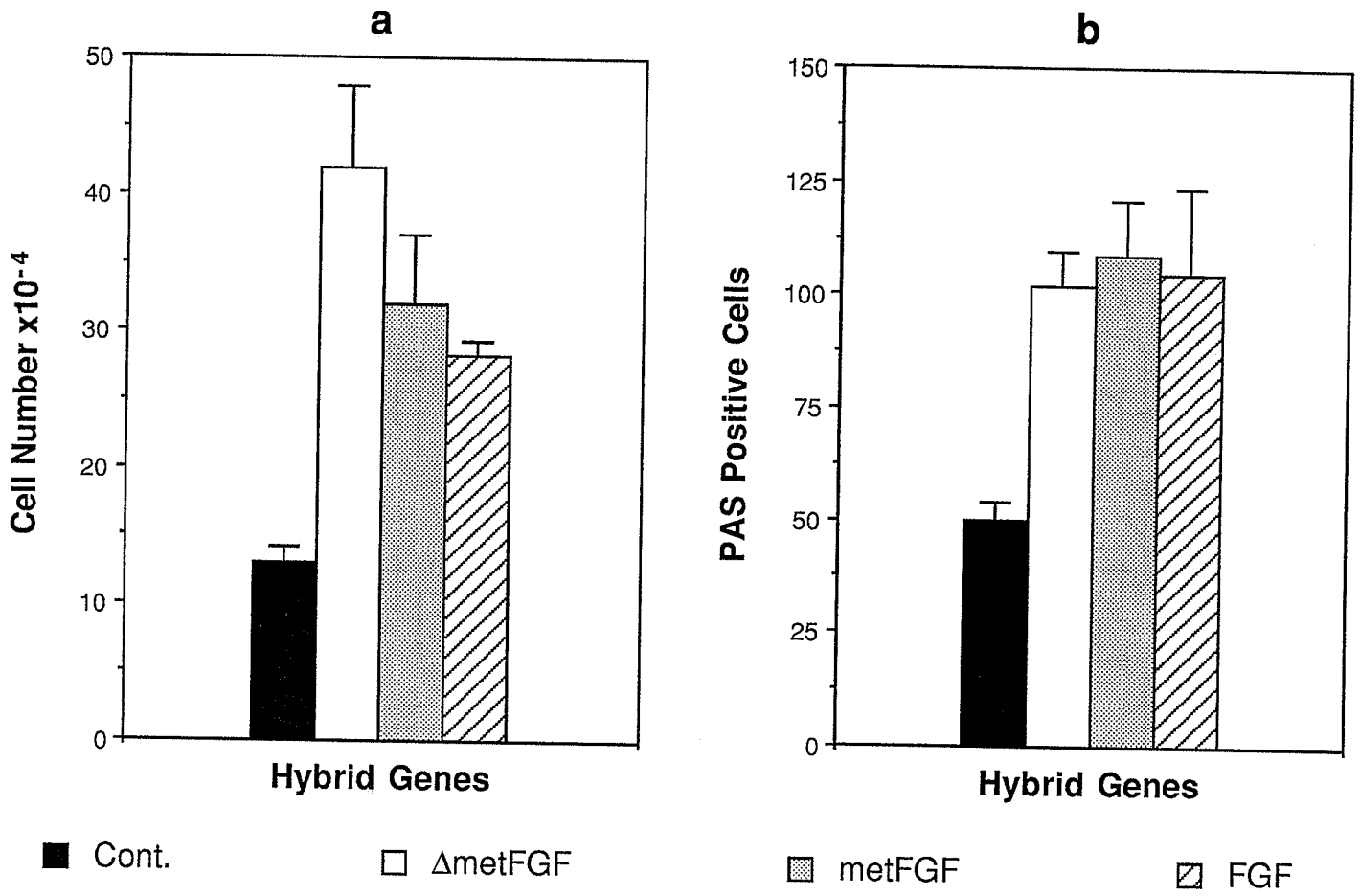
Further, PAS positive cells were scored in 12 random fields per coverslip (n=4) to determine whether the increase in total cell number reflects an increase in cardiac myocyte number. Significant increases in muscle cell number were also observed in cultures transfected with RSVp.ΔmetFGF (2.0 fold, $p<0.0005$), RSVp.metFGF (2.2 fold, $p<0.002$) and RSVp.FGF (1.5 fold, $p<0.05$) compared to cells transfected with the RSVp.CONT (Fig. 15b). There was no significant difference between the effect of ΔmetFGF versus metFGF overexpression.

5.5.5 Effect of wild type and modified FGF-2 cDNA expression on protein synthesis

Embryonic ventricular myocyte cultures transfected with or without FGF-2 sequences, were pulsed for 7 hours with ^{35}S-labelled methionine. Protein synthesis was assessed by measuring the incorporation of ^{35}S-labelled methionine into the cultures. The results from 2 experiments (n=5) are shown in Fig. 16a. A significant increase (~7.7 fold) in protein synthesis was observed in cultures transfected with RSVp.ΔmetFGF (8.2 fold, $p<0.05$), RSVp.metFGF (8.2 fold, $p<0.05$) and RSVp.FGF (6.7 fold, $p<0.05$) compared to cells transfected with the RSVp.CONT. There was no significant difference between the

Fig. 15

Fig. 15. Determination of cell number in embryonic cardiac myocyte cultures transfected with modified FGF-2 cDNAs. (a) Total cell number was determined in cardiac myocyte cultures after transfection with the hybrid genes indicated using a haemocytometer. The results (cell number x 10^{-4}) are expressed as the mean plus or minus the standard error of the mean from 6 determinations. Using an unpaired t-test, there is a significant (~2.6 fold) increase in cell number in cultures transfected with RSVp.ΔmetFGF, RSVp.metFGF and RSVp.FGF, compared to cells transfected with the RSVp.CONT. There is no significant difference between the effect of ΔmetFGF versus metFGF overexpression. (b) The number of PAS positive/glycogen-containing cells (myocytes) was determined by using a combination of *in situ* autoradiography and histochemistry. The results (PAS positive cells per coverslip) are expressed as the mean plus or minus the standard error of the mean from 4 determinations. Using an unpaired t-test, there is a significant (~1.9 fold) increase in cell number in cultures transfected with RSVp.ΔmetFGF, RSVp.metFGF and RSVp.FGF, compared to cells transfected with the RSVp.CONT. There is no significant difference between the effect of ΔmetFGF versus metFGF overexpression.


a
Cell Number x10-4
50
40
30
20
10
0
Hybrid Genes
Cont.
ΔmetFGF
b
PAS Positive Cells
150
125
100
75
50
25
0
Hybrid Genes
metFGF
FGF

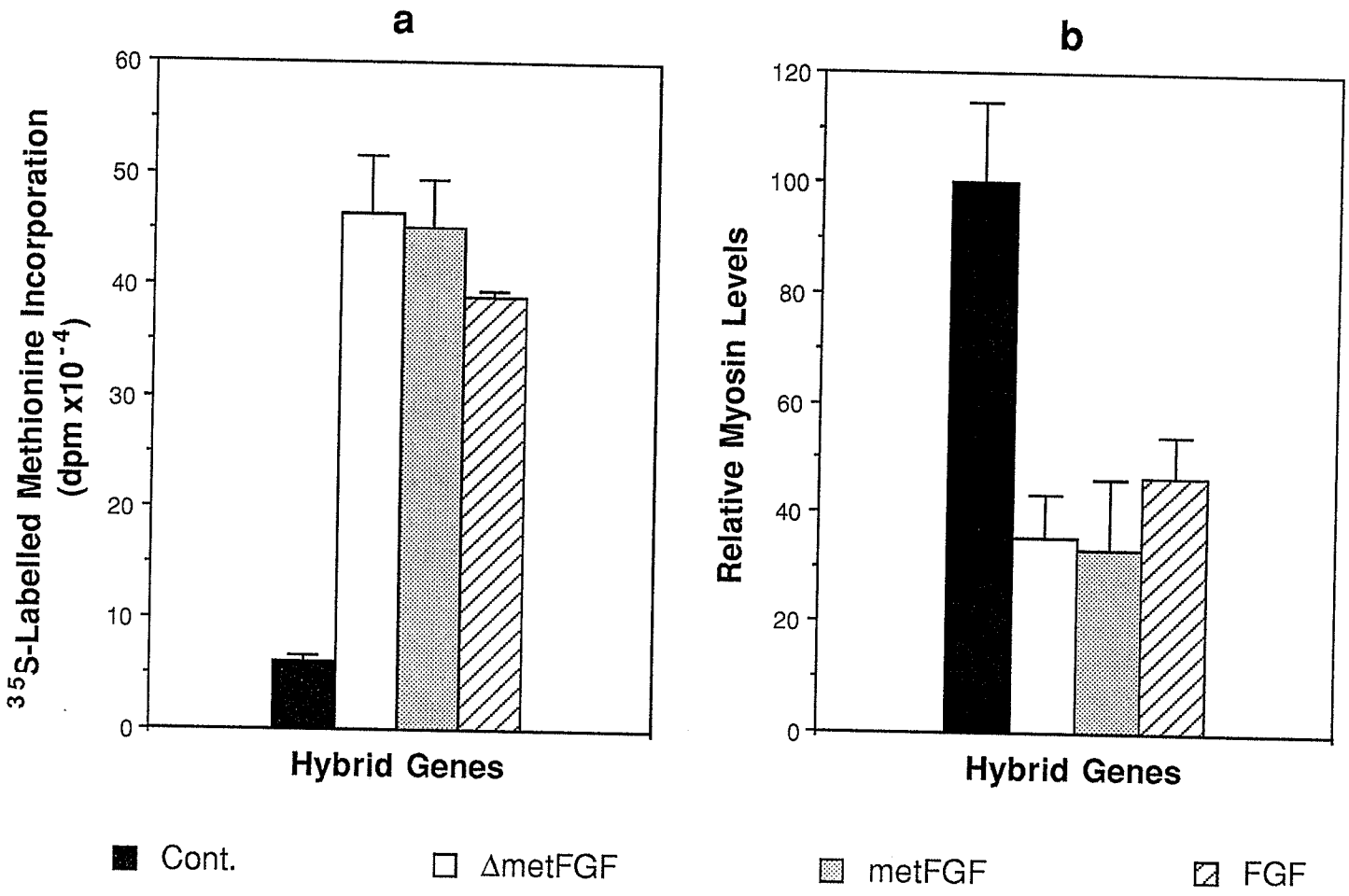
effect of ΔmetFGF versus metFGF overexpression. The rate of protein synthesis increases rapidly in cells preparing for mitosis during G1 phase to facilitate DNA replication and other cell division processes (Alberts *et al.*, 1989). Thus stimulation of cell proliferation could be secondary to the increases in overall protein synthesis in cardiac myocyte cultures overexpressing FGF-2.

It is possible that the increased proliferation of myocyte cultures overexpressing high or low molecular weight forms of FGF-2 can affect the differentiation status of these cultures. Therefore, myosin levels in the transfected cultures were assessed by SDS-PAGE in combination with immunoblotting with myosin antibodies. The 200 kD myosin bands detected in the lysates of cells transfected with RSVp.ΔmetFGF, RSVp.metFGF, RSVp.FGF and RSVp.CONT in 3 experiments (n=6-8) were scanned by densitometry. The myosin levels in the lysates of cultures transfected with FGF-2 cDNAs were compared to the myosin level in the RSVp.CONT lysate, which was arbitrarily set to 100 (Fig. 16b). A significant decrease (~2.7 fold) in myosin levels, compared to cells transfected with the RSVp.CONT, was observed in cultures transfected with RSVp.ΔmetFGF (2.8 fold, $p<0.002$), RSVp.metFGF (3.0 fold, $p<0.01$) and RSVp.FGF (2.2 fold, $p<0.005$). There was no significant difference between the effect of ΔmetFGF versus metFGF overexpression.

The reduction in myosin accumulation, in view of an overall augmentation in protein synthesis, is intriguing. This decrease in myosin levels was also indicated by a reduction in the intensity of anti-myosin staining of overexpressing cells compared to nonoverexpressing cells. This decrease in myosin accumulation with overexpression of high or low molecular weight FGF-2 could be explained by either a large increase in the proportion of non muscle cells, which is not supported by PAS staining results (Fig. 15b), or related to the increased proliferative state of myocytes and reflect the ultrastructural manifestation of this response. It is reported that FGF-2 can inactivate skeletal muscle specific transcription through phosphorylation of a protein kinase C site in myogenic helix-loop-helix proteins (Li *et al.*, 1992). Although less is known about cardiac myogenic proteins, a similar role for FGF-2 may exist in cardiac myocytes. It is possible that the release of FGF-2 or other biologically active stimuli from the cardiac myocyte cultures transfected with FGF-2 cDNAs might contribute to the effects on myocyte proliferation and

Fig. 16

Fig. 16. Effect of FGF-2 cDNAs on the protein synthesis in transfected embryonic cardiac myocyte cultures. (a) Assessment of new protein synthesis was done in cardiac myocyte cultures after transfection with the hybrid genes by pulsing with ^{35}S-labelled methionine (25 μCi/1.5 ml) for 7 hours. The results (dpm x 10^{-4}) are expressed as the mean plus or minus the standard error of the mean from 5 determinations. Using a paired t-test, there is a significant (~7.7 fold) increase in protein synthesis in cultures transfected with RSVp.ΔmetFGF, RSVp.metFGF and RSVp.FGF, compared to cells transfected with the RSVp.CONT. There is no significant difference between the effect of ΔmetFGF versus metFGF overexpression. (b) Myosin levels were determined in cardiac myocytes after transfection with the hybrid genes by immunoblotting with myosin antibodies, protein A autoradiography and by scanning the 200 kD myosin bands. The relative myosin levels are expressed as the mean plus or minus the standard error of the mean from 6-8 determinations. Using an unpaired t-test, there is a significant (~2.7 fold) decrease in protein synthesis in cultures transfected with RSVp.ΔmetFGF, RSVp.metFGF and RSVp.FGF, compared to cells transfected with the RSVp.CONT. There is no significant difference between the effect of ΔmetFGF versus metFGF overexpression.


a
b
^{35}S-Labelled Methionine Incorporation (dpm x10^{-4})
Relative Myosin Levels
0
10
20
30
40
50
60
0
20
40
60
80
100
120
Hybrid Genes
Hybrid Genes
Cont.
ΔmetFGF
metFGF
FGF

protein synthesis.

5.5.6 Distribution of FGF-2 in transfected embryonic ventricular myocytes

Embryonic ventricular myocytes were transfected with the RSV expression vector containing FGF-2 sequences and subsequently all cultures were processed for simultaneous localization of FGF-2 (rabbit anti-FGF-2 IgG), striated muscle myosin (mouse anti-myosin IgG) and nuclei (Hoechst 33342 stain), using triple immunofluorescence labelling and epifluorescence optics with appropriate filters (Kardami *et al.*, 1991b). Myocytes were identified by simultaneous myosin staining. Cardiac myocyte cultures transfected with the RSVp.CONT, as well as non transfected cultures, displayed low levels of FGF-2 staining (endogenous expression) under the experimental conditions described. Myocytes transfected with RSVp.FGF and RSVp.ΔmetFGF displayed predominantly nuclear FGF-2 localization which was confirmed by counterstaining for DNA with Hoechst 33342 dye (Fig. 17). Cardiac myocytes transfected with RSVp.metFGF (synthesizing only 18 kD FGF-2) displayed intense and uniform anti-FGF-2 staining in the cytoplasm as well as the nucleus (Fig. 18). These results are in agreement with the data from nonmuscle cells (COS-1) and previous reports on subcellular localization of high and low molecular weight forms of FGF-2 in different species (Baldin *et al.*, 1990; Bugler *et al.*, 1991; Powell and Klagsbrun, 1991).

5.5.7 Effect of wild type and modified FGF-2 cDNA expression on nuclear morphology

In contrast to their similar effects on overall cell proliferation, DNA and protein synthesis, overexpression of high and low molecular weight forms of FGF-2 in embryonic cardiac myocytes was associated with differences in nuclear morphology. The intense nuclear anti-FGF-2 staining observed in myocytes transfected with RSVp.FGF and RSVp.ΔmetFGF, was often associated with what appeared to be multiple nuclei of varying sizes. In the majority of cases (60%), DNA staining of these FGF-2-loaded nuclei revealed "clumps" resembling condensed chromatin (Fig. 19). These DNA "clumps", however, appeared distinct from the chromosomal patterns seen during the various stages of cell division (Cattini *et al.*, 1991; Kardami *et al.*, 1991a). The nuclear envelope was apparently

Fig. 17

Fig. 17. Distribution of FGF-2 and nuclear DNA in embryonic cardiac myocyte cultures transfected with RSVp.FGF (a,b,c) and RSVp.ΔmetFGF (d,e,f). Cells were stained for myosin (a,d), FGF-2 (b,e), and DNA (c,f). Closed and open arrows indicate myocytes overexpressing FGF-2 and displaying endogenous FGF-2 staining respectively. Note the co-incidence of overexpression of high molecular weight forms of FGF-2 and the DNA "clumps" (f). Bar is equivalent to 18 μm.

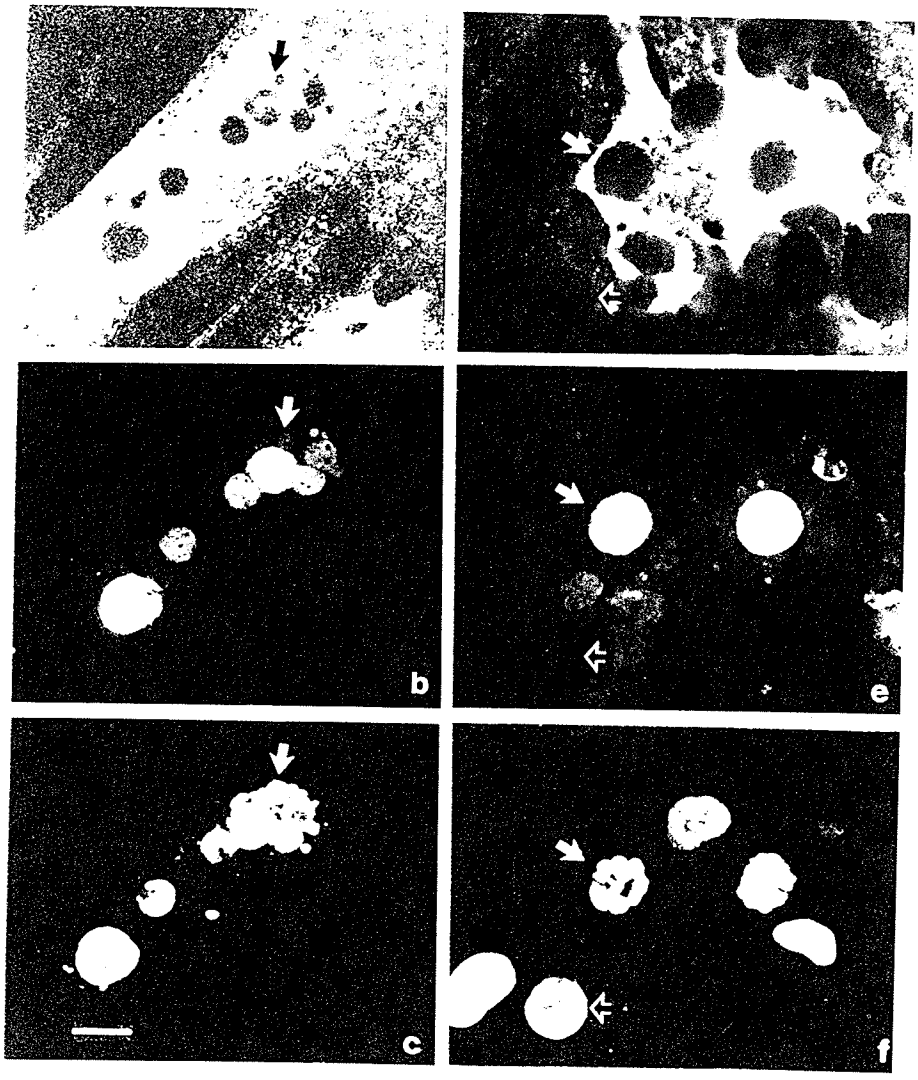

b
e
c
f

Fig. 18

Fig. 18. Distribution of FGF-2 and nuclear DNA in embryonic cardiac myocyte cultures transfected with RSVp.metFGF (a,b,c) and RSVp.CONT (d,e,f). Cells were stained for myosin (a,d), FGF-2 (b,e), and DNA (c,f). Closed and open arrows indicate myocytes overexpressing FGF-2 and showing levels of endogenous FGF-2 staining respectively. Intense FGF-2 staining of the nucleus and cytoplasm was observed in myocytes transfected with RSVp.metFGF. Cells transfected with RSVp.CONT revealed weak endogenous staining of FGF-2. Bar is equivalent to 36 μm.

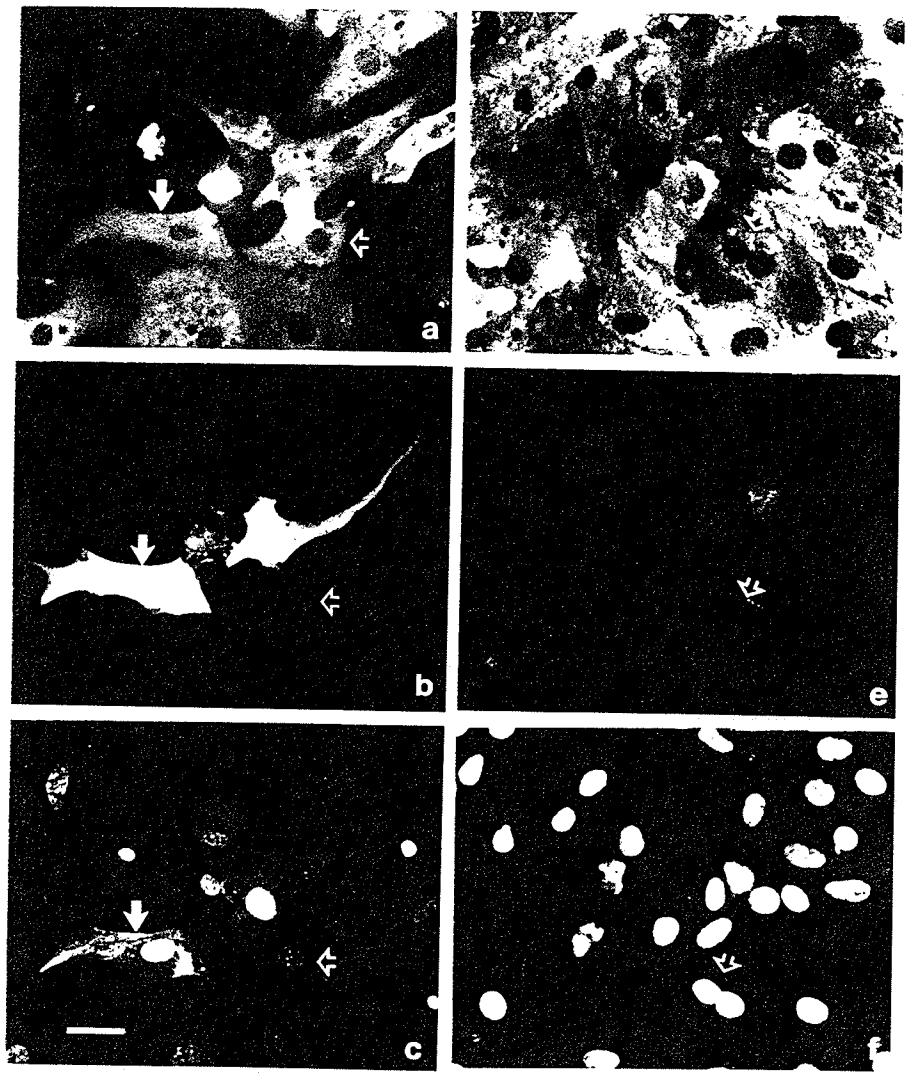
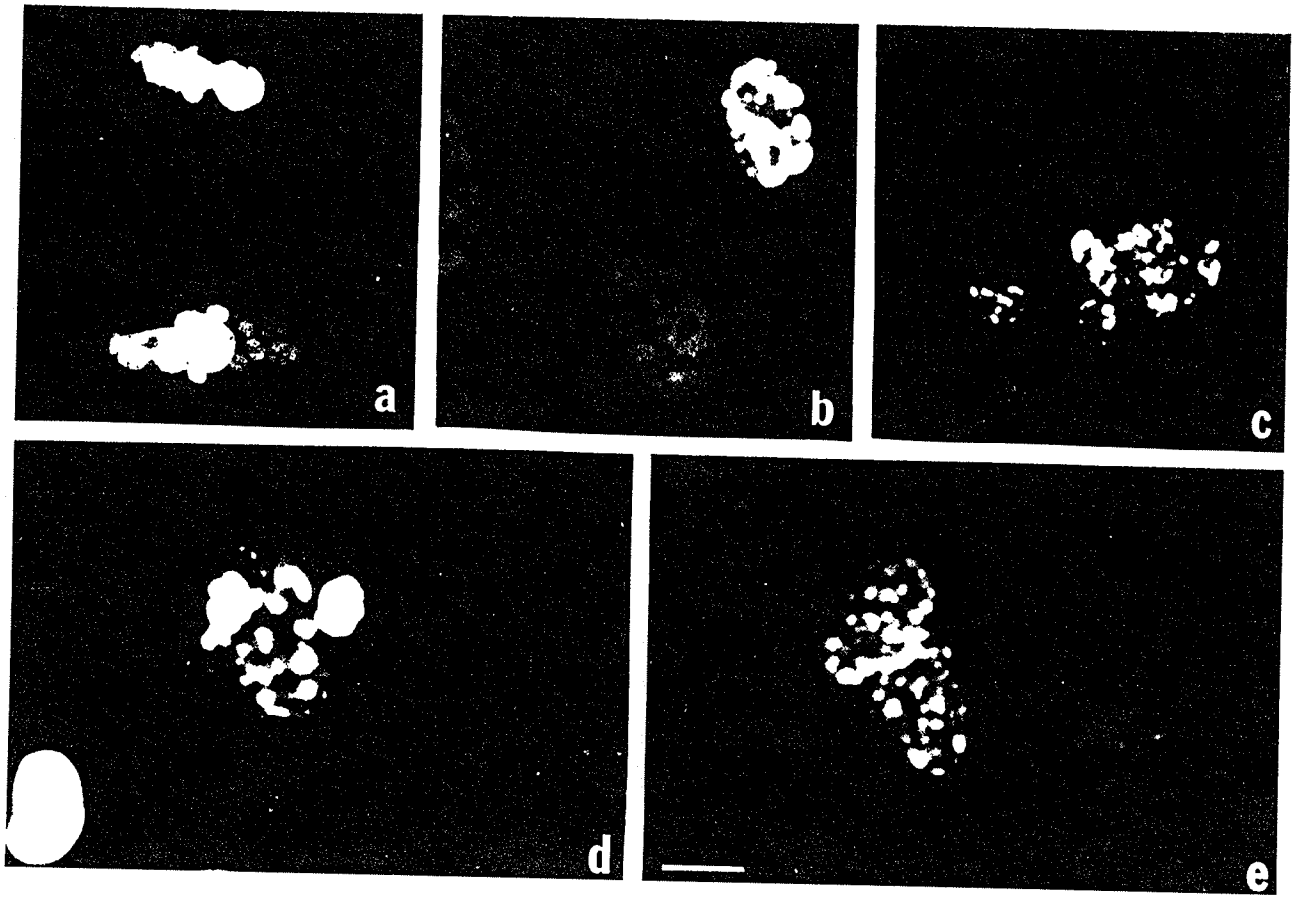

a
b
c
e
f

Fig. 19

Fig. 19. Overexpression of RSVp.ΔmetFGF or RSVp.FGF in embryonic cardiac myocyte cultures is associated with DNA "clumps" suggesting the presence of condensed chromatin. Light micrographs of nuclear DNA in embryonic cardiac myocytes stained with Hoechst Dye 33342. Cardiac myocytes overexpressing FGF-2 were identified by myosin and FGF-2 staining. Bar is equivalent to 20 μm, (a); 12.5 μm, (b,c and e); and 15 μm, (d).


a
b
c
d
e

still present as indicated by the exclusion of striated myosin staining from this compartment (Fig. 17). Bulging of the nuclear membrane was also frequently observed in cells overexpressing 22/21.5 kD FGF-2 (Fig. 17). None of the cells overexpressing the 18 kD FGF-2 displayed formation of DNA "clumps". Control cultures transfected with RSVp (Fig. 19), or non-transfected cultures (not shown), did not display the intense anti-FGF-2 staining of nuclei seen following overexpression of high or low molecular weight FGF-2 (Fig. 17).

The appearance of DNA "clumps" and condensed chromatin state in the majority of nuclei overexpressing high (ΔmetFGF and FGF) but not low (metFGF) molecular weight forms of FGF-2, could reflect the participation of high molecular weight species in the normal process of chromosomal condensation during prophase. This phenomenon is possibly related to the strong basic charge of 22/21.5 kD FGF-2, which is even more basic than the 18 kD form (Brigstock *et al.*, 1990). It should be pointed out that endogenous FGF-2 can be detected in the prophase nuclei of chicken cardiac myocytes (Kardami *et al.*, 1991a). The translocation of FGF-2 to the nucleus in late G_1 also supports a nuclear role for this protein (Baldin *et al.*, 1990). There is also a positive correlation between the mitotic potential of cardiac myocytes and levels of high molecular weight FGF-2. High molecular weight forms of FGF-2 are more abundant in the neonatal (less differentiated phenotype) than in the adult myocardium (differentiated phenotype), which has higher levels of the 18 kD FGF-2 species (Liu *et al.*, 1993).

It is unlikely that the DNA "clumps" resulted from the accumulation of a foreign protein in the nucleus, since this pattern was not observed in cells overexpressing 18 kD FGF-2 which also localizes to the nucleus. It is also unlikely that they were a consequence of a non-specific concentration difference, reflecting perhaps higher levels of the 22/21.5 kD FGF-2 compared to the 18 kD FGF-2 in the nuclear compartment. Assessment of FGF-2 in immunoblotting and immunofluorescence studies suggest higher levels of the 18 kD compared to the 22/21.5 kD forms in cells transfected with RSVp.metFGF and RSVp.ΔmetFGF, respectively. In ΔmetFGF construct, the modification of the AUG start site results in the replacement of one amino acid (Met) with three amino acids (Gln-Ala-Trp). It is possible that any modification, even the replacement of one amino acid, could result in a molecule with different biological properties. However, overexpression of the

RSVp.FGF construct, which produces wild type high molecular weight FGF-2, also resulted in DNA clumping in transfected chicken cardiac myocytes. Thus, this effect on nuclear morphology does not appear to be related to the mutation made in the ΔmetFGF cDNA.

However, it is also possible that the irregular nuclear morphology seen in chicken myocytes overexpressing high molecular weight forms of rat FGF-2 could reflect the heterologous system used, and be related to the few amino acid differences observed in the amino-terminus of the chicken and rat FGF-2 forms that could result in a related but different form of (rat) FGF-2 in a chicken cell (Fig. 20). Interestingly, a comparison of the amino terminal sequences containing the two putative leucine start sites upstream of the initiator methionine (responsible for the 18 kD forms) for both chicken and rat sequences, reveals some similarity but only when the downstream leucine start site in the rat sequence (-26; responsible for rat 21.5 kD form) is matched with the upstream leucine start site (-31) in the chicken sequence which is, presumably, responsible for the 22 kD chicken form (Fig. 20). As a consequence, the chicken 22 kD and rat 21.5 (not 22) kD forms are predicted to be the most related and the rat 22 kD FGF-2 could represent a related but different form in the transfected chicken cell.

5.5.8 Effect of wild type and modified FGF-2 cDNA expression on DNA ladders

The distinct effect of high molecular weight forms of FGF-2 on chromatin condensation and nuclear fragmentation could also represent a type of apoptosis or programmed cell death. Apoptosis is a tightly regulated process of cell death involving single cells which exhibit rapid reduction in cell volume, cell surface blebbing, compaction of cytoplasmic organelles, chromatin condensation and activation of nuclear endonucleases that can cleave chromatin at internucleosomal sites (Wyllie, 1987). Apoptosis has been suggested to play a role in the control of normal tissue development, regulation of cell number and removal of damaged or precancerous cells (Williams, 1991). Embryonic ventricular myocyte cultures overexpressing high and low molecular weight forms of FGF-2 were processed for DNA fragmentation/ladders which represent one of the criteria for the assessment of apoptosis. Distinct DNA ladders (Fig. 21) were observed in cultures

Fig. 20

Fig. 20. Comparison of amino acid sequences of high and low molecular weight forms of rat and chicken FGF-2. Triangles and circles are used to indicate the CUG (L, leucine) and AUG (M, methionine) initiation codons, respectively, in both rat (open symbols) and chicken (closed symbols) coding sequences. Shading indicates identical amino acids in the rat and chicken sequences. The amino terminal sequences containing the two putative leucine start sites upstream of the initiator methionine (responsible for the 18 kD forms) for both chicken and rat sequences, reveals 65% similarity when the downstream leucine start site in the rat sequence (-26; responsible for rat 21.5 kD form) is matched with the upstream leucine start site (-31) in the chicken sequence which is, presumably, responsible for the 22 kD chicken form. As a consequence, the chicken 22 kD and rat 21.5 (not 22) kD forms are predicted to be the most related to each other. Thus, in the transfected chicken cell, the rat 22 kD FGF-2 could represent a related but different form of FGF-2 due to the extension in the amino terminus.

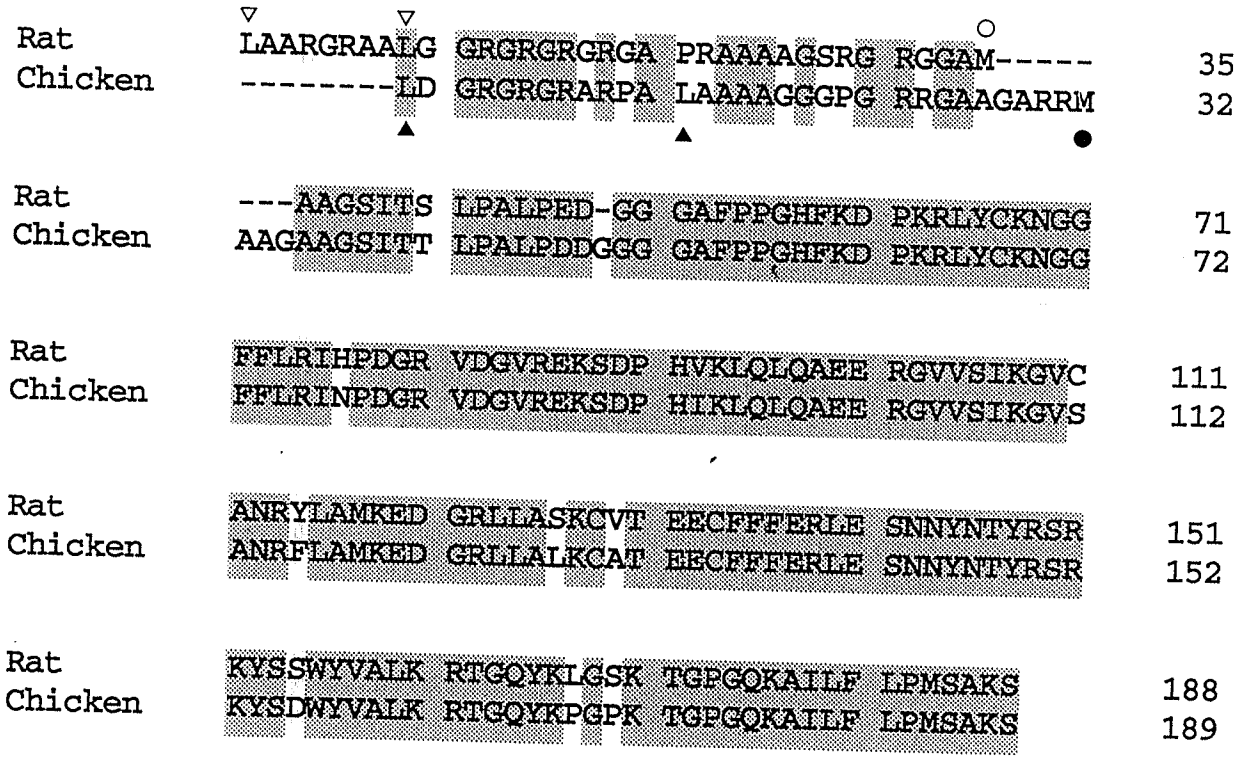
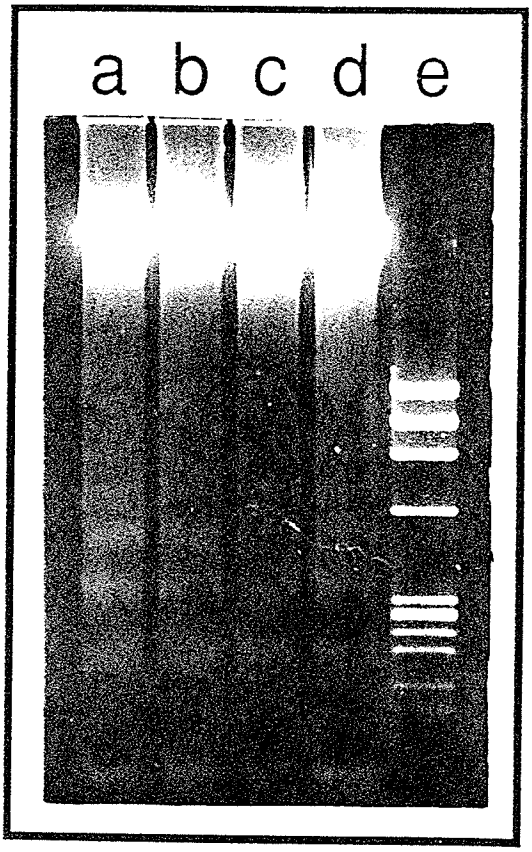

Rat
Chicken
LAARGRAALG GRGRGRGRGA PRAAAAGSRG RGGAM-----
--------LD GRGRGRARPA LAAAAGGGPG RRGAAGARRM
35
32
Rat
Chicken
---AAGSITS LPALPED-GG GAFPPGHFKD PKRLYCKNGG
AAGAAGSITT LPALPDDGGG GAFPPGHFKD PKRLYCKNGG
71
72
Rat
Chicken
FFLRIHPDGR VDGVREKSDP HVKLQLQAEE RGVVSIKGVC
FFLRINPDGR VDGVREKSDP HIKLQLQAEE RGVVSIKGVS
111
112
Rat
Chicken
ANRYLAMKED GRLLASKCVT EECFFFERLE SNNYNTYRSR
ANRFLAMKED GRLLALKCAT EECFFFERLE SNNYNTYRSR
151
152
Rat
Chicken
KYSSWYVALK RTGQYKLGSK TGPGQKAILF LPMSAKS
KYSDWYVALK RTGQYKPGPK TGPGQKAILF LPMSAKS
188
189

Fig. 21

Fig. 21. Elecrophoretic pattern of genomic DNA isolated from transfected embryonic ventricular myocyte cultures. Genomic DNA isolated from embryonic ventricular myocyte cultures transfected with RSVp.FGF (lane a), RSVp.ΔmetFGF (lane b), RSVp.metFGF (lane c) and expression vector without FGF-2 cDNA (lane d) was electrophoresed in a 1% agarose gel. The ϕX174/*Hae*III digest was used as a size marker (lane e). Note the presence of DNA ladders in cultures transfected with FGF-2 cDNAs (lanes a-c) as well as control plamid (d).


a b c d e

transfected with FGF-2 cDNAs (Fig. 21, lanes a,b,c) as well as control plasmid (Fig. 21, lane d) and the intensities of bands visualized by ethidium bromide staining were comparable between groups. These results suggest that a "background" level of apoptosis occurs in embryonic ventricular myocyte cultures and thus presumably, the high molecular weight FGF-2 induced nuclear fragmentation followed by cell death could be masked by "background" levels of apoptosis. A higher transfection efficiency (eg. viral transfections or transgenics) would resolve this problem. Apoptosis has been linked to the control of the primitive myocardial cell overgrowth associated with cardiac rhabdomyoma as well as removal of damaged cardiac myocytes after reperfusion injury (Gottileb *et al.*, 1994; Medioni *et al.*, 1994). Perhaps, the distinct effect of high molecular weight FGF-2 on chromatin condensation and nuclear fragmentation could serve as a mechanism for the removal of cells that were damaged during transfection procedure and this may be of physiological importance in the myocardium *in vivo*.

5.6 Concluding remarks

In conclusion, the results of present study indicate that: (i) overexpression of both high (22/21.5 kD) and low (18 kD) molecular weight forms of FGF-2 can stimulate proliferative potential, overall DNA and protein synthesis but decrease myosin levels in embryonic ventricular myocytes; (ii) low molecular weight FGF-2 localizes to the cytoplasm and nucleus whereas high molecular weight FGF-2 is found predominantly in the nucleus in the transfected embryonic ventricular myocytes; and (iii) overexpression of high but not low molecular weight FGF-2 leads to the formation of multiple DNA-containing "clumps" resembling condensed chromatin in embryonic cardiac myocyte nuclei. Although the significance of DNA "clumps" in myocytes overexpressing the 22/21.5 kD but not 18 kD FGF-2 is unclear, it provides evidence that different forms of FGF-2 may have different roles even within the same cellular compartments.

Chapter 6

Effects of overexpression of high and low molecular weight forms of FGF-2 on the proliferation of postnatal ventricular myocytes

6.1 Background

High and low molecular weight forms of basic fibroblast growth factor (FGF-2) were shown to play a vital role in the growth and differentiation of embryonic cardiac myocytes (see sections 5.5.3 to 5.5.5). However, the relative levels of different forms of FGF-2 were shown to be regulated during the development of the heart. Higher levels of high molecular weight forms (22/21.5 kD FGF-2) were present in the heart during the postnatal stage, whereas the low molecular weight form (18 kD FGF-2) was predominant in the adult (Liu *et al.*, 1993). Although the biological significance of high molecular weight forms of FGF-2 is unclear, this could be linked to the dramatic changes in nuclear events which occur during cardiac development. These include a reduction in the proliferative potential of rat cardiac myocytes as they develop through embryonic (dividing), postnatal (transitional) and adult (non dividing) stages (Clubb and Bishop, 1984; Zak, 1984). Thus, there appears to be a correlation between the expression of high molecular weight forms of FGF-2 and a proliferative phenotype of cardiac myocytes. In addition, a 25 kD high molecular weight form of FGF-2 was implicated in liver regeneration after partial hepatectomy (Presta *et al.*, 1989).

6.2 Rationale

Cardiac development soon after birth represents a transitional stage between embryonic (dividing) and adult (nondividing) phenotypes. The postnatal cardiac myocytes can undergo DNA synthesis and mitotic division during the first week but they fail to induce cell division and DNA synthesis at the end of the first and second weeks, respectively (Ueno *et al.*, 1988). Similarly, the nucleation pattern also changes during heart development such that cardiac myocytes are mononucleated during fetal and early postnatal

development and about 85% of them become binucleated by the third week after birth (Clubb and Bishop, 1984). There is scant information about the mechanisms regulating cardiac myocyte proliferation, binucleation and cessation of myocyte cell division. Previous studies established the importance of different forms of FGF-2 in the growth and differentiation of cardiac myocytes at the embryonic stage (see sections 5.5.3 to 5.5.5 and Pasumarthi *et al.*, 1994). Furthermore, there appears to be a correlation between the expression of high molecular weight forms of FGF-2 and a proliferative phenotype of cardiac myocytes (Liu *et al.*, 1993). Therefore, as a part of the long term goal to stimulate cell division in adult cardiac myocytes, the effects of overexpression of high and low molecular weight forms of FGF-2 on the growth of transitional stage postnatal cardiac myocytes (1 day old) were examined. In addition, the distinct effect of 22/21.5 kD FGF-2 on DNA clumping in embryonic chicken ventricular myocytes, was not considered established since it might have resulted from the heterologous system used (rat proteins in chicken cells), rendering potential physiological implications uncertain. Hence, the effects of high and low molecular weight FGF-2 overexpression on nuclear morphology were also reexamined in postnatal rat cardiac myocytes.

6.3 Hypothesis

Overexpression of high and low molecular weight forms of FGF-2 can affect the growth of postnatal ventricular myocytes in a differential manner.

6.4 Specific Aims

1. To express wild type and modified rat FGF-2 hybrid genes in postnatal ventricular myocytes and to examine the subcellular distribution of their expression products in transfected ventricular myocytes.
2. To examine the effects of overexpression of high and low molecular weight forms of FGF-2 on the proliferation of postnatal ventricular myocyte cultures.
3. To examine the effects of overexpression of high and low molecular weight forms of FGF-2 on binucleation in postnatal ventricular myocyte cultures.
4. To examine whether the the distinct effect of 22/21.5 kD FGF-2 on DNA clumping

observed in embryonic chicken ventricular myocytes is also seen in postnatal rat ventricular myocytes.

6.5 Results and Discussion

6.5.1 Expression of hybrid genes directed by RSV promoter in postnatal rat cardiac myocytes

Initially, the RSVp.ß-gal hybrid gene was introduced into postnatal ventricular myocyte cultures derived from newborn (1-2 day old) rat hearts, to assess the transfection efficiency. Staining for ß-galactosidase was detected in 18±4% of cells in cultures (n=6) transfected with RSVp.ß-gal compared to cultures transfected with the control (RSVp.CONT), where no staining was observed (Fig. 22a). Similar levels of transfection efficiency were obtained consistently when the percentage of cardiac myocytes overexpressing FGF-2 (after transfection with FGF-2 cDNAs) was determined by immunofluorescence microscopy. FGF-2 cDNAs were also introduced into postnatal rat ventricular myocyte cultures and their expression products were analyzed by immunoblotting. Overexpression of wild type FGF-2 cDNA (FGF) generated a pattern identical to that seen with ΔmetFGF and only 22 and 21.5 kD forms were observed (Fig. 22b). Modified rat FGF-2 cDNAs, ΔmetFGF and metFGF, expressed high (22 and 21 kD) or low (18 kD) molecular weight species, respectively (Fig. 22b). The level of 18 kD FGF-2 following overexpression of metFGF was consistently higher (>5 fold) than 22 and 21.5 kD FGF-2 generated from ΔmetFGF and FGF cDNAs (determined by densitometry). These results are in agreement with the expression patterns observed previously in embryonic ventricular myocytes (see section 5.5.1) as well as noncardiac COS-1 cells (see section 4.5.2). The overall levels of FGF-2 expression from endogenous as well as transfected genes appear to be lower in postnatal myocytes than that of embryonic myocytes (Fig. 22 vs 12) and this could be due to the differences in developmental stage/species, transfection efficiencies and methods (ECL vs ^{125}I-protein A) used for detection of FGF-2.

6.5.2 Subcellular localization of different forms of FGF-2 in transfected cardiac myocytes

Transfected cultures were processed for simultaneous localization of FGF-2, striated muscle myosin and nuclei, using triple fluorescence labelling. Control cultures transfected with RSVp.CONT (Fig. 23), or non transfected cultures (not shown), did not

Fig. 22

Fig. 22. RSV promoter can drive the expression of hybrid genes in postnatal rat cardiac myocyte cultures. (A) Postnatal rat cardiac myocyte cultures were transfected with RSVp.ß-gal for 24 hours and assessed for ß-galactosidase activity 48 hours later. Arrows indicate the myocytes overexpressing ß-galactosidase. (B) Postnatal cardiac myocyte cultures were also transfected with RSVp.FGF (lane a), RSVp.ΔmetFGF (lane b), RSVp.metFGF (lane c) and RSVp.CONT (lane d). Heparin-sepharose purified cardiac cell lysates from transfected cultures (lanes a-d) or non transfected cultures (lane e) were fractionated in a 12.5% SDS-PAGE gel, immunoblotted, probed with rabbit FGF-2 antibodies, and visualized using the ECL detection system. The mobilities of 22, 21.5 and 18 kD FGF-2 are indicated.

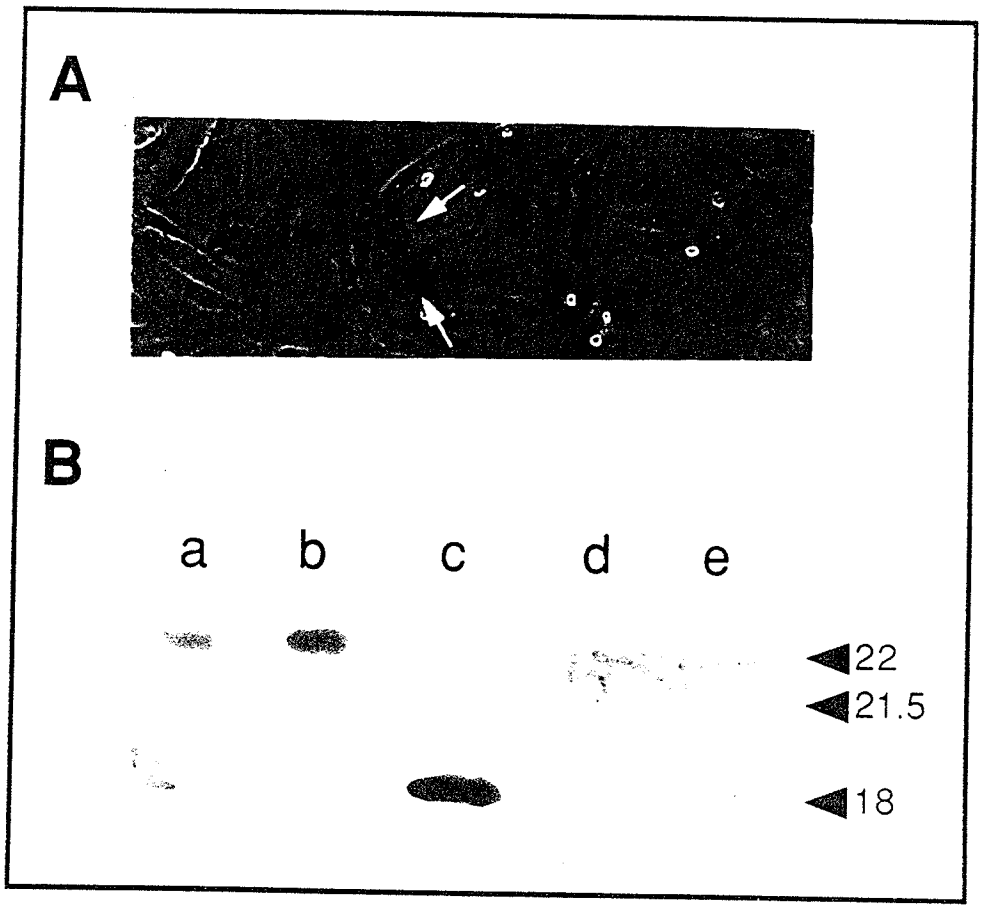
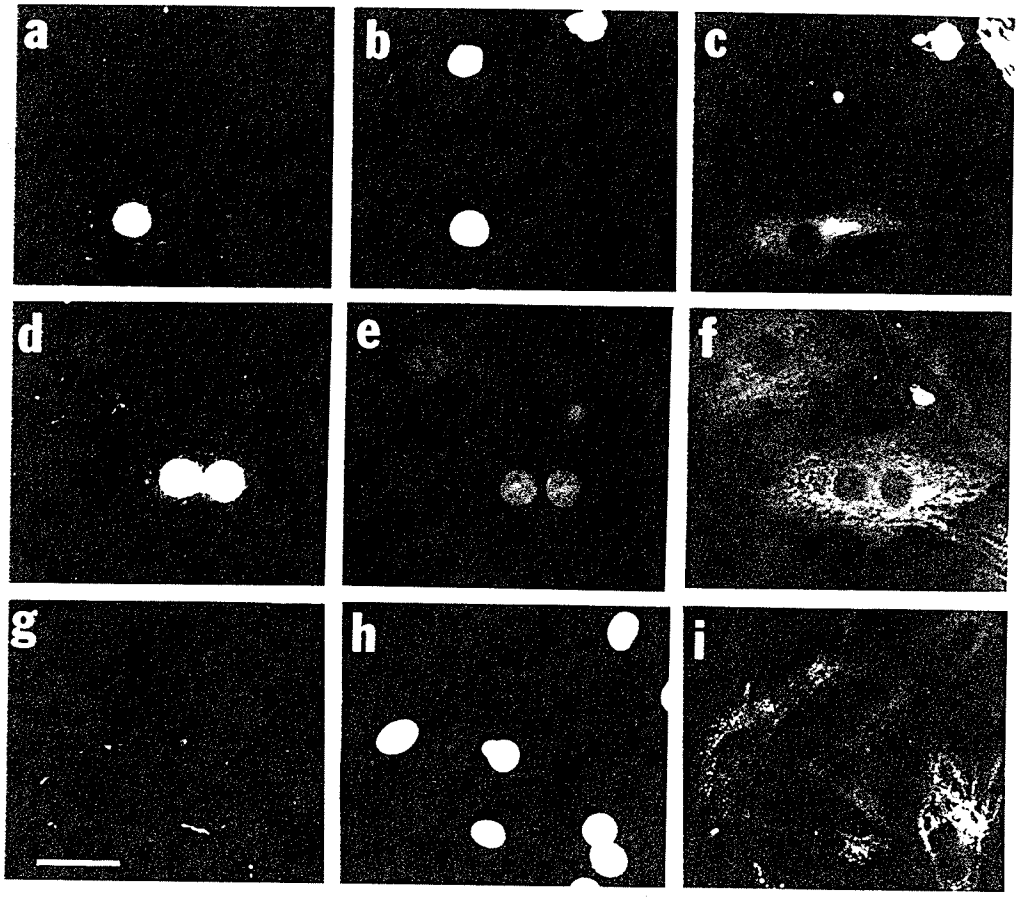

A
B
a
b
c
d
e
22
21.5
18

Fig. 23

Fig. 23. Localization of FGF-2 in postnatal rat cardiac myocytes transfected with RSVp.ΔmetFGF (a-f) and RSVp.CONT (g-i). Cells were stained for FGF-2 (a,d,g), DNA (b,e,h), and myosin (c,f,i). Predominant nuclear localization of FGF-2 was observed in mononucleated (a-c) and binucleated cardiac myocytes (d-f). An identical pattern of staining was seen in cultures transfected with RSVp.FGF. Bar is equivalent to 50 μm.


a
b
c
d
e
f
g
h
i

display the intense anti-FGF-2 staining of nuclei seen following overexpression of high or low molecular weight FGF-2 (Figs. 23 and 24). Subcellular localization of different forms of FGF-2 was examined in overexpressing cardiac myocytes. Myocytes transfected with RSVp.FGF and RSVp.ΔmetFGF displayed identical staining patterns. FGF-2 staining was localized predominantly to the nucleus or nuclei of mononucleated and binucleated cardiac myocytes, respectively, a finding which was confirmed by counterstaining DNA with Hoechst 33342 (Fig. 23). In contrast, cardiac myocytes transfected with RSVp.metFGF (that can generate only 18 kD FGF-2) displayed high levels of cytoplasmic and nuclear staining with a range of patterns including more intense nuclear or cytoplasmic staining (Fig. 24). However, the majority of cells displayed both cytoplasmic as well as nuclear staining of comparable intensity. The subcellular distribution of FGF-2 in the overexpressing rat cardiac myocyte cultures was identical to that observed with embryonic chicken myocytes (see section 5.5.6; Pasumarthi *et al.*, 1994) and non cardiac (kidney) COS-1 cells (see section 4.5.4). This in turn is consistent with the identical patterns of FGF-2 expression seen in these three cell types by immunoblotting.

6.5.3 Effect of FGF-2 on DNA synthesis

Two methods were employed to assess DNA synthesis in cardiac myocytes. In the first, a combination of *in situ* autoradiography ([^{3}H]thymidine incorporation) and PAS staining of glycogen/myocytes was used to obtain a Labelling Index (LI). The LI is the proportion of PAS+/glycogen-containing cells (myocytes) that also show the presence of [^{3}H]thymidine. The results are shown in Fig. 25, and are presented as fold differences relative to the control (RSVp.CONT) value (15.1±2.0%, n=8), which was arbitrarily set to 1.0. A significant increase in LI was observed in cultures transfected with RSVp.FGF (2.4 fold), RSVp.ΔmetFGF (3.2 fold) and RSVp.metFGF (3.0 fold) compared to cells transfected with RSVp.CONT ($p<0.001$). There was no significant difference between the effects of overexpression of high and low molecular weight FGF-2 on LI.

In a second approach, immunofluorescence staining with monoclonal antibodies to myosin and bromodeoxyuridine (BrdU) was used to confirm myocyte identity and assess the proportion of myocytes undergoing active DNA synthesis (S-phase nuclei) in cultures

Fig. 24

Fig. 24. Localization of FGF-2 in postnatal cardiac myocytes transfected with RSVp.metFGF. Cells were stained for FGF-2 (a,d), DNA (b,e), and myosin (c,f). Both nuclear and cytoplasmic localization of FGF-2 was observed in mononucleated (a-c) and binucleated cardiac myocytes (d-f). Note the range of nuclear and cytoplasmic staining observed metFGF overexpression. Bar is equivalent to 20 μm (a-c) and 50 μm (d-f).

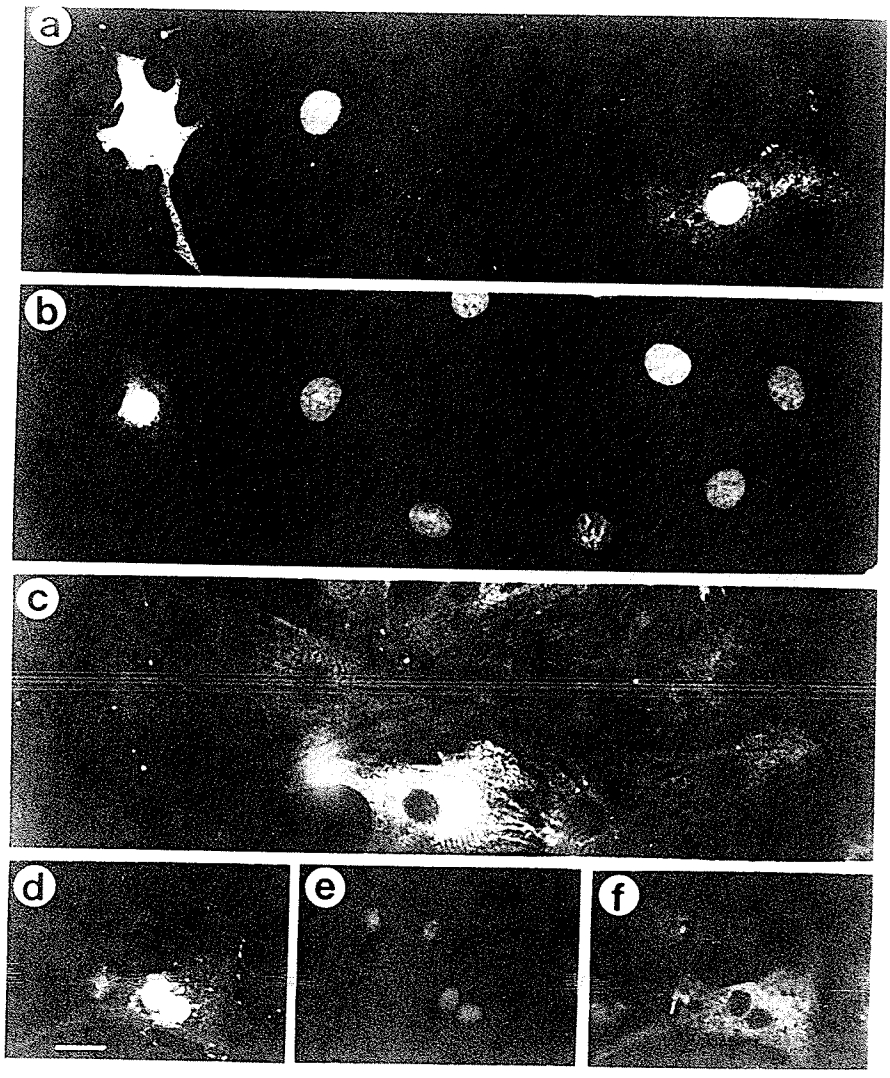
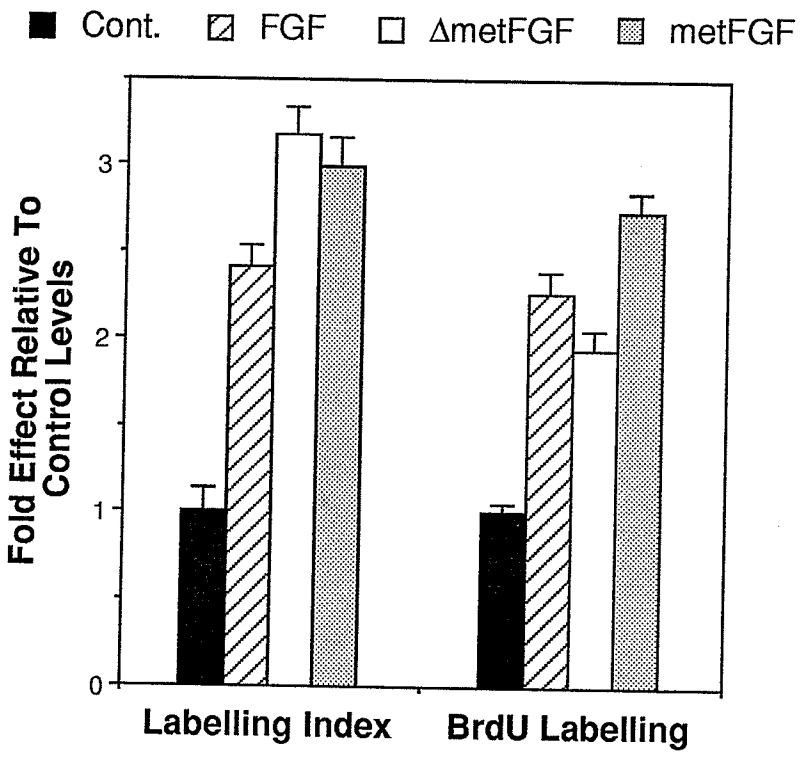

a
b
c
d
e
f

Fig. 25

Fig. 25. Overexpression of 22-21.5 kD and 18 kD FGF-2 in postnatal cardiac myocyte cultures stimulates DNA synthesis. DNA synthesis in transfected cardiac myocytes was assessed by [^{3}H]thymidine and BrdU incorporation. Labelling index (LI=radiolabelled PAS+ cells/total number of PAS+ cells) was determined (n=8) by using a combination of *in situ* autoradiography and histochemistry. Bromodeoxyuridine (BrdU) labelling was determined (n=11) by immunofluorescence staining with monoclonal antibodies to BrdU and myosin. The results are presented as fold differences relative to the control (RSVp.CONT) value, which was arbitrarily set to 1.0. Error bars are s.e.m. There are significant increases of LI and BrdU labelling in cultures transfected with RSVp.ΔmetFGF, RSVp.metFGF and RSVp.FGF, compared to cells transfected with the RSVp.CONT. There was no significant difference between the effects of high versus low molecular weight FGF-2 overexpression on LI. There was, however, a small statistically significant difference between the effects of overexpression of high (FGF, $p<0.05$ and ΔmetFGF, $p<0.001$) versus low molecular weight FGF-2 on BrdU staining.

Comparison	p value for LI	p value for BrdU
FGF vs Cont	<0.001	<0.001
ΔmetFGF vs Cont	<0.001	<0.001
metFGF vs Cont	<0.001	<0.001
FGF vs metFGF	>0.05 (ns)	<0.05
ΔmetFGF vs metFGF	>0.05 (ns)	<0.001

(ns = non significant)


Cont.
FGF
ΔmetFGF
metFGF
Fold Effect Relative To Control Levels
0
1
2
3
Labelling Index
BrdU Labelling

transfected with FGF hybrid genes or control plasmid. Anti-BrdU staining was confined to the nucleus, whereas anti-myosin staining was exclusively cytoplasmic in the rat cardiac myocytes. The results are shown in Fig. 25 and are presented as fold differences relative to the control (RSVp.CONT) value (18.8±1.1%, n=11), which was arbitrarily set to 1.0. There was a significant increase in the number of myocyte nuclei staining for BrdU in cultures transfected with RSVp.FGF (2.3 fold), RSVp.ΔmetFGF (1.9 fold) and RSVp.metFGF (2.7 fold) compared to cells transfected with RSVp.CONT ($p<0.001$). The difference between the effects of overexpression of high versus low molecular weight FGF-2 on BrdU staining was small (1.2 to 1.4 fold) but statistically significant (FGF, $p<0.05$ and ΔmetFGF, $p<0.001$).

The overall stimulation (~2.1 fold) of proliferative potential in postnatal rat ventricular myocyte cultures overexpressing high or low molecular weight forms of FGF-2, compares well with the ~2.6 fold stimulatory effect seen previously with embryonic chicken ventricular myocytes (see section 5.5.4). The LI for rat cardiac myocytes was reported to decline from day 15 of gestation and approaches zero by the end of third week after birth (Marino *et al.*, 1991). The LI (15.1%) obtained with postnatal rat cardiac control cultures (transfected with RSVp.CONT) in this study, is in agreement with LI values reported previously for postnatal rat hearts (15.4%; Clubb and Bishop, 1984) as well as cardiac myocyte cultures (12.5%; Marino *et al.*, 1991). Similarly, the LI for postnatal rat myocyte cultures transfected with rat FGF-2 cDNAs (~43.1%), is comparable to the LI of 32.5% and 30.6% determined for embryonic cardiac myocytes *in vitro* and *in vivo*, respectively (Marino *et al.*, 1991; Rumyantsev, 1991). Thus, these results suggest that the overexpression of both high and low molecular weight forms of rat FGF-2 in postnatal cardiac myocyte cultures can increase the basal levels of DNA synthesis to those associated with the embryonic phenotype.

The stimulatory effects of high (2.3 and 2.0 fold for FGF and ΔmetFGF, respectively) and low (2.3 fold for metFGF) molecular weight forms of FGF-2 on DNA synthesis as determined by [^{3}H]thymidine incorporation and PAS staining were comparable (Fig. 25). Any differences in the potency of high versus low molecular weight FGF-2 may have been masked by the higher levels of 18 kD FGF-2 expression (see Fig. 22b). However, assessment of DNA synthesis by a combination of BrdU incorporation

and anti-myosin staining revealed a small but significant difference in the degree of stimulation by 22/21.5 kD FGF-2 (2.3 and 1.9 fold for FGF and ΔmetFGF, respectively) versus 18 kD FGF-2 (2.7 fold). The reason for the discrepancy in the results obtained with thymidine versus BrdU incorporation is unclear, but is possibly related to the different methods used to identify myocytes. Regardless, no significant difference was observed between the stimulatory effects of high and low molecular weight forms of FGF-2 on cell number and mitotic index (MI).

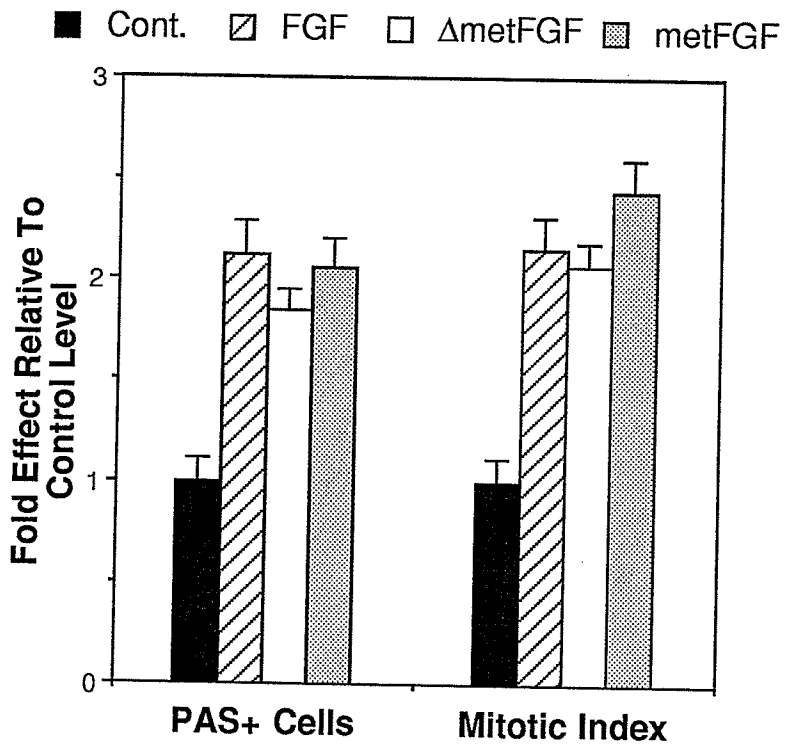
6.5.4 Effect of FGF-2 on cell number and MI

To determine the effects of overexpression of high or low molecular weight forms of FGF-2 on cardiac myocyte proliferation, the PAS+ cells (myocytes) were scored in random fields from cultures transfected with FGF-2 cDNAs and RSVp.CONT. The results are shown in Fig. 26, and are presented as fold differences relative to the control (RSVp.CONT) value, which was arbitrarily set to 1.0. There was a significant increase in PAS+ cells in cultures transfected with RSVp.FGF (2.1 fold), RSVp.ΔmetFGF (1.8 fold) and RSVp.metFGF (2.1 fold) compared to cultures transfected with RSVp.CONT ($p<0.001$). There was no significant difference between the effects of overexpression of high and low molecular weight FGF-2 on the number of PAS+ staining cells.

The Mitotic Index (MI) was also determined as a further indicator of proliferative potential in the transfected cultures. The fraction of cardiac myocytes in three readily identifiable stages of mitosis (metaphase, anaphase and telophase) was assessed in random fields from cultures transfected with FGF-2 cDNAs and RSVp.CONT. The chromosomes were easily identified by a combination of Hoechst staining for DNA and immunofluorescence microscopy. The results are shown in Fig. 26 and are presented as fold differences relative to the control (RSVp.CONT) value (4.6±0.5%, n=13), which was arbitrarily set to 1.0. There was a significant increase in MI of cultures transfected with RSVp.FGF (2.2 fold), RSVp.ΔmetFGF (2.1 fold) and RSVp.metFGF (2.4 fold) compared to cultures transfected with RSVp.CONT ($p<0.001$). There was no significant difference between the effects of overexpression of high and low molecular weight FGF-2 on overall MI. Thus, these increases in cell number and MI of cultures transfected with FGF-2 cDNAs substantiate the previously observed increases in DNA synthesis (Fig. 25).

Fig. 26

Fig. 26. Overexpression of 22-21.5kDa and 18 kDa FGF-2 in postnatal rat cardiac myocyte cultures is associated with increases in cell number and mitotic index. Cardiac myocyte proliferation was determined by scoring PAS+ cell number as well as readily visible mitotic figures (by Hoechst staining) in random fields (n=8-13) in cultures transfected with FGF-2 cDNAs and RSVp.CONT. The results are presented as fold differences relative to the control (RSVp.CONT) value, which was arbitrarily set to 1.0. Error bars are s.e.m. There are significant increases in PAS+ cell number ($p<0.001$) and mitotic index ($p<0.001$) of cultures transfected with RSVp.ΔmetFGF, RSVp.metFGF and RSVp.FGF. There is no significant difference between the effects of high versus low molecular weight FGF-2 overexpression on PAS+ cell number and mitotic index.


Cont.
FGF
ΔmetFGF
metFGF
Fold Effect Relative To Control Level
3
2
1
0
PAS+ Cells
Mitotic Index

6.5.5 Proximity dependent effect of FGF-2 on DNA synthesis

In the process of assessing MI, it was observed that cardiac myocytes undergoing mitosis were often found in close proximity to myocytes overexpressing either high or low molecular weight form of FGF-2 (Fig. 27). This observation suggested that FGF-2 was released from the overexpressing myocytes to act on the surrounding cells, presumably through a receptor mediated pathway. If this is the case, surrounding cardiac myocytes should be able to express high affinity receptors for FGF-2 and neutralizing antibodies specific for FGF-2 should be able to block the effects of released FGF-2 on the mitosis of surrounding cells.

6.5.5.1 Expression of high affinity receptors for FGF-2 in postnatal cardiac myocytes

RT-PCR assay was used to test whether postnatal rat ventricular myocytes express high affinity receptors (FGFR-1) for FGF-2. Two fragments of 610 bp and 343 bp sizes were amplified from the postnatal rat ventricular myocyte RNA, using primers specific for the extra cellular domain (Fig. 28). These fragments (610 bp and 343 bp) correspond to long and short forms of FGFR-1 respectively. Long and short isoforms of FGFR-1 exist due to the presence or absence of first Ig- like domain (Fig. 28) via alternate splicing from the same mRNA (Johnson and Williams, 1993) and both forms were shown to bind FGF-2 (Jaye *et al.*, 1992). Long form of FGFR-1 was the predominant species in postnatal rat ventricular myocytes in contrast to the adult ventricular myocytes in which short form was the major species (Liu *et al.*, 1995). Although it is not clear whether these individual forms of FGFR-1 activate different signalling pathways, a correlation between the expression of long form of FGFR-1 and proliferative potential of cardiac myocytes was reported (Jin *et al.*, 1994; Pasumarthi *et al.*, 1995). Thus these results satisfied one of the requirements for the proximity-dependent paracrine action of FGF-2 in the transfected cardiac myocyte cultures.

6.5.5.2 Effect of neutralizing antibodies for FGF-2 on the incidence of mitosis in the proximity of overexpressing myocytes

To test whether the effect of FGF-2 on DNA synthesis is mediated in a proximity-

Fig. 27

Fig. 27. Mitotic cardiac myocytes in close proximity to a myocyte overexpressing FGF-2. Light micrographs showing three examples (a-c and d,e and f,g) of myocytes in various stages of mitosis (arrow heads) in close proximity to a cardiac myocyte overexpressing FGF-2 (arrows). Double or triple staining was done for FGF-2 (a,d,f), DNA (b,e,g) and myosin (c). Bar is equivalent to 50 μm (a-c) or 20 μm (d-g).

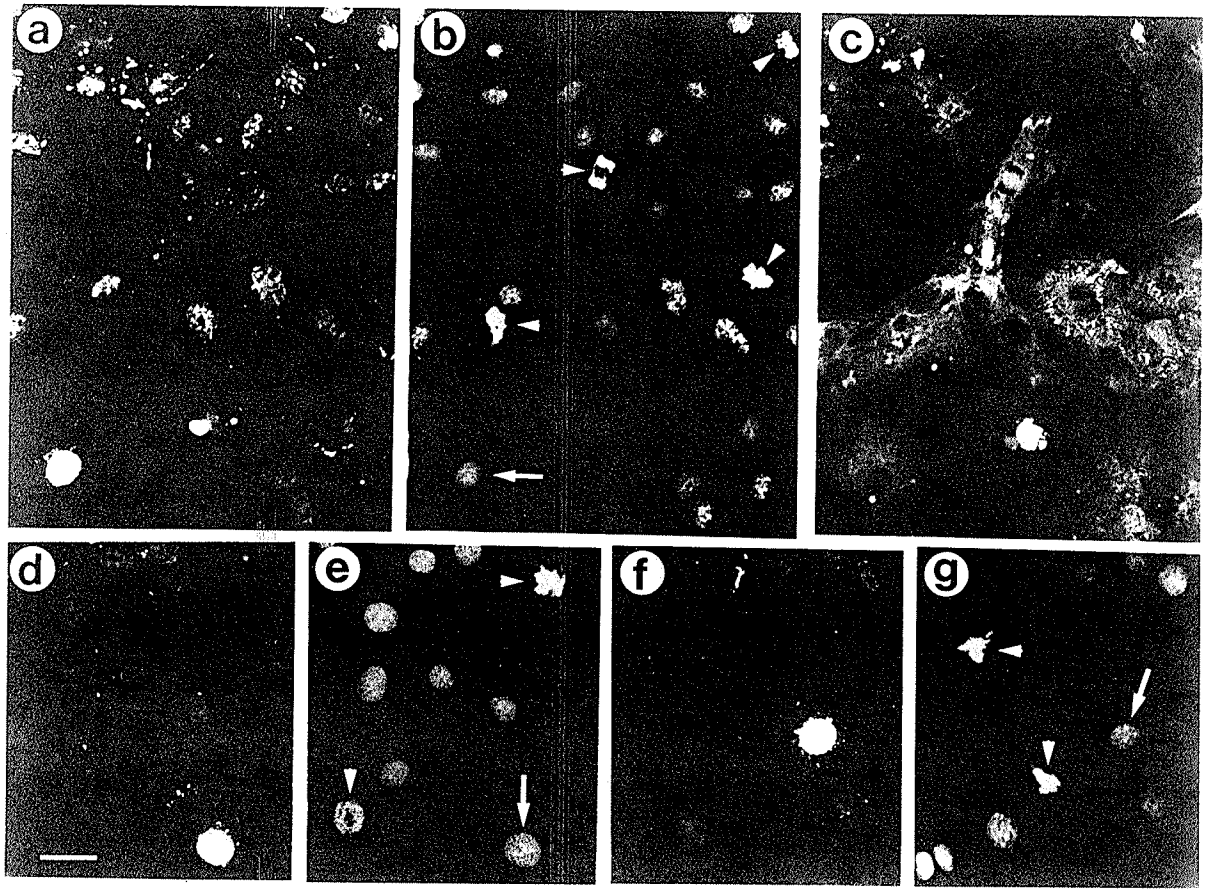
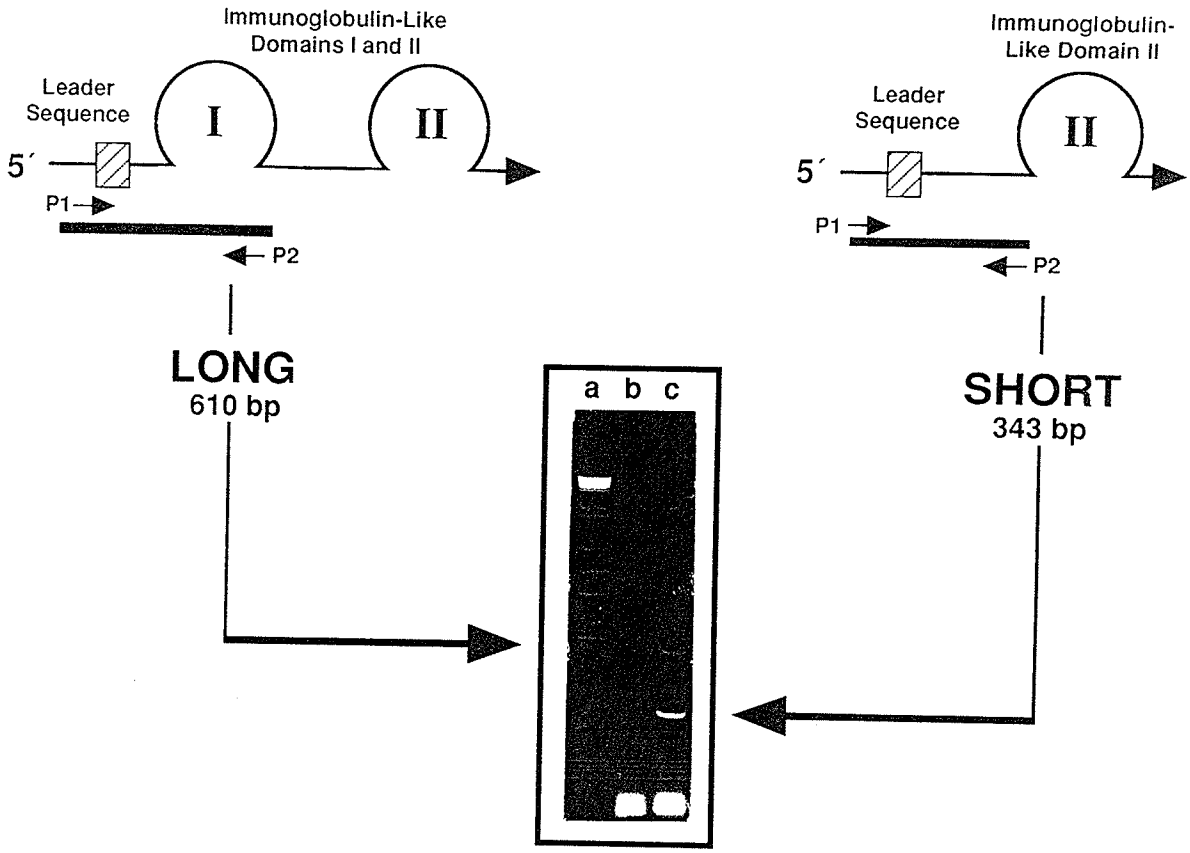

a
b
c
d
e
f
g

Fig. 28

Fig. 28. Detection of the transcripts for short and long isoforms of FGFR-1 in postnatal rat cardiac myocytes by RT-PCR amplification. Extracellular region containing two or three Ig-like domains of rat FGFR-1 was amplified using specifc oligonucleotide primers (based on sequences reported by Kim *et al.*, 1993) in the absence (lane b) or presence (lane c) of reverse transcriptase by RT-PCR. Amplification products (lanes b and c) were fractionated together with a ϕX174/*Hae*III marker in a 2% agarose gel. Arrows indicate 343- and 610- bp products which correspond to short and long isoforms of FGFR-1 respectively.


Immunoglobulin-Like
Domains I and II
Leader
Sequence
I
II
5´
P1
P2
LONG
610 bp
Immunoglobulin-
Like Domain II
Leader
Sequence
II
5´
P1
P2
SHORT
343 bp
a b c

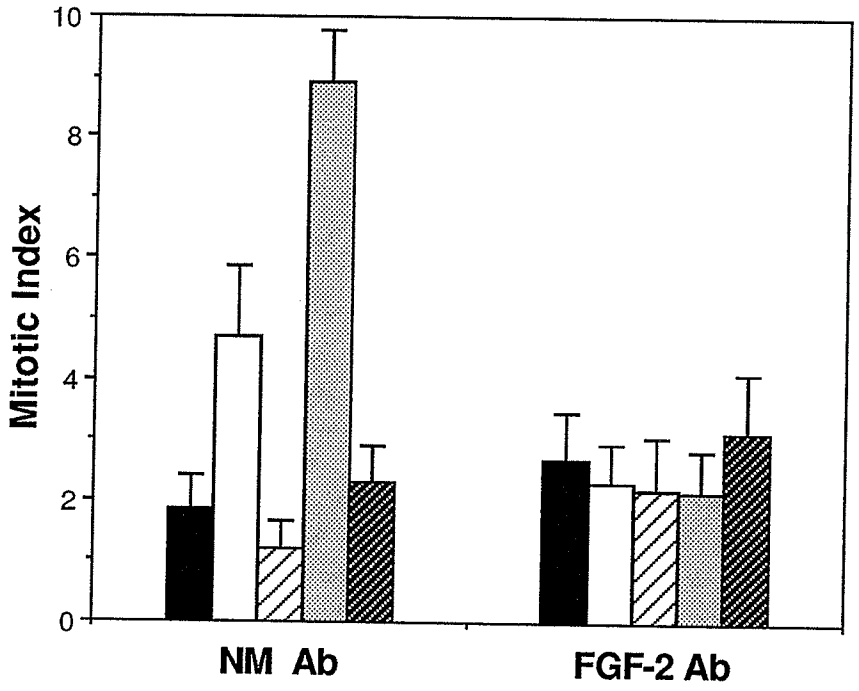
dependent manner, transfected postnatal rat ventricular myocyte cultures were maintained in the presence or absence of neutralizing antibodies for FGF-2. Incidence of mitotic figures in a field corresponding to a 5 cell radius (~125 μm), centred around a cardiac myocyte overexpressing high (ΔmetFGF) or low molecular weight FGF-2 (metFGF), was compared to that of an identical field centered around a non overexpressing myocyte within the same culture dish (n=15-20). Indeed, there was a significant increase (3.9 fold) in cells visibly undergoing mitosis in a field centered around a FGF-2 overexpressing myocyte compared to an identical field centered around a non overexpressing myocyte, in the absence of FGF-2 neutralizing antibodies (Fig. 29; see values obtained in the presence of NM Ab; high molecular weight FGF-2: $p<0.02$ or low molecular weight FGF-2: $p<0.001$). However, this apparent proximity-dependent effect on mitosis was inhibited in the presence of FGF-2 antibodies (Fig. 29). There was no significant difference between the incidence of mitosis near a FGF-2 overexpressing and non overexpressing myocyte in the presence of neutralizing FGF-2 antibodies. Further, this level was similar to "background" levels obtained from cultures transfected with the RSVp.CONT and maintained with either NM Ab or FGF-2 antibodies (Fig. 29). The "background" level of mitosis would be presumably induced by factors other than extracellular FGF-2 as it was not blocked by FGF-2 neutralizing antibodies. Interestingly, the proximity-dependent effect of low molecular weight FGF-2 overexpression on DNA synthesis was significantly higher (1.9 fold, $p<0.01$) than that of high molecular weight FGF-2 in transfected cultures (Fig. 29). This could be due to the higher level of expression seen with the low molecular weight FGF-2 compared to high molecular weight form (Fig. 22b).

6.5.5.3 Effect of conditioned medium from the transfected myocyte cultures on the growth of FBHE cells

During FGF-2 neutralizing antibody experiments, it was necessary to ensure that sufficient levels of antibodies were present in the medium of transfected ventricular myocyte cultures to neutralize the FGF-2 released from cells. Therefore, at the end of the neutralizing antibody experiments, conditioned medium from the transfected cultures was used to perform a growth assay on FBHE cells. Conditioned medium containing either FGF-2 antibodies or NM-Ab from each transfected rat ventricular myocyte culture dish was

Fig. 29

Fig. 29. A proximity-dependent increase in mitosis seen with both high (ΔmetFGF) and low molecular weight FGF-2 (metFGF) is inhibited in the presence of neutralizing antibodies to FGF-2. Mitotic indices for postnatal rat cardiac myocytes were determined by scoring the percentage number of cells with readily visible mitotic figures (by Hoechst staining) in areas corresponding to a 5 cell radius (n=15) containing or not containing an overexpressing cell, from the same culture, transfected with either RSVp.ΔmetFGF or RSVp.metFGF. This experiment was done with cells maintained in the presence of normal mouse antibodies (NM Ab) or FGF-2 antibodies. Cultures transfected with RSVp.CONT were also used to determine "background" control levels (n=15-20). A significant increase was observed in cells visibly undergoing mitosis in the presence versus absence of a FGF-2 overexpressing cardiac myocyte in the same culture, whether transfected with high (ΔmetFGF; $p<0.02$) or low molecular weight FGF-2 (metFGF; $p<0.0001$). This proximity-dependent stimulation of mitosis was inhibited when cardiac myocytes were maintained with FGF-2 antibodies after transfection.



■ "Background"

□ ΔmetFGF/within a 5 cell radius of an overexpressing myocyte

▨ ΔmetFGF/within a 5 cell radius of a non overexpressing myocyte

▩ metFGF/within a 5 cell radius of an overexpressing myocyte

▨ metFGF/within a 5 cell radius of a non overexpressing myocyte

transferred into a 60 mm dish plated with FBHE cells and recombinant human FGF-2 was added (3 ng/ml) to each plate. FBHE cells require FGF-2 for their growth/survival (Gospadarowicz *et al.*, 1976). FBHE cells maintained in the presence of conditioned medium, initially containing 10 μg/ml FGF-2 antibodies, revealed a significant inhibition of FGF-2-dependent growth compared to the cells maintained in conditioned medium containing 10 μg/ml NM Ab in 5 days, as assessed by microscopy (Fig. 30). Further, the cells were counted using a Coulter counter at the end of the experiment and the results indicated that the number of cells maintained in the presence of FGF-2 antibodies was significantly less (~2.5 fold) than the number of cells maintained in the presence of NM Ab (Fig. 31). However, the blunting effect of FGF-2 antibodies on the growth of cells maintained in conditioned medium transferred from cultures transfected with high (3.0 fold, n=3, $p<0.01$) and low (2.7 fold, n=3, $p<0.03$) molecular weight forms of FGF-2 was significantly higher than the blunting effect seen with that of RSVp.CONT group (1.7 fold, n=3, $p<0.01$). This could be due to the fact that the cultures transfected with FGF-2 cDNAs express and release higher levels of FGF-2 into the medium compared to the control cultres. These results confirmed that the FGF-2 neutralizing antibodies did not loose their activity during the course of neutralizing antibody experiments using cardiac myocytes.

Therefore, the ~2.2 fold increase in MI seen in cultures overexpressing 22/21.5 and 18 kD FGF-2 (Fig. 26) is consistent with the release of FGF-2 from overexpressing cells, stimulating mitosis in a proximity-dependent paracrine manner which occurs through a receptor-mediated pathway. Although FGF-2 lacks a signal sequence for its secretion, there is evidence to suggest that it can be actively released and bind to the immediate extracellular matrix (D' Amore, 1990). It has also been shown that the contraction-induced transient, survivable disruptions in the plasma membrane of cardiac myocytes can release both FGF-1 and FGF-2 to promote their autocrine growth (Clarke *et al.*, 1995). Recently, it has been implicated that the endogenous ouabain-like compounds may regulate the natural release of FGF-2 possibly through an active transport mechanism via Na^+/K^+ ATPase, during injury repair, inflammation and angiogenesis (Florkiewicz *et al.*, 1996). Therefore, it is possible that a contribution to the effects on DNA synthesis and cell division occurs through the release of FGF-2 from damaged cells. Regardless, these effects are mediated by FGF-2 as

Fig. 30

Fig. 30. Conditioned medium containing neutralizing antibodies for FGF-2, can blunt the FGF-2 dependent growth of FBHE cells. Conditioned medium containing normal mouse antibodies (NM Ab) or neutralizing antibodies for FGF-2 (FGF-2 Ab) was collected from transfected (eg. Cont. and ΔmetFGF) postnatal cardiac myocyte cultures at the end of the experiment and transferred into a 60 mm dish plated with FBHE cells. FBHE cells were stimulated with recombinant human 18 kD FGF-2 (3 ng/mL) and maintained for 5 days. Light micrographs show that condition medium containing FGF-2 Ab but not NM Ab decreases FBHE cell number. Note the detrimental effect of FGF-2 Ab on the growth of FBHE cells as indicated by development of vacuoles in the cytoplasm.

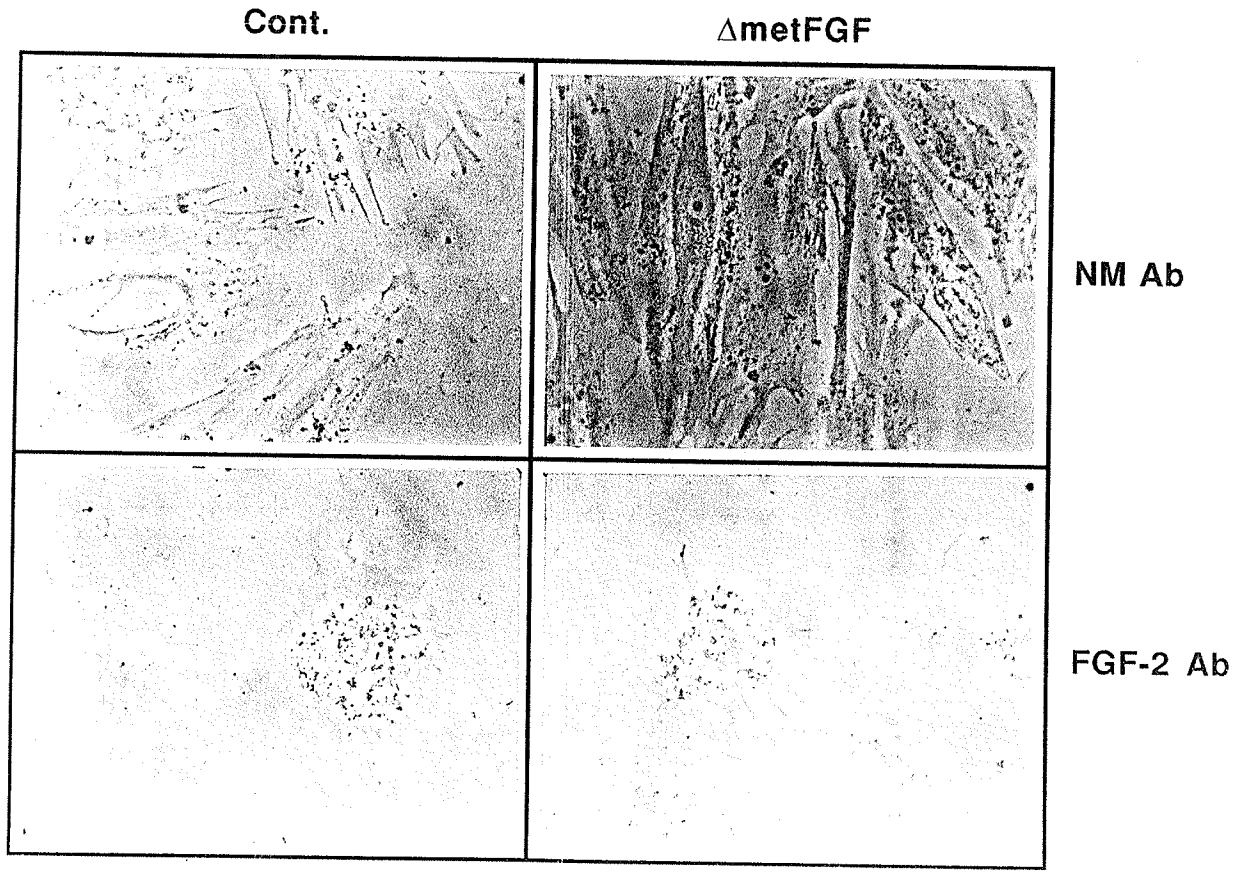
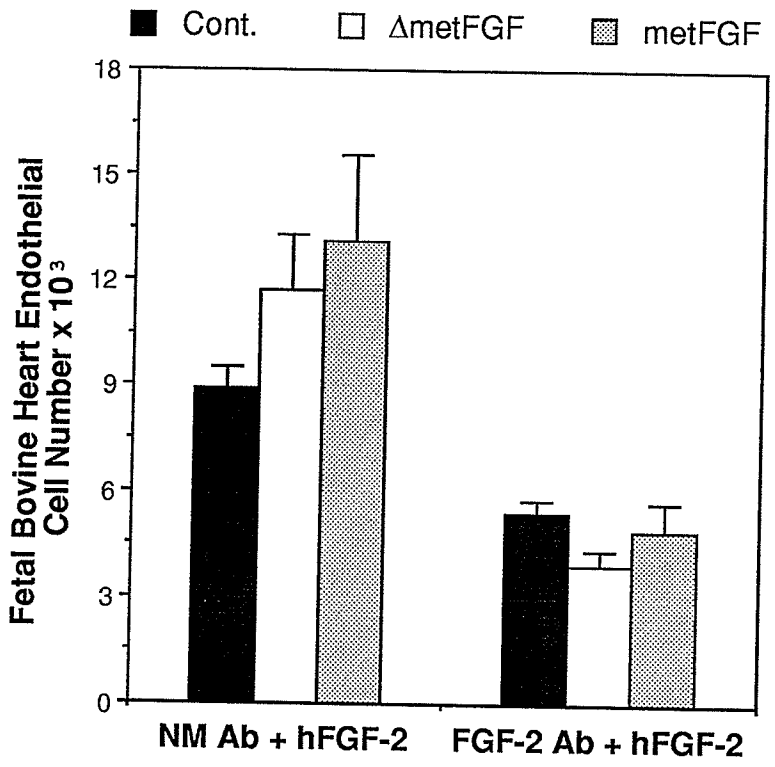

Cont.
ΔmetFGF
NM Ab
FGF-2 Ab

Fig. 31

Fig. 31. Determination of cell number in fetal bovine heart endothelial (FBHE) cell cultures. FBHE cells were maintained in the presence of recombinant human 18 kD FGF-2 and conditioned medium (containing NM Ab or FGF-2 Ab) transferred from transfected postnatal cardiac myocyte cultures (Cont., ΔmetFGF and metFGF) for 5 days. Total cell number was determined in FBHE cultures (n=3) using a Coulter counter. There were significant decreases (~2.5 fold) in the total number of FBHE cells maintained in the presence of FGF-2 Ab compared to the cells maintained in the presence of NM Ab. However, there was also a significant difference between the blunting effects of conditioned medium from cultures transfected with ΔmetFGF (3.0 fold, $p<0.01$) or metFGF (2.7 fold, $p<0.03$) compared to the Cont. plasmid (1.7 fold, $p<0.01$).


Cont.
ΔmetFGF
metFGF
Fetal Bovine Heart Endothelial Cell Number x 10³
18
15
12
9
6
3
0
NM Ab + hFGF-2
FGF-2 Ab + hFGF-2

opposed to the transfection process, since results were significantly different from those obtained with control cultures transfected with RSVp.CONT.

6.5.6 Effect of FGF-2 on binucleation

Rat cardiac myocytes are mononucleated during fetal and early postnatal development and about 85% of cardiac myocytes become binucleated by the third week after birth (Clubb and Bishop, 1984). Binucleation was considered to be an early marker of cardiac myocyte growth hypertrophy and there is no information about the factors responsible for this process (Clubb and Bishop, 1984).

6.5.6.1 Effect of FGF-2 on total degree of binucleation in the presence or absence of neutralizing antibodies

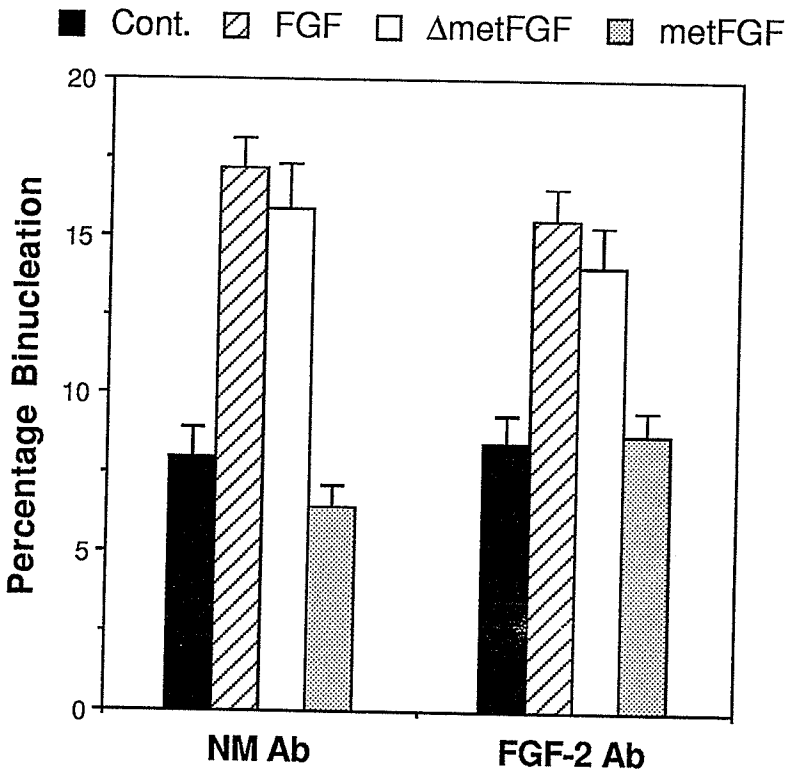
To test whether high and low molecular weight forms of FGF-2 influence the degree of binucleation, the fraction of binucleated cardiac myocytes was determined in random fields from cultures transfected with FGF-2 cDNAs and RSVp.CONT. The nuclei of cardiac myocytes were easily identified by a combination of Hoechst staining for DNA, antibodies to myosin and immunofluorescence microscopy. Since antibodies to FGF-2 were able to inhibit the effect of high as well as low molecular weight FGF-2 on mitosis, the percentage of binucleation was also assessed in the transfected cultures treated with or without neutralizing antibodies to FGF-2 (Fig. 32). There was a significant increase in binucleation in cultures transfected with RSVp.FGF (2.2 fold, $p<0.001$) and RSVp.ΔmetFGF (2.0 fold, $p<0.001$) but not RSVp.metFGF when compared to the control value (6.6±0.6%, n=33) obtained from cultures transfected with RSVp.CONT (Fig. 32). However, there was no significant effect of FGF-2 antibodies on the stimulation of binucleation observed with overexpression of high molecular weight FGF-2 (FGF or ΔmetFGF; Fig. 32).

6.5.6.2 Correlation between overexpression of FGF-2 and binucleation

Triple staining for myosin, FGF-2 and DNA suggested that a significant proportion of cardiac myocytes overexpressing FGF-2 were also binucleated. Therefore the level of binucleation was also assessed as a percentage of cardiac myocytes overexpressing

Fig. 32

Fig. 32. Stimulation of total degree of binucleation seen with 22-21.5 kD but not 18 kD FGF-2 is unaffected in the presence of neutralizing antibodies to FGF-2. Cardiac myocyte binucleation was determined by scoring binucleated cells (by myosin and Hoechst staining) in random fields (n=30) in cultures transfected with FGF-2 cDNAs and RSVp.CONT and then maintained in the presence of normal mouse antibodies (NM Ab) or neutralizing FGF-2 antibodies (FGF-2 Ab). Error bars represent SEM. There were significant increases in overall binucleation of cultures transfected with RSVp.FGF (2.2 fold, $p<0.001$) and RSVp.ΔmetFGF (2.0 fold, $p<0.001$) but not RSVp.metFGF. There was no significant effect of FGF-2 antibodies on the stimulation of binucleation observed with overexpression of high molecular weight FGF-2 (FGF or ΔmetFGF).


Cont.
FGF
ΔmetFGF
metFGF
Percentage Binucleation
20
15
10
5
0
NM Ab
FGF-2 Ab

FGF-2 in cultures transfected with RSVp.FGF, RSVp.ΔmetFGF or RSVp.metFGF. Binucleation was seen in 33% and 40% of overexpressing cardiac myocytes in cultures transfected with RSVp.FGF and RSVp.ΔmetFGF, respectively; the difference between these results is not considered significant. In contrast, only 9% of cardiac myocytes overexpressing RSVp.metFGF were binucleated, which is significantly different from the value obtained with either RSVp.FGF ($p<0.0001$) or RSVp.ΔmetFGF ($p<0.0001$) (Fig. 33). Further, the percentage level of binucleation of cardiac myocytes overexpressing FGF, ΔmetFGF or metFGF was unaffected by the presence of neutralizing FGF-2 antibodies added to the culture medium (not shown).

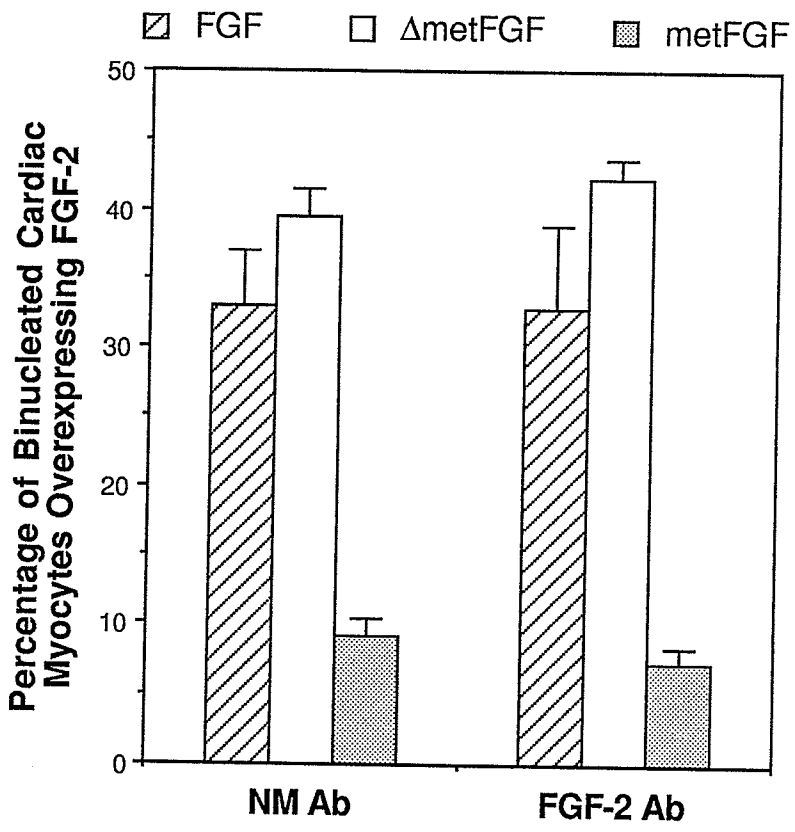
In contrast to the paracrine (cell surface receptor-mediated) pathway indicated for hyperplastic growth, the results were consistent with a distinct intracellular effect of high molecular weight FGF-2 on binucleation in rat postnatal cardiac myocytes in culture. Further evidence for an intracellular effect of high molecular weight FGF-2 was reported recently based on phenotypic changes occurring in mouse 3T3 cells overexpressing dominant negative FGFR-1 (Bikfalvi *et al.*, 1995). It is possible that these intracrine effects of FGF-2 may be mediated through specific intracellular receptors (Kim *et al.*, 1993). It was proposed that some binucleated cardiac myocytes might divide into two by formation of new intercalated discs (Shozawa *et al.*, 1990). Thus, binucleated cardiac myocytes may serve as potential sources of "new" cells in pathological states. This concept is further substantiated by a recent report indicating that binucleation in hepatocytes decreases during regenerative, hyperplastic and neoplastic growth (Gerlyng *et al.*, 1992). It is intriguing to note that high molecular weight forms of FGF-2 exert two distinct effects on postnatal rat cardiac myocyte growth by stimulating cell proliferation and binucleation in paracrine and intracrine manners, respectively. It is also possible that the increase in binucleation associated with the overexpression of high molecular weight forms of FGF-2 may serve as a "potential source" of new cells during disease states.

6.5.6.3 A possible mechanism for high molecular weight FGF-2 induced binucleation

Lines of nuclear cleavage were observed with both FGF-2 and DNA staining in about 5% of cardiac myocytes overexpressing high molecular weight FGF-2, suggesting

Fig. 33

Fig. 33. Increased incidence of binucleation in postnatal cardiac myocytes overexpressing 22-21.5 kD but not 18 kD FGF-2. A positive correlation was observed between overexpression of 22-21 kD but not 18 kD FGF-2 and incidence of binucleation. The level (presented as a percentage) of binucleation in cardiac myocytes overexpressing FGF-2 following transfection with RSVp.FGF, RSVp.ΔmetFGF or RSVp.metFGF is shown. Error bars represent SEM. There is a significant difference between the percentage of binucleation seen in cardiac myocytes overexpressing 18 kD FGF-2 (RSVp.metFGF) versus 22-21.5 kD FGF-2 with either RSVp.FGF ($p<0.0001$) or RSVp.ΔmetFGF ($p<0.0001$).


FGF
ΔmetFGF
metFGF
Percentage of Binucleated Cardiac Myocytes Overexpressing FGF-2
50
40
30
20
10
0
NM Ab
FGF-2 Ab

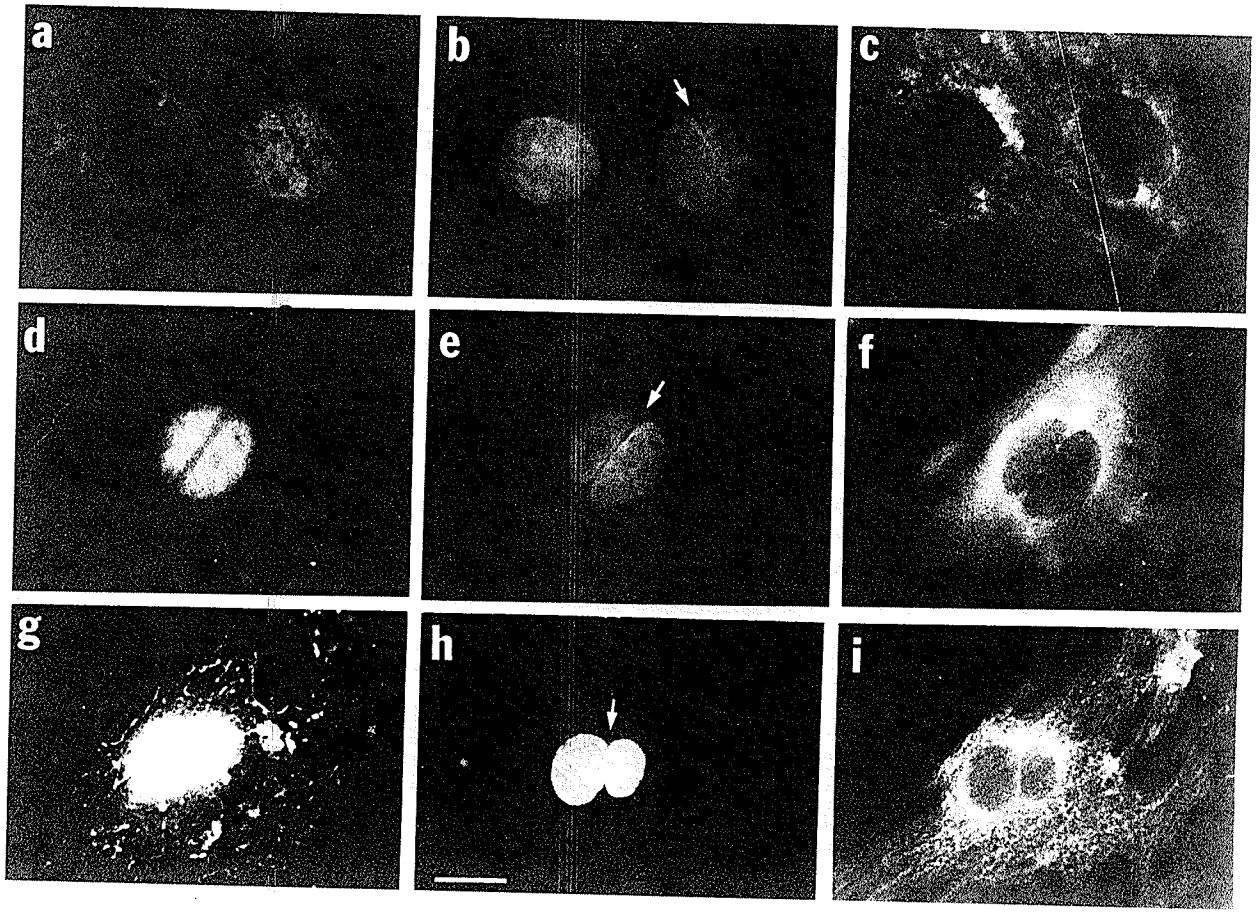
that they were undergoing amitotic division (Fig. 34) compared to <0.1% of cells overexpressing 18 kD FGF-2 or transfected with RSVp.CONT. These cleavage lines appeared to divide the nucleus in both symmetrical and asymmetrical manners and were still apparent in the presence of neutralizing antibodies to FGF-2. This is consistent with the notion that 22/21.5 kD FGF-2 mediates its effect on nucleation of cardiac myocytes through amitosis, presumably, in an intracrine manner. Binucleation in cardiac myocytes is believed to result from amitotic division (nucleus of a cell is constricted into two by a process, not involving mitosis) or karyokinesis without cytokinesis (nuclear mitotic division without cytoplasmic seperation) (Brodsky, 1991; Clubb and Bishop, 1984; Katzberg *et al.*, 1977; Rumyantsev, 1991). My results do not rule out karyokinesis without cytokinesis as a mechanism for binucleation in cardiac myocytes overexpressing FGF-2. In addition, the estimated percentage of nuclear cleavage would be an underestimate of the overall extent of amitosis, since it represents a "snapshot" of the whole process and does not include cells with fully separated nuclei; unless binucleated cells are the product of a clearly asymmetric nuclear division (i.e. containing two nuclei of different sizes), it would not be possible to differentiate between karyokinesis and amitosis. Amitosis could represent a culture phenomenon, however, a few nuclear divisions in cardiac myocytes were reported to occur during postnatal cardiomyogenesis through amitosis (Rumyantsev, 1991). Binucleation maintains the normal nuclear versus cytoplasmic ratio in cardiac myocytes during their physiological hypertrophic growth (Brodsky, 1991; Engelmann *et al.*, 1986). Therefore, it is possible that the amitosis induced by high molecular weight FGF-2 may serve this purpose in cardiac myocytes during hypertrophy.

6.5.7 Effect of wild type and modified FGF-2 cDNA expression on nuclear morphology

Clumping of the DNA, resembling chromatin condensation, was seen in 20% of cardiac myocytes overexpressing high but not low molecular weight FGF-2. These "clumps" were observed in both mononucleated and binucleated cardiac myocytes (Fig. 35) and appeared distinct from the chromosomal patterns seen during the various stages of cell division (Cattini *et al.*, 1991; Kardami *et al.*, 1991a). A similar effect on DNA clumping was observed in ~60% of embryonic (chicken) cardiac myocytes overexpressing high molecular weight forms of rat FGF-2 and it was speculated that this could be a

Fig. 34

Fig. 34. Nuclear cleavage in cardiac myocytes overexpressing 22/21.5 FGF-2. Triple staining was done for FGF-2 (a,d,g), DNA (b,e,h) and myosin (c,f,i). Light micrographs showing three stages of nuclear cleavage. Nuclear furrowing is detectable (a-c). Furrow is clearly visible (d-f) by FGF-2 or DNA staining (d,e) and traces of myosin can be detected in the furrow (f). After what appears to be cleavage, the nuclei continue to stain intensely for FGF-2 (g,h) and myosin staining between nuclei (in this case of different sizes) is evident (i). Arrows indicate plane of cleavage furrow. Bar is equivalent to 10 μm (a-f) or 15 μm (g-i).


a
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c
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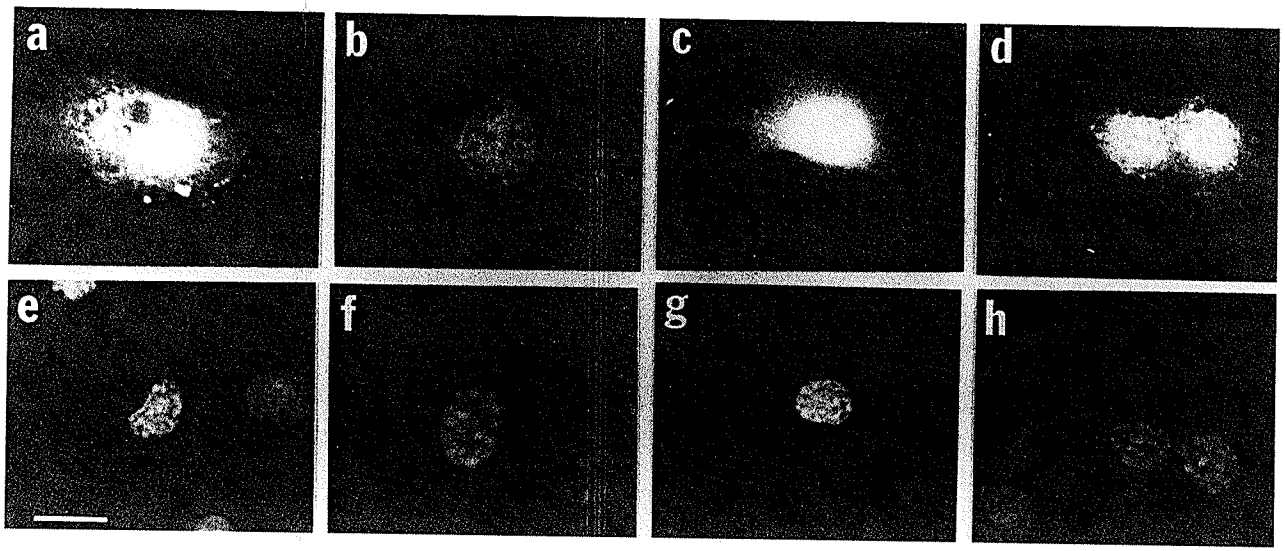
e
f
g
h
i

Fig. 35

Fig. 35. Overexpression of 22-21.5 kD FGF-2 in postnatal rat cardiac myocyte cultures is associated with DNA "clumps". Light micrographs showing four paired examples of mononucleated and binucleated cardiac myocytes, stained for FGF-2 (a-d) and DNA (e-h), displaying DNA clumping. The intensity and pattern of DNA staining is suggestive of chromatin condensation. Bar is equivalent to 20 μm.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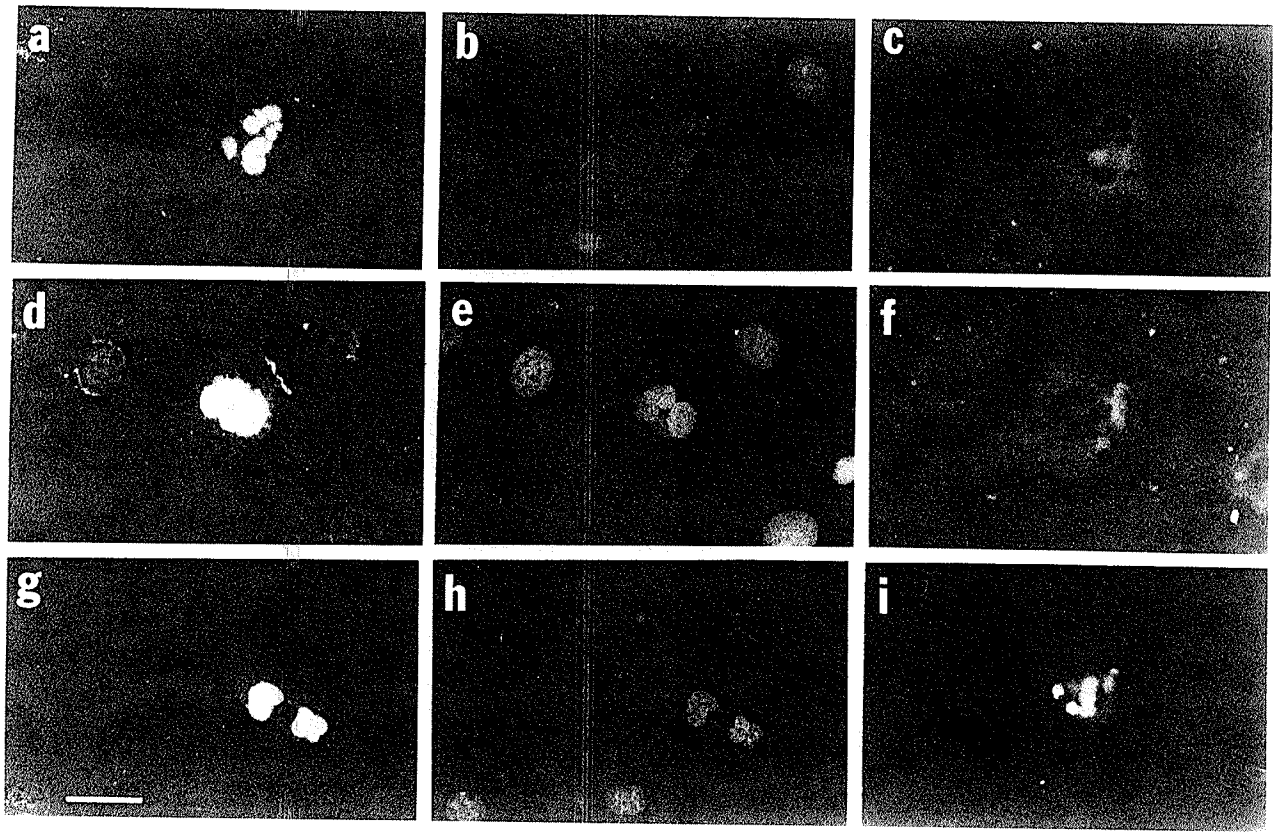
heterologous effect due to the species difference (Pasumarthi *et al.*, 1994). However, the present study ruled out this possibility of heterologous effect as the rat protein was overexpressed in a rat cell. The DNA clumping observed in postnatal cardiac myocytes was still apparent in the presence of neutralizing antibodies to FGF-2, suggesting an intracrine role for high molecular weight form of FGF-2. It could be physiologically significant as similar results were obtained with two different species. In addition, there appears to be some degree of cell/tissue specificity to this event, as the overexpression of high molecular weight FGF-2 in non cardiac COS-1 cells (derived from African green monkey kidney) did not lead to the DNA clumping. FGF-2 is known to bind to chromatin (Gualandris *et al.*, 1992) and is capable of modifying gene transcription *in vitro* (Nakanishi *et al.*, 1992). The high molecular weight species would be expected to associate with chromatin at a higher affinity than 18 kD FGF-2 due to the additional basic amino acids present in the amino terminal extension (Brigstock *et al.*, 1990). Higher incidence of DNA clumping in embryonic cardiac myocytes (~60%) compared to postnatal cardiac myocytes (~20%) can be attributed to the differences in the transfection efficiency.

In addition, a small number of cardiac myocytes (less than 1%) overexpressing high but not low molecular weight FGF-2 contained, what appeared to be, multiple nuclei of varying sizes with patterns suggestive of nuclear partitions, fragmentation and nuclear chains (Fig. 36). Cardiac myocytes containing three or more nuclei (multinucleated) were reported to constitute about 5% of ventricular myocytes in the adult rat heart (Kellerman *et al.*, 1992) and examples were also seen in the adult human heart (Shozawa *et al.*, 1990). It is possible that the DNA clumping seen in cardiac myocytes overexpressing 22/21.5 kD FGF-2 (Fig 4; Pasumarthi *et al.*, 1994) represent an aspect of the nuclear partition, however, chromatin condensation and nuclear fragmentation are also features of apoptosis. Apoptosis has been linked to the control of primitive myocardial cell overgrowth associated with cardiac rhabdomyoma as well as removal of damaged cardiac myocytes following reperfusion injury (Medioni *et al.*, 1994; Gottlieb *et al.*, 1994). Therefore, it is possible that the DNA clumping and nuclear fragmentation induced by high molecular weight form FGF-2 may be involved in the removal of damaged cardiac myocytes during disease states.

Fig. 36

Fig. 36. Postnatal cardiac myocytes containing nuclei of varying sizes in cultures overexpressing 22/21.5 kD FGF-2. Triple staining was done for FGF-2 (a,d,g), DNA (b,e,h) and myosin (c,f,i). Three examples of cardiac myocytes overexpressing FGF-2 and containing multiple nuclei of different sizes in cultures transfected with RSVp.ΔmetFGF (a-c) and RSVp.FGF (d-i) are shown. Bar is equivalent to 20 μm.


a
b
c
d
e
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g
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i

6.6 Concluding remarks

In conclusion, the results of present study indicate that: (i) high molecular weight FGF-2 is found predominantly in the nucleus while low molecular weight FGF-2 localizes to both the cytoplasm and nucleus of transfected postnatal rat ventricular myocytes, (ii) overexpression of both high and low molecular weight forms of FGF-2 can stimulate hyperplastic growth of postnatal rat cardiac myocytes, (iii) the effect of FGF-2 on DNA synthesis is mediated, at least in part, in a proximity-dependent paracrine manner on adjacent cardiac myocytes and is of a similar magnitude for both forms, (iv) overexpression of high but not low molecular weight FGF-2 leads to a significant increase in binucleation by an intracrine pathway, (v) nuclear amitotic cleavage might contribute to the process of binucleation induced by high molecular weight FGF-2, and (vi) overexpression of high but not low molecular weight FGF-2 leads to changes in nuclear morphology which include DNA clumping and nuclear fragmentation through an intracrine pathway.

Chapter 7

Effects of overexpression of high and low molecular weight forms of FGF-2 on the differentiation of postnatal rat cardiac myocytes

7.1 Background

Both proliferation and differentiation can occur simultaneously in cardiac myocytes (Rumyantsev, 1977) unlike skeletal muscle cells in which these events can be uncoupled (Endo and Nadal-Ginard, 1986; Nguyen *et al.*, 1983). Cardiac myocytes express lower levels of differentiation proteins at the embryonic stage (eg. desmin; Gamiz *et al.*, 1993) and remain proliferative (Rumyantsev, 1977). By contrast, adult cardiac myocytes express higher levels of differentiation markers and lose their proliferative potential (Ueno *et al.*, 1988). In addition, postnatal growth of cardiac myocytes is accompanied by switches in the expression pattern of muscle-specific isoforms (see section 2.2.1; Table 1). For instance, ß-myosin heavy chain (ß-MHC) is expressed predominantly during embryonic stage in rodents, while α-myosin heavy chain (α-MHC) is expressed in the adult myocardium (Lompre *et al.*, 1984). Embryonic isoforms MLC-1a and ß-Tropomyosin are down regulated in the adult heart (Hirzel *et al.*, 1985; Izumo *et al.*, 1988). Similarly, the expression of atrial natriuretic factor (ANF) is down regulated in adult ventricular myocytes (Lattion *et al.*, 1986a,b). However, several fetal markers (eg. ß-MHC, ANF and α-skeletal actin) are re-expressed in adult cardiac myocytes during hypertrophy (Parker *et al.*, 1990; Parker and Schneider, 1991). The mechanisms regulating the proliferation and differentiation of cardiac myoctyes are largely unknown. Both FGF-1 and FGF-2 were shown to inhibit the differentiation of skeletal muscle cells in culture (Clegg *et al.*, 1987; Lanthrop *et al.*, 1985) by inactivating the factors necessary for muscle gene transcription (Li *et al.*, 1992). FGF-2 plays a vital role in the growth and differentiation of cardiac myocytes (Claycomb and Moses, 1988; Kardami, 1990; Sugi *et al.*, 1993). It was shown to stimulate DNA synthesis and proliferation of cardiac myocytes, when added exogenously (Kardami, 1990). Overexpression of both high or low molecular weight

forms of rat FGF-2 in cardiac myocyte cultures increased DNA synthesis, mitosis and cell proliferation but only the high molecular weight form was able to increase binucleation (see sections 5.5.3; 5.5.4; 6.5.3 to 6.5.6 and Pasumarthi *et al.*, 1996).

7.2 Rationale

Adult cardiac myocytes are differentiated and loose their ability to divide, posing a problem with regard to their regenerative response during cardiac damage. The mechanisms regulating growth and differentiation of cardiac myocytes are largely unknown. Studies aimed at understanding these processes would facilitate the efforts to convert differentiated postnatal cardiac myocytes into less differentiated and or more proliferative embryonic phenotype. Both high and low molecular weight forms of FGF-2 were shown to stimulate DNA synthesis as well as proliferation of postnatal cardiac myocytes similar to the levels seen with embryonic cardiac myocyte cultures (see sections 5.5.3; 5.5.4; 6.5.3 to 6.5.5). However, it is not known whether the increased proliferative potential of postnatal myocyte cultures overexpressing either high or low molecular weight FGF-2 correlates with a decrease in the differentiation of cardiac myocytes and or reappearance of an embryonic phenotype. Therefore, the effects of overexpression of high and low molecular weight forms of FGF-2 on the synthesis and expression pattern of muscle-specific protein isoforms in postnatal cardiac myocyte cultures were examined.

7.3 Hypothesis

Overexpression of high and low molecular weight forms of FGF-2 can decrease the differentiation state and stimulate the expression of embryonic isoforms of muscle-specific proteins in postnatal ventricular myocytes.

7.4 Specific Aims

1. To examine the subcellular distribution of endogenous myosin and FGF-2 in dividing and nondividing postnatal rat ventricular myocytes.
2. To examine the effects of overexpression of FGF-2 on muscle-specific protein synthesis in postnatal ventricular myocyte cultures.

3. To examine the effects of overexpression of FGF-2 on the RNA levels of some muscle-specific proteins in postnatal ventricular myocyte cultures.

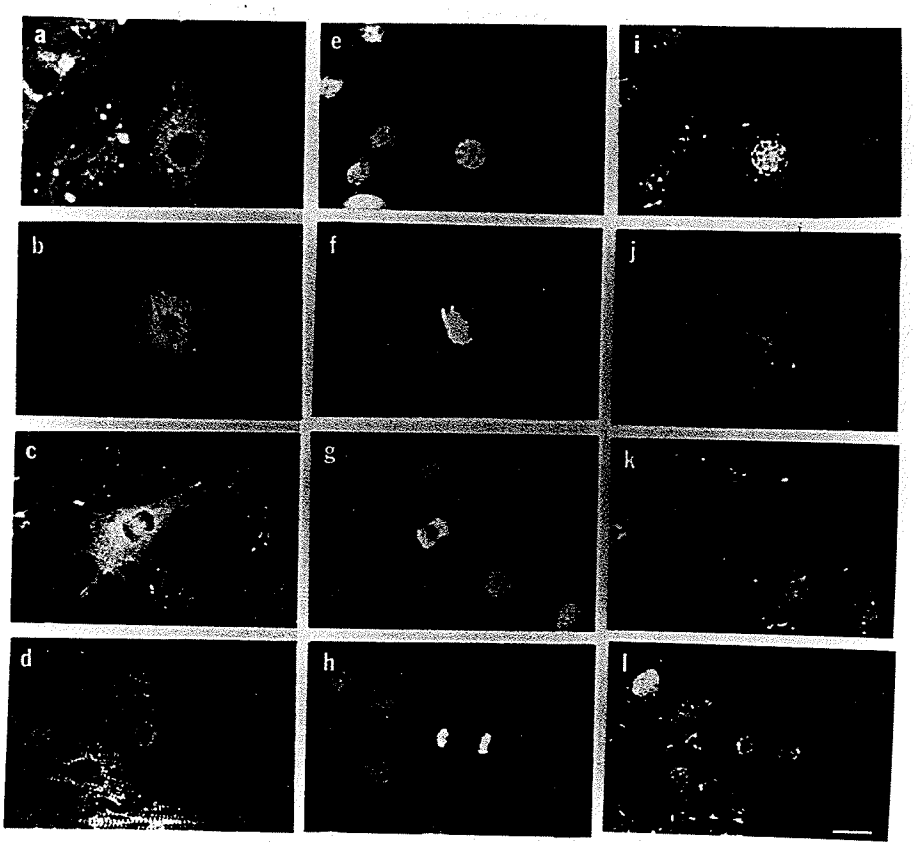
7.5 Results and Discussion

7.5.1 Distribution of myosin and FGF-2 in dividing and non dividing postnatal rat cardiac myocytes

As a first step in understanding proliferation and differentiation of cardiac myocytes, the subcellular distribution of myosin as well as FGF-2 was examined in postnatal rat cardiac myocyte cultures (n=3) transfected with RSVp.CONT, using triple immunofluorescence labelling. Cardiac myocytes in S-phase were identified by simultaneous labelling with BrdU and myosin antibodies. Cardiac myocytes in interphase, S-phase (DNA synthesis) and prophase revealed well organized sarcomeres when stained with myosin antibodies. Myosin staining was confined to the cytoplasm and excluded from the nuclear compartment of these cells (Fig. 37a). FGF-2 staining in these cells was predominantly perinuclear (Fig. 37i). There was a switch in the myosin staining pattern from sarcomeric to both diffuse and intense cytoplasmic staining, as the cardiac myocytes traverse through prophase to metaphase (Fig. 37b). It is possible that the increased levels of certain factors in cardiac myocytes during the transition between prophase and metaphase can stimulate the expression of endogenous proteases that are responsible for the conversion of sarcomeric myosin into monomeric myosin. Certainly, FGF-2 is known to stimulate the expression of metalloproteases that are responsible for the conversion of plasminogen into plasmin (Isacchi *et al.*,1991). It was postulated that a Z-band specific protease is activated to facilitate the breakdown of sarcomeres in dividing amphibian cardiac myocytes (Rumyantsev, 1991). Similar to the myosin staining pattern, FGF-2 staining was also diffuse and cytoplasmic, followed by the disappearance of perinuclear staining in cardiac myocytes undergoing metaphase (Fig. 37j). Both myosin and FGF-2 antibodies did not stain the metaphase plate. In contrast, staining of the equatorial axis was observed with both myosin and FGF-2 antibodies in myocytes undergoing anaphase (Fig. 37c,k). During telophase, myosin staining appeared to mobilize towards poles away from the central axis (Fig. 37d). Perinuclear staining of FGF-2 started to reappear, following the formation of

Fig. 37

Fig. 37. Distribution of myosin and FGF-2 during different phases of cell cycle in postnatal rat cardiac myocytes. Cells were stained for myosin (a-d), nuclei (e-h) and FGF-2 (i-l). Note the differential distribution of myosin and FGF-2 in myocytes undergoing prophase (a,e,i), metaphase (b,f,j), anaphase (c,g,k) and telophase (d,h,l). Bar is equvalent to 20 μm.


a
e
i
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h
l

daughter nuclei (Fig. 37l). After the completion of karyokinesis, myosin staining was also apparent in the cleavage furrow during cytokinesis in cardiac myocytes (Fig. 38a). Furthermore, FGF-2 staining reappeared around the nuclei of daughter cells (Fig. 38c). In the case of binucleated cardiac myocytes sarcomeric myosin staining was apparent in the internuclear zone (Fig. 38d). Thus, these results suggest that the transfection process does not interfere with myocyte cell division. Also, these data suggest that relative distribution of myosin and FGF-2 staining can vary depending on the stage of the cell cycle in cardiac myocytes.

7.5.2 Effects of high and low molecular weight forms of FGF-2 on the sarcomeric structure in rat cardiac myocytes

In light of variations in the relative distribution of endogenous myosin and FGF-2 during myocyte cell division, the effects of overexpression of both high and low molecular weight forms of FGF-2 on the sarcomeric structure were examined in neonatal rat cardiac myocytes after gene transfer. Cells staining intensely for FGF-2 indicating overexpression were observed in cultures transfected with FGF-2 cDNAs, in contrast to cultures transfected with the RSVp.CONT and untransfected cultures. Myocytes overexpressing either high or low molecular weight FGF-2 cDNAs displayed nuclear FGF-2 staining (Fig. 39) except that myocytes overexpressing the low molecular weight form also revealed intense and uniform anti-FGF-2 staining in the cytoplasm (Fig. 39c). Myosin staining was diffuse and cytoplasmic in about 50% of cardiac myocytes (n=3) overexpressing either high or low molecular weight forms of FGF-2 (Fig. 39e,f) compared to characteristic sarcomeric staining observed in non overexpressing myocytes (Fig. 39d). These results suggest that overexpression of either high or low molecular weight FGF-2 could decrease the relative levels of myosin in cardiac myocytes. Similar to this, a recent report also indicated that the treatment of adult rat ventricular myocyte cultures exogenously with FGF-2 (recombinant low molecular weight form) led to a restriction in myofibrillar staining for F-actin and myomesin (myosin binding protein present at the central M line of muscle thick filament) with a sharp boundary to the perinuclear region compared to the normal cytoplasmic staining observed in the control myocytes (Harder *et al.*, 1996).

Fig. 38

Fig. 38. Distribution of myosin, DNA and FGF-2 staining in cardiac myocytes undergoing cytokinesis (a-c) and binucleation (d-f). Triple staining was done for myosin (a,d), DNA (b,e) and FGF-2 (c,f). Note the reappearance of perinuclear FGF-2 staining in the daughter cells (c) and presence of sarcomeric myosin staining in the internuclear zone of binucleated cardiac myocyte (d). Bar is equvalent to 20 μm.

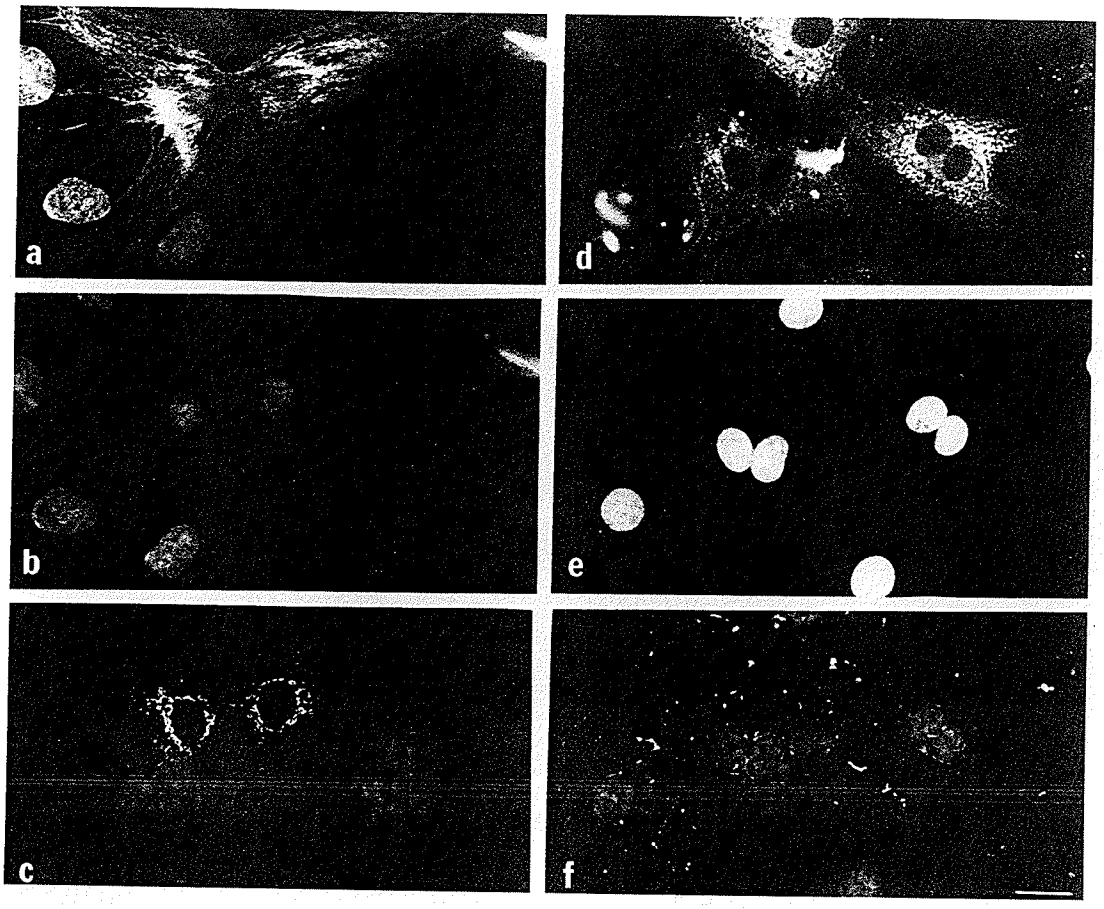
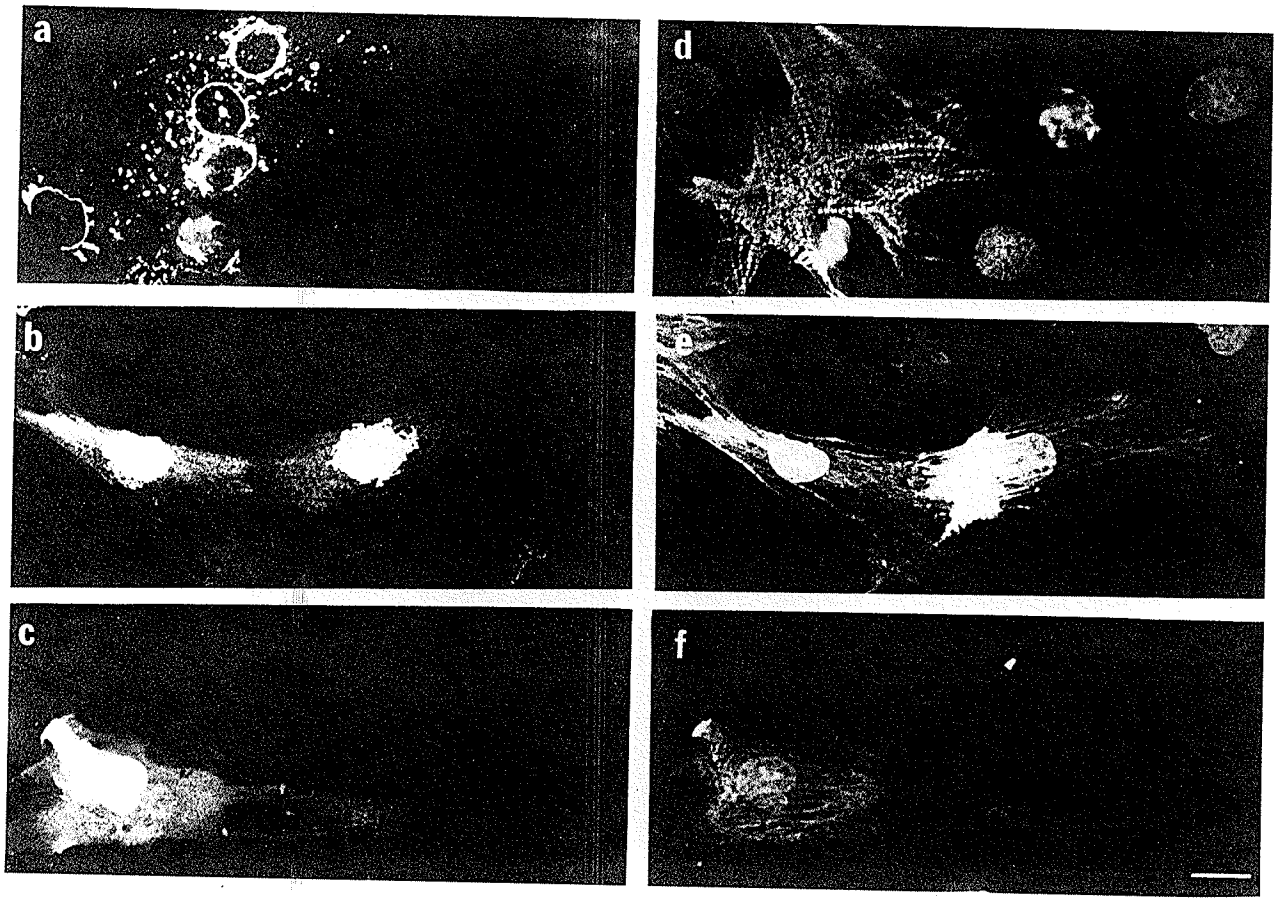

a
d
b
e
c
f

Fig. 39

Fig. 39. Overexpression of high or low molecular weight forms of FGF-2 leads to disorganization of sarcomeric structure in transfected postnatal rat cardiac myocytes. Triple staining was done for FGF-2 (a-c) or myosin and bromodeoxyuridine (d-f). Cardiac myocytes from cultures transfected with RSVp.CONT (a,d), RSVp.FGF (b,e) and RSVp.metFGF (c,f). Note the presence of sarcomeric structure in myocytes transfected with RSVp.CONT (d) in contrast to the disorganization of sarcomeric structure in myocytes overexpressing either high (e) or low (f) molecular weight forms of FGF-2. Bar is equvalent to 20 μm.


a
b
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e
f

7.5.3 Effects of high and low molecular weight forms of rat FGF-2 on myocyte specific protein synthesis

The effects of overexpression of high and low molecular weight forms of rat FGF-2 on the levels of myocyte-specific proteins were also assessed in postnatal cardiac myocyte cultures after gene transfer. The levels of myosin, desmin and sarcomeric actin (muscle differentiation markers) in the transfected cultures were determined using SDS-PAGE followed by immunoblotting with specific antibodies (Fig. 40). The myosin (200 kD), desmin (55 kD) and sarcomeric actin (45 kD) bands detected in the lysates of transfected cultures (n=5-8) were scanned by densitometry. The levels of differentiation markers in the lysates of cultures transfected with FGF-2 cDNAs (Fig. 40; lanes b,c,d) were compared to the levels in the RSVp.CONT lysate (lane a), which was arbitrarily set to 100% (Fig. 41). Significant decreases (~ 2 fold) in myosin levels were observed in cultures (Fig. 41; n=8) transfected with RSVp.FGF (45%, $p<0.01$), RSVp.ΔmetFGF (45%, $p<0.01$) and RSVp.metFGF (50%, $p<0.01$) compared to cells transfected with the RSVp.CONT (100%). Desmin levels were also decreased (Fig. 41) significantly (~ 2.5 fold) in cultures (n=5) transfected with RSVp.FGF (41%, $p<0.03$), RSVp.ΔmetFGF (36%, $p<0.01$) and RSVp.metFGF (44%, $p<0.03$) compared to cells transfected with the RSVp.CONT (100%). However, sarcomeric actin levels did not vary significantly in cultures (Fig. 41; n=5) transfected with either high or low molecular weight FGF-2 cDNAs compared to the control cultures. There was no significant difference between the effects of overexpression of high and low molecular weight FGF-2 on both myosin and desmin synthesis in rat cardiac myocytes.

These results are consistent with my previous observations that the overexpression of high or low molecular weight FGF-2 in embryonic ventricular myocyte cultures also led to a significant decrease in myosin levels (see section 5.5.5). It is possible that the decreases in myosin and desmin levels in postnatal cardiac myocyte cultures overexpressing either high or low molecular weight FGF-2 could be mediated in a paracrine manner. FGF-2 was shown to repress myosin heavy chain synthesis in G1 myoblast cultures (MM14) in the presence or absence of serum (Clegg *et al.*, 1987). In addition, FGF-2 was shown to inactivate muscle-specific transcription through phosphorylation of a protein kinase C site in myogenic helix-loop-helix proteins (Li *et*

Fig. 40

Fig. 40. Levels of differentiation markers myosin, desmin and α-sarcomeric actin in transfected postnatal cardiac myocyte cultures. Total lysates (~10 μg) from transfected cultures (n=5-8) were fractionated in a 7.5% (for myosin) or 12.5% (for desmin and actin) SDS-PAGE gel, immunoblotted, probed with specific antibodies to visualize myosin (200 kD), desmin (55 kD) and α-sarcomeric actin (45 kD) bands. Lysates from cultures transfected with RSVp.CONT, lane a; RSVp.FGF, lane b; RSVp.ΔmetFGF, lane c; RSVp.metFGF, lane d. Although, the level of α-sarcomeric actin in lane a, appears to be more compared to that of other lanes (b-d), cumulative results from 5 independent transfections (see Fig. 41) revealed no significant difference between α-sarcomeric actin levels of cultures transfected with high or low molecular weight FGF-2 cDNAs and control plasmid.

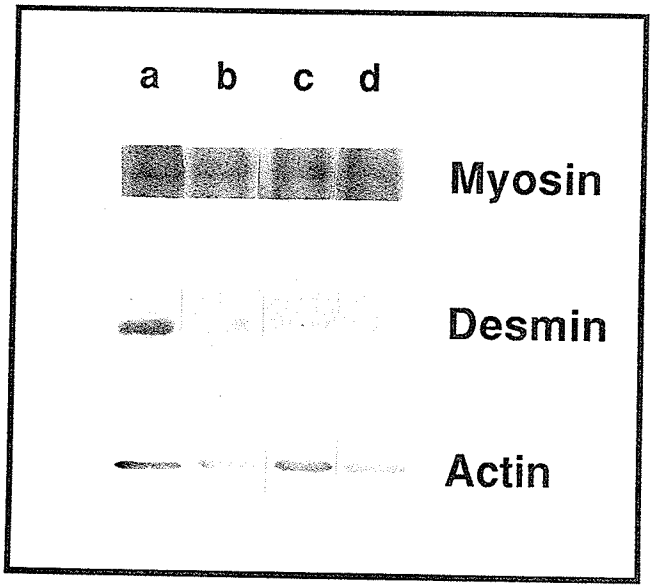
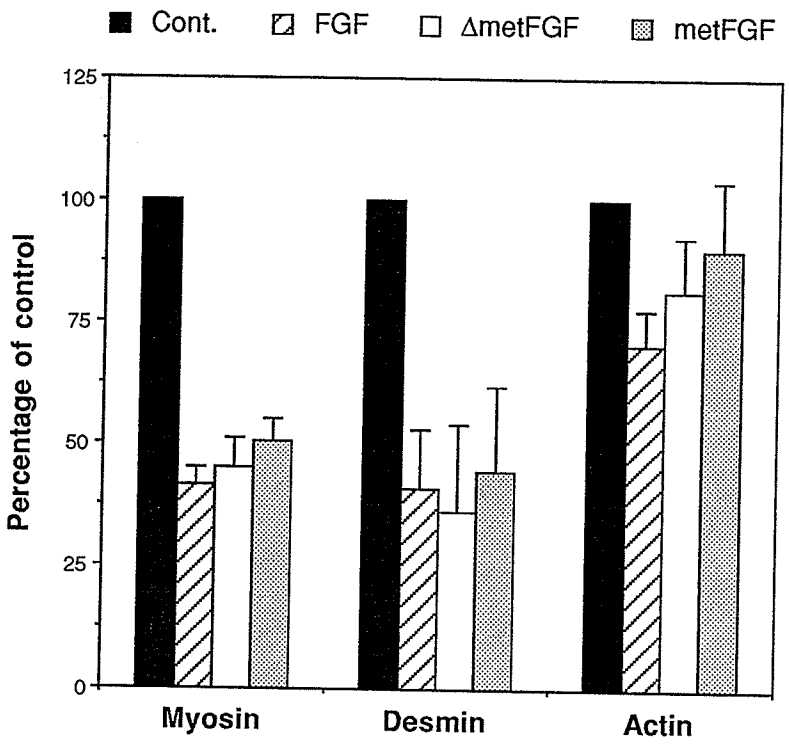

a b c d
Myosin
Desmin
Actin

Fig. 41

Fig. 41. Overexpression of high or low molecular weight forms of FGF-2 decreases the levels of differentiation markers in transfected postnatal rat cardiac myocyte cultures. Myosin, desmin and α-sarcomeric actin bands in transfected cardiac myocyte cultures were visualized by immunoblotting with specific antibodies and their levels were determined by scanning 200, 55, and 45 kD bands respectively. The levels of differentiation markers in cultures transfected with FGF-2 cDNAs were compared to the levels in the control cultures which were arbitrarily set to 100. Significant decreases in the levels of myosin (~2.1 fold, $p<0.05$, n=8) and desmin (~2.4 fold, $p<0.05$, n=5) but not alpha sarcomeric actin (n=5) were observed in cultures transfected with FGF-2 cDNAs compared to the controls. There is no significant difference between the effects of overexpression of high and low molecular weight FGF-2 on both myosin and desmin levels.


Cont.
FGF
ΔmetFGF
metFGF
Percentage of control
125
100
75
50
25
0
Myosin
Desmin
Actin

al., 1992). Although less is known about cardiac myogenic proteins (Olson and Srivastava, 1996), it is possible that a similar role for FGF-2 exists in cardiac myocytes. Furthermore, it was postulated that a Z-band-specific protease is activated to facilitate the breakdown of sarcomeres in dividing cardiac myocytes (Rumyantsev, 1991). Thus, it is possible that the decreased levels of myosin and desmin in rat cardiac myocyte cultures overexpressing high or low molecular weight FGF-2 could be consequences of the increased proliferative state of transfected myocytes (see sections 6.5.3 to 6.5.5). Similarly desmin expression can also be influenced by the proliferative state of cardiac myocytes. Embryonic chicken cardiac myocytes (Hamburger and Hamilton's stage 17) are less differentiated and reported to be desmin-negative compared to the differentiated cardiac myocytes (stage 25) which show a sharp increase in desmin levels (Gamiz *et al.*, 1993). It is interesting to note that alpha sarcomeric actin levels are not significantly influenced by overexpression of either high or low molecular weight forms of FGF-2. The effect of overexpression of FGF-2 on myosin and desmin but not actin implies a selective mechanism for regulation of sarcomeric proteins/structure in cardiac myocytes. Interestingly, exogenous administration of FGF-2 in adult rat ventricular myocyte cultures was shown to increase the levels of both α-sarcomeric actin (~1.3 fold) and α-smooth muscle actin (~6 fold) compared to the control cultures (Harder *et al,* 1996). The overall increase in all isoforms of actin (α-sarcomeric and α-smooth muscle actins) in addition to a dense packing of α-smooth muscle actin surrounding the myofibrillar apparatus was believed to restrict the myofibrillar growth to the perinuclear region in adult cardiac myoctes treated with FGF-2 (Harder *et al.*, 1996). Although exogenous FGF-2 treatment led to a smaller increase in α-sarcomeric actin (Harder *et al.*, 1996), my results did not reveal any significant difference in the levels of α-sarcomeric actin between transfected and control cultures and this discrepancy raises the possibility that the exogenously added FGF-2 and FGF-2 that is overexpressed within the transfected myocyte may utilize different mechanisms for the regulation of myocyte growth and differentiation.

7.5.4 Effects of low molecular weight form of FGF-2 on the RNA levels of some muscle-specific proteins

It is possible that the decreased levels of differentiation markers in postnatal myocyte cultures overexpressing FGF-2 may also reflect changes in the expression pattern of muscle-specific protein isoforms and thus a possible recapitulation of embryonic phenotype. In order to investigate this possibility, total RNA was isolated from the cultures transfected with RSVp.metFGF and RSVp.CONT. As an additional control, RNA was also isolated from the cultures treated exogenously with recombinant human FGF-2 (10 ng/ml; UBI, Lake Placid, NY). In addition, the transfected cultures were maintained in the presence of either 10μg/ml anti-bovine FGF-2 type 1 antibodies or 10 μg/ml normal mouse (NM) antibodies for 48 hours before harvesting for RNA, to investigate whether the effects (if any) are mediated through either paracrine or intracrine pathways similar to the effects of FGF-2 on DNA synthesis and binucleation (see sections 5.5.3. to 5.5.6). RSVp.metFGF was chosen because (i) the effects of both high and low molecular weight forms of FGF-2 on muscle protein synthesis were similar as indicated by immunofluorescence (Fig. 39) as well as immunoblotting data (Figs. 40, 41), (ii) expression of low molecular weight FGF-2 from RSVp.metFGF was consistently higher (>5 fold) than the expression of high molecular weight forms from either RSVp.FGF or RSVp.ΔmetFGF (see Fig. 22b; section 6.5.1) and (iii) the paracrine mediated effects of low molecular weight FGF-2 on DNA synthesis were significantly higher (1.9 fold, $p<0.01$) than those of high molecular weight FGF-2 (see Fig. 29; section 6.5.5.2).

The RNA levels of α-MHC, ß-MHC and ANF in the transfected cultures or treated exogenously with human FGF-2 (n=3) were assessed by northern blotting using specific radiolabelled probes (Fig. 42). RNA loading was normalized by probing with a radiolabelled cDNA for 28S. A band of ~6.0 kb was detected with both α-MHC and ß-MHC probes and a band of ~0.9 kb was detected with an ANF probe in the RNA samples isolated from the transfected cultures (n=3). The bands were scanned by densitometry and the levels of RNA in cultures transfected with RSVp.metFGF were compared to the levels in the RSVp.CONT cultures after normalizing with values obtained using 28S probe (Figs. 43, 44). Significant decreases in α-MHC RNA levels were observed in cultures overexpressing low molecular weight form of FGF-2 (1.8 fold, $p<0.03$) compared to

Fig. 42

Fig. 42. RNA levels of differentiation markers α-MHC, ß-MHC and ANF in transfected postnatal cardiac myocyte cultures. Total RNA was isolated from cultures (n=3) transfected with RSVp.CONT (lanes a,b) or RSVp.metFGF (lanes c,d) and maintained in the presence of normal mouse antibodies (lanes a,c) or neutralizing antibodies to FGF-2 (lanes b,d) as well as from cultures treated exogenously with 10 ng/mL of recombinant human 18 kD FGF-2 (lane e). RNA (~15 μg) was fractionated in a 1.5% agarose gel containing formaldehyde, blotted to nitrocellulose and assessed with radiolabelled probes for α-MHC, ß-MHC and ANF. RNA loading was normalized by probing with a radiolabelled cDNA for 28S. Transcripts were visualized by autoradiography.

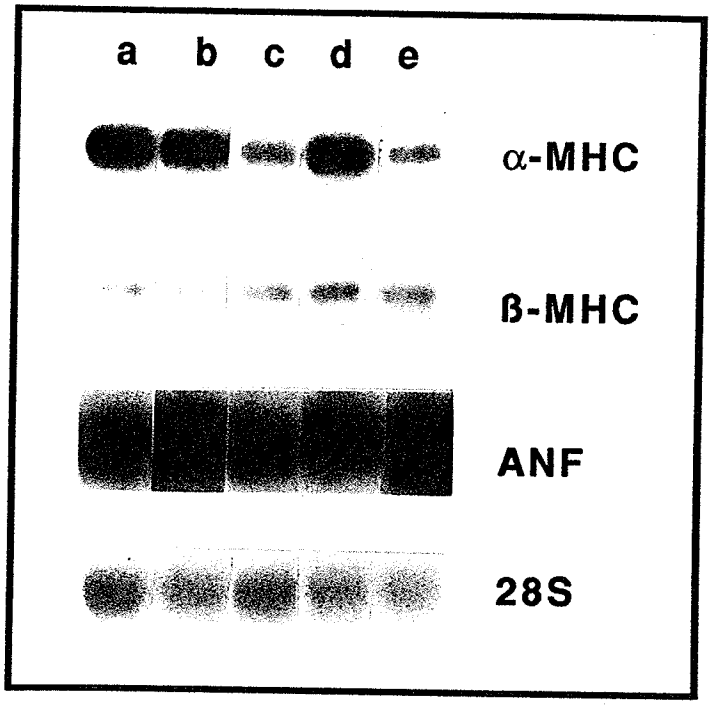

a b c d e
α-MHC
ß-MHC
ANF
28S

Fig. 43

Fig. 43. Both overexpression of rat 18 kD FGF-2 as well as exogenous treatment of recombinant human 18 kD FGF-2 decrease the expression of α-MHC but not ß-MHC in postnatal cardiac myocyte cultures. Transcripts of α-MHC and ß-MHC in postnatal cardiac myocyte cultures were visualized by RNA blotting with specific probes and their relative levels were determined by densitometry. RNA loading was normalized by using the signals obtained with 28S. There was a significant decrease (1.8 fold, $p<0.03$) in α-MHC RNA levels of cultures (n=3) transfected with RSVp.metFGF compared to RSVp.CONT in the presence of NM Ab and this decrease was blocked in the presence of neutralizing antibodies to FGF-2. Similarly treatment of cardiac myocytes exogenously with 10 ng/mL of recombinant human 18 kD FGF-2 resulted in a significant decrease (2.3 fold, $p<0.02$) in α-MHC RNA levels compared to the control cultures. However, both transfected rat 18 kD FGF-2 or exogenously treated human 18 kD FGF-2 did not effect the RNA levels of ß-MHC compared to the control values.

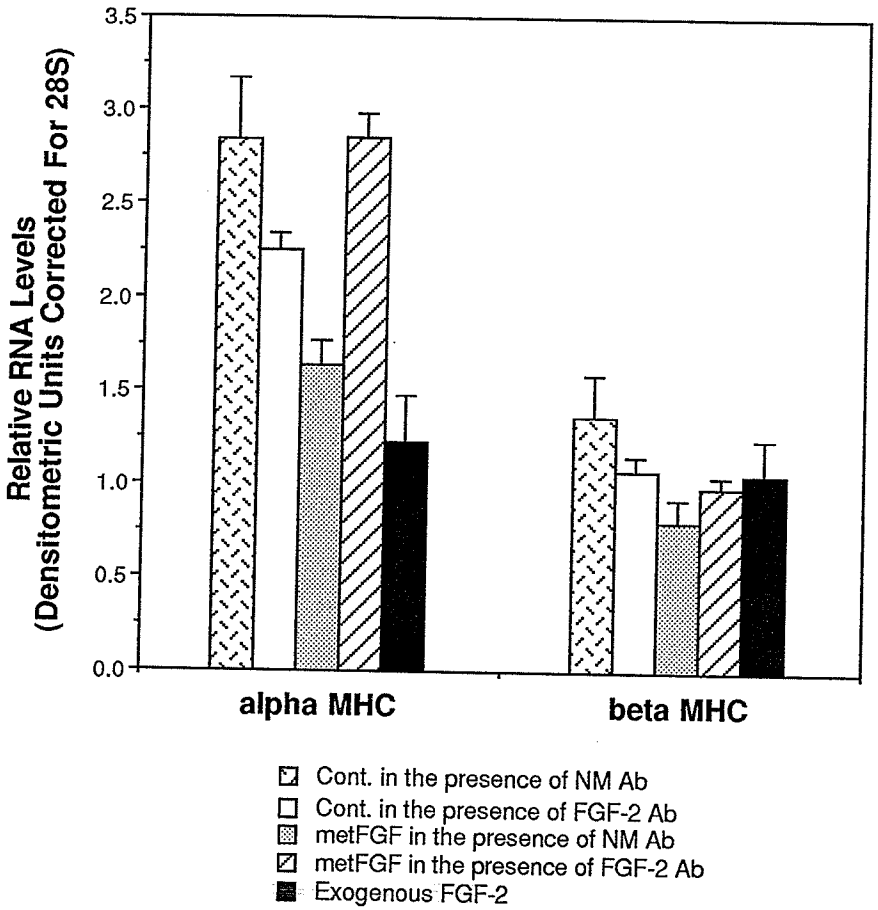
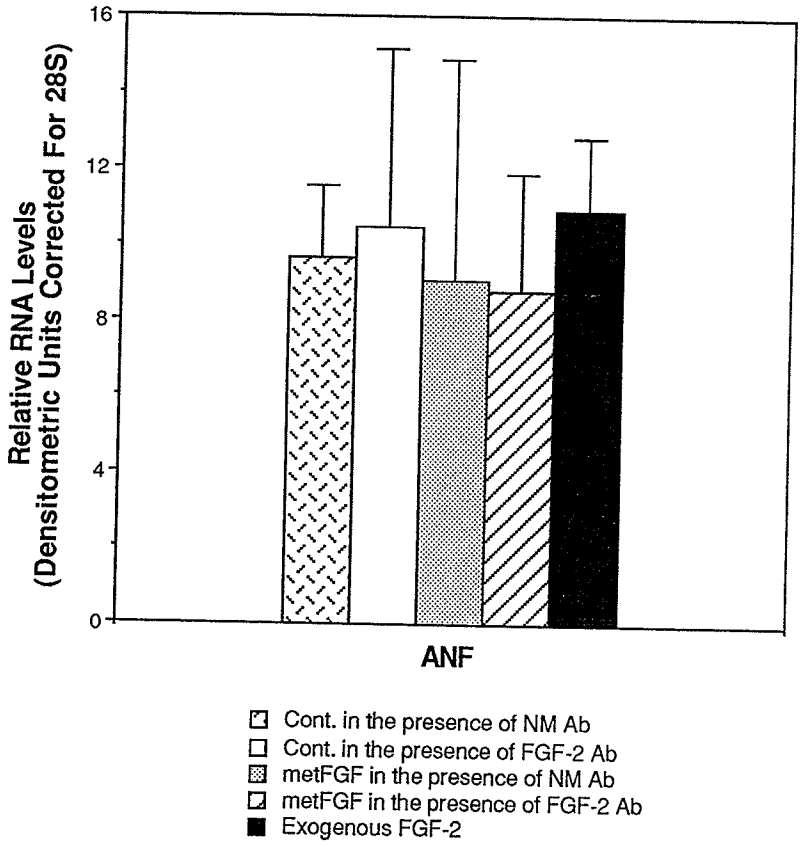

Relative RNA Levels
(Densitometric Units Corrected For 28S)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
alpha MHC
beta MHC
Cont. in the presence of NM Ab
Cont. in the presence of FGF-2 Ab
metFGF in the presence of NM Ab
metFGF in the presence of FGF-2 Ab
Exogenous FGF-2

Fig. 44

Fig. 44. Overexpression of rat 18 kD FGF-2 did not effect the expression of ANF in postnatal cardiac myocyte cultures. Expression of ANF RNA in postnatal cardiac myocyte cultures (n=3) was visualized by RNA blotting with a specific radiolabelled cDNA probe and the relative levels were determined by densitometry. RNA loading was normalized by using the signals obtained with 28S. Both overexpression of rat 18 kD FGF-2 and exogenous treatment of human 18 kD FGF-2 did not effect the RNA levels of ANF either in the presence or absence of neutralizing antibodies to FGF-2 compared to the control values.


16
12
8
4
0
Relative RNA Levels
(Densitometric Units Corrected For 28S)
ANF
Cont. in the presence of NM Ab
Cont. in the presence of FGF-2 Ab
metFGF in the presence of NM Ab
metFGF in the presence of FGF-2 Ab
Exogenous FGF-2

cultures transfected with the RSVp.CONT (Fig. 43). This decrease in α-MHC RNA levels of RSVp.metFGF cultures was blocked in the presence of neutralizing antibodies for FGF-2 (Fig. 43). Further, there was no significant difference between the RNA levels of RSVp.metFGF cultures in the presence of FGF-2 antibody and that of RSVp.CONT cultures in the presence or absence of FGF-2 antibody. Thus, these results suggest that low molecular weight form of FGF-2 can decrease the RNA levels of α-MHC by acting in a paracrine pathway. These data obtained from the transfected cultures were also consistent with a significant decrease (2.3 fold, $p<0.02$) in α-MHC RNA levels of cultures treated exogenously with human FGF-2 (Fig. 43) as well as with decreased levels of total myosin protein in the transfected cultures (see section 7.5.3; Figs. 40, 41).

After normalization with 28S ribosomal RNA signal, RNA levels of ß-MHC and ANF did not vary significantly in cultures transfected with RSVp.metFGF or cultures treated with exogenous FGF-2 compared to the RSVp.CONT cultures (Figs. 43 and 44). It was shown that the exogenous administration of FGF-2 in rat cardiac myocyte cultures induced expression of ß-MHC (Parker *et al.*, 1990a), ANF (Parker *et al.*, 1990b) and decreased the levels of α-MHC (Parker *et al.*, 1990a). However, this discrepancy between my results and the previously published studies (Parker *et al.*, 1990a,b) could be due to differences in the RNA analysis methods as well as culture conditions. Although, ethidium bromide stained 28S RNA was shown for comparison in their studies, Parker *et al.*, expressed their results relative to the expression in control cells without any normalization to 28S RNA levels. A similar comparison of the absolute RNA levels (without normalization) of ß-MHC and ANF in cultures transfected with RSVp.metFGF and RSVp.CONT would certainly indicate an increase in the expression of ß-MHC in cultures overexpressing 18 kD FGF-2 (Fig. 42). In addition, Parker *et al.* (1990a) performed their northern blot analysis using RNA pooled from four cultures and this may not necessarily represent the variations between experiments. Furthermore, the discrepancy between my results and published studies (Parker *et al.*, 1990a,b) could be attributed to different amounts of FGF-2 (10 ng/ml versus 25 ng/ml) used for exogenous stimulation as well as developmental stage (1 day versus 2 day postnatal) of hearts used for ventricular myocyte cultures. Similarly, it is possible that a higher level of low molecular weight FGF-2 expression may influence the levels of ß-MHC and ANF in transfected cultures. Although

not established rigorously, my RNA analyses results suggest that the ventricular myocyte cultures transfected with RSVp.metFGF reveal decreased levels of α-MHC with no changes in ß-MHC and ANF RNA levels.

7.6 Concluding remarks

In summary, these results suggest that (i) subcellular distribution of endogenous myosin as well as FGF-2 in cardiac myocytes vary during cell division (ii) sarcomeric structure of myosin disorganizes during cardiac myocyte division, (iii) overexpression of both high and low molecular weight forms of FGF-2 in postnatal rat cardiac myocytes can lead to significant decreases in the protein levels of myosin and desmin but not α-cardiac actin, (iv) overexpression of low molecular weight form of FGF-2 in postnatal rat cardiac myocytes can lead to significant decreases in the RNA levels of α-MHC in a paracrine manner, and (v) overexpression of FGF-2 in cardiac myocyte cultures can lead to a decreased state of differentiation as indicated by decreases in the protein levels of myosin and desmin as well as RNA levels of α-MHC.

Chapter 8

Cloning and sequencing of the 5´-flanking region of rat FGF-2 gene

8.1 Background

Basic fibroblast growth factor (FGF-2) is a potent mitogen (Baird, 1994) and known to regulate normal physiological processes which include morphogenesis, organogenesis, neovascularization and responses to wounding (Baird and Bohlen, 1990). FGF-2 plays a vital role in the cardiogenesis (Sugi *et al.*, 1993) and is shown to stimulate the formation of collateral vessels in the experimental infarct models due to its angiogenic property (Yanagisawa-Miwa *et al.*, 1992). This growth factor has been shown to possess cardioprotective properties during ischemic insults or myocardial infarctions (Battler *et al.*, 1993; Harada *et al.*, 1994; Padua and Kardami, 1993; Yanagisawa-Miwa *et al.*, 1992). In addition, the data from my previous studies (chapters 5 and 6) and other studies (Harder *et al.*, 1996; Kardami *et al.*, 1993; Sugi *et al.*, 1993) suggest that FGF-2 is involved in the growth and differentiation of embryonic as well as postnatal cardiac myocytes. All these properties make FGF-2 a potential candidate to stimulate cardiac myocyte growth and offer protection during myocardial damage. FGF-2 has also been implicated in tissue regeneration (Baird, 1994; Presta *et al.*, 1989; Sievers *et al.*, 1987). It is abundant in the developing and adult brain (Powell *et al.*, 1991; Riva and Mocchetti, 1991) and required for the origin and growth of neuronal and glial cells (Sherman *et al.*, 1991; Vescovi *et al.*, 1993). Glial cells produce high levels of FGF-2, as well as its high affinity receptor and thus proliferation of these cells is stimulated in an autocrine manner (Gerdes *et al.*, 1992). FGF-2 produced from glial cells was also shown to promote the survival and function of neurons in a paracrine manner (Morrison *et al.*, 1986; Walicke *et al.*, 1986). Abnormal levels of FGF-2 have been linked with neurodegenerative diseases such as Alzheimer's disease (Cotman and Gomez-Pinilla, 1991), Huntingtons's disease (Tooyama *et al.*, 1993a) and Parkinson's disease (Tooyama *et al.*, 1993b). Clearly, this potent mitogen must be tightly regulated in the normal conditions to avoid uncontrolled proliferation.

8.2 Rationale

FGF-2 is involved in the growth and differentiation of several tissues including those of cardiovascular and central nervous systems. This growth factor has several desirable properties to stimulate cardiac myocyte proliferation, angiogenesis and neuronal survival during disease conditions. Thus, application of FGF-2 either in the form of purified protein or by gene transfer may bring about a therapeutic modality for diseases involving those tissues. However, this approach may not be suitable for diseases in which FGF-2 levels are upregulated above the physiological levels [eg. Alzheimer's and Huntington's diseases (Cotman and Gomez-Pinilla, 1991; Tooyama *et al.*, 1993a), diabetic complications (Karpen *et al.*, 1992) and gliomas (Takahashi *et al.*, 1991, 1992)] and identification of factors that can regulate the endogenous levels of FGF-2 may offer a solution in such cases. However, there is scant information about the regulatory mechanisms governing the endogenous levels of FGF-2. Recent studies employing rat astroglial and hippocampal primary culture cells as well as the rat C6 glioma cell line, indicated that transcriptional activation of the FGF-2 gene occurs in response to mitogenic stimuli such as the addition of serum or phorbol ester (Flott-Rahmel *et al.*, 1992; Powell and Klagsbrun, 1993). With a view to understanding the regulation of FGF-2 gene transcription, the 5′-flanking region of the rat FGF-2 gene was cloned, sequenced and the promoter region was characterized. In addition, there was a discrepancy between the reported 5′ ends of rat brain (Kurokawa *et al.*, 1988) and ovarian (Shimasaki *et al.*, 1988) FGF-2 cDNA sequences. Hence, the 5′ end of rat FGF-2 mRNA was also mapped using the probes specific for 5′-flanking and coding regions as well as primer extension analysis.

8.3 Hypothesis

The 5′-flanking region of rat FGF-2 gene contains regulatory elements that respond to mitogenic stimuli (eg. serum, protein kinase C activators). As a prerequisite to testing this hypothesis, the 5′-flanking region of the rat FGF-2 gene has to be cloned.

8.4 Specific Aims

1. To clone the 5′-flanking region of rat FGF-2 gene.
2. To identify the transcriptional initiation site(s) in rat FGF-2 gene.

3. To sequence the 5′-flanking region of rat FGF-2 gene.

4. To partially characterize the regulatory elements present in the 5′-flanking region of rat FGF-2 gene.

8.5 Results and Discussion

8.5.1 Cloning of the 5′-flanking region of rat FGF-2 gene

The rat FGF-2 cDNA (~1 kb, Shimasaki *et al.*, 1988) and a 350 bp *Xho* 1/*Sma* 1 fragment from the 5′ end of FGF-2 cDNA were used as probes to screen a Sprague Dawley rat testis genomic library. Five positive clones (λrFGF2-c1 to λrFGF2-c5) were obtained with the full length FGF-2 cDNA probe and only three of them (λrFGF2-c3 to λrFGF2-c5) hybridized to 350 bp *Xho* 1/*Sma* 1 fragment from the 5′ end of cDNA (see Fig. 45) suggesting that these three clones were more likely to contain the 5′-flanking sequences of rat FGF-2 gene. Partial restriction mapping revealed that the clone λrFGF2-c4 contained the largest insert (~10 kb, Fig. 46) and therefore, this clone was selected for further analysis. Restriction endonuclease digestion of phage DNA from λrFGF2-c4 with *Bam*H1 revealed 5 bands (B1-B5), ranging in size from ~1 to 5 kb by gel electrophoresis (see Figs. 46, 47 and 48). Following DNA (Southern) blotting, a strong hybridization signal was seen with a band (B2) of 1.4 kb using a radiolabelled FGF-2 cDNA or *Xho* 1/*Sma* 1 fragment probes (Fig. 46, lanes c,g,k). Both probes hybridized to a 4 kb band in the *Xho* 1 digest of clone λrFGF2-c4 (Fig. 46, lane h) but not with that of clone λrFGF2-c3 or 5 (lanes d,l). From these partial restriction mapping results it is conceivable that the 4 kb *Xho* 1 fragment in clone λrFGF2-c4 contains at least a part or all of the B2 sequence and therefore can be used for further mapping of sequences flanking B2 fragment (see section 8.5.2 below). The FGF-2 cDNA probe also hybridized to a 1 kb band (B1) weakly even after high stringency washings (Fig. 47, lane g). Based on the restriction mapping results, the immediate 5′-flanking region of the rat FGF-2 cDNA (Shimasaki *et al.*, 1988) is most likely to be found in the 1.4 kb (B2; Fig. 47, lane c) fragment and 1 kb (B1; Fig. 47, lane c) fragment may contain a part of rat FGF-2 coding sequence. Therefore, the 1.4 kb (B2) and 1 kb (B1) fragments were subcloned into the *Bam*H1 site of the plasmid pUC119 (Fig. 47, lanes a,b).

Fig. 45

Fig. 45. Isolation of genomic clones that can hybridize to both full length and 5′ end specific probes of the rat FGF-2 cDNA. A rat testis genomic library was screened with a 1 kb rat FGF-2 cDNA (Shimasaki *et al.*, 1988) and a 350 bp fragment from the 5′ end of cDNA probes. Five clones (λrFGF2-c1 to λrFGF2-c5) were positive with 1 kb cDNA and only three (λrFGF2-c3 to λrFGF2-c5) of them hybridized to the 5′ end of cDNA probe. The figure shows paired examples of genomic filters screened with both probes. λrFGF2-c1 clone showed positive hybridization with only 1 kb cDNA probe, in contrast, λrFGF2-c4 clone was positive for both cDNA as well as 5′ end specific probes.

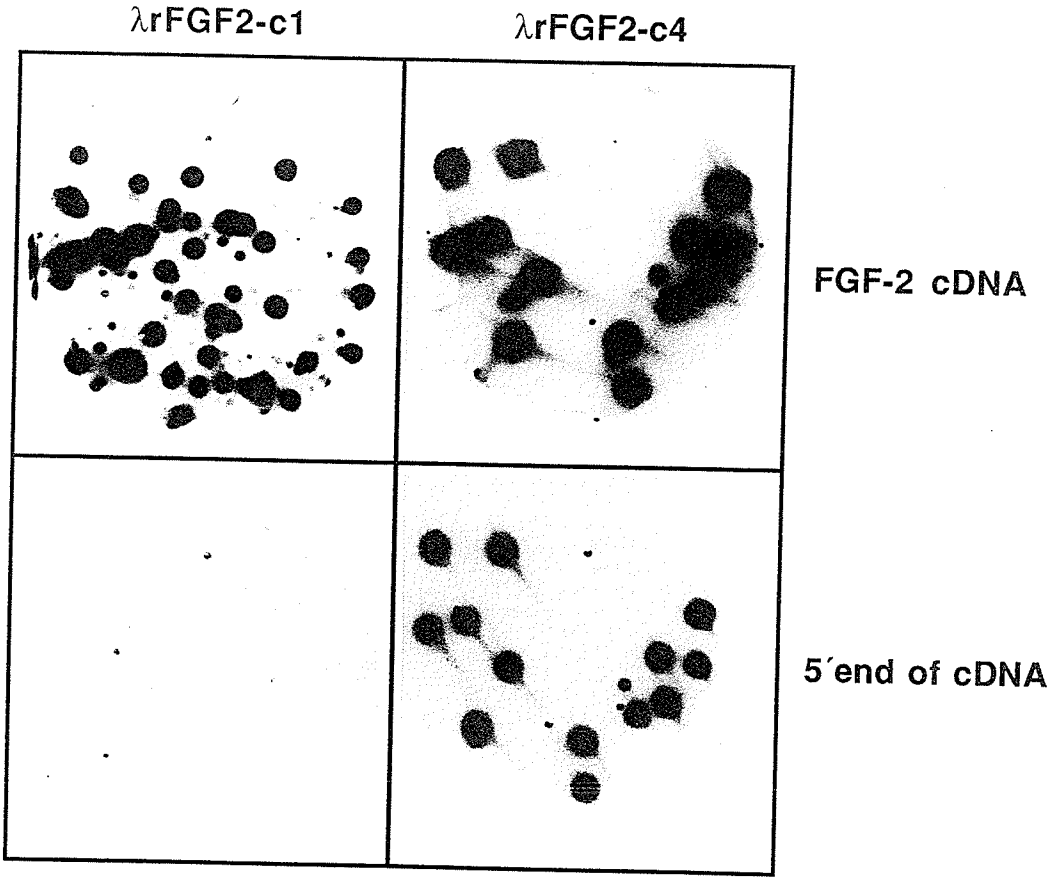

λrFGF2-c1
λrFGF2-c4
FGF-2 cDNA
5´end of cDNA

Fig. 46

Fig. 46. Partial restriction mapping of three genomic clones (λrFGF2-c3 to λrFGF2-c5) that were positive for hybridization with the 5′ end of rat FGF-2 cDNA. Genomic DNA (25 μg) from three clones was digested with: *Bam*H1 (a,e,i) or *Xho*1 (b,f,j) restriction endonucleases. The DNA was electrophoresed in a 1% agarose gel (lanes a,b,e,f,i,j) and transferred to nitrocellulose (lanes c,d,g,h,k,l). The 1 kb rat FGF-2 cDNA or 5′ end of cDNA were radiolabelled by random priming and used for hybridization. Solid arrowheads indicate the position of a 1.4 kb (B2) band in *Bam*H1 digests of all clones (lanes c,g,k), that hybridized strongly with both probes. Open arrow head indicates the position of a 4 kb band in *Xho*1 digest of λrFGF2-c4 clone (lanes f,h). Molecular sizes were derived from λ/*Hin*dIII digests.

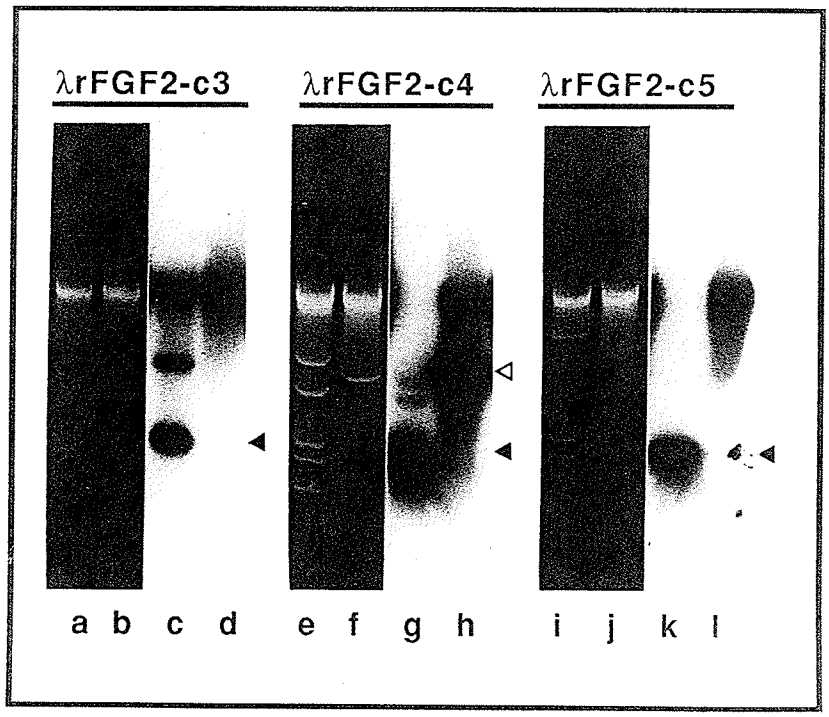
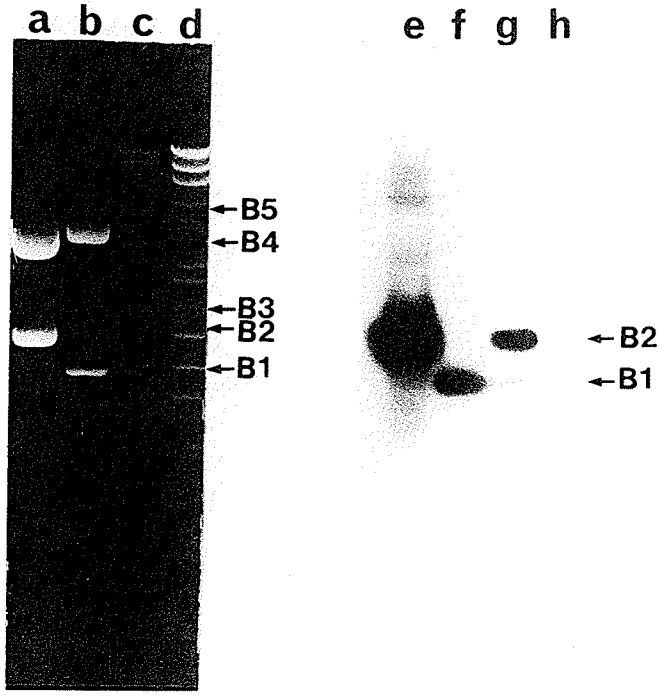

λrFGF2-c3
λrFGF2-c4
λrFGF2-c5
a b c d e f g h i j k l

Fig. 47

Fig. 47. Subcloning of 1 kb (B1) and 1.4 kb (B2) fragments of λrFGF2-c4 clone which showed positive hybridization with rat FGF-2 cDNA. The 1 kb (B1) and 1.4 kb (B2) fragments of λrFGF2-c4 clone were gel purified and subcloned in *Bam*H1 site of pUC119. Plasmid DNA (~1 μg) from B2 (lane a) and B1 (lane b) subclones as well as genomic DNA (~25 μg) from λrFGF2-c4 clone (lane c) were digested with *Bam*H1. The DNA was electrophoresed in a 1% agarose gel (lanes a,b,c) together with a molecular size marker λ/*Hin*dIII digest (lane d) and transferred to nitrocellulose (lanes e,f,g,h). The 1 kb rat FGF-2 cDNA was radiolabelled by random priming and used for hybridization. The positions of B1 (1 kb), B2 (1.4 kb), B3 (1.6 kb), B4 (3.3 kb) and B5 (4.3 kb) fragments in *Bam*H1 digest of λrFGF2-c4 (lane c) were indicated. Both1.4 and 1 kb bands in *Bam*H1 digests of B2 (lane a) and B1 (lane b) subclones hybridized strongly with rat FGF-2 cDNA (lanes e,f). Although, B2 fragment in *Bam*H1 digest of λrFGF2-c4 clone (lane g) hybridized strongly with cDNA probe, B1 fragment showed a weak hybridization signal (lane g).


a b c d
e f g h
B5
B4
B3
B2
B1
B2
B1

8.5.2 Identification of sequences flanking the 1.4 kb rat FGF-2 genomic fragment

In order to identify both 5′ and 3′ sequences that are flanking the 1.4 kb B2 fragment in λrFGF2-c4, phage DNA was digested with *Xho*1 based on partial restriction mapping results (see section 8.5.1), and hybridized to radiolabelled B1-B5 probes. Digestion of phage DNA from λrFGF2-c4 with *Xho*1 revealed 2 bands of 4 kb and 0.7 kb by gel electrophoresis (Fig. 46, lane f). The 4 kb band in the *Xho*1 digest revealed positive hybridizations with B1 (1 kb), B2 (1.4 kb) and B3 (1.6 kb) probes but not with B4 (~3.3 kb) or B5 (~4.3 kb) probes (see Fig. 47, lane c), suggesting that B1, B2 and B3 are contiguous sequences. Based on hybridization results (see Figs. 46, 47), B2 must be located 5′ to the B1 fragment as B2 but not B1 hybridizes to the 5′ end of cDNA. Partial restriction mapping of B1 and B2 subclones indicates that B1 has at least one *Xho*1 site but not B2. Therefore, the B3 sequence can not be contiguous to or downstream of the B1 sequence in the 4 kb *Xho*1 fragment of λrFGF2-c4 and, thus, must be located 5′ to the B2 sequence. A physical map of the genomic clone (λrFGF2-c4) and a partial restriciton map of the B2 fragment are shown in Fig. 48.

8.5.3 Genomic organization of the 5′-flanking region of rat FGF-2 gene

To establish the identity of 1.4 kb fragment (B2) in rat genomic sequences, Sprague Dawley rat liver DNA was digested with *Bam*H1, *Eco*R1 and *Xba*1, transferred to nitrocellulose and probed with radiolabelled 1.4 kb B2 fragment. As a positive control, genomic DNA from λrFGF2-c4 was also digested with the same enzymes and probed with B2. Based on the restriction map (Fig. 48b), bands of predicted sizes (1.4 kb with *Bam*H1; 1.2 kb and 0.2 kb with *Bam*H1/*Eco*R1; 1.15 kb and 0.25 kb with *Bam*H1/*Xba*1,) were detected in rat genomic DNA (Fig. 49). Additional bands of 6.6 and 2 kb were observed following *Eco*R1 digestion and, presumably, result from the restriction sites located upstream and downstream of the 1.4 kb fragment in the rat genome. Similar results were obtained with the genomic DNA from λrFGF2-c4 (Fig. 49). Evaluation of the DNA blotting results indicates that rat FGF-2 gene exists as a single copy in the genome, similar to its human counterpart (Abraham *et al.*, 1986b).

Fig. 48

Fig. 48. Schematic representation of the genomic clone (λrFGF2-c4) isolated from a Sprague Dawley rat testis genomic library. (A) Partial *Bam*H1 restriction map of the phage λrFGF2-c4 insert: The 1.4 kb B2 fragment was detected using a rat FGF-2 probe containing 5′ untranslated sequences, and is flanked by 1.6 kb B3 and 1 kb B1 fragments upstream and downstream, respectively. **(B)** Physical map of the 1.4 kb B2 fragment which was divided into different regions (1-9) by convenient restriction sites.

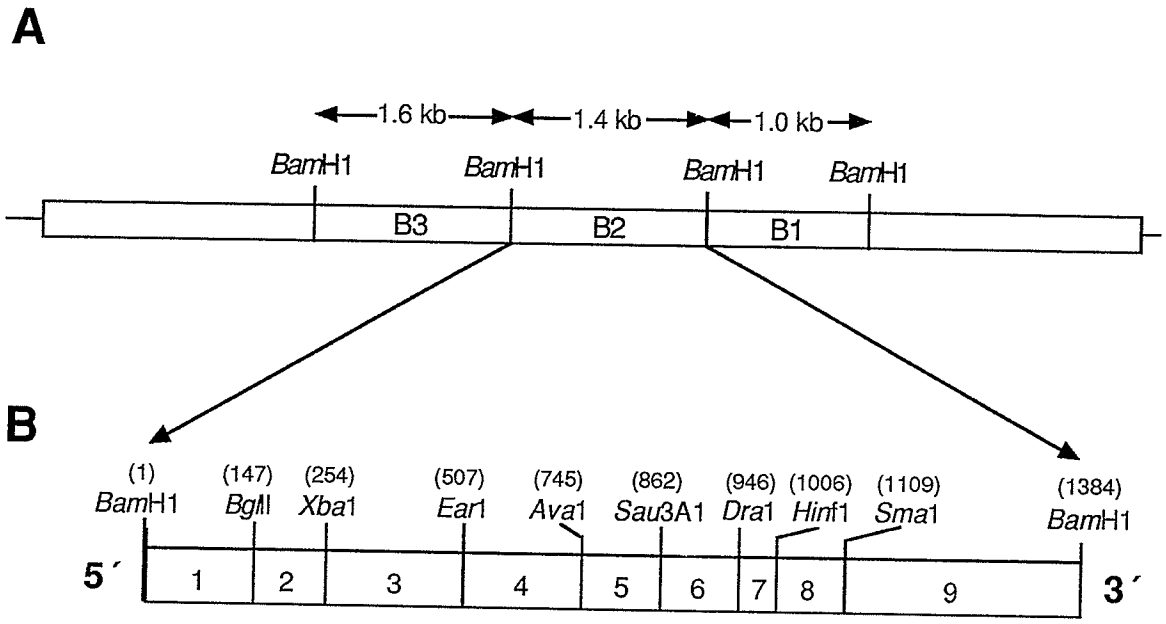
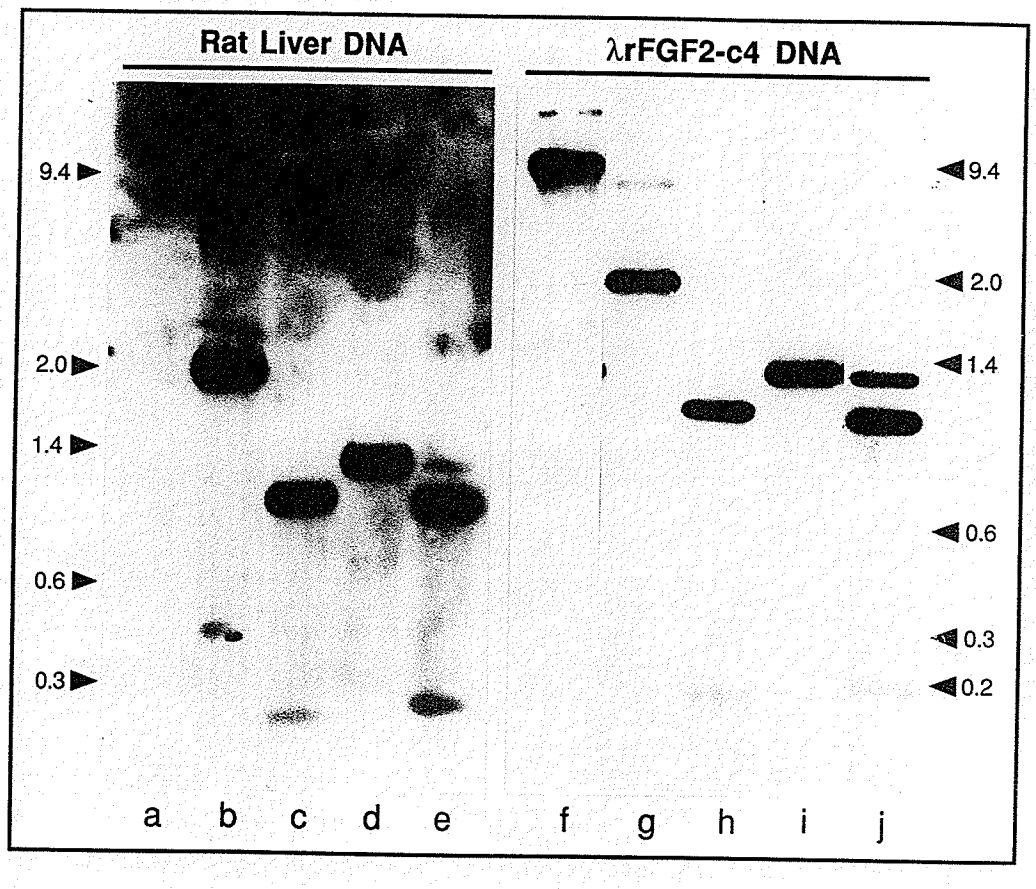

A
1.6 kb
1.4 kb
1.0 kb
BamH1
BamH1
BamH1
BamH1
B3
B2
B1
B
(1)
BamH1
(147)
BglI
(254)
Xba1
(507)
Ear1
(745)
Ava1
(862)
Sau3A1
(946)
Dra1
(1006)
Hinf1
(1109)
Sma1
(1384)
BamH1
5´
1
2
3
4
5
6
7
8
9
3´

Fig. 49

Fig. 49. DNA (Southern) blot analysis of the 5′-flanking region of the rat FGF-2 gene. Sprague Dawley rat liver (30 μg) or λrFGF2-c4 DNA (22 μg) was left uncut (a,f) or digested with: *Eco*R1 (b,g); *Eco*R1/*Bam*H1 (c,h); *Bam*H1 (d,i) or *Bam*H1/*Xba*1(e,j)restriction endonucleases. The DNA was electrophoresed in a 1.7% (rat liver DNA) or 1% (λrFGF2-c4 DNA) agarose gel and transferred to nitrocellulose. The 1.4 kb B2 fragment was radiolabelled by random priming and used for hybridization. Arrowheads indicate the molecular sizes derived from λ/*Hin*dIII or ϕX174/*Hae*III digests.


Rat Liver DNA
λrFGF2-c4 DNA
9.4
2.0
1.4
0.6
0.3
9.4
2.0
1.4
0.6
0.3
0.2
a b c d e f g h i j

8.5.4 Analysis of 5′-end of rat FGF-2 mRNA by northern blotting

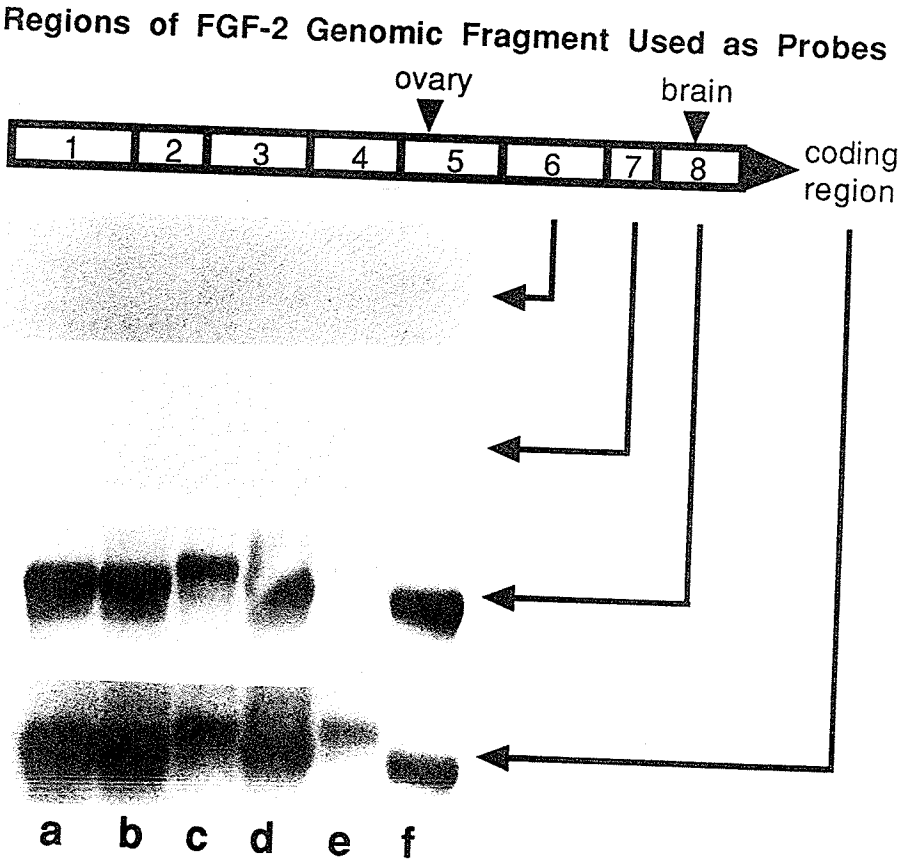
Full length rat FGF-2 cDNAs have been cloned from brain (Kurokawa *et al.*, 1988) and ovary (Shimasaki *et al.*, 1988). However, the ovarian cDNA is reported to contain ~280 bp of additional 5′ untranslated sequence (regions 5-8; Fig. 48b) compared to the brain cDNA. This could be due to either premature termination of the brain cDNA by reverse transcriptase during cloning, or use of alternative (tissue-specific) transcription initiation sites (Shimasaki *et al.*, 1988). In light of the discrepancy between the brain and ovarian cDNAs, we used a series of restriction fragments from the 5′-flanking region as probes to identify the 5′ end of the FGF-2 RNA in various rat tissues and cell lines. Regions 1-7 of the B2 fragment as well as the B3 fragment (Fig. 50) failed to detect RNA, but hybridization of the same blot with either region 8 of B2 or rat FGF-2 cDNA revealed ~6 kb FGF-2 transcript in all tissues except in rat heart and U87-MG cells which revealed ~7 kb FGF-2 transcripts (lanes c and e, Fig. 50). Size differences of FGF-2 transcripts can result due to use of multiple poly A signals (Kurokawa *et al.*, 1987), varying lengths of 5′- and 3′-untranslated sequences (Prats *et al.*, 1989), and alternative splicing (Borja *et al.*, 1993). As a control for labelling as well as hybridizations, all the probes obtained from B2 or B3 fragments were hybridized to rat DNA (Southern) blots separately and were positive for 1.4 kb or 1.6 kb bands generated by *Bam*H1 digestion (see Fig. 47) of genomic DNA. Although regions 5-7 are part of the published ovarian FGF-2 cDNA sequence, they did not hybridize to RNA (0.1 mg) from rat tissues including the ovary (Fig. 50, lane a). Thus, our RNA hybrdization results indicate that the major transcription initiation site(s) of the rat FGF-2 gene are located in region 8 (*Hinf* 1/*Sma* 1) or "just" upstream (but not sufficiently far to allow successful detection by hybridization) into region 7 (*Dra* 1/*Hinf* 1; see Fig. 48b). Furthermore, it appears that the 5′ end reported for the brain cDNA is more representative of FGF-2 RNA detected in all cell types examined.

8.5.5 Identification of major transcription initiation site(s) in rat FGF-2 gene

Primer extension was employed to further localize a transcription initiation site (or sites) for the rat FGF-2 gene. Two antisense oligonucleotides, FGFLS3 and B2LS1 were selected based on the sequences of brain and ovarian FGF-2 cDNAs as well as partial sequence of the B2 fragment. B2LS1 primer can bind to the sequences located just upstream of the ovarian FGF-2 cDNA end (see Fig. 50) and thus was chosen to identify if there are any alternatively spliced transcripts that may not contain regions 5-7 of the

Fig. 50

Fig. 50. Identification of the upstream transcribed region in the FGF-2 B2 fragment using RNA (northern) blot analysis. Total RNA (100 μg) from (a) rat ovary, (b) rat brain, (c) rat heart, (d) rat H9c2 myoblast cells, (e) human U87-MG astrocytoma cells and (f) rat C6 glioma cells, was resolved in a 1.5% denaturing agarose gel containing formaldehyde, blotted to nitrocellulose and hybridized sequentially to regions 6, 7 and 8 of the B2 fragment (Fig. 1B) as well as the rat FGF-2 cDNA. FGF-2 transcripts of ~5 kb were visualized by autoradiography. The arrowheads indicate the regions of the B2 fragment that correspond to the location of the 5′ end of cDNAs reported for rat ovary and brain.


Regions of FGF-2 Genomic Fragment Used as Probes
ovary
brain
1
2
3
4
5
6
7
8
coding region
a
b
c
d
e
f

genomic sequence (see Fig. 48b). Primer extension of total RNA from rat heart using the FGFLS3 primer revealed two major bands of 87 bp (P_0), 123 bp (P_1) and an additional less prominent band of 166 bp (P_2) (Figs. 51, lane f). By contrast, primer extension of total RNA from rat brain using the FGFLS3 primer revealed three major bands of 87 bp (P_0), 123 bp (P_1) and 166 bp (P_2) as well as additional less prominant bands (Figs. 52a, lane e). As assessed by densitometry, band intensity was $P_0>P_1>P_2$ in rat hear RNA (Fig. 51) and $P_0>P_2>P_1$ in rat brain RNA (Fig. 52a). Although there appears to be some tissue specificity with the usage of P_1 and P_2 start sites in brain and heart, the primer extension results from both tissues clearly indicate that the P_0 is the predominant start site of FGF-2 gene in both tissues (Figs. 51, 52a). However, primer extension of rat heart or brain RNA using B2LS1 primers did not reveal any major start sites beyond those identified with the FGFLS3 primer. Furthermore, P_0, P_1 and P_2 initiation sites fall into and just upstream of region 8 (*Hinf* 1/*Sma* 1; Fig. 48b) which showed positive hybridization with rat RNA samples (Fig. 50) and thus are consistent with the RNA blotting data. These results suggest that Po, P_1, P_2 located at nucleotides +1, -38, and -79 respectively, represent the most likely candidates for transcription initiation sites that are used efficiently in the rat heart and brain tissues.

P_0, P_1 and P_2 were also detected when the FGFLS3 oligonucleotide was used as a primer to identify the FGF-2 transcription start sites in C6 glioma cells (Fig. 52b). FGF-2 gene transcription is reported to respond to phorbol ester treatment in C6 glioma (Powell and Klagsbrun, 1993) and human U87MG astrocytoma (Murphy *et al.*, 1988b) cells. RNA was isolated from C6 cells treated with or without 100 nM PMA and assessed by primer extension. Although Po levels are relatively low compared to P2 under serum free (-PMA) conditions (Fig. 52b, lane e), a clear increase (~2.0 fold, n=2) in Po intensity was observed in the presence of PMA (lane f). PMA treatment also resulted in the induction of transcription from a "new" site, Pi, located at nucleotide +20 (Fig. 52b, lane f). The appearance of a "new" start site, suggests that FGF-2 gene transcription can occur through alternative transcription initiation sites in response to various stimuli. Similarly, the human FGF-1 gene transcription was shown to be modulated via an alternative promoter in response to phorbol ester and serum stimulation (Chotani *et al.*, 1995). It is worth noting that the ovarian FGF-2 cDNA (which contains an additional 280 bp of 5′ untranslated

Fig. 51

Fig. 51. Mapping of the transcription start site(s) in the rat FGF-2 gene by primer extension of rat heart RNA. Fifty micrograms of (f) total rat heart RNA or (e) yeast tRNA was hybridized with ^{32}P-end-labelled FGFLS3 oligonucleotide and extended by AMV reverse transcriptase. Primer extended products were resolved in an 8% polyacrylamide/8 M urea gel and detected by autoradiography. Major products (P_0, P_1 and P_2) are indicated by arrowheads. Sizes were determined by comparison with (a-d) unrelated DNA sequence ladder and (g) radiolabelled size markers (pBR322 cut with *Msp*1).

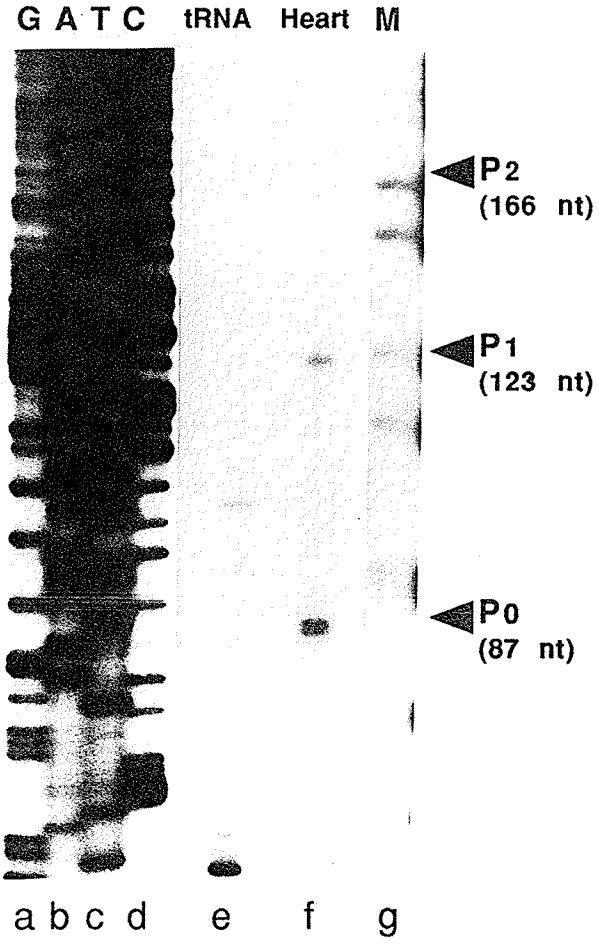
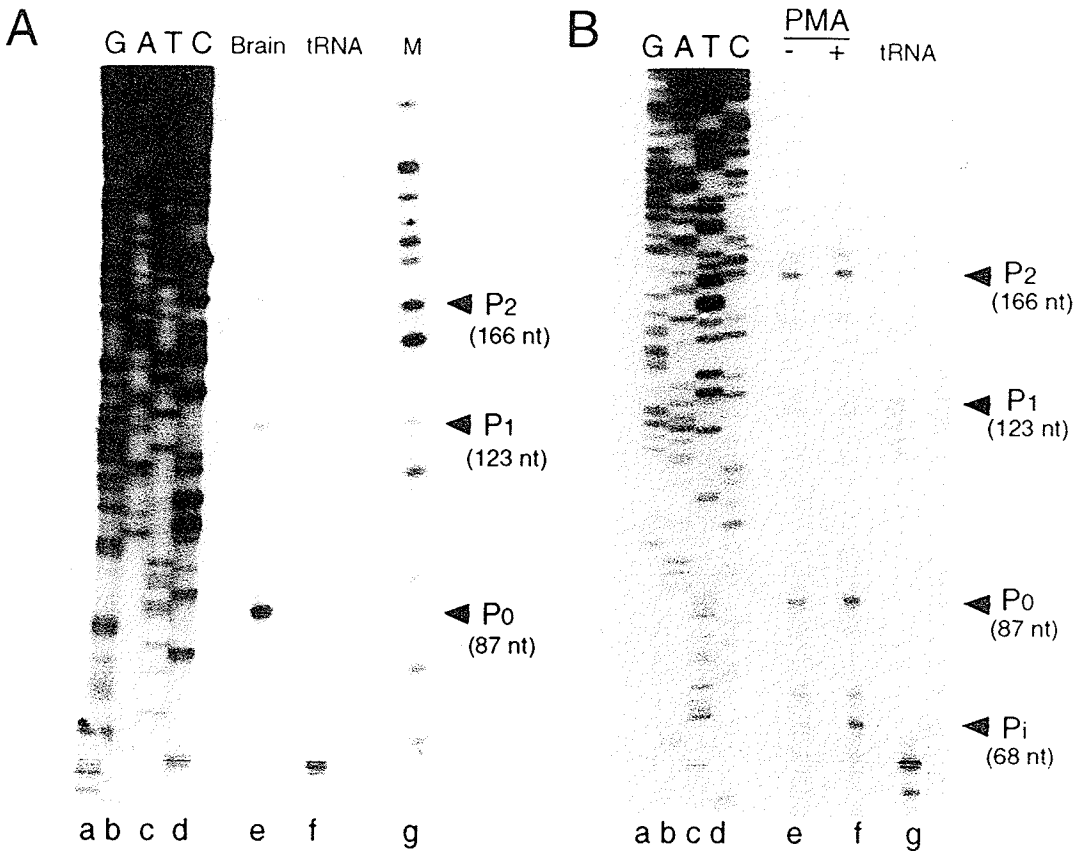

G A T C
tRNA
Heart
M
P2
(166 nt)
P1
(123 nt)
P0
(87 nt)
a b c d
e
f
g

Fig. 52

Fig. 52. Mapping of the transcription start site(s) in the rat FGF-2 gene by primer extension of RNA isolated from cells of the central nervous system. **(A)** Fifty micrograms of (e) total rat brain RNA or (f) yeast tRNA was hybridized with ^{32}P-end-labelled FGFLS3 oligonucleotide and extended by AMV reverse transcriptase. Primer extended products were resolved in an 8% polyacrylamide/8 M urea gel and detected by autoradiography. Major products (P_0, P_1 and P_2) are indicated by arrowheads. Sizes were determined by comparison with (a-d) unrelated DNA sequence ladder and (g) radiolabelled size markers (pBR322 cut with *Msp*1). **(B)** Total RNA (50 µg) isolated from C6 glioma cells treated (e) without or (f) with 100 nM PMA for 1 hour, as well as (g) yeast tRNA, was extended using the ^{32}P-end-labelled FGFLS-3 oligonucleotide primer. Major products (P_0, P_1, P_2 and Pi) detected with the C6 RNA samples are indicated by arrowheads. Sizes were determined by comparison with (a-d) an unrelated DNA sequence ladder. Note the induction of Pi in response to PMA treatment.


A
G A T C
Brain
tRNA
M
P2
(166 nt)
P1
(123 nt)
P0
(87 nt)
a b c d e f g
B
G A T C
PMA
- +
tRNA
P2
(166 nt)
P1
(123 nt)
P0
(87 nt)
Pi
(68 nt)
a b c d e f g

sequence compared to the brain cDNA) was isolated from a pregnant mare serum gonadotropin (PMSG)-stimulated rat ovarian cDNA library (Shimasaki *et al.*, 1988) and it is possible that the FGF-2 transcript used for cDNA synthesis in this case, was induced by PMSG from an upstream alternative start site. As a result the P_0, P_1 and P_2 start sites would be included in the ovarian cDNA sequence. However, P_0 appears to be the "bonafide" start site as it is (i) a prominant band in heart, brain and C6 RNA samples (Figs. 51, 52a,b), (ii) located upstream within 4 nucleotides from the 5′- end of rat brain FGF-2 cDNA sequence (Kurokawa *et al.*, 1988) and (iii) stimulated by PMA (~2.0 fold, n=2; Fig. 52b, lane f). Appearance of additional less prominant bands during the primer extension could be due to the nature of secondary structure associated with those regions (Figs. 51, 52).

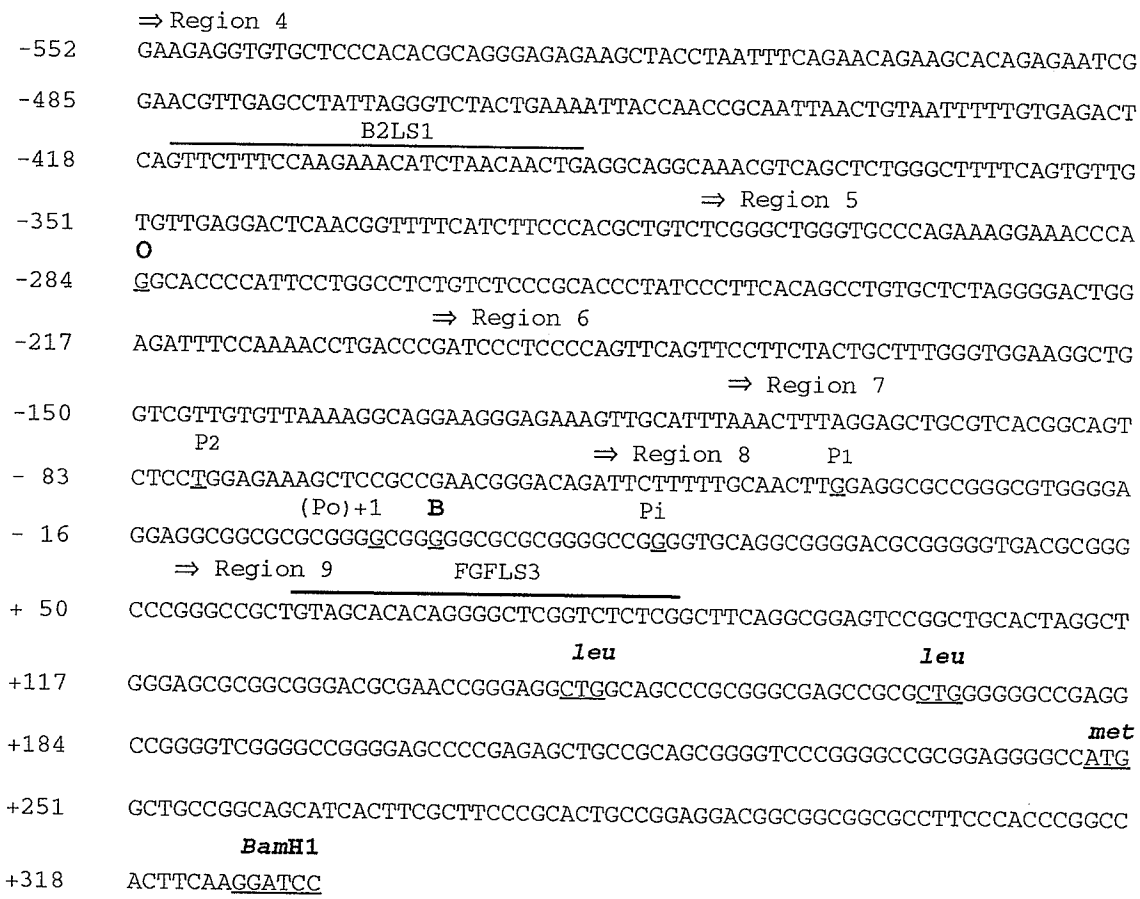
8.5.6 Sequence of the rat FGF-2 promoter region

The sequence of the rat FGF-2 promoter region from nucleotide -552 to +330 is shown in Fig. 53. This includes sequence from regions 4-8 of the B2 fragment (Fig. 48b) and the putative transcription start sites Pi, P_0, P_1 and P_2 (Figs. 51, 52). The sequence analysis indicates that B2 extends into the coding region and contains about 146 to 248 bp of 5′ untranslated DNA, assuming P_0 represents the bonafide transcription initiation site (+1) and accounting for the presence of both upstream leucine (+147/+149) and methionine (+249/+251) translation start sites. By subtraction, the B2 fragment contains at least 1058 bp of FGF-2 5′-flanking DNA. Based on a comparison of common genomic and reported cDNA sequences, the 5′ end for both brain (**B**) and ovarian (**O**) FGF-2 cDNAs are shown, as well as the location of the initiator methionine (*Met*) and leucines (*Leu*) (Fig. 53). Although the location of the 5′ end of the brain cDNA is consistent with the RNA analyses and within 4 nucleotides downstream of the P_0 start site determined by primer extension, it should be noted that P_0, P_1 and P_2 are contained within the ovarian cDNA.

The 5′-flanking sequence of rat FGF-2 lacks conventional TATA and CAAT boxes, but contains GC rich as well as AT rich regions that are located within 35-40 bp upstream of P_0 and P_2 start sites (Fig. 53). Transcription of several house keeping genes which include human monoamine oxidase A (Denny *et al.*, 1994), human ATP synthase alpha subunit (Akiyama *et al.*, 1994), rat xanthine dehydrogenase/oxidase (Chow *et al.*,

Fig. 53. Nucleotide sequence of -552/+330 of the rat FGF-2 gene. The open arrows indicate the relative locations of regions 4-9 of the B2 fragment (Fig. 48B) and Pi, P0, P1 and P2 indicate transcription start sites detected by primer extension analysis (Figs. 51 and 52). Nucleotides were numbered with reference to the major transcription initiation site (P0 or +1). Sequences that correspond to the oligonucleotides (B2LS1 and FGFLS3) used for primer extension are "overlined". The reported 5´ ends for both brain (**B**) and ovarian (**O**) FGF-2 cDNAs are shown, as well as the location of the initiator methionine (***Met***) and leucines (***Leu***) residues.

Fig. 53


⇒ Region 4
-552 GAAGAGGTGTGCTCCCACACGCAGGGAGAGAAGCTACCTAATTTCAGAACAGAAGCACAGAGAATCG
-485 GAACGTTGAGCCTATTAGGGTCTACTGAAAATTACCAACCGCAATTAACTGTAATTTTTGTGAGACT
B2LS1
-418 CAGTTCTTTCCAAGAAACATCTAACAACTGAGGCAGGCAAACGTCAGCTCTGGGCTTTTCAGTGTTG
⇒ Region 5
-351 TGTTGAGGACTCAACGGTTTTCATCTTCCCACGCTGTCTCGGGCTGGGTGCCCAGAAAGGAAACCCA
O
-284 GGCACCCCATTCCTGGCCTCTGTCTCCCGCACCCTATCCCTTCACAGCCTGTGCTCTAGGGGACTGG
⇒ Region 6
-217 AGATTTCCAAAACCTGACCCGATCCCTCCCCAGTTCAGTTCCTTCTACTGCTTTGGGTGGAAGGCTG
⇒ Region 7
-150 GTCGTTGTGTTAAAAGGCAGGAAGGGAGAAAGTTGCATTTAAACTTTAGGAGCTGCGTCACGGCAGT
P2
⇒ Region 8 P1
- 83 CTCCTGGAGAAAGCTCCGCCGAACGGGACAGATTCTTTTTGCAACTTGGAGGCGCCGGGCGTGGGGA
(Po)+1 B Pi
- 16 GGAGGCGGCGCGCGGGGCGGGGGCGCGCGGGGCCGGGGTGCAGGCGGGGACGCGGGGGTGACGCGGG
⇒ Region 9 FGFLS3
+ 50 CCCGGGCCGCTGTAGCACACAGGGGCTCGGTCTCTCGGCTTCAGGCGGAGTCCGGCTGCACTAGGCT
leu leu
+117 GGGAGCGCGGCGGGACGCGAACCGGGAGGCTGGCAGCCCGCGGGCGAGCCGCGCTGGGGGGCCGAGG
met
+184 CCGGGGTCGGGGCCGGGGAGCCCCGAGAGCTGCCGCAGCGGGGTCCCGGGGCCGCGGAGGGGCCATG
+251 GCTGCCGGCAGCATCACTTCGCTTCCCGCACTGCCGGAGGACGGCGGCGGCGCCTTCCCACCCGGCC
BamH1
+318 ACTTCAAGGATCC

1994), rat/human synapsin I (Sauerwald *et al.*, 1990) and rat insulin like growth factor binding protein-2 (Kutoh *et al.*, 1993), that do not contain a TATA box in their 5′-flanking region, appears to rely on GC rich motifs. An "initiator sequence" was also proposed for the transcriptional initiation of TATA-less genes (Smale and Baltimore, 1989). Rat FGF-2 gene transcription initiation sites Pi, P_0, P_1 and P_2 were mapped to the motifs GGGCCG**G**G, G**G**CGG, **G**GAGG and a palindrome TCTCC**T**GGAGA respectively. The pentanucleotide motif GGCGG was already reported to be the putative transcription initiation site of mouse fibroblast growth factor receptor (*bek*/KGFR) gene (Avivi *et al.*, 1992) and also corresponds to the consensus binding site of the transcription factor Sp1 (Briggs *et al.*, 1986). Furthermore, the major start site P_0 (G**G**CGG motif) of the rat FGF-2 gene has been conserved in the human FGF-2 genomic sequence (-138 to -135; see Fig. 54). However, a start site was mapped to a **C**CGCC motif in the human FGF-2 gene (Shibata *et al.*, 1991), and it is interesting to note that GGCGG motif of rat the FGF-2 gene is the reverse complement of human counterpart.

Alignment of -552/+53 of the rat FGF-2 gene and -678/-86 of reported human FGF-2 genomic sequences revealed an overall similarity of ~60% (Fig. 54). A comparable GC content between human (48.8%) and rat (50.8%) sequences also was observed. Greater similarity with human FGF-2 was observed in the more proximal rat FGF-2 promoter region (67% for -250/+1 versus 52% for -500/-251), and "proximally conserved domains" (PCDs) of 17 bp or more with 82-92% similarity could be identified (Fig. 54). The conservation of PCDs might be of evolutionary importance and related to specific function. Analysis of the sequence for the binding sites of known transcription factors revealed potential sites for CREB (Montaminy and Bilezikjian, 1987; Sassone-Corsi *et al.*, 1988), Egr-1 (Biesiada *et al.*, 1996), Ets (Wasylyk *et al.*, 1990; Ho *et al.*, 1990), LBP-1 (Parada *et al.*, 1995; Yoon *et al.*, 1994), NF-E1 (Palzkill and Newlon, 1988; Wall *et al.*, 1988), PERE (Ardati and Nemer, 1993) and Sp1 (Briggs *et al.*, 1986) transcription factors (see Table 5; Faisst and Meyer, 1992).

8.5.7 Mapping of the first exon-intron boundary in rat FGF-2 gene

The 1 kb (B1) fragment was initially sequenced using M13 forward and reverse primers, by the dideoxy method (see Fig. 4; section 3.19). Sequence of the 1 kb genomic

Fig. 54

Fig. 54. Alignment/comparison of upstream rat (-552/+53) and human (-678/-86) FGF-2 genomic sequences. Pi, P_0, P_1 and P_2 indicate transcription start sites detected by primer extension analysis (Figs. 51 and 52). The reported 5´ ends for both brain (**B**) and ovarian (**O**) FGF-2 cDNAs are shown, as well as the location of the initiator methionine (***Met***) and leucines (***Leu***) residues. Rat FGF-2 sequence is aligned to the 5´-flanking sequence of the human FGF-2 gene reported by Shibata *et al.*, 1991, and colons (:) were introduced in both sequences to obtain the best match. Nucleotides in the human FGF-2 sequence not aligning with the rat FGF-2 sequence are indicated by lower case letters. Three domains (PCD-1, -2 and -3) of 17 or more base pairs located in the proximal promoter region and displaying more than 80% conservation between the rat and human sequences are boxed.

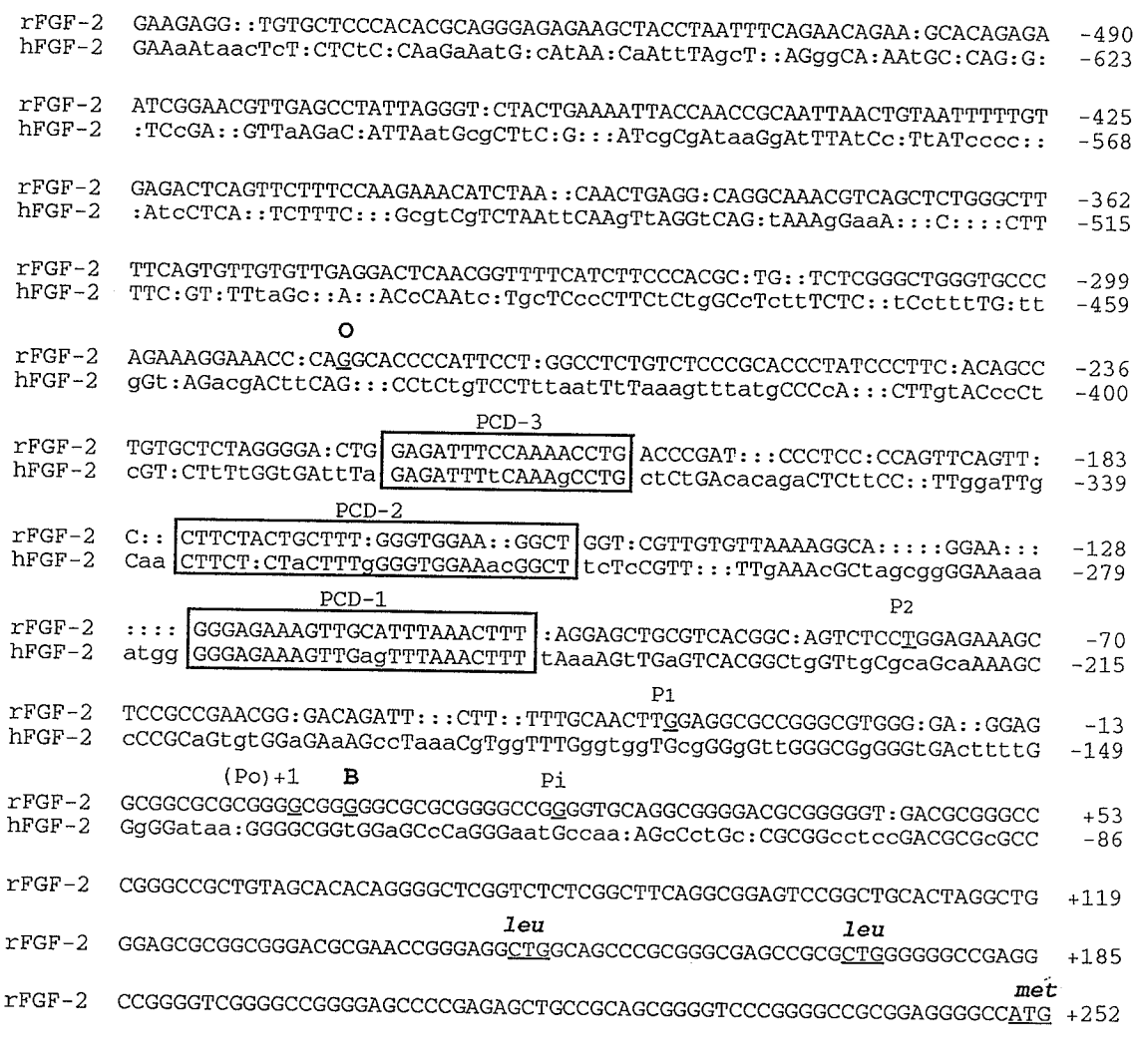

rFGF-2 GAAGAGG::TGTGCTCCCACACGCAGGGAGAGAAGCTACCTAATTTCAGAACAGAA:GCACAGAGA -490
hFGF-2 GAAaAtaacTcT:CTCtC:CAaGaAatG:cAtAA:CaAttTAgcT::AGggCA:AAtGC:CAG:G: -623
rFGF-2 ATCGGAACGTTGAGCCTATTAGGGT:CTACTGAAAATTACCAACCGCAATTAACTGTAATTTTTGT -425
hFGF-2 :TCcGA::GTTaAGaC:ATTAatGcgCTtC:G:::ATcgCgAtaaGgAtTTAtCc:TtATcccc:: -568
rFGF-2 GAGACTCAGTTCTTTCCAAGAAACATCTAA::CAACTGAGG:CAGGCAAACGTCAGCTCTGGGCTT -362
hFGF-2 :AtcCTCA::TCTTTC:::GcgtCgTCTAAttCAAgTtAGGtCAG:tAAAgGaaA:::C::::CTT -515
rFGF-2 TTCAGTGTTGTGTTGAGGACTCAACGGTTTTCATCTTCCCACGC:TG::TCTCGGGCTGGGTGCCC -299
hFGF-2 TTC:GT:TTtaGc::A::ACcCAAtc:TgcTCccCTTCtCtgGCcTcttTCTC::tCctttTG:tt -459
O
rFGF-2 AGAAAGGAAACC:CAGGCACCCCATTCCT:GGCCTCTGTCTCCCGCACCCTATCCCTTC:ACAGCC -236
hFGF-2 gGt:AGacgACttCAG:::CCtCtgTCCTttaatTtTaaagtttatgCCCcA:::CTTgtACccCt -400
PCD-3
rFGF-2 TGTGCTCTAGGGGA:CTG GAGATTTCCAAAACCTG ACCCGAT:::CCCTCC:CCAGTTCAGTT: -183
hFGF-2 cGT:CTtTtGGtGAttTa GAGATTTtCAAAgCCTG ctCtGAcacagaCTCttCC::TTggaTTg -339
PCD-2
rFGF-2 C:: CTTCTACTGCTTT:GGGTGGAA::GGCT GGT:CGTTGTGTTAAAAGGCA:::::GGAA::: -128
hFGF-2 Caa CTTCT:CTaCTTTgGGGTGGAAacGGCT tcTcCGTT:::TTgAAAcGCtagcggGGAAaaa -279
PCD-1 P2
rFGF-2 :::: GGGAGAAAGTTGCATTTAAACTTT :AGGAGCTGCGTCACGGC:AGTCTCCTGGAGAAAGC -70
hFGF-2 atgg GGGAGAAAGTTGagTTTAAACTTT tAaaAGtTGaGTCACGGCtgGTtgCgcaGcaAAAGC -215
P1
rFGF-2 TCCGCCGAACGG:GACAGATT:::CTT::TTTGCAACTTGGAGGCGCCGGGCGTGGG:GA::GGAG -13
hFGF-2 cCCGCaGtgtGGaGAaAGccTaaaCgTggTTTGggtggTGcgGGgGttGGGCGgGGGtGActtttG -149
(Po)+1 B Pi
rFGF-2 GCGGCGCGCGGGGCGGGGGCGCGCGGGGCCGGGGTGCAGGCGGGGACGCGGGGGT:GACGCGGGCC +53
hFGF-2 GgGGataa:GGGGCGGtGGaGCcCaGGGaatGccaa:AGcCctGc:CGCGGcctccGACGCGcGCC -86
rFGF-2 CGGGCCGCTGTAGCACACAGGGGCTCGGTCTCTCGGCTTCAGGCGGAGTCCGGCTGCACTAGGCTG +119
leu leu
rFGF-2 GGAGCGCGGCGGGACGCGAACCGGGAGGCTGGCAGCCCGCGGGCGAGCCGCGCTGGGGGGCCGAGG +185
met
rFGF-2 CCGGGGTCGGGGCCGGGGAGCCCCGAGAGCTGCCGCAGCGGGGTCCCGGGGCCGCGGAGGGGCCATG +252

Table 5

Table 5. Analysis of the 5´-flanking sequence of the rat FGF-2 gene for potential binding sites of known transcription factors. Relative locations of the putative *cis*-elements in the sense (+) or antisense (-) strands are indicated with reference to the transcription initiation site (+1 or P0).

Cis-element	Consensus binding site	Sequence in the 5´-flanking region	Strand	Location
AT-rich sequence	-	GCATTTAAACTTTAGG GGACATTTATCC	+ +	-116/-101 -993/-982
CREB	TGACGTYW	TGACGTTT	-	-380/-373
Egr-1	CGCCCCCGC	CGCCCCCGC	-	+1/+9
Ets	C/GA/CGGAA/TGC/T	CAGGAAGG	+	-133/-126
LBP-1	WCTGG	ACTGG TCTGG ACTGG TCTGG	+ + + +	-222/-218 -370/-366 -1021/-1017 -1028/-1024
NF-E1	AGATAT/C	AGATAC AGATAT	+ +	-622/-617 -1042/-1038
PERE	AGGGAGGG	AGGGAGGG	+ +	-761/-754 -778/-771
Sp1	GGCGG	GGCGG	+ + +	+27/+31 +1/+4 -13/-9

fragment (B1) includes a stretch of 98 bp at its 5′ end that matches with the first exonic sequence of both brain (Kurokawa *et al.*, 1988) and ovarian (Shimasaki *et al.*, 1988) FGF-2 cDNAs, in addition to the non matching putative intervening region (Fig. 55). The 98 bp region of the B1 subclone is contiguous to the exonic sequence contained in the 3′ end of B2 sequence (Fig. 53) and this suggests that the B1 fragment is located 3′ of B2 fragment in λrFGF2-c4. Further, S1 nuclease protection assay (Fig. 5, see section 3.22) was performed to define the first exon-intron boundary more precisely using rat brain RNA. A 130 bp *Bam*H1 and *Acc*1 fragment containing the putative exon-intron boundary (Fig. 55) was labelled at the *Bam*H1 end and used as a probe for solution hybridization with total RNA from rat brain. Rat brain RNA was chosen because, FGF-2 RNA was reported to be abundant in brain (Shimasaki *et al.*, 1988). S1 nuclease protection assay of rat brain total RNA using the radiolabelled *Bam*H1 and *Acc*1 fragment revealed one major band (Fig. 56, lane b) and the size of this protected fragment was found to be 98 bases by comparing to the radiolabelled size markers (pBR322 cut with *Msp*1) and an unrelated DNA sequence ladder. However, the presence of 130 bp probe in brain sample (lane b) could be due to either reannealing of the double stranded probe or due to hybridization with unspliced heterogeneous nuclear RNA (hnRNA). Many cloning artifacts due to hnRNA were reported to interfere with cloning of the rat FGF-2 cDNA (Shimasaki *et al.*, 1988). Alignment of the rat genomic FGF-2 sequence (B1) and corresponding human FGF-2 genomic sequence (Shibata *et al.*, 1991) revealed an 89% homology (12 mismatches out of 98 nucleotides) in the coding regions with a conservation of the exon-intron boundary (5′-CACGgtgagt-3′ of rat versus 5′-CACAgtgagt-3′ of human) (Fig. 55). However, there was no homology between the intronic sequences of rat and human FGF-2 genomic sequences (Fig. 55). Furthermore, boundaries for exon 1, 2, and 3 of all the known genes of the FGF family (FGF-2, FGF-3, FGF-4 and FGF-5) align perfectly except that the exon 1 boundary for FGF-2 is shifted by three nucleotides. This suggests that their genes are derived from a single ancestral gene through the processes of duplication and evolutionary divergence (Baird and Bohlen 1990; Gospadarowicz *et al.*, 1987).

Fig. 55

Fig. 55. Partial nucleotide sequence of 1 kb B1 genomic fragment which contains the putative exon-intron boundary of rat FGF-2 gene. The 5′ end of 1 kb rat FGF-2 genomic fragment in the B1 subclone was partially sequenced. The *Bam*H1 and *Acc* 1 cleavage sites are underlined. Alignment of the available B1 sequence (288 nucleotides) with rat brain FGF-2 cDNA sequence (Kurokawa *et al.*, 1988) revealed a perfect match between the first 98 nucleotides of B1 (indicated by upper case letters) and 340/428 of cDNA (indicated by dots) in addition to a non matching putative intronic sequence (indicated by lower case letters). Also these 98 nucleotides were contiguous with the coding sequence found in -552/+306 of B2 fragment (see Fig. 53). Comparison of rat B1 genomic sequence (1/288) with the human FGF-2 genomic sequence (Shibat *et al.*, 1991) revealed an 89% homology between the first 98 nucleotides of B1 and 1663/1761 of the human sequence in addition to a high degree of conservation in the exon-intron boundaries (indicated by an arrowhead). Nucleotides in the human FGF-2 sequence not aligning with the rat FGF-2 sequence (upper case letters), intronic sequences (lower case letters) and similarities (dots) are indicated.

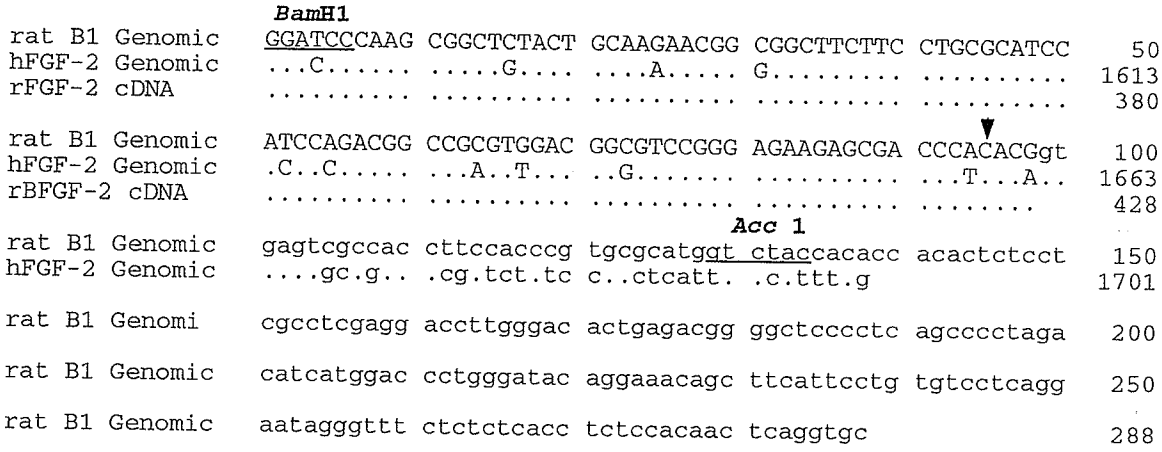
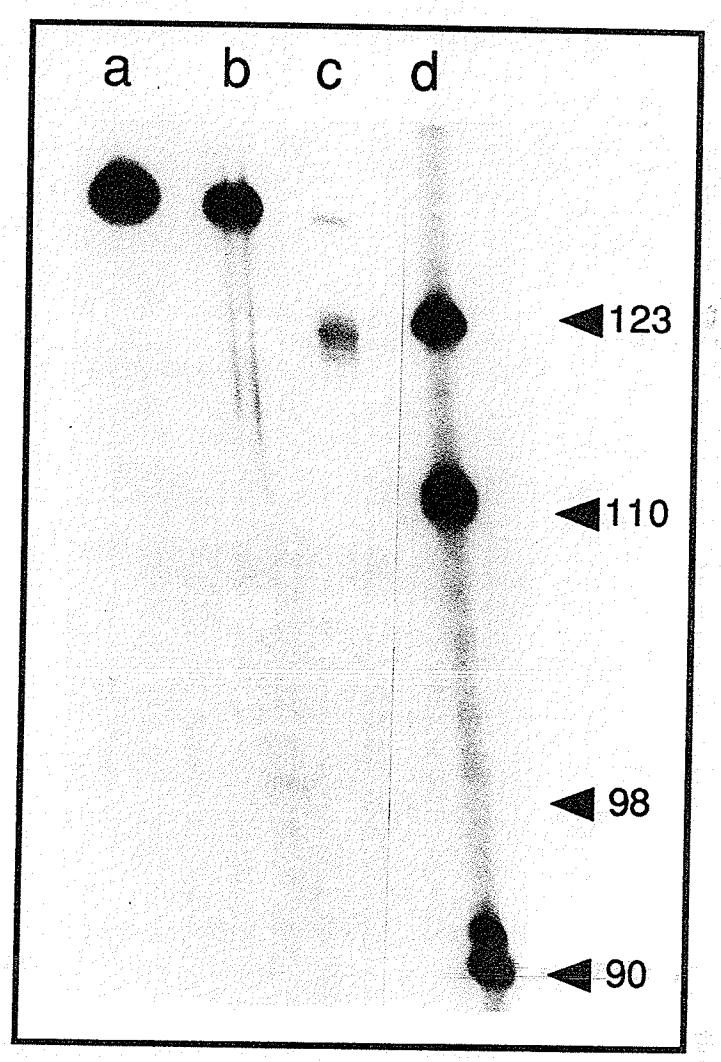

BamH1
rat B1 Genomic GGATCCCAAG CGGCTCTACT GCAAGAACGG CGGCTTCTTC CTGCGCATCC 50
hFGF-2 Genomic ...C..... .....G.... ....A..... G..... 1613
rFGF-2 cDNA 380
rat B1 Genomic ATCCAGACGG CCGCGTGGAC GGCGTCCGGG AGAAGAGCGA CCCACACGgt 100
hFGF-2 Genomic .C..C..... ...A..T... ..G..... ....T...A.. 1663
rBFGF-2 cDNA 428
Acc 1
rat B1 Genomic gagtcgccac cttccacccg tgcgcatggt ctaccacacc acactctcct 150
hFGF-2 Genomic ....gc.g.. .cg.tct.tc c..ctcatt. .c.ttt.g 1701
rat B1 Genomi cgcctcgagg accttgggac actgagacgg ggctcccctc agcccctaga 200
rat B1 Genomic catcatggac cctgggatac aggaaacagc ttcattcctg tgtcctcagg 250
rat B1 Genomic aatagggttt ctctctcacc tctccacaac tcaggtgc 288

Fig. 56

Fig. 56. Identification of exon-intron boundary in the 1 kb (B1) rat FGF-2 genomic fragment. The 1 kb B1 fragment was radiolabelled at both *Bam*H1 ends, digested with *Acc* 1 and a 130 bp *Bam*H1/*Acc* 1 fragment radiolabelled at *Bam*H1 end was gel purified (see Figs. 5 and 55). The 130 bp *Bam*H1/*Acc* 1 radiolabelled fragment was left undigested (lane a) or digested ater solution hybridization with either 100 μg of total rat brain RNA (lane b) or yeast tRNA (lane c) using S1 nuclease. The products were resolved together with radiolabelled size markers (pBR322 cut with *Msp*1; lane d) in an 8% polyacrylamide/8 M urea gel and detected by autoradiography. Sizes of the S1 nuclease protected band (98 bp, lane b) as well as radiolabelled markers are indicated. Note the presence of 130 bp probe in the brain sample (lane b) either due to reannealing or due to hybridization with unspliced heterogenous nuclear RNA.


a
b
c
d
123
110
98
90

8.6 Concluding remarks

In summary, a 1.4 kb region of rat genomic DNA was cloned that: (i) has overlap at the 3´ end with rat FGF-2 cDNA sequences; (ii) contains start sites for transcription in the rat brain, heart and glioma C6 cells; (iii) contains domains of sequence in the proximal promoter region which are conserved in human FGF-2 5´-flanking DNA; and (iv) contains putative regulatory elements.

Chapter 9

Characterization of the 5′-flanking region of rat FGF-2 gene and its response to mitogenic stimuli

9.1 Background

FGF-2 is widely distributed in the embryonic as well as postnatal brain (Powell *et al.*, 1991; Riva and Mocchetti, 1991) and its abundance is consistent with an important role for this growth factor in the development of the brain (Baird, 1994). It is reported to elicit potent multipotential trophic effects on neurons, endothelial cells and glia of the central nervous system (Baird, 1994; Powell *et al.*, 1991; Riva and Mocchetti, 1991). Although neurons are the key players in the mammalian nervous system, they are outnumbered by a supporting cast of glial cells (Alberts *et al.*, 1989). The glial cells express high levels of FGF-2 and its high affinity receptor (FGFR-1) and also serve as a reservoir for FGF-2 that can enhance the survival and differentiated functions of neurons during normal and disease conditions (Morrison, 1991). FGF-2 from the glial cell was shown to enhance the survival of cerebral and hippocampal neuronal cells (Morrison *et al.*, 1986; Walicke *et al.*, 1986). Furthermore, altered expression of FGF-2 has been implicated in the disease process of several neurodegenerative disorders (Tooyama *et al.*, 1993a,b). In this context, administration of FGF-2 was shown to prevent neuronal death *in vivo* (Riva and Mocchetti, 1991) and this suggests that the increased availability of FGF-2 may serve as a specific treatment regimen to slow or regress the progression of neurodegenerative diseases. Clearly, this potent mitogen must be tightly regulated in the central nervous system (CNS) to avoid uncontrolled proliferation of cells (Riva and Mocchetti, 1991). However, there is scant information about the transcriptional regulation of FGF-2 gene.

Studies employing rat astroglial and hippocampal primary cell cultures as well as the rat C6 glioma cell line, indicated that transcriptional activation of the FGF-2 gene occurs in response to mitogenic stimuli such as the addition of serum or phorbol ester, inspite of abundant intracellular stores of FGF-2 (Flott-Rahmel *et al.*, 1992; Powell and Klagsbrun, 1993). These data clearly implicate FGF-2 gene transcription in the mitogenic response.

With a view to understanding the regulation of FGF-2 gene transcription, the previously cloned 5′-flanking region of the rat FGF-2 gene was further characterized in terms of its responsiveness to mitogenic stimuli (serum and phorbol ester).

9.2 Rationale

FGF-2 has been implicated in neuronal (Sievers *et al.*, 1987) and hepatic (Presta *et al.*, 1989) regeneration. It is also involved in the process of wound healing (Baird and Walicke, 1989) and protection of neuronal (Baird, 1994; Finklestein *et al.*, 1990) and cardiac (Padua and Kardami, 1993; Yanagisawa-Miwa *et al.*, 1992) tissues against injury. Recent studies implicate the involvement of FGF-2 in diabetic complications (Karpen *et al.*, 1992) as well as neurodegenerative diseases such as Alzheimer's disease (Cotman and Gomez-Pinilla, 1991), Huntington's disease (Tooyama *et al.*, 1993a) and Parkinson's disease (Tooyama *et al.*, 1993b). These disorders impact enormously on the public health and therefore, we sought to increase our knowledge related to the therapeutic agents that can regulate the endogenous levels of FGF-2 with a view to prevent/protect as well as treat the affected individuals.

9.3 Hypothesis

Endogenous levels of FGF-2 expression can be modulated at the transcriptional level in brain derived glioma C6 cells in response to various mitogenic stimuli.

9.4 Specific Aims

1. To examine the promoter activity of rat FGF-2 gene in rat glial and non glial cell types.
2. To examine the promoter activity of rat FGF-2 gene in human astroglial cells.
3. To test the responsiveness of 5′-flanking region of rat FGF-2 gene to mitogenic stimuli in glioma C6 cells.
4. Attempts to identify the regions of the rat FGF-2 gene that respond to mitogenic stimuli.

9.5 Results and Discussion

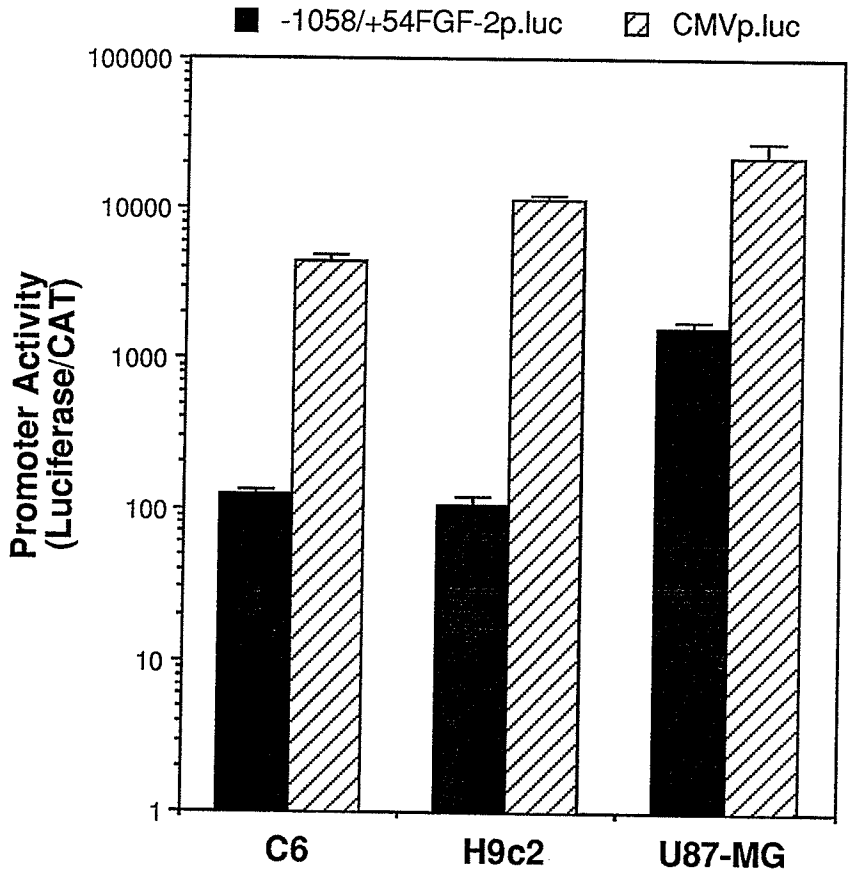
9.5.1 Promoter activity of the rat FGF-2 gene in glial and non glial cell lines derived from rat and human

The 5′-flanking region of the rat FGF-2 gene was introduced upstream of the translation start site of luciferase reporter sequence in the promoterless luciferase plasmid (pXP1) to generate a hybrid FGF-2/luciferase gene -1058/+54FGF-2p.*luc*. (Fig. 6, see section 3.25). This hybrid gene was used to transiently transfect and to assess the promoter activity of the cloned fragment in rat glioma C6, rat heart myoblast H9c2 as well as human astroglioma U87-MG cell lines. The promoterless luciferase plasmid pXP1 (-p.*luc*) was also used to transfect cells as a control for random transcription initiation. These cells were all co-transfected with the chloramphenicol acetyl transferase (CAT) gene directed by the Rous sarcoma virus promoter (RSVp.CAT) as a control for DNA uptake which was used subsequently to correct values for luciferase activity. Therefore, promoter function was measured in terms of luciferase activity and normalized using CAT activity. For comparison, all three cell types were also transfected with a heterologous hybrid luciferase gene directed by Cytomegalo virus promoter (CMVp.*luc*).

Significant promoter activities were observed with -1058/+54FGF-2p.*luc*. compared to the promoterless luciferase plasmid (-p.*luc*) in both glial (~30 fold, $p<0.0001$; C6 and U87-MG) and non glial (~15 fold, $p<0.0002$; H9c2) cells of rat and human origin (Fig. 57). However, the absolute promoter activity of -1058/+54FGF-2p.*luc* was significantly higher (~14 fold, $p<0.0001$) in human U87-MG cells compared to rat C6 or H9c2 cells but of similar magnitude in the rat cell types (Fig. 57). It is important to consider the variations between experimental conditions and or transfection efficiencies when comparing the activity of -1058/+54FGF-2p.*luc* in different cell types. These considerations may be resolved by comparing FGF-2/luciferase hybrid gene activity to an internal standard such as CMVp.*luc* in each cell type. The promoter activity of CMVp.*luc* was significantly less in C6 glial cells compared to that of U87-MG astroglial cells (~5 fold, $p<0.02$) and non glial H9c2 cells (~3 fold, $p<0.0002$). There was no significant difference in the promoter activity of CMVp.*luc* between U87-MG cells and H9c2 cells (Fig. 57). Although there was no significant difference in the absolute promoter activity of -1058/+54FGF-2p.*luc* in rat glial (C6) and non glial (H9c2) cells, when expressed as a

Fig. 57

Fig. 57. Promoter activity of a hybrid luciferase gene directed by 1058 bp of rat FGF-2 upstream flanking sequences (-1058/+54FGF-2p.*luc*) in glial and non glial cells derived from rat and human. Both glial (rat C6; human U87-MG) and non glial (rat heart myoblast H9c2) cells were transfected with -1058/+54FGF-2p.*luc* or a promoterless luciferase gene (-p.*luc*) and co-transfected with RSVp.CAT for 24 hours. For comparisons, cells were also transfected with CMVp.*luc*. Cells were maintained for a further 48 hours before harvesting and assessment of reporter gene activities. The promoter activities (luciferase/CAT) for -1058/+54FGF-2p.*luc* and CMVp.*luc* are shown for each cell type and represent the mean from at least 6-8 determinations after subtraction of -p*luc* activity which serves as a measure of random transcription initiation. Basal levels for -p*luc* in rat (C6, H9c2) and human (U87-MG) cells were 4.46±0.37, 7.47±1.27 and 48.52±12.22, respectively. The absolute promoter activity of -1058/+54FGF-2p.*luc* was significantly higher (~14 fold, $p<0.0001$) in human U87-MG cells compared to rat C6 or H9c2 cells, but of similar magnitude in the rat cell types. The promoter activity of CMVp.*luc* was significantly less in C6 glial cells compared to that of U87-MG astroglial cells (~5 fold, $p<0.02$) and non glial H9c2 cells (~3 fold, $p<0.0002$). Although there was no signficant difference in the absolute promoter activity of -1058/+54FGF-2p.*luc* in rat glial (C6) and nonglial (H9c2) cells, when expressed as a percentage of CMVp.*luc* activity, the promoter activity of -1058/+54FGF-2p.*luc* was higher in glial cells (~7% in U87-MG; ~3% in C6) compared to non glial cells (~1% in H9c2). Bars represent standard error of the mean.


-1058/+54FGF-2p.luc
CMVp.luc
Promoter Activity
(Luciferase/CAT)
100000
10000
1000
100
10
1
C6
H9c2
U87-MG

percentage of CMVp.*luc* activity, the promoter activity of -1058/+54FGF-2p.*luc* was higher in glial cells (~7% in U87-MG; ~3% in C6) compared to non glial cells (~1% in H9c2). Thus, these results suggest that the rat FGF-2/luciferase gene is expressed both in rat and human cell types and the level of expression is significantly higher in glial cells (U87-MG, C6) compared to non glial cells (heart myoblast H9c2). These results are also consistent with previous reports that the FGF-2 gene is highly transcribed in cells derived from CNS compared to other tissues (Shimasaki *et al.*, 1988). However, the higher levels of rat FGF-2 promoter activity in human astroglial U87-MG cells compared to that in rat glioma C6 cells could be due to either differences in species/cell types or due to a higher transcriptional rate of the endogenous gene in U87-MG cells which may facilitate a better transcriptional milieu for the transfected rat gene in human U87-MG cells compared to rat C6 cells. In addition, comparison of the rat FGF-2 sequences with the published human FGF-2 genomic sequences revealed a 78% homology in the coding regions of exon I and 60% homology in the 5′-flanking regions (see section 8.5.6; Fig. 54). RNA blotting results suggest that *Hinf*1/*Sma*1 fragment (region 8; -53 to +54) but not the upstream fragments from rat FGF-2 gene hybridize to U87-MG total RNA (see section 8.5.4; Fig. 50). Furthermore, the major start site Po (G**G**CGG motif) of the rat FGF-2 gene which falls into *Hinf*1/*Sma*1 fragment (region 8; -53 to +54) has been well conserved in the human FGF-2 genomic sequence (-138 to -135; see section 8.5.6; Fig. 54) and is also the reverse complement of the human FGF-2 gene start site (**C**CGCC motif, Shibata *et al.*, 1991).

9.5.2 Response of the 5′-flanking region of rat FGF-2 gene to mitogenic stimuli in C6 glioma cells

The hybrid FGF-2/luciferase reporter gene (-1058/+54FGF-2p.*luc*, Fig. 6; see section 3.25) which contained all the available upstream sequences present in the B2 fragment, was used initially to transfect rat glioma C6 cells to assess the cloned fragment of FGF-2 5′-flanking DNA for responsiveness to mitogenic stimuli. After gene transfer, C6 cells were treated (see section 3.3) with the following agents in triplicate plates: (i) no serum, (ii) 10% FBS and (iii) phorbol ester (100 nM PMA). A promoterless luciferase gene (-p.*luc*) was also used to transfect cells to provide a level for random transcription

initiation. All values were normalized for variation in DNA uptake using RSVp.CAT, and it was also assumed that this viral promoter was constitutively expressed under the various experimental conditions used. The -1058/+54FGF-2p.*luc* gene demonstrated significant promoter activity when compared to -p.*luc* in the absence of serum, and was stimulated (>50 fold) in the presence of 10% FBS (Fig. 58). In contrast, treatment with phorbol ester resulted in a modest (1.6 fold) but significant ($p<0.005$) increase in -1058/+54FGF-2p.*luc* activity (Fig. 58). This is consistent with the primer extension data in which the putative transcription initiation site Po was stimulated (~2 fold) by PMA treatment at the transcriptional level (see section 8.5.5, Fig. 52b).

9.5.3 Localization of regions in the rat FGF-2 gene that respond to mitogenic stimuli in C6 glioma cells by deletion analysis

Additional hybrid genes -911/+54FGF-2p.*luc*, -313/+54FGF-2p.*luc* and -110/+54FGF-2p.*luc* (Fig. 6; see section 3.25) were generated and transiently transfected in rat glioma C6 cells to further localize serum and phorbol ester responsive regions of the rat FGF-2 gene. All the hybrid genes demonstrated significant increases in their promoter activities when compared to -p.*luc* in the absence of serum (Fig. 59). A repressor activity was localized to -1058/-911 region, as the promoter activity of -911/+54FGF-2p.*luc* was significantly higher (4.6 fold, $p<0.0001$) than that of -1058/+54FGF-2p.*luc* in the absence of mitogenic stimuli (Fig. 59). By contrast, promoter activities of all the hybrid genes were stimulated (>50 fold) in the presence of 10% FBS (Fig. 60) similar to -1058/+54FGF-2p.*luc* gene (see Figs. 58, 60). However, there was no significant difference between promoter activities of -1058/+54FGF-2p.*luc* and -911/+54FGF-2p.*luc* suggesting that the repressor activity associated with -1058/-911 region in the absence of serum (Fig. 59) was removed in the presence of 10% FBS (Fig. 60). In addition, the promoter activity of -313/+54FGF-2p.*luc* was significantly higher (1.5 fold, $p<0.01$) compared to the promoter activity of -1058/+54FGF-2p.*luc* but not -911/+54FGF-2p.*luc* in the presence of serum (Fig. 60). Further, deletion of -313/-110 region resulted in a significant decrease (~3 fold, $p<0.0001$) in the promoter activity of -110/+54FGF-2p.*luc* compared to -313/+54FGF-2p.*luc*, suggesting that at least a part of the positive effect of serum on FGF-2 gene transcription can be localized to -110/+54 region. Also, gene transfer studies suggested that

Fig. 58

Fig. 58. A hybrid luciferase gene directed by 1058 bp of rat FGF-2 upstream flanking sequences (-1058/+54FGF-2p.*luc*) is responsive to treatment with phorbol ester or serum in C6 glioma cells after gene transfer. Rat glioma C6 cells were transfected with -1058/+54FGF-2p.*luc* or a promoterless luciferase gene (-p.*luc*) and co-transfected with RSVp.*cat* for 24 hours. Cells were then maintained for 48 hours in (i) serum-free conditions (-serum), (ii) serum-free conditions but with 100 nM PMA for the final 24 hours (+PMA), and (iii) 10% FBS (+serum) before harvesting and assessment of reporter gene activities. The promoter activity (luciferase/CAT) for -1058/+54FGF-2p.*luc* under each of the conditions tested are indicated and represent the mean from at least 6 determinations after subtraction of -p*luc* activity which serves as a measure of random transcription initiation. Basal levels for -p.*luc* under conditions of -serum, +PMA and +serum were 0.07±0.04, 0.05±0.01 and 4.47±0.38, respectively. Bars represent standard error of the mean.

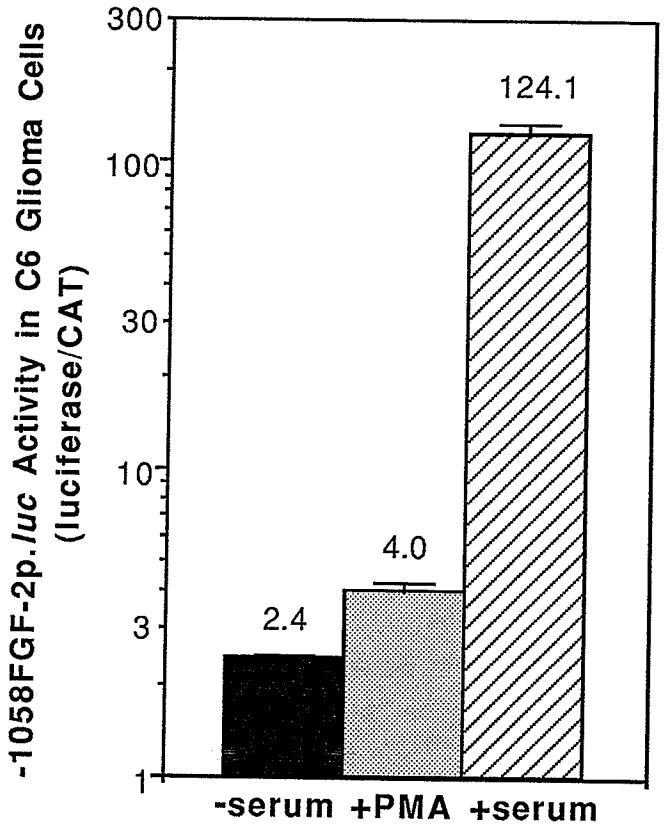

-1058FGF-2p.luc Activity in C6 Glioma Cells
(luciferase/CAT)
300
100
30
10
3
1
124.1
4.0
2.4
-serum
+PMA
+serum

Fig. 59

Fig. 59. Evidence for a repressor activity in -1058/-911 region of the rat FGF-2 gene in the absence of mitogenic stimuli. Rat glioma C6 cells were transfected with hybrid FGF-2p/luciferase genes (-1058/+54FGF-2p.*luc*; -911/+54FGF-2p.*luc*, -313/+54FGF-2p.*luc* and -110/+54FGF-2p.*luc* ; Fig. 6) or a promoterless luciferase gene (-p.*luc*) and co-transfected with RSVp.CAT for 24 hours. Cells were then maintained for further 48 hours in serum-free conditions before harvesting and assessment of reporter gene activities. The promoter activities (luciferase/CAT) for all hybrid gene are shown as the mean from at least 6-12 determinations after subtraction of -p.*luc* activity which serves as a measure of random transcription initiation. Basal level for -p.*luc* under serum free conditions was 0.07±0.04. The promoter activity of -911/+54FGF-2p.*luc* was significantly higher (4.6 fold, $p<0.0001$) than that of -1058/+54FGF-2p.*luc* in the absence of mitogenic stimuli. Bars represent standard error of the mean.

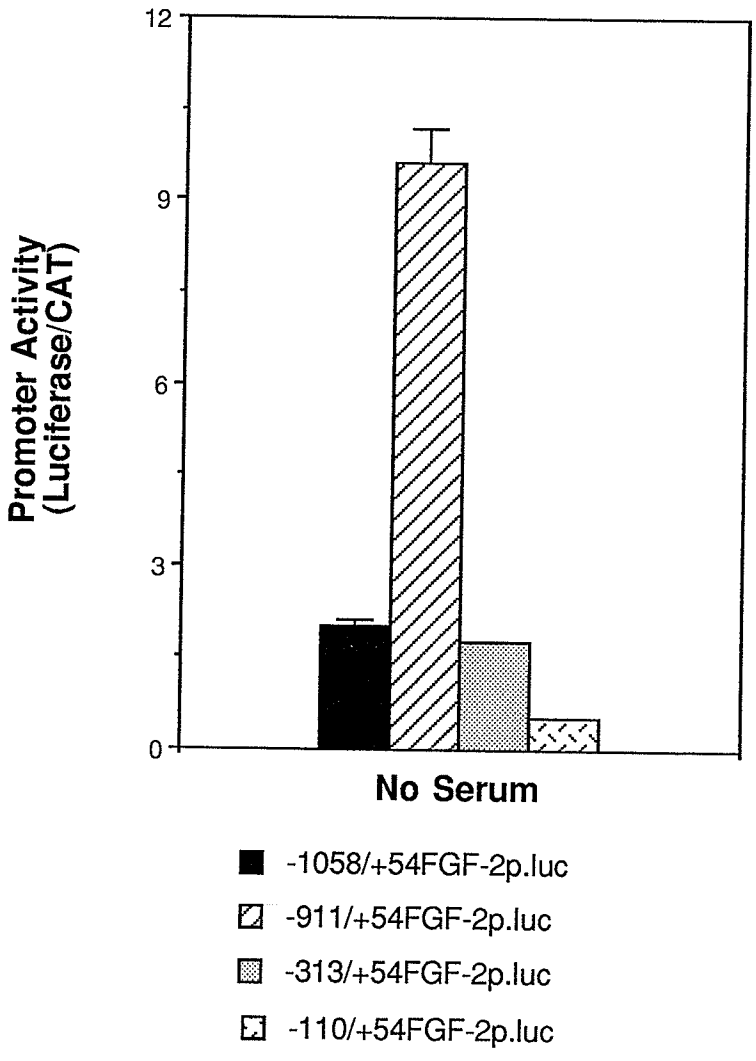
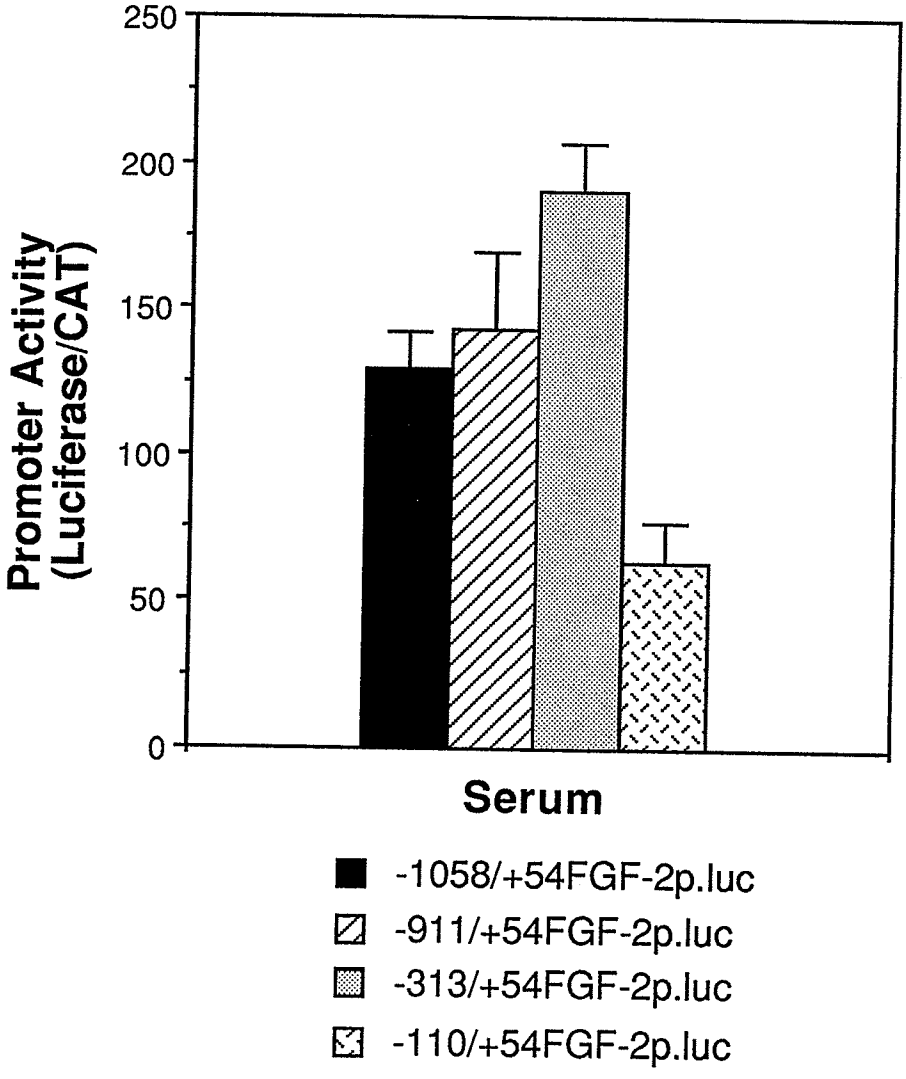

12
9
6
3
0
Promoter Activity
(Luciferase/CAT)
No Serum
-1058/+54FGF-2p.luc
-911/+54FGF-2p.luc
-313/+54FGF-2p.luc
-110/+54FGF-2p.luc

Fig. 60

Fig. 60. Evidence for the removal of repressor activity from the region -1058/-911 of the rat FGF-2 gene in the presence of mitogenic stimuli. Rat glioma C6 cells were transfected with hybrid FGF-2p/luciferase genes (-1058/+54FGF-2p.*luc*; -911/+54FGF-2p.*luc*, -313/+54FGF-2p.*luc* and -110/+54FGF-2p.*luc* ; Fig. 6) or a promoterless luciferase gene (-p.*luc*) and co-transfected with RSVp.CAT for 24 hours. Cells were then maintained for a further 48 hours in medium containing 10% FBS (+serum) before harvesting and assessment of reporter gene activities. The promoter activities (luciferase/CAT) for all hybrid gene are shown as the mean from at least 8 determinations after subtraction of -p.*luc* activity which serves as a measure of random transcription initiation. Basal level for -p.*luc* in serum treated cells was 4.5±0.37. There was no significant difference between promoter activities of -1058/+54FGF-2p.*luc* and -911/+54FGF-2p.*luc* in the presence of serum (mitogenic stimuli). The promoter activity of -313/+54FGF-2p.*luc* was significantly higher (1.5 fold, $p<0.01$) compared to that of -1058/+54FGF-2p.*luc* but not -911/+54FGF-2p.*luc* in the presence of serum. Bars represent standard error of the mean.


250
200
150
100
50
0
Promoter Activity
(Luciferase/CAT)
Serum
-1058/+54FGF-2p.luc
-911/+54FGF-2p.luc
-313/+54FGF-2p.luc
-110/+54FGF-2p.luc

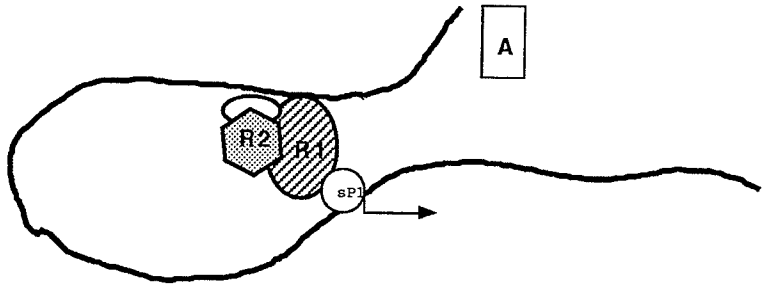
~164 nucleotides (-110 to +54) of the 5´-flanking region of the rat FGF-2 gene are sufficient to promote expression of hybrid luciferase gene in C6 cells (Fig. 60). Thus the functional data is consistent with the RNA hybridization (see section 8.5.4; Fig. 50) and primer extension (see 8.5.5; Fig. 51, 52) results in that the major transcriptional start site(s) of rat FGF-2 gene could be located in *Dra*1/*Sma*1 (-110 to +54) fragment (see section 8.5.6; Fig. 53).

The repressor activity associated with -1058/-911 region suggests that the basal transcription of FGF-2 gene would be repressed in the absence of any stimulus (Fig. 61) and this repression can be relieved by serum perhaps through modulation of the binding properties of the putative repressor protein(s). Thus these results implicate a "negative element/derepressor mechanism" for the tight regulation of FGF-2 gene to avoid uncontrolled effects of such a potent molecule (Fig. 61). Sequence analysis of rat FGF-2 gene in between -1058 and -911 nucleotides, revealed two binding sites for a transcription factor LBP-1 (WCTGG, -1028 to -1024 and -1021 to -1017 ; see section 8.5.6; Table 5). LBP-1 is known to control the transcription of growth regulatory genes in both positive (Jones *et al.*, 1988; Yoon J-B *et al.*, 1994) and negative (Parada *et al.*, 1995; Yoon J-B *et al.*, 1994) manners and it is possible that this factor is involved in the negative regulation of rat FGF-2 gene. Serum induced transcription of many eukaryotic genes is largely due to binding of a transcription factor, serum response factor (SRF) to an AT-rich serum response element (SRE, *aka* CArG box; CC[A/T]$_6$GG) and its interaction with TFIIF in the promoter region (Hill *et al.*, 1994). The SRF was also shown to mediate serum-inducible transcription by binding to the unconventional AT-rich elements in several genes (Muscat *et al.*, 1988; Papadopoulos and Crow, 1993). However, rat FGF-2 promoter does not appear to contain any SRE-like sequence other than a few AT-rich stretches (GGACATTTATCC; -993 to -982 and GCATTTAAACTTTAGG; -116 to -101). The motif GCATTTAAACTTTAGG is largely conserved in the human FGF-2 5´-flanking DNA. The AT-rich stretch (5´-TTTAAA-3´) located at nucleotides -113/-108 has been described as a promoter element in the mouse whey acidic protein gene and was shown to interact with nuclear protein (Lubon and Hennighausen, 1987).

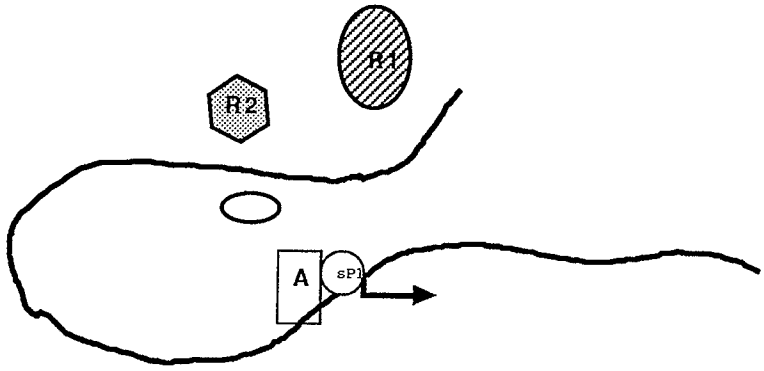
The hybrid FGF-2/luciferase genes, -911/+54FGF-2p.*luc*, -313/+54FGF-2p.*luc* and -110FGF-2p.*luc* were also transfected into C6 cells and tested for their responsiveness

Fig. 61

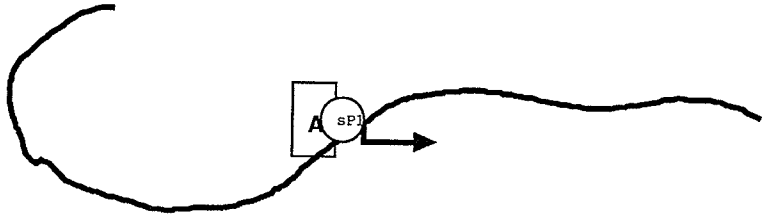
Fig. 61. A hypothetical model for the regulation of rat FGF-2 gene in the presence or absence of mitogenic stimuli. A=activator; R1=repressor 1; R2=repressor 2; Sp1=transcription factor. Repressed or weak transcription is indicated by a thin arrow. Active transcription is indicated by solid arrows.



No Mitogen



+ Mitogen



No repressor
binding region

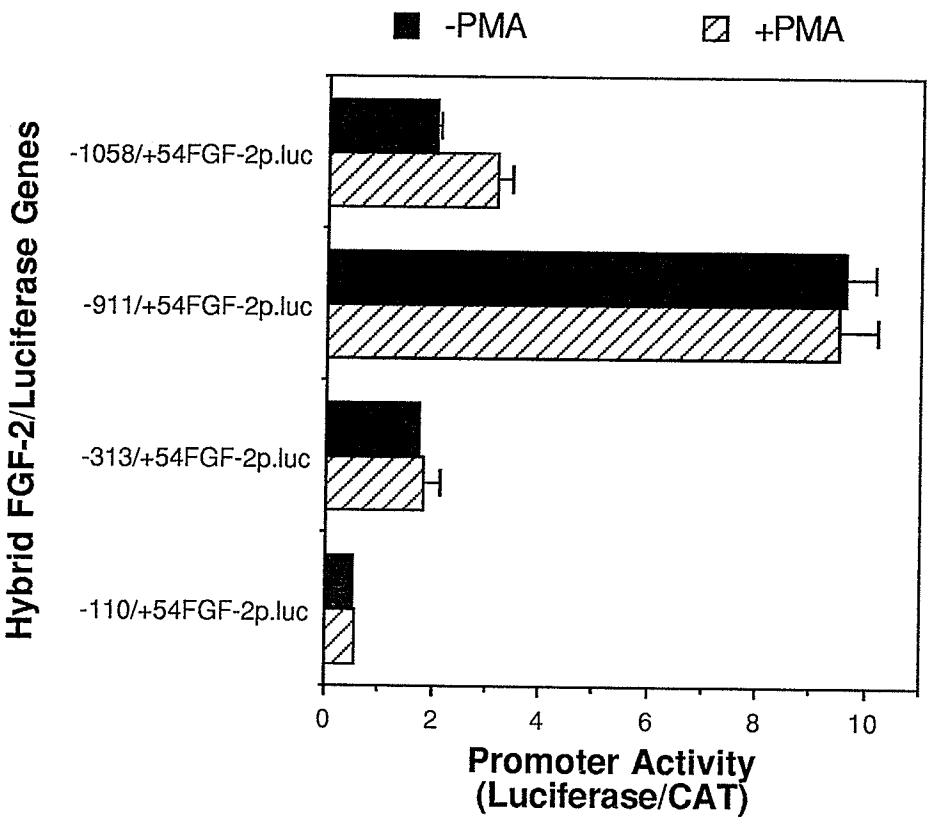
to PMA. Compared to the promoter activity in untreated cells, there was no significant increase in the activities of these hybrid genes in PMA treated cells (Fig. 62) unlike the effect seen with -1058/+54FGF-2p.*luc* gene (Figs. 58, 62). These results suggest that the deletion of 147 bp from nucleotide -1058 to -911 was sufficient for loss of phorbol ester responsiveness. However, the PMA induced increase in the promoter activity of -1058/+54FGF-2p.*luc* was not sufficient to remove the repressor activity associated with -1058/-911 region (Figs. 59, 62) unlike the effect seen with serum (Fig. 60). This could be either due to differences in the potencies or due to the use of alternative stimulatory mechanisms by serum and PMA. Phorbol ester is known to induce protein kinase C (PKC) which stimulates gene transcription via AP-1 or AP-2 transcription factors (Auwerx and Sassone-Corsi, 1991; Mitchell *et al.*, 1987). PKC can also activates the MAP kinase pathway resulting in phosphorylation of the Ternary Complex Factors (TCFs) that can bind to the Ets consensus sequence C/GA/CGGAA/TGC/T (Faisst and Meyer, 1992; Hill *et al.*, 1994). Phosphorylation of TCFs in turn potentiate their ability to activate transcription in conjunction with SRF (Hill *et al.*, 1994). The 5′-flanking region of rat FGF-2, unlike its human counterpart, does not contain AP-2 (or AP-1) sites, but an Ets-like sequence (5′-CAGGAAGg-3′; -133 to -126) is located just in front of an AT-rich motif (5′GCATTTAAACTTTAGG 3′; -116 to -101; see section 8.5.6; Fig. 53 and Table 5). It is unlikely that the PMA induced transcription of rat FGF-2 gene may be due to either of these mechanisms, as the promoter activity of the truncated construct -313/+54FGF-2p.*luc*. containing Ets-like and AT-rich sequences, was not induced by PMA treatment in C6 cells (Fig. 62). Clearly, these data do not exclude the possibility that AP-1 or AP-2 sites are contained in sequences outside of those reported in this study.

9.5.4 Characterization of protein interactions with -1058/-911 region of the FGF-2 gene in the presence or absence mitogenic stimuli

In light of the serum as well as phorbol ester responsiveness and suggestion of repressor activity (see section 9.5.3; Figs. 59 to 62), gel mobility shift assays were used to assess the patterns of interaction between radiolabelled region -1058/-911 (*Bam*H1/*Bgl*II fragment; see section 8.5.2; Fig. 48b), and nuclear proteins isolated from C6 glioma cells grown in the absence of serum as well as presence of 100 nM PMA or 10% FBS. The

Fig. 62

Fig. 62. Localization of phorbal ester response in 5´-flanking region of the rat FGF-2 gene and an evidence for the partial removal of repressor activity associated with -1058/-911 region. Rat glioma C6 cells were transfected with hybrid FGF-2p/luciferase genes (-1058/+54FGF-2p.*luc*; -911/+54FGF-2p.*luc*, -313/+54FGF-2p.*luc* and -110/+54FGF-2p.*luc* ; Fig. 6) or a promoterless luciferase gene (-p.*luc*) and co-transfected with RSVp.CAT for 24 hours. Cells were then maintained for 24 hours in serum-free conditions and then in the presence or absence of 100 nM PMA (±PMA) for the final 24 hours before harvesting and assessment of reporter gene activities. The promoter activities (luciferase/CAT) for all hybrid gene are shown as the mean from at least 6-12 determinations after subtraction of -p.*luc* activity which serves as a measure of random transcription initiation. Basal levels for -p.*luc* in the presence or absence of PMA were 0.05±0.01 and 0.07±0.04, respectively. There was a significant increase (1.6 fold, $p<0.002$) in the promoter activity of -1058/+54FGF-2p.*luc* in cells treated with PMA compared to untreated cells. However, there was no significant difference between the promoter activities of hybrid genes -911/+54FGF-2p.*luc*, -313/+54FGF-2p.*luc* and -110/+54FGF-2p.*luc* in presence or absence of PMA. Bars represent standard error of the mean.


-PMA
+PMA
Hybrid FGF-2/Luciferase Genes
-1058/+54FGF-2p.luc
-911/+54FGF-2p.luc
-313/+54FGF-2p.luc
-110/+54FGF-2p.luc
0
2
4
6
8
10
Promoter Activity
(Luciferase/CAT)

presence of a low mobility/large complex was evident with increasing levels of nuclear protein from C6 cells grown in the absence of phorbol ester or serum (Fig. 63, lanes b,c,d). By contrast, nuclear protein from cells treated with 100 nM PMA (lanes e,f,g) or 10% FBS (lanes h,i,j) gave rise to a similar pattern of DNA/protein interaction but the major complex was reduced in size (increased in mobility) in comparison to that seen with no phorbol ester or serum (Fig. 63). It could be that the protein interactions in -1058/-911 region of FGF-2 gene are modulated in response to serum in C6 glioma cells possibly to relieve a repressor protein(s). Thus these results are in agreement with the functional data (see section 9.5.3; Figs. 59, 60, 62) which supports the notion of "negative element/derepressor mechanism" (Fig. 61).

Further, nuclease protection assays were performed using radiolabelled region -1058/-911 (*Bam*H1/*Bgl*II fragment) to identify sites of DNA and C6 nuclear protein interactions indicated by the results of the gel mobility shift assays, as well as to investigate a possible alteration in DNA binding resulting from treatment with mitogenic stimuli. Three regions in the radiolabelled -1058/-911 fragment were relatively protected from DNase I digestion [DNase I resistant regions (DRR) I to III] in the presence of nuclear proteins from C6 cells maintained in serum free conditions (lane 3, Fig. 64) but not with nuclear proteins from C6 cells treated with 10% FBS (lane 2, Fig. 64). These regions were mapped to the sequences (i) 5′-AACTGATGGGGTTGAGGACATTTATCCCCAAA-3′ (DRR-I; -1008 to -977), (ii) 5′-AC<u>TCTGG</u>AG<u>ACTGG</u>C-3′ (DRR-II; -1030 to -1016) and (iii) 5′-ACTGAAGAAGATATTAAT-3′ (DRR-III; -1050 to -1033) using chemical sequencing reactions. Comparison of these sequences with the known transcription factor consensus sequences revealed two binding sites for LBP-1 (<u>WCTGG</u>, -1028 to -1024 and -1021 to -1017; Parada *et al.*, 1995; Yoon *et al.*, 1994) in DRR-II. It is possible that one or more nuclear proteins (including LBP-1) from C6 glioma cells can bind to these sequences in the absence of mitogenic stimuli (lane 3, Fig. 64) and binding properties of these proteins may be altered or interfered in response to serum treatment (lane 2, Fig. 64). These differential DNA protein interactions are consistent with different sizes of complexes observed during gel mobility shift assays (Fig. 63) as well as differences in the promoter activities (see section 9.5.3; Figs. 59, 60) in the absence or presence of serum. However, the DNase I digestion pattern of radiolabelled -1058/-911 fragment in the presence of C6 cells treated

Fig. 63

Fig. 63. Assessment of DNA protein interactions in -1058/-911 region of the rat FGF-2 gene with nuclear proteins isolated from C6 glial cells grown in the presence or absence mitogenic stimuli. Radiolabelled -1058/-911 fragment of the rat FGF-2 gene (lane a) was incubated with 4 μg (lanes b,e,h), 8 μg (lanes c,f,i) and 12 μg (lanes d,g,j) of nuclear protein isolated from rat C6 glial cells grown in the absence of serum (lanes b,c,d) or presence of serum (lanes h,i,j) and PMA (lanes e,f,g) and resolved on a 4% polyacrylamide gel. A low mobility/large complex (solid arrow) was seen in the absence of serum or phorbol ester (lane c) in contrast to a major complex with increased mobility (open arrow) in the presence of serum (lane j) and phorbol ester (lane g). Small arrow indicates the position of a common complex observed in the absence of serum (lane b) as well as presence of PMA (lanes e,f) and serum (lanes h,i).

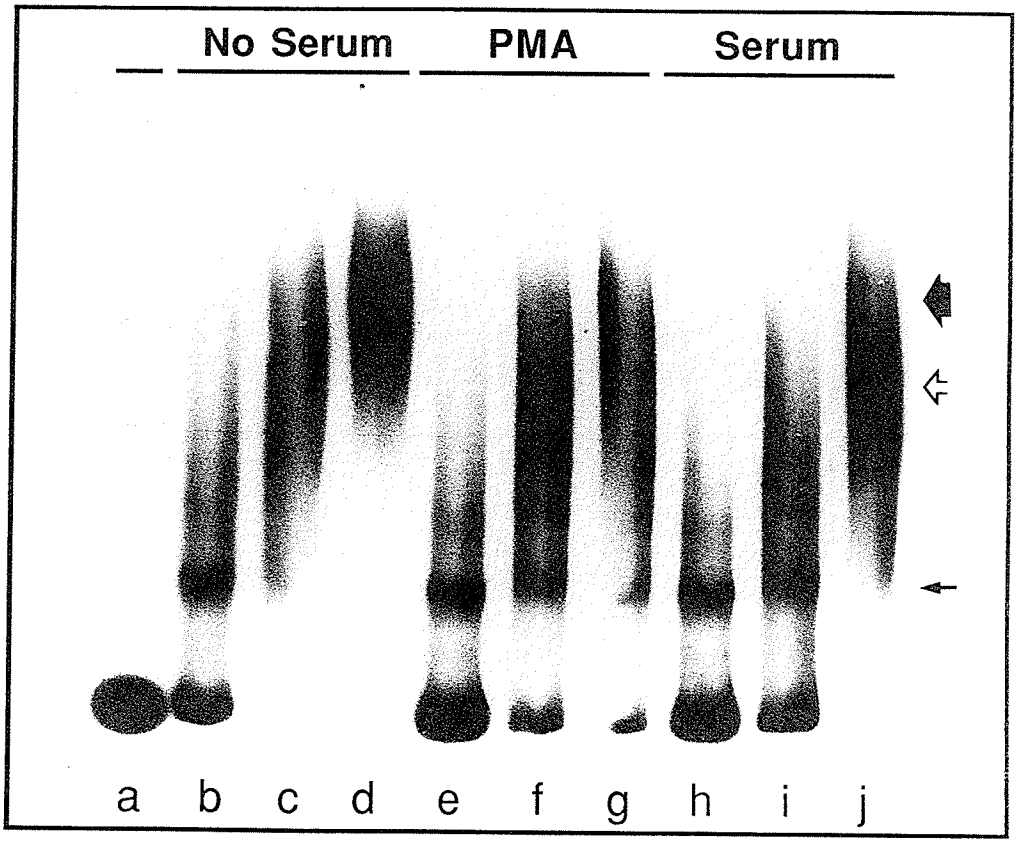
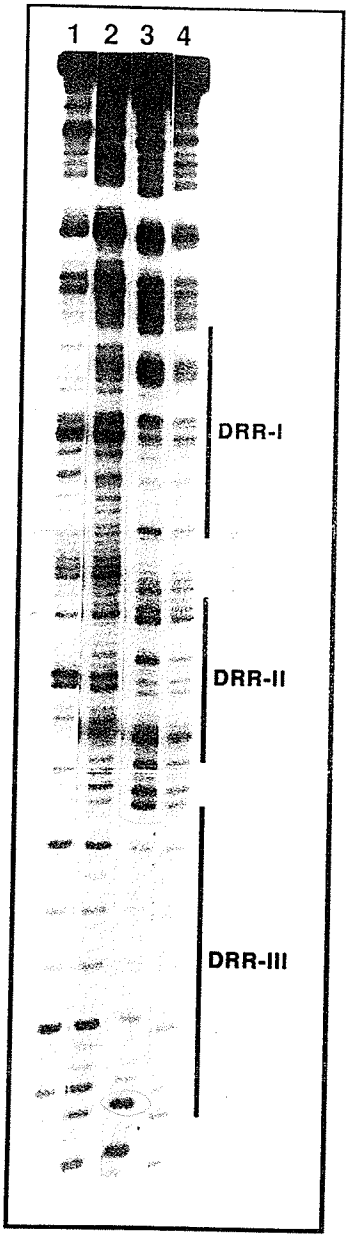

No Serum
PMA
Serum
a b c d e f g h i j

Fig. 64

Fig. 64. Identification of sequences responsible for differential DNA protein interactions in -1058/-911 region of the rat FGF-2 gene in the presence or absence mitogenic stimuli. DNase I protection assay was employed to identify the sites of DNA protein interactions in the the -1058/-911 region of the rat FGF-2 gene. The -1058/-911 fragment was isolated as an *Eco*RI/*Hin*dIII fragment that was radiolabelled at *Eco*RI end from the plasmid B2Δbgx (see Fig. 4) and incubated without (lane 1) or with (lanes 2-4) 15 μg of nuclear protein isolated from rat C6 glial cells grown in the presence (lane 2) or absence (lane 3) of serum and presence of PMA (lane 4). The samples were digested with DNase I and resolved together with chemical sequencing reactions (G and G+A) in an 8% polyacrylamide/8 M urea gel. Nuclease protection sites (DRR-1 to DRR-III) were visualized by autoradiography.


1 2 3 4
DRR-I
DRR-II
DRR-III

with 100 nM PMA (lane 4, Fig. 64) did not reveal any apparent interaction when compared to the patterns observed in the absence (lane 3, Fig. 64) or presence (lane 2, Fig. 64) of serum. It could be that the stimulation of glioma C6 cells by PMA may not alter the binding events in -1058/-911 region of the rat FGF-2 gene as efficiently as serum. This is also consistent with the functional data that PMA was able to stimulate modestly (1.6 fold) the promoter activity of -1058/+54FGF-2p.*luc* but not upto the level of activity seen with -911/+54FGF-2p.*luc* (see section 9.5.3; Fig. 62). Alternatively, PMA mediated stimulation of rat FGF-2 gene may utilize entirely a different mechanism. However, the precise functions of DRRs (Fig. 64) in the context of FGF-2 gene regulation need to be determined by using site directed mutagenesis studies. It is possible that the mutations in either one or all three of these DRRs in -1058/-911 region may relieve the repressor effect on the rat FGF-2 gene transcription in the absence of mitogenic stimuli.

9.6 Concluding remarks

In summary, these results suggest that: (i) the 5′-flanking region of rat FGF-2 gene, when placed upstream of luciferase sequence, can promote the expression of hybrid luciferase gene in rat glioma C6, heart myoblast H9c2 and human astrocytoma U87-MG cells after gene transfer in a non tissue specific manner, (ii) the promoter activity of the FGF-2/luciferase hybrid gene is higher in glial cells (C6 and U87-MG) compared to non glial (H9c2) cells, (iii) ~164 (-110/+54) nucleotides of upstream sequences are sufficient to maintain the minimal promoter activity in the transient expression assays, (iv) FGF-2 promoter activity can be increased in response to mitogenic stimuli (phorbol ester and serum) in glioma C6 cells, (v) a repressor activity is localized to -1058/-911 region of the rat FGF-2 gene in the absence of mitogenic stimuli and it can be relieved only in part by PMA but completely in response to serum treatment (vi) at least a part of the serum mediated stimulation of the rat FGF-2 gene transcription can be localized to -313/-110 region, (vii) deletion of -1058/-911 region was sufficient for the loss of phorbol ester responsiveness of the rat FGF-2 gene in glioma C6 cells, (viii) specific and differential DNA protein interactions occur between -1058/-911 region and nuclear protein from C6 cells grown in the presence or absence of mitogenic stimuli (serum and phorbol esters), (ix)

DNA protein interactions occurring at three regions (DRR-I to III) in -1058/-911 region can be modulated in response to mitogenic stimuli in C6 cells, (x) FGF-2 promoter activity in C6 cells can be increased in response to mitogenic stimuli, possibly through relieving a repressor activity and a "derepressor mechanism" exists in C6 glial cells for the tight regulation of FGF-2 gene to avoid uncontrolled production of such a potent molecule.

Chapter 10

Final statement

The normal processes of cellular growth and differentiation are impaired during several disease conditions including diseases of cardiovascular and central nervous systems. Both cardiovascular and neurological disorders are known to have a tremendous impact on the public health. Heart diseases are complicated by the inability of adult cardiac myocytes to divide following myocardial cell loss, while neurodegenerative diseases are characterized by a marked decrease in the neuronal survival. These drawbacks have stimulated a large interest in biomedical research to identify the factors that are involved in the proliferation and differentiation of cells of cardiovascular as well as central nervous systems which will facilitate the efforts to stimulate cell growth or survival in the affected individuals. Potential means of therapies include direct application of such factors as well as introduction of their genes or cells that are genetically programmed to overexpress these factors into the affected tissues. Alternatively, the exploration of means that can modulate the transcription of specific genes with a view to alter the endogenous levels of therapeutically important factors, would also be beneficial for the affected patients.

Basic fibroblast growth factor (FGF-2) is a potent mitogen and exists in high and low molecular weight forms because of alternate initiation of translation from the same mRNA (Florkiewicz and Sommer, 1989). FGF-2 mediates various biological responses by binding to cell surface receptors of the tyrosine kinase family (Baird, 1994; Mason, 1994). Low molecular weight form of FGF-2 purified through recombinant DNA technology, has been shown to stimulate regeneration of nerve and retinal cells (Baird and Walicke, 1989; Sievers *et al.*, 1987). In addition, a high molecular weight form of FGF-2 has been implicated in hepatic regeneration (Presta *et al.*, 1989). FGF-2 is shown to play a vital role in cardiogenesis, growth of myocardium and cardioprotection (Padua and Kardami, 1993; Sugi *et al.*, 1993; Yanagisawa-Miwa *et al.*, 1992). Loss of proliferative potential in adult cardiac myocytes is reported to correlate with a loss of high molecular weight forms of FGF-2 (Liu *et al.*, 1993). Thus, it is possible that the differentiated phenotype of adult cardiac myocytes can be reverted back to proliferative/less differentiated embryonic

phenotype by increasing the levels of either high and or low molecular weight forms of FGF-2. Therefore, in the first part of my doctoral studies, I examined the effects of overexpression of high and or low molecular weight forms of FGF-2 on cardiac myocyte growth and differentiation with a view to stimulate myocyte growth during cardiac damage.

Modified rat FGF-2 cDNAs that can preferentially express high (22-21.5 kD) or low molecular weight forms (18 kD) of FGF-2 were generated and introduced into cardiac as well as non cardiac cell types by gene transfer. Expression studies indicated that the translation of low molecular weight form of FGF-2 is repressed by sequences located upstream of AUG codon in the rat FGF-2 mRNA. Similar repressor effect for the sequences located upstream of AUG codon in the human FGF-2 mRNA was reported (Prats *et al.*, 1992). Identification of these "repressor sequences" in the rat FGF-2 mRNA through mutation studies would provide an insight into the mechanism behind the differential regulation of translation from AUG vs CUG codons as well as developmental regulation of different forms of FGF-2 in heart, brain and other tissues (Liu *et al.*, 1993; Giordano *et al.*, 1992). Immunolocalization studies indicated differences in the subcellular distribution of high and low molecular weight forms in transfected cells. High molecular weight FGF-2 was found predominantly in the nucleus whereas low molecular weight FGF-2 localized to the cytoplasm as well as nucleus of transfected cells, suggesting that these forms may have different functions. Further, the effects of high and low molecular weight forms of FGF-2 on the growth and differentiation of embryonic and postnatal cardiac myocytes were analysed after gene transfer. Significant increases in DNA synthesis and proliferation of cardiac myocytes were observed in cultures overexpressing either high or low molecular weight forms of FGF-2. These studies also provided an evidence that the stimulation of mitosis by 22-21.5 or 18 kD FGF-2 represents a proximity-dependent paracrine effect. In addition, overexpression of high or low molecular weight FGF-2 in postnatal ventricular myocyte cultures resulted in disorganization of sarcomeric structure as well as significant decreases in the differentiation markers myosin and desmin suggesting a shift in the differentiation status of transfected myocyte cultures more towards proliferative/embryonic phenotype. Changes in the proliferation and differentiation potentials of cardiac myocytes can be linked to the dramatic nuclear events which occur during cardiac development. These include a gradual decrease in the proliferative potential

of cardiac myocytes as they traverse through embryonic (dividing), neonatal (transitional) and adult (non dividing) stages (Clubb and Bishop, 1984; Zak, 1984). In addition, majority (~85%) of the adult rat cardiac myocytes become binucleated and thus binucleation is considered as an early marker of cardiac myocyte differentiation (Clubb and Bishop, 1984). Although, the process of binucleation is believed to occur through nuclear division without cytokinesis, there is scant information about the mechanisms governing this process (Clubb and Bishop, 1984). My studies provided the first evidence that overexpression of high but not low molecular weight FGF-2 favoured an increase in the binucleation of postnatal cardiac myocyte cultures in a cell surface receptor-independent pathway. It was also observed that high molecular weight FGF-2 mediated increase in binucleation occurs via amitosis. However, my studies do not rule out the possibility of karyokinesis as an alternative mechanism for binucleation.

In contrast to their similar effects on cardiac myocyte proliferation, overexpression of 22-21.5 kD but not 18 kD FGF-2 was also associated with clumping of the DNA and nuclear fragmentation which resemble features of apoptosis or programmed cell death in embryonic as well as postnatal ventricular myocyte cultures. By contrast to the nuclear changes in apoptotic cells, necrotic cell death is characterized by loss of plasma membrane integrity and severe changes in the cytoplasmic components (Arends *et al.*, 1990). The DNA clumping/nuclear fragmentation associated with the overexpression of 22-21.5 kD FGF-2 appeared distinct from the process of cell death due to necrosis as indicated by exclusion of myosin staining in the nuclear compartment, intact cell and nuclear membranes as well as from the normal stages of cell cycle (Cattini *et al.*, 1991; Kardami *et al.*, 1991a) and represented the first report of a differential effect of high versus low molecular weight forms of FGF-2 (Pasumarthi *et al.*, 1994; Pasumarthi *et al.*, 1996). Although, the physiological significance of this process is yet to be investigated, similar nuclear morphology/chromatin condensation was reported in rat cardiac myocytes subjected for H_2O_2 injury (Janero *et al.*, 1991). In addition, a transient increase in high molecular weight forms of FGF-2 was detected in the rat hearts during isoproterenol-induced cardiac myocyte injury (Padua and Kardami, 1993). It is possible that the 22-21.5 kD FGF-2 binds to the chromatin and initiates events of apoptosis (eg. chromatin condensation and DNA fragmentation) by interacting with other proteins (eg. proteases, nucleases) in the

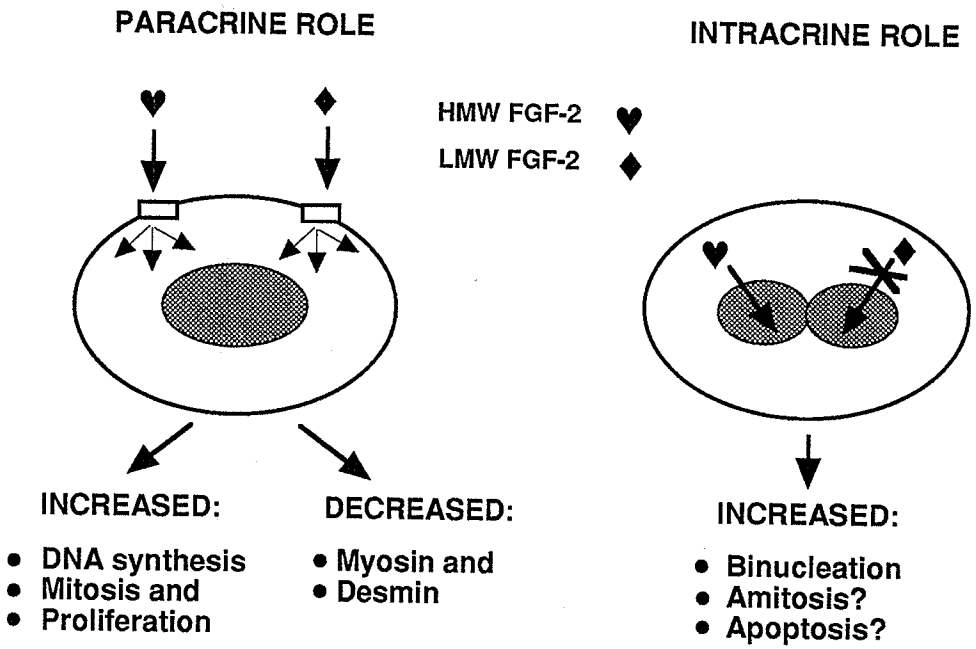
nuclei of damaged cardiac myocytes through a cell surface receptor-independent nuclear pathway. In deed, it was shown that FGF-2 can bind to nuclear chromatin with a high affinity (Gualandris *et al.*, 1993) and interact with several nuclear proteins (Amalric *et al.*, 1994). The effects of 22-21.5 kD FGF-2 on the nuclear morphology were also seen in the presence of neutralizing antibodies to FGF-2 suggesting that this is an intracrine mediated process. Therefore, it is conceivable that the DNA clumping and nuclear fragmentation induced by high molecular weight form FGF-2 may be involved in the removal of damaged cardiac myocytes during myocardial injury. Prior to testing this assumption, it is also important to establish the *in situ* localization of high molecular weight FGF-2 in the ischemia-reperfusion myocardial injury models using specific antibodies that can recognise 22-21 kD but not 18 kD FGF-2. Programmed cell death/ apoptosis of myocytes has been linked to ischemia reperfusion injury as well as excessive mechanical forces associated with increased ventricular loading (Kajstura *et al.*, 1996) and thus plays an important role in cardiac pathology. Although, my studies have provided an evidence that the high molecular weight forms of FGF-2 can initiate a form of apoptosis in the rat cardiac myocytes, it would be necessary to investigate further about the type of 22-21 kD FGF-2 induced apoptosis (eg. states of chromatin, nuclear membrane and contents of the cytoplasm) by examining the myocytes overexpressing 22-21 kD FGF-2 under a higher resolution using electron microscopy. It would also be interesting to examine whether 22-21 kD FGF-2 induced apoptosis in cardiac myocytes is accompanied by changes in the expression of Bax, Fas gene products (involved in the promotion of cell death; Oltavi *et al.*, 1993; Tanaka *et al.*, 1994) and Bcl-2 gene product (involved in the protection of cells against apoptosis; Reed, 1994) as well as whether the myocytes can be rescued from apoptosis by overexpression of Bcl-2. Information obtained through these studies should provide an insight into the measures that can offer protection against myocardial damage or ischemia-reperfusion injuries incurred during surgical procedures.

In summary, my studies indicated that both high and low molecular weight forms of FGF-2 can stimulate proliferation (as revealed by increases in LI, BrdU index, mitotic index and cell number) and decrease differentiation (as revealed by decreases in myosin and desmin levels) of ventricular myocytes in a proximity-dependent paracrine manner (Fig. 65). However, only high but not low molecular weight FGF-2 can act in an intracrine

Fig. 65

Fig. 65. Schematic diagram showing the paracrine or intracrine effects of high and low molecular weight forms of rat FGF-2 in cardiac myocytes.

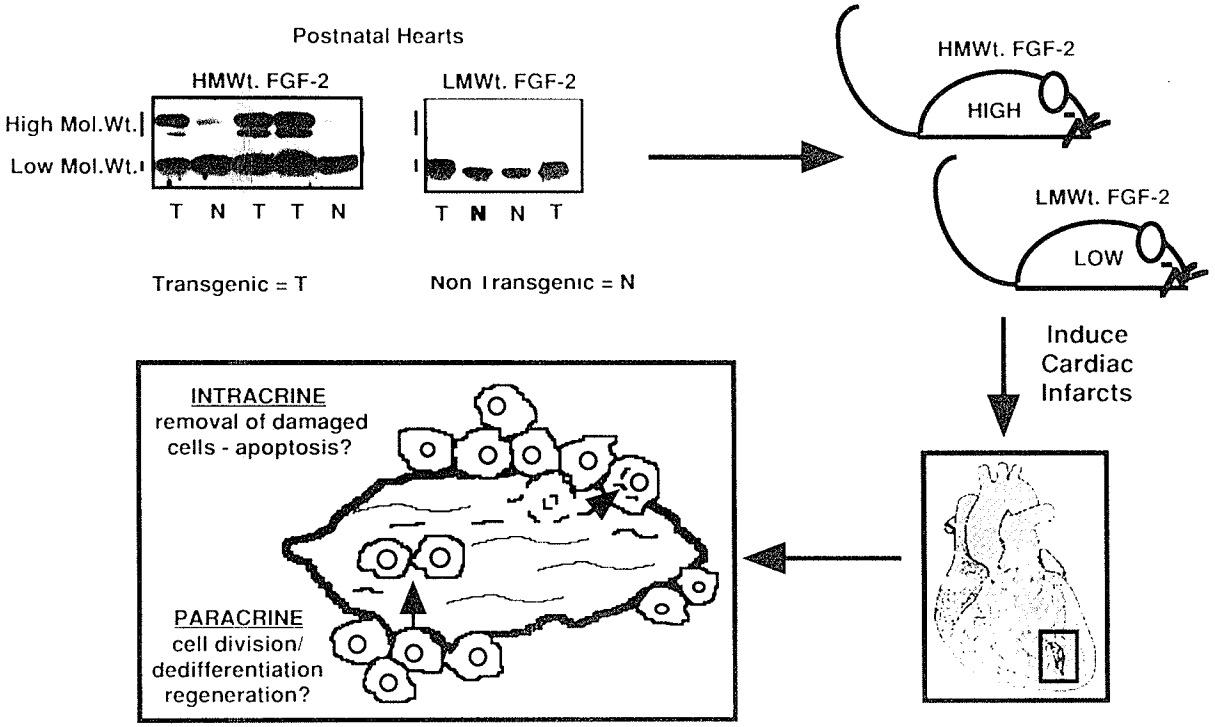
Effects of FGF-2 on Postnatal Cardiac Myocyte Growth



manner to increase DNA clumping/nuclear fragmentation and binucleation in ventricular myocytes (Fig. 65). Thus, these *in vitro* gene transfer studies provided substantial evidence that FGF-2 can be used to stimulate postnatal cardiac myocyte proliferation *in vivo*. However, it should be noted that these studies were performed on 1 day old postnatal cardiac myocytes, as similar studies on terminally differentiated cardiac myocytes are not feasible due to the paucity of methodologies to maintain them in culture for prolonged periods. Therefore, with a view to testing our hypothesis *in vivo,* we generated transgenic mice carrying modified FGF-2 cDNAs (ΔmetFGF or metFGF) under the control of RSV promoter, that are designed to overexpress either high or low molecular weight forms of FGF-2 specifically in the heart and these models are currently being characterized (Fig. 66). These transgenic mice would enable us to examine the effects of overexpression of different forms of FGF-2 at much later stages of postnatal cardiac development, as the RSV promoter was shown to be constitutively active in the transgenic mice hearts (Jackson *et al.*, 1990).

Using these *in vivo* models, it would be interesting to examine (i) whether the proliferative potential of adult cardiac myocytes can be increased in mice overexpressing either high or low molecular weight forms of FGF-2 (ii) whether the regenerative growth of postnatal cardiac myocytes can be stimulated after myocardial injury and (iii) the mechanisms governing the DNA clumping/nuclear fragmentation associated with the overexpression of high molecular weight FGF-2 and its possible involvement in the removal of damaged cardiac myocytes from the myocardium. Effects of overexpression of FGF-2 on cardiac myocyte proliferation can be assessed in the transgenic mice hearts *in situ* as well as in cardiac myocyte cultures generated from transgenic mice hearts *in vitro* by ^{3}H-thymidine and BrdU labelling methodologies (Marino *et al.*, 1991; Rumyantsev, 1991). Similarly, the levels of differentiation markers myosin and desmin can be assessed in transgenic versus non transgenic mice hearts. Although, there was a significant decrease in the RNA levels of α-MHC in postnatal rat myocyte cultures overexpressing FGF-2, my studies did not reveal any significant changes in the RNA levels of fetal markers ß-MHC and ANF. However, it was reported that exogenous treatment of rat cardiac myocyte cultures with FGF-2 upregulated both ß-MHC and ANF RNA levels (Parker and Schneider, 1991). Hence, it would be of interest to reexamine whether overexpression of

Fig. 66. An *in vivo* model for testing the regenerative potential and cardioprotective properties of high and low molecular weight forms of FGF-2 in an adult heart.


Postnatal Hearts
HMWt. FGF-2
LMWt. FGF-2
High Mol.Wt.
Low Mol.Wt.
T N T T N
T N N T
Transgenic = T
Non Transgenic = N
HMWt. FGF-2
HIGH
LMWt. FGF-2
LOW
Induce
Cardiac
Infarcts
INTRACRINE
removal of damaged
cells - apoptosis?
PARACRINE
cell division/
dedifferentiation
regeneration?

high or low molecular weight forms of FGF-2 has a similar effect *in vivo* on the levels of fetal markers ß-MHC and ANF as well as α-MHC in adult transgenic mice hearts. Higher levels of FGF-2 and re-expression of the embryonic marker, vimentin, were found in the cardiac lesions induced by isoproterenol. In addition, my studies provided additional evidence that overexpression of FGF-2 in rat cardiac myocytes can increase the proliferative potential. Therefore, the regenerative potential of cardiac myocytes in transgenic mice overexpressing FGF-2 can be tested after inducing myocardial infarctions by using isoproterenol (Padua and Kardami, 1993). Regenerative response of cardiac myocytes can be correlated with increases in the number of mitotic figures or ^{3}H-thymidine and BrdU labelling indices in the lesioned area of transgenic heart compared to the non lesioned area from a non transgenic mouse heart. Isoproterenol-induced injury model can also be use to test whether there is any correlation between overexpression of high molecular weight FGF-2 and removal of damaged cardiac myocytes surrounding lesions in transgenic mice hearts. It would be interesting to see if the number of myocytes undergoing chromatin condensation and nuclear fragmentation increase in the lesioned area in addition to increases in apoptosis indicator proteins Bax, Fas and Bcl-2.

FGF-2 also plays a vital role in the morphogenesis of the central nervous system (CNS) and exerts potent multipotential trophic effects on neurons, endothelial cells and glia of the CNS (Powell *et al.*, 1991; Riva and Mocchetti, 1991). It is required for the origin and growth of neuronal and glial cells as well as nerve cell regeneration (Baird, 1994). Glial cells produce high levels of FGF-2, that can stimulate autocrine growth as well as the survival and differentiated functions of neurons in a paracrine manner. Clearly, this potent mitogen must be tightly regulated to avoid uncontrolled proliferation of cells. Altered expression of FGF-2 has been implicated in the disease process of several neurodegenerative disorders. While increased levels of FGF-2 were found in the astroglial cells of severely affected areas in Alzheimer's and Huntington's diseases (Cotman and Gomez-Pinilla, 1991; Tooyama *et al.*, 1993a), decreased levels of FGF-2 were associated with the dopaminergic neurons of the midbrain in Parkinson's disease (Tooyama *et al.*, 1993b). Although neurons are the key players in the mammalian nervous system, they are outnumbered by a supporting cast of glial cells (Alberts *et al.*, 1989). Thus, it is possible that the regulation of endogenous levels of FGF-2 in glial cells may alleviate at least some

of the problems associated with neurodegenerative diseases.

Transcription of an eukaryotic gene is regulated by certain transcription factors which can bind to specific *cis*-elements that are located in the 5′-flanking region of that gene. The relative levels or availability of these transcription factors in cells can be stimulated in both positive and negative manners by certain chemotherapeutic agents. For example steroid hormones (glucocorticoids, progestins, mineralocorticoids and androgens) can bind to their respective receptors and these hormone-receptor complexes can recognise specific responsive elements in the target genes to activate their expression (Cato *et al.*, 1986; Cato *et al.*, 1987; Arriza *et al.*, 1987). Transcription factor NF-1kB is required for the expression of several cellular genes (eg. interleukin-1, interleukin-6, interferon-ß, TNF-α) that are involved in inflammation and infection (Grill *et al.*, 1993). The anti-inflammatory drugs sodium salicylate and aspirin are known to decrease the availability of transcription factor NF-1kB by inhibiting the degradation of NF-1kB inhibitor protein and thus facilitate the retention of NF-1kB in the cytoplasm (Kopp and Ghosh, 1994). Thus, it is possible to regulate the endogenous levels of FGF-2 gene product via specific chemotherapeutic agents provided we know the information about the types of *cis*-elements present in the 5′-flanking region of FGF-2 gene, factors binding to those sites and mechanisms that can regulate the transcription of FGF-2 gene. Although, the 5′-flanking region of the human FGF-2 gene has been cloned (Shibata *et al.*, 1991), there is no information about its regulation in the CNS, especially during neurodegenerative diseases. This is mainly because of the practical problems associated with the availability of tissue samples from patients with neurodegenerative disorders. Studies conducted on human cell lines may solve this problem to some extent, but information obtained through such attempts will ultimately have to be tested *in vivo* on the diseased patients which again raises ethical concerns. All these constraints can be overcome by the use of experimental animal models such as rodents. There are several well established animal models for neurodegenerative disorders (Anger, 1991; Price *et al.*, 1991; Ripps *et al.*, 1995; Sengstock *et al.*, 1994) which may facilitate the efforts to test suitable therapeutic agents identified through *in vitro* studies, with regard to regulating endogenous levels of FGF-2 in the context of lesions associated with the CNS. However, the 5′-flanking region of the rodent (rat or mice) FGF-2 gene is not available for such studies. Therefore, in the second

part of my doctoral thesis, I cloned the 5′-flanking region of the rat FGF-2 gene and attempted to increase our current knowledge about the mechanisms regulating endogenous levels of FGF-2 in glial cells with a view to promote neuronal survival during neurodegenerative diseases.

A Sprague Dawley rat testis genomic library was screened using rat FGF-2 cDNA specific probes to isolate potential clones containing the 5′-flanking region of the rat FGF-2 gene. Based on partial restriction mapping and DNA blot analysis of isolated genomic clones, 5′-flanking region as well as 5′ end of cDNA sequence were most likely to be found in two genomic fragments of 1.4 kb (B2) and 1 kb (B1) sizes. Hence, B2 and B1 fragments were subcloned and sequenced. Sequence analysis revealed that B2 fragment contains the 5′-flanking region in addition to an overlap at its 3′ end with the coding region (including methionine codon) of previously reported FGF-2 cDNA sequences (Kurokawa *et al.*, 1988; Shimasaki *et al.*, 1988). By contrast, the first 98 nucleotides of B1 fragment share an overlap with the coding sequence which is contiguous to that of B2 fragment. The remaining sequence of B1 did not reveal any homology with the rat FGF-2 cDNA sequence and thus represents the intervening sequence. However, there is a discrepancy between the 5′ ends of previously reported rat brain (Kurokawa *et al.*, 1988) and ovarian (Shimasaki *et al.*, 1988) FGF-2 sequences. The ovarian cDNA is reported to contain an additional 280 bp of 5′ untranslated sequence compared to the brain cDNA and this additional sequence is also found in the 1.4 kb (B2) genomic fragment. It was suggested that the shorter cDNA sequence reported for rat brain FGF-2 could have resulted from either premature termination of reverse transcription during cDNA synthesis or from the use of alternative transcription initiation sites in brain versus ovary in a tissue specific manner (Shimasaki *et al.*, 1988). In order to resolve this discrepancy between the 5′ ends of rat brain and ovarian cDNAs, I employed RNA blotting as well as primer extension experiments using RNA samples from various rat cell types including brain and ovary. Results of RNA blotting studies indicated that the probes derived from the additional 280 bp sequence found in ovarian cDNA, did not hybridize to any RNA samples obtained from rat tissues including ovary. By contrast, the probe containing the 5′ end of brain cDNA hybridized to all RNA samples. This suggests that the 5′ end reported for the brain cDNA is more representative of FGF-2 RNA detected in all tissues (including ovary) examined. Primer extension results

were also consistent with the RNA blotting results and revealed multiple transcription initiation sites (P_0, P_1, and P_2) which lie within 45 nucleotides upstream of the 5′ end of rat brain cDNA in the genomic sequence (see section 8.5.6; Fig. 53).

Transcription of FGF-2 gene is reported to respond to mitogenic stimuli such as serum and phorbol esters in rat astroglial and hippocampal primary cell cultures as well as the rat C6 glioma cell line (Flott-Rahmel *et al.*, 1992; Powell and Klagsbrun, 1993). Therefore, I assessed the effect of phorbol ester on the stimulation of transcription from initiation sites P_0, P_1, and P_2 in glioma C6 cells, using primer extension analysis. Phorbol ester treatment resulted in the stimulation of transcription from P_0 site in addition to the induction of a "new" site (P_i) in C6 glioma cells. Similarly, the human FGF-1 gene transcription was shown to be modulated via an alternative promoter in response to phorbol ester and serum stimulation (Chotani *et al.*, 1995). Thus my results suggest that the rat FGF-2 gene transcription can also occur through alternative transcription initiation sites in response to various stimuli. If this assumption is true, my results also offer a possible explanation for the discrepancy between brain and ovarian FGF-2 cDNA sequences. The rat ovarian cDNA was isolated from a pregnant mare serum gonadotropin (PMSG)-stimulated rat ovarian cDNA library (Shimasaki *et al.*, 1988). Thus, it is possible that the FGF-2 transcript used for cDNA synthesis in this case, was induced by PMSG from an upstream alternative start site. However, under physiological conditions, FGF-2 gene transcription is initiated from $P_0 > P_2 > P_1$ in all rat tissues including ovary and as a result the additional ovarian cDNA sequence located further beyond these sites, can not be detected in the normal tissues. It is possible that these additional sequences found in the ovarian FGF-2 cDNA isolated from PMSG-stimulated ovaries may regulate the stability or half-life of FGF-2 RNA. Existence of such regulatory sequences in the 3′ end of rat brain FGF-2 have been recently reported (el Husseini *et al.*, 1992). Gonadotropin induced superovulation is known to trigger extensive growth response in ovarian cells as well as angiogenesis to facilitate folliculogenesis and ovulation (Rone and Goodman, 1990). FGF-2 is known to be a strong promoter of both cell proliferation and new blood vessel formation (Slavin, 1995). FGF-2 has been implicated to play a vital role in the estrus cycle (Guthridge *et al.*, 1992) and growth of ovarian cells (Lavranos *et al.*, 1994). However the RNA stability of several growth factors including FGF-2 was shown to be compromised at the

posttrasnscriptional level (Akashi *et al.*, 1991; Brawerman, 1987; el-Husseini *et al.*, 1992; Kimelman and Kirschner, 1989). It could be that the ovarian cellular machinery, when stimulated by hormones (eg. PMSG) uses an alternative FGF-2 transcript with higher stability to meet the increased growth requirements (Fig. 67). Analysis of RNA samples from superovulated and non superovulated ovaries for the presence of this longer FGF-2 transcript, using the additional 280 bp ovarian sequence may be a compelling experiment to prove this hypothesis. It would also be interesting to see whether this longer FGF-2 transcript exists in ovarian cells during estrus cycle and normal ovulation period.

Further, sequence analysis of the 5′-flanking region of the rat FGF-2 gene revealed potential binding sites for known transcription factors CREB, Ets, LBP-1, NF-E1, PERE, and Sp1 (see section 8.5.6; Table 5). Although, my preliminary experiments using forskolin (a promoter of cyclic AMP which binds to CREB) in glioma C6 cells did not stimulate the hybrid FGF-2/luciferase activity, it would be interesting to see if FGF-2 gene transcription can be stimulated in other cell types. Alignment of 5′-flanking sequence of the rat FGF-2 gene with that of reported human FGF-2 genomic sequence revealed an overall similarity of ~60%. However, a greater overlap (~67%) was observed in the proximal promoter region compared to the overlap (51%) in distal sequences. By contrast, comparison of coding regions of rat and human FGF-2 sequences revealed a homology of ~89% at nucleotide level (Kurokawa *et al.*. 1987; 1988). Similarly, the first exon-intron boundary between rat and human FGF-2 genes (see section 8.5.7; Fig. 55) has been well conserved. This feature of higher homology in coding regions and a considerable degree of diversity in the 5′-flanking regions between rat and human FGF-2 sequences can be frequently found in many eukaryotic genes. For example, the 5′-flanking sequences of rat (Barta *et al.*, 1981) and human (DeNoto *et al.*, 1981) growth hormone genes also share an overall homology of ~66% with a greater overlap (~74%) in the proximal promoter region compared to the overlap in distal promoter region (~57%) (Fig. 68). However, comparison of rat (Seeburg *et al.*, 1977) and human growth hormone (Martial *et al.*, 1979) coding sequences revealed a homology of ~76% at the nucleotide level. Perhaps, diversity in the 5′-flanking regions of a particular gene between species might have occurred during evolution through point mutations, crossovers, transpositions, gene duplications and rearrangements (Miller and Eberhardt, 1983), to accommodate subtle differences in the

Fig. 67

Fig. 67. Schematic diagram showing the possible mechanisms involved in the regulation of FGF-2 gene.

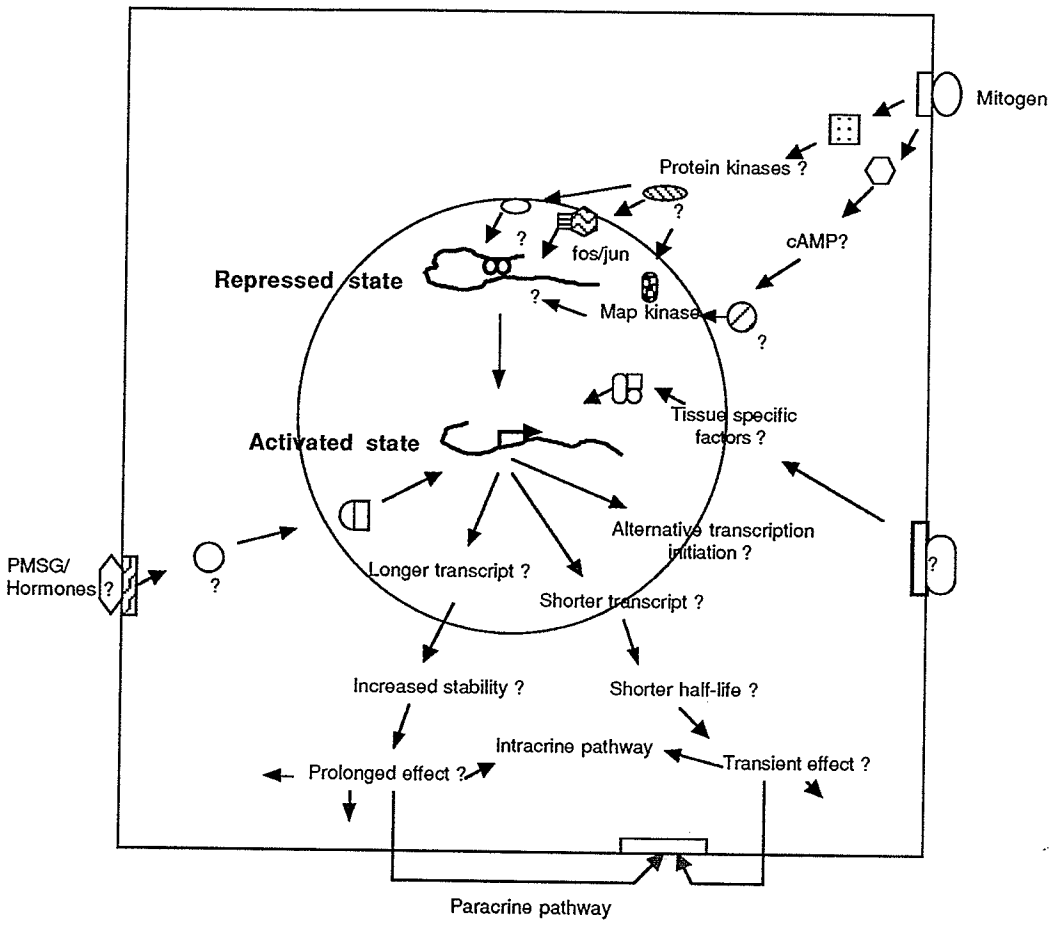
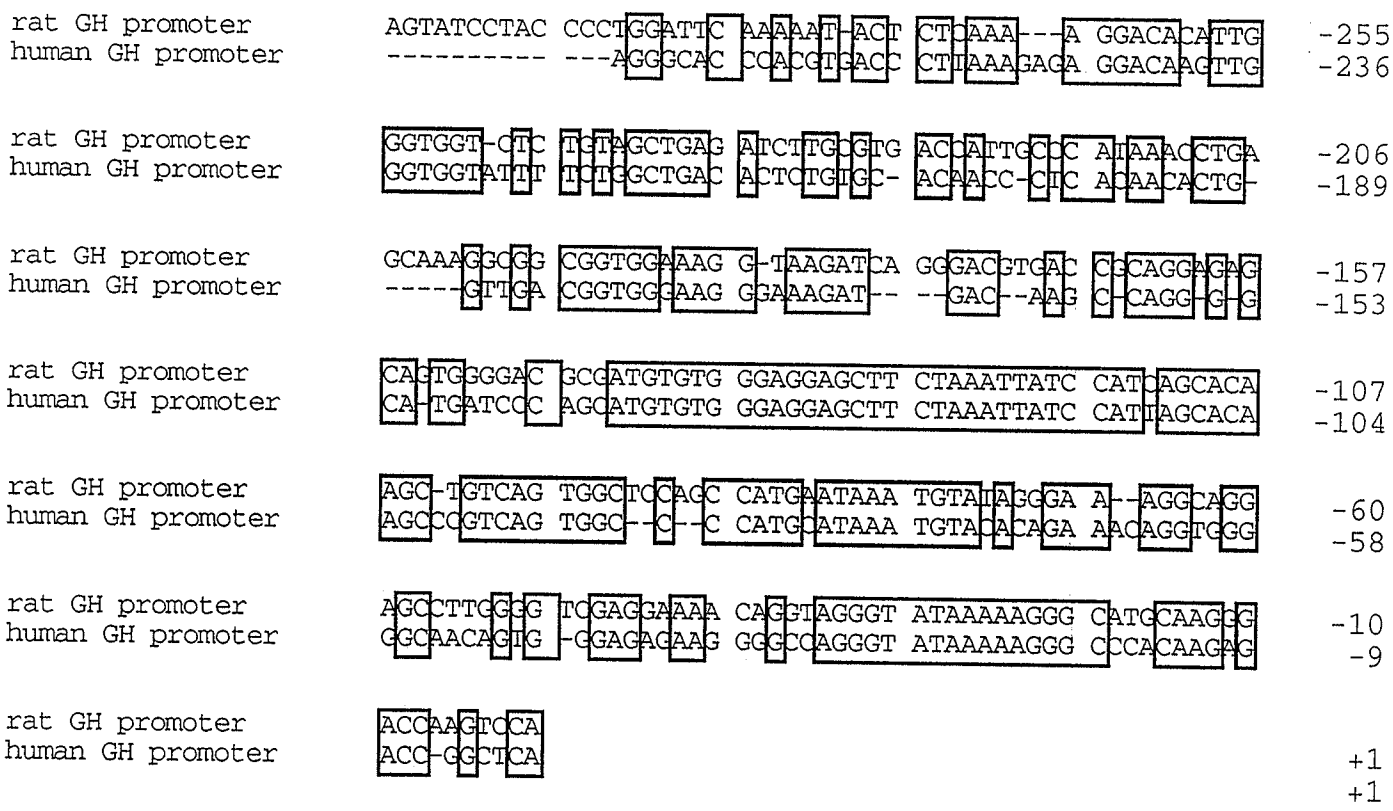

Mitogen
Protein kinases ?
?
?
cAMP?
fos/jun
Repressed state
?
Map kinase
?
Tissue specific factors ?
Activated state
Alternative transcription initiation ?
PMSG/ Hormones ?
?
Longer transcript ?
Shorter transcript ?
?
Increased stability ?
Shorter half-life ?
Intracrine pathway
Transient effect ?
Prolonged effect ?
Paracrine pathway

Fig. 68

Fig. 68. Alignment/comparison of upstream rat (+1/-300; Barta *et al.*, 1981) and human (+1/-272; DeNoto *et al.*, 1981) growth hormone genomic sequences.


rat GH promoter
human GH promoter
AGTATCCTAC CCCTGGATTC AAAAAAT-ACT CTCAAA---A GGACACATTG
---------- ---AGGGCAC CCACGTGACC CTTAAAGAGA GGACAAGTTG
-255
-236
rat GH promoter
human GH promoter
GGTGGT-CTC TGTAGCTGAG ATCTTGCGTG ACCATTGCCC AIAAACCTGA
GGTGGTATTT TCTGGCTGAC ACTCTGTGC- ACAACC-CTC ACAACACTG-
-206
-189
rat GH promoter
human GH promoter
GCAAAGGCGG CGGTGGAAAG G-TAAGATCA GGGACGTGAC CGCAGGAGAG
-----GTTGA CGGTGGCAAG GGAAAGAT-- --GAC--AAG C-CAGG-G-G
-157
-153
rat GH promoter
human GH promoter
CAGTGGGGAC GCGATGTGTG GGAGGAGCTT CTAAATTATC CATCAGCACA
CA-TGATCCC AGCATGTGTG GGAGGAGCTT CTAAATTATC CATTAGCACA
-107
-104
rat GH promoter
human GH promoter
AGC-TGTCAG TGGCTCCAGC CATGAATAAA TGTATAGGGA A--AGGCAGG
AGCCCGTCAG TGGC--C--C CATGCATAAA TGTACACAGA AACAGGTGGG
-60
-58
rat GH promoter
human GH promoter
AGCCTTGGGG TCGAGGAAAA CAGGTAGGGT ATAAAAAGGG CATGCAAGGG
GGCAACAGTG -GGAGACAAG GGGCCAGGGT ATAAAAAGGG CCCACAAGAG
-10
-9
rat GH promoter
human GH promoter
ACCAAGTCCA
ACC-GGCTCA
+1
+1

physiological and anatomical features of those species. Nevertheless, some of the regulatory pathways for each gene have been conserved between species to execute a similar function. For example, regulation of growth hormone production in pituitary by transcription factor Pit-1 in a tissue specific manner, is similar in rat and human and it was shown that the promoter regions of both rat and human were active in rat anterior pituitary tumor (GC) cells compared to non pituitary cells after gene transfer (Cattini *et al.*, 1986a,b; 1987). Further, this activity can be explained by a high degree of conservation of human and rat Pit 1 sequences (~96%) at the protein level (Lew and Elsholtz, 1991). A careful examination of the 5′-flanking sequences of rat and human FGF-2 genes for highly conserved motifs revealed three "proximally conserved domains" (PCDs) of 17 bp or more with 82-92% similarity (see section 8.5.6; Fig. 54). The conservation of PCDs in rat and human FGF-2 genes might be of evolutionary importance and related to specific function. Although a homology search for these conserved PCDs with the known DNA sequences in Genbank did not reveal any sequences containing such domains, an AT-rich stretch (5′-TTTAAA-3′) located in PCD-1 has been described as a promoter element in the mouse whey acidic protein gene and was shown to interact with nuclear protein (Lubon and Hennighausen, 1987). It would be an interesting experiment to see whether similar nuclear proteins bind to these PCDs with nuclear proteins from rat and human cells by using gel mobility and nuclease protection assays. Site-directed mutagenesis can also be employed to obtain a quick insight into the functional significance of these PCD sequences. Positive results from these experiments may compel us to use these PCDs as probes to screen cDNA expression libraries derived from rat and human tissues in order to isolate novel transcription factors that may play a vital role in the regulation of FGF-2 gene.

In order to assess the promoter activity of cloned fragment, I generated a hybrid FGF-2/luciferase reporter gene by subcloning all the available 5′-flanking sequence (-1058/+54) of rat FGF-2 gene into a promoterless luciferase plasmid. Transient transfection of this hybrid gene into glial (C6 and U87-MG) and non glial (H9c2) cells of rat and human origin revealed higher promoter activity in glial cells compared to non glial cells. This is in agreement with the previous reports that FGF-2 is gene expression in higher in brain and other tissues of the CNS (Shimasaki *et al.*, 1988). In addition, rat FGF-2 promoter activity was found to be significantly higher in human U87-MG glial cells

compared to rat C6 glial cells. It is not surprising to see that the rat promoter is functional in a human cell type, as the sequence analysis showed a high degree of conservation in the proximal promoter regions of rat and human FGF-2 genes. However, it is surprising to see that the rat promoter is more active in human glial cells compared to rat glial cells. Although the reason for this could be a difference in the rates of endogenous FGF-2 gene transcription, it should be considered as a positive aspect because the data obtained from rat studies can be always verified in human cells with an ultimate goal of uncovering potentials to regulate FGF-2 gene expression during neurodegeneration. Towards this goal, I also examined the responsiveness of rat FGF-2/luciferase hybrid gene to mitogenic stimuli such as serum and phorbol ester (a protein kinase C activator) in glioma C6 cells. Transient gene transfer studies indicated that the rat FGF-2 promoter activity was stimulated by both serum and phorbol ester compared to the basal promoter activity in the absence of those mitogens in glioma C6 cells. Further, I performed deletion analysis of the 5′-flanking region of rat FGF-2 gene to localize the mitogenic responsive regions. Deletion analysis results suggested that the rat FGF-2 gene is repressed in the absence of mitogenic stimuli and this repressor activity was localized to -1058/-911 region. In addition, the phorbol ester response was also localized to -1058/-911 region, while a part of the serum response was localized to -313/-110 region (see section 9.5.3). However, the 5′-flanking sequence does not appear to contain any serum response or AP1 elements which suggest that the responsiveness of rat FGF-2 gene to serum and phorbol ester could be through alternative pathways. Further deletions as well as characterization of DNA protein interactions in these regions (-1058/-911 and -313/-110) are required to identify novel elements in the rat FGF-2 gene that respond to mitogenic stimuli (phorbol ester and serum).

The repressor activity associated with -1058/-911 region was relieved completely in response to serum but only in part by phorbol ester stimulation (see section 9.5.3, Figs. 59, 60, 62). These results are also consistent with the differential binding events of -1058/-911 region with nuclear proteins isolated from glioma C6 cells treated with or without mitogenic stimuli as indicated by gel mobility and nuclease protection assays (see section 9.5.4; Figs. 63, 64). Similarity of nuclease protection patterns of -1058/-911 regions in the absence or presence of phorbol ester is in turn consistent with the modest effect of phorbol ester in relieving the repressor effect (see sections 9.5.3 and 9.5.4). Three potential

sequences (DRRs-I to III) were identified by nuclease protection assay, which could be the most likely regions responsible for repression of rat FGF-2 gene in the absence of mitogenic stimuli in glioma C6 cells. One of these regions (DRR-II) contained two binding sites for the transcription factor LBP-1 which is shown to be involved in the regulation of several genes in both positive and negative manners (Jones *et al.*, 1988; Parada *et al.*, 1995; Yoon J-B *et al.*, 1994). It is possible that the rat FGF-2 gene is repressed in the absence of mitogenic stimuli due to the binding of LBP-1 and or other factors to DRRs of rat FGF-2 gene. It would be interesting to see whether the repression of rat FGF-2 gene in the absence of mitogenic stimuli can be permanently relieved in glioma C6 cells by mutating DRR sequences through site-directed mutagenesis. With regards to the mechanism behind mitogenic (serum and phorbol ester) activation of rat FGF-2 gene in glioma C6 cells, it is possible that the mitogens activate certain second messengers (eg. protein kinase C, protein kinase A, cAMP) through cell surface receptors of glial cells. These second messengers may in turn activate their substrates (eg. serine-threonine kinases, MAP kinase, products of oncogenes: *fos*, *jun, Egr-1*) which can alter the interactions of repressor proteins bound to -1058/-911 region of rat FGF-2 gene either by reducing their binding affinity (eg. phosphorylation) or by directly competing for their binding sites (eg. *fos* and *jun*) (Fig. 67). In addition to the relief of repressor proteins (Fig. 61, 67), activation of certain positive factors must also contribute to the positive effect of mitogens on FGF-2 gene transcription as the deletion of -313/-110 region revealed a significant decrease in the promoter activity of FGF-2/luciferase hybrid gene. A combination of site-directed mutagenesis studies as well as stimulation of glial cells with agonists and antagonists for a variety of second messengers should be helpful in further identifying the players involved in mitogenic activation of rat FGF-2 gene.

With regards to the significance of rat FGF-2 gene repression in the absence of mitogenic stimuli, it could be that the cellular machinery prefers to maintain such a potent growth factor at low levels to avoid uncontrolled effects. Transcriptional control of FGF-2 gene may represent one of many fine controls for the regulation of endogenous FGF-2 levels, such as regulation by heparin sulphate proteoglycans (Baird and Walicke, 1989) and anti sense transcripts (Kimelman and Kirschner, 1989; Murphy and Knee, 1994). Alternatively, the repression of rat FGF-2 gene in the absence of mitogenic stimuli in glial

cells could be physiologically important merely in those cell types. Rat glioma C6 cells were derived from rat brain tumors induced by *N*-nitrosomethylurea, and reported to possess properties of astrocytes by their ability to produce high levels of S-100 protein (Benda *et al.*, 1968). However, their precise cellular origin is not clear. It is possible that they were immortalized during the conversion of neuroprogenitor cells into either astrocytes or oligodendrocytes and may represent an "intermediary stage". It was shown that the progenitor cells in culture can become either astrocytes in the presence of serum or oligodendrocytes in the absence of serum (Raff *et al.*, 1983; Stallcup and Beasley, 1987). If the assumption that glioma C6 cells represent an "intermediary stage" is true, then these cells must have the potential to produce markers specific for both astrocytes (eg. glial fibrillary acidic protein) as well as oligodendrocytes (eg. basic myelin protein) in presence or absence of serum respectively. FGF-2 was shown to inhibit the differentiation of progenitor cells of the CNS into oligodendrocytes and promote their conversion into astrocytes (McKinnon *et al.*, 1990; 1991). It is possible that glioma C6 cells tend to regulate their endogenous FGF-2 gene expression in the absence of mitogenic stimuli (eg. serum) to promote the expression of oligodendrocyte specific markers. However, FGF-2 gene transcription is activated in C6 cells in the presence of serum to promote the expression of astrocyte specific markers. This premise can be tested by analysing RNA samples isolated from C6 glioma cells grown in the presence or absence of serum for astrocyte or oligodendrocyte specific markers. These results may also provide an explanation for the presence or absence of repressor effect on the promoter activity of FGF-2/luciferase hybrid gene in C6 cells in the absence or presence of serum respectively.

In summary, my results suggested that the FGF-2 gene transcription can be increased in response to mitogenic stimuli, serum and phorbol esters in C6 glial cells and this may have a potential to promote neuronal survival and growth during disease conditions. It would be interesting to test the effects of various agonists (eg. phorbol ester, diacyl glycerol) and antagonists (eg. chelerythrine, polymyxin B and H-7) of PKC in the experimental animal models harbouring neurodegenerative conditions. Availability of the 5′-flanking sequences of FGF-2 gene should also enable the efforts to explore several other mechanisms that are important in the regulation of FGF-2 levels in specific brain areas as a therapeutic intervention in acute and chronic neurodegerative diseases. The drug L-deprenyl

was shown to promote neuronal survival in response to mechanical lesions in experimental models and a recent report has implicated increased levels of FGF-2 in astrocytes surrounding the lesioned area, as a possible mechanism for L-deprenyl-induced neuroprotection (Biagini *et al.*, 1994). This premise can also be verified at the transcriptional level, by testing whether the rat FGF-2/luciferase hybrid gene activity can be stimulated in response to L-deprenyl. In addition to its role in neuronal survival, FGF-2 has also been strongly implicated in tissue repair, angiogenesis, cardioprotection, hepatic regeneration and retinal epithelial survival *in vivo*. Thus, understanding the mechanisms regulating such a potent growth factor may have an enormous impact on ameliorating diseases associated with a number of tissues. We have generated transgenic mice carrying luciferase gene under the control of 5´-flanking region of rat FGF-2 gene, with a view to testing the effects of various therapeutic agents on the expression of hybrid FGF-2/luciferase gene. The information obtained through such studies can be used to stimulate the growth and or survival of different cell types during disease conditions.

In conclusion, these doctoral studies indicated that (i) both high and low molecular weight forms of FGF-2 can be used to stimulate the proliferative potential of postnatal cardiac myocytes and (ii) the endogenous levels of FGF-2 can be regulated in glial cells by mitogenic stimuli and thus, suggested a therapeutic value for FGF-2 in diseases associated with cardiovascular and central nervous systems. These studies also raised the possibility that high molecular weight FGF-2 may be involved in the removal of damaged cardiac myocytes through apoptosis and proposed novel mechanisms for cardiac myocyte binucleation as well as derepression of FGF-2 gene in response to mitogenic stimuli in glial cells. In addition, this work also led to the development of tools (hybrid genes that can preferentially express high or low molecular weight forms of FGF-2 and the 5´-flanking region of FGF-2 gene as well as transgenic mice carrying these genes) that can be used by other researchers to address specific questions.

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