ADRENERGIC REGULATION OF RAT FGF-2 GENE EXPRESSION IN THE HEART

BY

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(née REMPEL)

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

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FACULTY OF GRADUATE STUDIES

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Adrenergic Regulation of Rat FGF-2 Gene Expression in the Heart

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of MASTER OF SCIENCE

In memory of my grandfather

Norman William ("Bill") Radforth, PhD, FRSC

1912-1999

whose wisdom and understanding of the Universe

taught me that there is room in the heart of a scientist

for a relationship with her Creator.

"In the beginning was the Word, and the Word was with God, and the Word was God. He was with God in the beginning. Through him all things were made; without him nothing was made that has been made. In him was life, and that life was the light of men. The light shines in the darkness, and the darkness has not understood it."

The Gospel according to John, the first chapter.

Abstract

Fibroblast growth factor-2 (FGF-2) is a potent ubiquitous, multifunctional peptide growth factor, whose effects in cardiovascular cells include stimulation of DNA synthesis and cell proliferation, angiogenesis, negative inotropy, hypotension, and cardioprotection. Regulation of FGF-2 production and activity is controlled at all levels, ranging from transcription to modulation of ligand-receptor interactions between FGF-2 and its currently known high affinity receptor in the heart (FGFR1). The myocardium releases FGF-2 with every contraction through transient disruptions in the sarcolemma of cardiac myocytes. Such release of FGF-2 can be increased with adrenergic stimulation. This led to the hypothesis that *de novo* synthesis of FGF-2 may accompany an induced increase in its release, specifically by adrenergic regulation at the transcriptional level. Other pathways may also exist which link FGF-2 release to its synthesis. The 5′-flanking region of the rat FGF-2 gene has been cloned in our laboratory. Like its human homologue, the rat FGF-2 promoter has no TATA box and instead contains G/C-rich regions typical of a housekeeping gene. In Chapter 3, RNA blotting and transient transfection (of a hybrid luciferase gene directed by 1,058 bp of rat FGF-2 5′-flanking DNA) in cultured neonatal rat cardiac myocytes were used to show that FGF-2 gene expression was increased by norepinephrine (NE) after 6 h, specifically and primarily through α_1-adrenergic receptors. Deletion analysis of the 1,058 bp of rat FGF-2 5′-flanking DNA showed that an increase in FGF-2 transcription using phenylephrine (PE, an α_1-specific agonist) for 48 h could be elicited with only 313 bp of upstream sequence. This deletion excluded an A/G-rich region containing a tandem repeat of an 8 bp element (5′-AGGGAGGG-3′) which is similar to a phenylephrine-responsive element in the rat ANF promoter. The PE response

of the rat FGF-2 5′-flanking region also occurred independently of myocyte contraction or Ca^{2+} entry via L-type Ca^{2+} channels. In two lines of transgenic mice into which the FGF-2/luciferase hybrid gene had been introduced, intraperitoneal injection of PE increased luciferase after 6 h. In Chapter 4, the functional significance of the A/G-rich region of the FGF-2 promoter was assessed. A 37-bp oligonucleotide encompassing the tandem repeat of the 8 bp element was inserted upstream of a heterologous viral promoter and found to confer an enhancer effect on transcription in neonatal rat cardiac myocytes, as well as in human and rat glioma cell lines. Gel mobility shift assays using the 37 bp oligonucleotide and nuclear protein isolated from neonatal rat hearts showed the formation of four specific complexes representing DNA/protein interactions of varying affinities. The same four complexes were seen with nuclear protein isolated from cultured neonatal rat cardiac myocytes, but these complexes did not change with stimulation with NE for 6 h prior to the isolation of nuclear protein. In Chapter 5, a working model of Langendorff perfusion of murine hearts for the study of the relationship between FGF-2 release and synthesis, particularly in the context of ischemia-reperfusion injury, is introduced. In conclusion, these studies demonstrated that 1,058 bp of rat FGF-2 5′-flanking DNA was sufficient to invoke a positive response in cardiac myocytes to catecholamine stimulation specifically through α_1-adrenergic receptors, and that this response occurred *in vitro*, independently of myocyte contraction and Ca^{2+} influx, as well as *in vivo* in the adult heart. Although the A/G-rich region was shown not to play a role in this response, this region does carry enhancer activity and does bind nuclear protein with high affinity and specificity. The relationship between adrenergic stimulation and a role for FGF-2 in responding to physiological stress is discussed.

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Finally, to my mate, Gilles Réal Joseph: for your unconditional love. I only hope that I can be for you what you have been for me. "Two are better than one, for they receive a good return for their work...". With you all the way!

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List of Abbreviations

A	adenosine
aa	amino acids
AC	adenylate cyclase
ADP	adenosine-5′-diphosphate
ANF/ANP	atrial natriuretic factor/atrial natriuretic peptide
approx.	approximately
AR	adrenergic receptor
Arg	arginine
ATP	adenosine-5′-triphosphate
BAPTA-AM	1,2 Bis(2-aminophenoxy)ethane N, N, N′, N′-tetraacetic acid
BDM	butanedione monoxime
BNP	brain natriuretic peptide
bp	base pairs
bpm	beats per minute
C	cytosine
°C	degrees centigrade
cAMP	cyclic adenosine 5′-monophosphate
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
CMV	cytomegalovirus
cpm	counts per minute

CRE	cAMP response element
CREB	CRE-binding protein
CREM	CRE modulator
DAG	1,2-diacyl-sn-glycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
Egr-1	early growth response protein-1
EGTA	ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid
FBS	fetal bovine serum
FGF-2	fibroblast growth factor-2 or basic fibroblast growth factor
FGFR	FGF receptor
g	gram(s) OR gravity equivalents
G	guanosine
GAG	glycosaminoglycan
Gly	glycine
GTP	guanosine 5′-triphosphate
GuanSCN	guanidine isothiocyanate
h	hour(s)
HEBS	HEPES-buffered saline
HEPES	N-[2-hydroxyethyl] piperazine-N′[2-ethanesulfonic acid]

HMW	high molecular weight
HSPG	heparan sulfate proteoglycan
HSV-TK	herpes simplex virus thymidine kinase
Hz	Hertz
ID	identification
ip	intraperitoneally
IP_3	inositol-1,4,5-triphosphate
JNK	Janus kinase
kb	kilobase pair(s)
kDa	kilodaltons
kg	kilogram(s)
LDH	lactate dehydrogenase
LMW	low molecular weight
MAPK	mitogen-activated protein kinase
βMHC	β-myosin heavy chain
mg	milligram(s)
MLC	myosin light chain
MOPS	3-[N-Morpholino] propane sulfonic acid
min	minute(s)
ml/μl	millilitre/microlitre
mm	millimetre
mM/μM/nM	millimolar/micromolar/nanomolar
mmHg	millimetres mercury

mRNA	messenger RNA
ms	milliseconds
NE	norepinephrine
nt	nucleotides
PE	phenylephrine
PERE	phenylephrine-responsive element
PIP_2	phosphatidyl inositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RLU	relative light units
rpm	revolutions per minute
RSV	Rous sarcoma virus
RTEF	related to transcription enhancer factor
sec	second(s)
SDS	sodium dodecyl sulfate
Ser	serine
SKA	skeletal α-actin
SR	sarcoplasmic reticulum
SRE	serum response element
SSC	saline sodium citrate

T	thymidine
TEF-1	transcription enhancer factor-1
Thr	threonine
Tris	Tris [hydroxymethyl] aminomethane
U	units *or* uracil
UV	ultraviolet
V	volts
v/v	volume per volume
vs.	versus
VSMC	vascular smooth muscle cells
wt.	weight

Chapter 1. Introduction

1.1. FGF-2 and the Fibroblast Growth Factor Family

Basic fibroblast growth factor (bFGF or FGF-2) is a prototype member of a multi-gene family consisting of 18 characterized members to date (Szebenyi and Fallon, 1999; Miyake et al., 1998; Hoshikawa et al., 1998; Ohbayashi et al., 1998). This diverse family of peptide growth factors plays multiple roles in cell replication, angiogenesis, cell survival, apoptosis, adhesion, motility, and differentiation, and well as mediating complex biological events such as tumorigenesis, morphogenesis, and blood vessel remodeling (reviewed in Szebenyi and Fallon, 1999). These effects are mediated by a family of high affinity FGF receptors (FGFR1-4). All FGFRs are tyrosine kinases, each with a single membrane-spanning domain, which bind the different FGFs with varying affinities (Ornitz et al., 1996). Each of the four FGFR genes gives rise to multiple isoforms by alternative splicing. Most isoforms are cell-surface proteins with a single membrane spanning domain, but some are secreted.

FGF-2 was originally purified from brain and pituitary as a 16-18 kDa monomeric peptide (Gospodarowicz et al., 1984). Multiple molecular weight isoforms exist, arising from alternate translation initiation sites on the same mRNA, to produce 18 kDa low molecular weight (LMW) or 21-25 kDa high molecular weight (HMW) FGF-2 isoforms (see sections 1.2 and 1.3.2). FGF-2 orthologs (human, bovine, ovine, and rat) share a 89-95% identity in amino acid sequence for 18 kDa FGF-2 (Basilico and Moscatelli, 1992). Three exons each encode an anti-parallel β-sheet, and the three sheets are arranged in a

trigonal pyramidal structure, with no disulfide bonds (Ericksson et al., 1991; Zhu X. et al., 1991). The HMW isoforms differ from LMW FGF-2 by an N-terminal extension of varying length, depending on species and the CUG start codon used. The N-terminal extension contains several glycine (Gly)-arginine (Arg) repeats. The Arg residues in these repeats are highly methylated (Bikfalvi et al., 1997; references therein). Although it is known that the N-terminal extension contains a nuclear localization signal (Quarto et al., 1991), the role of methylation in nuclear transport is not known. HMW FGF-2 is localized to the nucleus and LMW FGF-2 to the cytoplasm in most cell types. The functional differences among the isoforms and their localization will be discussed.

1.2. Biological Roles for FGF-2 in the Cardiovascular System

FGF-2 has many effects on many tissues throughout embryonic development and into adulthood (reviewed in Bikfalvi et al., 1997). In the cardiovascular system, FGF-2 and FGF receptor signaling also play multiple and related roles. During early cardiac development, FGF-2 along with activin-A mimics and may mediate the effect of the anterior endoderm on mesodermal precardiac cells (Sugi and Lough, 1995). Later, FGF-2 also mediates the transformation of epithelial cells to mesenchyme during the formation of the cardiac cushions (Markwald et al., 1996), a process involving a loss of cell-cell interactions and increased migratory potential. Signaling through FGFR1 is required for cardiac myocyte proliferation during embryonic development (Mima et al., 1995). The ability of FGF-2 to promote cell migration and affect differentiation is also evident in its effects on the development of other organs, including skeletal muscle (Gerber et al., 1997; Hannon et al., 1996). FGF-2 also promotes cell survival during organogenesis,

demonstrated in many tissues such as neurons (Grothe and Wewetzer, 1996), endothelial cells (Miao et al., 1997), and vascular smooth muscle cells (Fox and Shanley, 1996).

The effects of FGF-2 on DNA synthesis and cell proliferation are also evident in most, if not all, cardiovascular cell types. FGF-2 is a potent angiogen, stimulating the proliferation of both endothelial and smooth muscle cells. Anti-sense inhibition of FGF-2 in murine embryos disrupted vascular development (Leconte et al., 1998), and overexpression of human FGF-2 in transgenic mice led to increased DNA synthesis in isolated vascular smooth muscle cells (Davis et al., 1997). This effect is associated with its role in vascular remodeling and lumen narrowing in response to altered blood flow or vascular injury (Bryant et al., 1999). This angiogenic as well as atherosclerotic potential leads to a ying-and-yang model for therapeutic use: an ischemic area could be reperfused with the addition of FGF-2 to promote angiogenesis (Lazarous et al., 1995; Ueno et al., 1997), while inhibiton of FGF-2 activity could be used to treat restenosis (Yuwaka et al., 1998), or tumorigenesis (Maret et al., 1995). In addition to this growth and remodeling effect, FGF-2 appears to affect vascular function. It has vasodilatory (Cuevas et al., 1991) and negative inotropic (Padua et al., 1998) properties, and transgenic mice deficient in FGF-2 had impaired vascular smooth muscle contractility and were hypotensive (Zhou et al., 1998).

FGF-2 has also been shown to have multiple effects in cardiac myocytes. These effects can be divided according to two signal transduction pathways initiated by binding of FGF-2 to FGFR1, followed by receptor dimerization and autophosphorylation (Kardami

et al., 1999). First, FGF-2 stimulates cardiac myocyte proliferation in both chicken and rat (Kardami, 1990; Pasumarthi et al., 1994; Pasumarthi et al., 1996). This effect is mediated by the Ras/Raf/mitogen-activated protein kinase cascade, resulting in changes in the cell nucleus (Kardami et al., 1999). Another signaling pathway mediated by FGF-2 through FGFR1, is the activation of phospholipases β and γ_1, leading eventually to translocation and activation of protein kinase C isoforms, (Kardami et al., 1999). This activity of FGFR1 induces the serine phosphorylation of connexin 43, a component of cardiomyocyte gap junctions (Doble et al., 1996) This phosphorylation, which was tyrosine-phosphorylation dependent, was associated with decreased gap junction permeability, resulting in decreased metabolic coupling between neighbouring myocytes, and may be important in the proliferative response (Kardami and Doble, 1998).

Another effect of FGF-2 that is mediated by protein kinase C is cardioprotection. In cultured neonatal rat cardiac myocytes, exogenous FGF-2 reduced the damage caused by hydrogen peroxide, measured by lactate dehydrogenase release (Kardami et al., 1993). In isolated perfused rat hearts, exogenous addition of FGF-2 resulted in improved functional (contractile) recovery following global ischemia-reperfusion injury (Padua et al., 1995), an effect mediated by the novel protein kinase C isoforms δ and ε (Padua et al., 1998). Using a transient coronary ligation protocol, addition of FGF-2 also improved functional recovery following ischemia-reperfusion injury *in vivo* in the rat (Cuevas et al., 1997). Improved myocardial function with FGF-2 treatment was also observed in a porcine model of chronic myocardial ischemia (Harada et al., 1994). Whether or not cardioprotection could be achieved through endogenous production of FGF-2 is currently

under study. However, there is evidence that FGF-2 expression is upregulated during ischemic injury in rat brain (Finkelstein et al., 1988; Lin et al., 1997) and retinal tissue (Miyashiro et al., 1998). Also, in the rat heart, FGF-2 accumulates at focal regions of isoproterenol-induced injury (Padua et al., 1993). FGF-2 is well established in other tissues as an injury response molecule, being localized to areas of wounding in the skin (Kurita et al., 1992) and ulcers of the digestive tract (Folkman et al., 1991). Knockout mice lacking any endogenous FGF-2 showed deficiencies in wound healing (Ortega et al., 1998). The healing effect of FGF-2 has been associated with its angiogenic potential. In the heart, FGF-2 also promotes angiogenesis in models of chronic myocardial ischemia (Harada et al., 1994; Lazarous et al., 1995; Yanagisawa-Miwa, 1992). However, the cardioprotective nature of FGF-2 does not seem to be entirely dependent on its ability to revascularize an area, since FGF-2 lends direct protection to the cardiac myocyte in culture (Kardami et al., 1993) and in isolated hearts during ischemia and reperfusion through a PKC-mediated pathway (Padua et al., 1998).

As mentioned above, FGF-2 exists in high and low molecular weight isoforms as a result of alternative translation start sites on the same RNA. The functional significance of the various isoforms is not well understood. It is generally accepted that LMW FGF-2 is preferentially exported from the cell or localized to the cytoplasm, and HMW FGF-2 is directed to the nucleus. This difference in subcellular localization implies a difference in function. In embryonic chicken cardiac myocytes, both high and low molecular weight species increases DNA synthesis, proliferation and protein synthesis, but only HMW FGF-2 seemed to have an effect on chromatin in the nucleus (Pasumarthi et al., 1994). In

the rat heart, there is a switch in the predominant isoform from HMW during development to LMW in the adult (Liu et al., 1993). In neonatal rat cardiomyocytes, the mitotic index increased with overexpression of either LMW or HMW isoforms; however, only HMW isoform overexpression was associated with an increase in myocyte binucleation (Pasumarthi et al., 1996). The localization of HMW vs. LMW isoforms in other cell types is more polarized and less overlapping (Bikfalvi et al., 1995). In CHO cells, the different isoforms are associated with different intracellular protein complexes (Patry et al., 1997). It is not known whether the same is true for cardiac cells.

1.3. Post-transcriptional Regulation of FGF-2

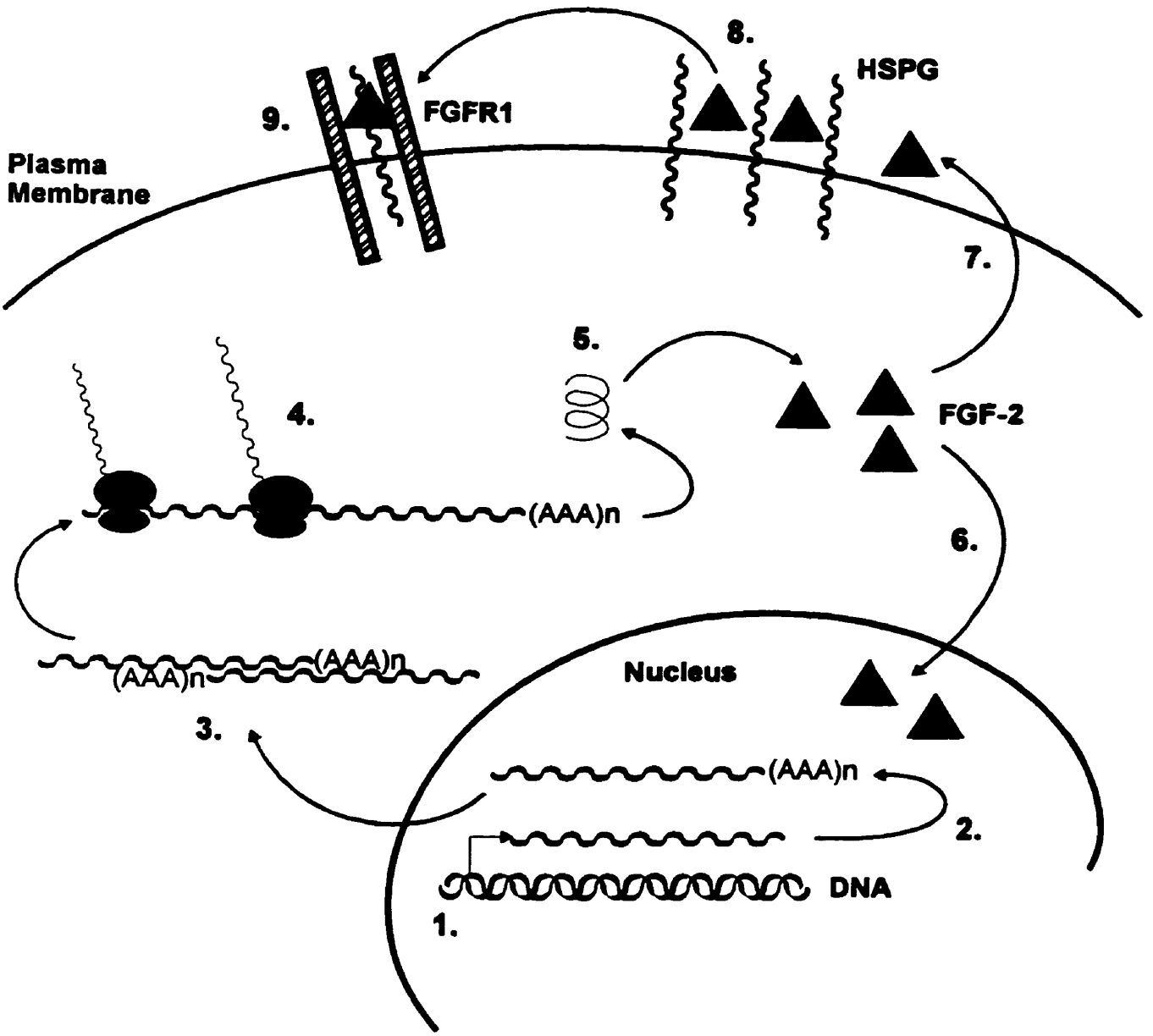
The regulation of FGF-2 is extremely complex, involving influence at all levels (Figure 1): transcription, mRNA processing (splicing, polyadenylation, mRNA stability), translation, post-translational modifications, intracellular trafficking, secretion/export, bioavailability, and ligand-receptor interactions. FGF-2 transcription will be dealt with in more detail in the next section, while the remaining levels will be discussed here in brief.

Figure 1.

Control of FGF-2 production and activity is exerted at multiple levels.

The synthesis and availability of FGF-2 is tightly regulated at all levels, and is described in sections 1.3 and 1.4 of the text. The levels discussed include:

1. Transcription
2. RNA processing: splicing, polyadenylation
3. Antisense RNA: mRNA stability and translational control
4. Translation
5. Post-translational modifications: ribosylation, phosphorylation
6. Intracellular trafficking (nuclear localization)
7. Release/Export to extracellular space
8. Sequestration in the extracellular matrix
9. Ligand-Receptor interactions


8.
HSPG
9.
FGFR1
Plasma
Membrane
7.
5.
4.
FGF-2
(AAA)n
6.
(AAA)n
(AAA)n
Nucleus
3.
(AAA)n
2.
DNA
1.

1.3.1. Diversity in mRNA Structure

The FGF-2 gene, like most other members of the FGF family, is divided into three exons which are spliced together during mRNA processing. The full length human FGF-2 mRNA is 6,774 nt long, and 90% of it is composed of untranslated regions (Arnaud et al., 1999). Transcripts of 7.0, 3.7, 3.5, 2.8, 2.2 and 1.8 kb have been reported for human FGF-2 (Murphy et al., 1988), and sizes of 6.0, 3.7, 2.5, 1.8, 1.6 1.4 and 1.0 kb have been reported from rat (Powell and Klagsbrun, 1991). The variation in transcript size arises from the use of alternative polyadenylation sites within the very large 3′-untranslated region (Basilico and Moscatelli, 1992). In the chicken, alternative splicing of exon 1 has been reported (Borja et al., 1993), giving rise to AltFGF-2, containing a completely different N-terminal domain. AltFGF-2 is expressed in highly specific regions during embryonic development and appears to function in neural tube development (Borja et al., 1996). Such alternative splicing has not been reported for other species.

An antisense FGF-2 mRNA has been reported in Xenopus (Kimelman and Kirschner, 1989) chicken (Savage and Fallon, 1995), human (Murphy and Knee, 1994) and rat (Knee et al., 1997). These transcripts are thought to be involved in the regulation of FGF-2 mRNA stability, either through enzymatic modification of double-stranded RNA molecules, or through other post-transcriptional mechanisms including inhibition of transcript processing, transport, or translation (Knee et al., 1997; Knee and Murphy, 1997). FGF-2 mRNA stability may also be regulated through variation in the 3′-untranslated region of the various transcripts (el-Husseini et al., 1992; Knee and Murphy, 1997), which gives rise to the multiple transcript lengths described above.

1.3.2. Alternative Translation of the FGF-2 mRNA.

The high and low molecular weight isoforms of FGF-2 arise from translation intiation from multiple start codons on the same mRNA. LMW (18 kDa) FGF-2 coding begins at a canonical AUG (methionine) start codon, while HMW isoforms (20-25 kDA, depending on species) originate from two or more upstream CUG (leucine) unconventional start codons. Translation of the human FGF-2 mRNA occurs from one AUG codon or three CUG codons through an internal ribosome entry site (Vagner et al., 1995). Several cis-acting regulatory elements were identified in the 5′-untranslated region of this mRNA, which could potentially act through trans-acting factors which recognize secondary or tertiary nucleic acid structures (Prats et al., 1992). More recently, a larger, 34-kDa HMW isoform was identified in HeLa cells which is synthesized from an even more distal CUG codon on the same mRNA, this time in a cap-dependent manner (Arnaud et al., 1999). Translational control of the human FGF-2 gene is thus very complex, and preferential translation from certain start codons could reflect differences in isoform function. A rat homologue to the 34-kDa human FGF-2 has not yet been identified. Indeed, the N-terminal extensions in rat HMW FGF-2 are shorter than in human, and there is a stop codon just upstream from the two CUG start codons (Pasumarthi et al., 1997; see also Discussion).

1.3.3. Post-Translational Modifications, Trafficking and Release

FGF-2, along with several other members of the FGF family, lacks a classical signal sequence and is not processed for secretion by an ER/Golgi-dependent mechanism (Mignatti et al., 1992). However, the FGF-2 protein is a substrate for post-translational ADP-ribosylation at arginine residues (Boulle et al., 1995), and is phosphorylated in two

locations (Ser-72 and Thr-120) by protein kinase C and protein kinase A, respectively (Basilico and Moscatelli, 1992). The functional significance of either ADP-ribosylation or phosphorylation is not known.

The N-terminal extensions of HMW FGF-2 isoforms contain highly methylated Arg-Gly repeats known to drive nuclear localization (Basilico and Moscatelli, 1992). The mechanism of trafficking is not well understood, though high and low molecular weight forms of FGF-2 are associated with distinct intracellular protein complexes (Patry et al., 1997). Although the dogma exists that LMW FGF-2 is cytoplasmic and HMW FGF-2 is nuclear, this compartmentalization is more defined in some tissues than in others, and 18-kDa FGF-2 has been observed in the nuclei of some cells, including cardiac myocytes (Pasumarthi et al., 1994; Pasumarthi et al., 1996).

Generally, HMW FGF-2 is considered to be entirely intracellular (although paracrine effects, sensitive to neutralizing antibodies, have been reported [Pasumarthi et al., 1996]), while LMW FGF-2 may be intracellular or exported to the extracellular matrix. Because it lacks an identifiable secretory signal, the mechanism by which 18 kDa FGF-2 is released from intracellular stores has been a subject of considerable interest. Quantitative export from COS-1 cells appears to occur via a unique cellular pathway in an energy-dependent manner, separate from Golgi-mediated processing (Florkiewicz et al., 1995). This novel mode of export was blocked with ouabain and other cardenolides (Florkiewicz et al., 1998), implicating a role for the Na^+, K^+-ATPase. Although co-immunoprecipitation gave evidence for interaction between the catalytic subunit of the

ATPase and FGF-2 (Florkiewicz et al., 1998), experiments using the yeast two-hybrid system indicate that the interaction may be indirect, possibly involving a novel protein complex (Oh and Lee, 1998).

In the heart, FGF-2 is released through disruption of the plasma membrane in endothelial cells (Ku and D'Amore, 1995), vascular smooth muscle cells (Cheng et al., 1997), and cardiac myocytes (Kaye et al., 1996). Non-lethal plasma membrane disruption is a common occurrence in tissues that are subjected to mechanical stretch, such as skeletal muscle, gut epithelium, the vasculature, and the myocardium (McNeil and Steinhardt, 1997). Cardiac myocytes in culture and in isolated perfused rat heart preparations are subjected to this non-lethal wounding under normal mechanical load (Kaye et al., 1996; Clarke et al, 1995). During such membrane disruptions, FGF-2 (as well as FGF-1) is released from the cell, and then can signal back through its receptor (FGFR1) in an autocrine manner. An increased level of mechanical activity, achieved either through pacing of cells (Kaye et al., 1996) or β-adrenergic stimulation of the isolated myocardium (Clarke et al., 1995) results in increased release of FGF-2. The "wound hormone" hypothesis, then, stipulates that factors released in such a graded manner would mediate cellular events related both to normal homeostasis and to the adaptation to increased work (McNeil and Steinhardt, 1997). It is also noteworthy that release of FGF-2 and other angiogenic factors was demonstrated in a canine model of coronary artery occlusion (Weihrauch et al., 1998). FGF-2 was detected in the myocardial interstitial fluid in a time-dependent manner (12-14 days following the initiation of occlusion). The delay preceding the release of FGF-2 indicates that this release may be via a mechanism other

than plasma membrane disruption as described above. It is possible, therefore, that the energy-dependent export pathway described for COS-1 cells (Florkiewicz et al., 1995) also occurs in the myocardium.

1.3.4. Bioavailability and Ligand-Receptor Interaction: HSPGs

The final level at which FGF-2 activity may be regulated involves mechanisms by which the signaling capacity of an FGF-2 molecule is activated or subdued. FGF-2 released into the extracellular matrix is held in place by heparan sulfate proteoglycans (HSPG). Proteoglycans are macromolecules consisting of a protein core to which are covalently attached polysaccharide chains known as glycosaminoglycans (GAG). Proteoglycans are named according to the disaccharide repeat contained in the GAG. The GAGs of HSPGs contain a repeat similar to that which is found in heparin. FGF-2, along with all FGFs, bind heparin as well as HSPGs. The degree and type of sulfation of an HSPG alters its affinity for FGF-2 (Aviezer et al., 1994). HSPG binding to FGF-2 will enhance or may even be absolutely required for FGF-2 signaling to occur (Szebenyi and Fallon, 1999). Indeed, FGFR1 also contains a heparin-binding domain, and an accepted model for FGF-2 signaling through FGFR1 consists of a ternary complex, where ligand-receptor interaction is facilitated and stabilized by the presence of HSPG at the cell surface (Klint and Claesson-Welsh, 1999). Furthermore, there is substantial evidence that these low-affinity FGF-2 receptors play a more direct role in signal transduction (Kardami et al., 1999, and references therein).

In addition to their contribution to signaling, HSPGs also increase the half life of FGF-2 in the extracellular matrix, thus protecting it from degradation and serving as a storage

reservoir for the molecule until the cell is triggered accordingly (Klagsbrun and Baird, 1991; Szebenyi and Fallon, 1999). Protein storage is likely to be important in FGF-2 biology, since the protein is present in tissues at much higher levels than its mRNA, which is relatively unstable (Abraham et al., 1986). Finally, HSPGs, particularly syndecan, may play a role in FGF-2 internalization (Quarto and Amalric, 1994).

1.4. FGF-2 Transcription

1.4.1. Gene Structure and Species Homology

The human FGF-2 gene spans at least 38 kb of genomic DNA on chromosome 4 (Basilico and Moscatelli, 1992). It is organized into three exons separated by two large introns, and, as mentioned earlier, an exceedingly large and variable 3′-untranslated region. The FGF-2 gene, or parts of it, have been cloned from human, opposum, bovine, rat, chick, mouse, sheep, *Xenopus*, and newt (Szebenyi and Fallon, 1999). The FGF-2 protein is highly conserved among mammalian species, with a 155-aa open reading frame in all three of human, mouse, and rat, differing in predicted amino acid sequence at only 7 positions (95.5% identity, Figure 2A). The N-terminal extensions that are found in HMW isoforms are more divergent between rat and human (Figure 2B). When aligned, it becomes apparent that insertional changes occurred over time to increase the length of the N-terminal sequence in human relative to rat. However, even in light of these changes, the overall Gly-Arg repeat pattern is conserved. The homology among ortholog proteins is reflected in the cDNA sequence, which is highly conserved between rodent and human within the coding region. Beyond the coding region, however, the sequence diverges considerably (Figure 2C), a trend which holds for introns, untranslated regions of the cDNAs, as well as for the 5′flanking regions. The nature of the FGF-2 promoters from human and rat will be discussed in the following section.

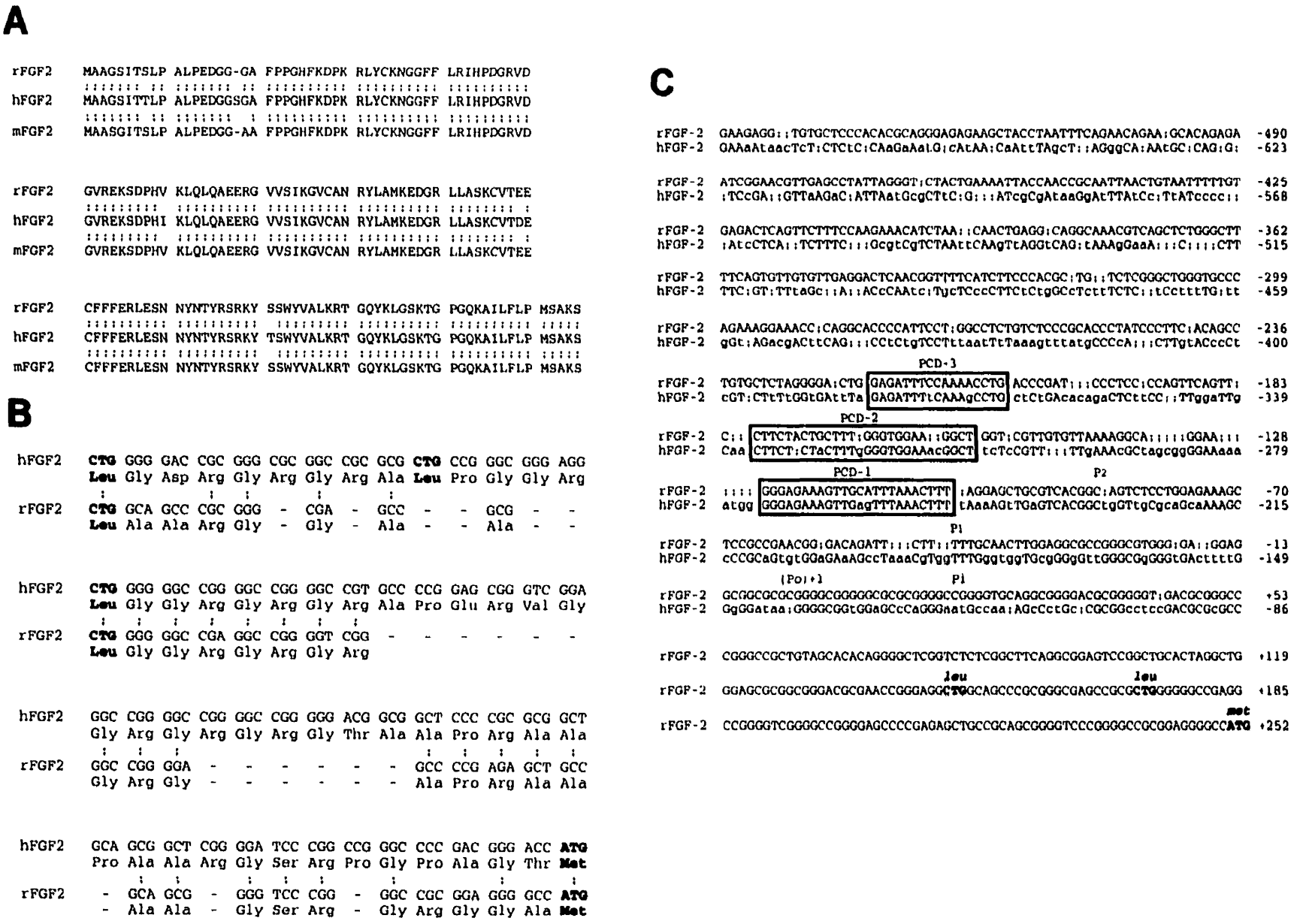
Figure 2.

Sequence homology between rodent and human FGF-2.

(A) Alignment of amino acid sequences for 18 kDa FGF-2 from rat (rFGF-2), human (hFGF-2), and mouse (mFGF-2). A colon (:) indicates amino acid identity between adjacent sequences, and a dash (-) indicates a gap was inserted to adjust the alignment. Excluding the single insertion, there is 96% amino acid identity among the three orthologs. Genbank accession numbers: M22427 (rFGF2); M27968 (hFGF2); M30644 (mFGF2).

(B) Alignment of protein and DNA sequences from the N-terminal extensions of high molecular weight FGF-2 from human (hFGF-2) and rat (rFGF-2). The three-letter symbol for the amino acid is shown below its corresponding codon. The colon and dash symbols are as in **(A)** above. Mismatched nucleotides are underlined in the rat sequence. Excluding insertions, there is 83% total amino acid homology and 82% total nucleotide homology in this region of the FGF-2 gene. Genbank accession numbers: M27968 (hFGF2); M22427 (rFGF2).

(C) Alignment of the FGF-2 5′-flanking regions from rat (rFGF-2) and human (hFGF-2) genomic clones. Insertions are indicated by a colon (:) and mismatched nucleotides are marked in lowercase in the human sequence. Nucleotide positions relative to established transcription start sites (+1) are indicated on the right. In this region, homology is much reduced, limited in essence to 3 regions labeled as proximal conserved domains (PCD). The four transcription start sites for the rat gene are marked (P_i, P_o, P_1, P_2). Adapted from Pasumarthi et al., 1997. Genbank accession numbers: U78079 (rFGF-2); S81809 (hFGF-2).


A
rFGF2 MAAGSITSLP ALPEDGG-GA FPPGHFKDPK RLYCKNGGFF LRIHPDGRVD
hFGF2 MAAGSITTLP ALPEDGGSGA FPPGHFKDPK RLYCKNGGFF LRIHPDGRVD
mFGF2 MAASGITSLP ALPEDGG-AA FPPGHFKDPK RLYCKNGGFF LRIHPDGRVD
rFGF2 GVREKSDPHV KLQLQAEERG VVSIKGVCAN RYLAMKEDGR LLASKCVTEE
hFGF2 GVREKSDPHI KLQLQAEERG VVSIKGVCAN RYLAMKEDGR LLASKCVTDE
mFGF2 GVREKSDPHV KLQLQAEERG VVSIKGVCAN RYLAMKEDGR LLASKCVTEE
rFGF2 CFFFERLESN NYNTYRSRKY SSWYVALKRT GQYKLGSKTG PGQKAILFLP MSAKS
hFGF2 CFFFERLESN NYNTYRSRKY TSWYVALKRT GQYKLGSKTG PGQKAILFLP MSAKS
mFGF2 CFFFERLESN NYNTYRSRKY SSWYVALKRT GQYKLGSKTG PGQKAILFLP MSAKS
B
hFGF2 CTG GGG GAC CGC GGG CGC GGC CGC GCG CTG CCG GGC GGG AGG
Leu Gly Asp Arg Gly Arg Gly Arg Ala Leu Pro Gly Gly Arg
rFGF2 CTG GCA GCC CGC GGG - CGA - GCC - - GCG - -
Leu Ala Ala Arg Gly - Gly - Ala - - Ala - -
hFGF2 CTG GGG GGC CGG GGC CGG GGC CGT GCC CCG GAG CGG GTC GGA
Leu Gly Gly Arg Gly Arg Gly Arg Ala Pro Glu Arg Val Gly
rFGF2 CTG GGG GGC CGA GGC CGG GGT CGG - - - - - -
Leu Gly Gly Arg Gly Arg Gly Arg
hFGF2 GGC CGG GGC CGG GGC CGG GGG ACG GCG GCT CCC CGC GCG GCT
Gly Arg Gly Arg Gly Arg Gly Thr Ala Ala Pro Arg Ala Ala
rFGF2 GGC CGG GGA - - - - - - GCC CCG AGA GCT GCC
Gly Arg Gly - - - - - - Ala Pro Arg Ala Ala
hFGF2 GCA GCG GCT CGG GGA TCC CGG CCG GGC CCC GAC GGG ACC ATG
Pro Ala Ala Arg Gly Ser Arg Pro Gly Pro Ala Gly Thr Met
rFGF2 - GCA GCG - GGG TCC CGG - GGC CGC GGA GGG GCC ATG
- Ala Ala - Gly Ser Arg - Gly Arg Gly Gly Ala Met
C
rFGF-2 GAAGAGG;;TGTGCTCCCACACGCAGGGAGAGAAGCTACCTAATTTCAGAACAGAA;GCACAGAGA -490
hFGF-2 GAAaAtaacTcT;CTCtC;CAaGaAalG;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;TycTCccCTTCtCtgGCcTcttTCTC;;tCctttTG;tt -459
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 P1
rFGF-2 GCGGCGCGCGGGGCGGGGGCGCGCGGGGCCGGGGTGCAGGCGGGGACGCGGGGGT;GACGCGGGCC +53
hFGF-2 GgGGataa;GGGGCGGtGGaGCcCaGGGnatGccaa;AGcCctGc;CGCGGcctccGACGCGcGCC -86
rFGF-2 CGGGCCGCTGTAGCACACAGGGGCTCGGTCTCTCGGCTTCAGGCGGAGTCCGGCTGCACTAGGCTG +119
leu leu
rFGF-2 GGAGCGCGGCGGGACGCGAACCGGGAGGCTGGCAGCCCGCGGGCGAGCCGCGCTGGGGGGCCGAGG +185
met
rFGF-2 CCGGGGTCGGGGCCGGGGAGCCCCGAGAGCTGCCGCAGCGGGGTCCCGGGGCCGCGGAGGGGCCATG +252

1.4.2. The FGF-2 Promoter: A Housekeeping Gene

The 5′-flanking regions of the FGF-2 gene have been cloned from the human (Shibata et al., 1991) and rat (Pasumarthi et al., 1997) genomes. The promoter from either species does not contain TATA or CCAAT boxes, characteristic of the so-called "housekeeping" genes of the cell. Housekeeping gene promoters are characterized by the lack of canonical TATA or CCAAT elements, the presence of multiple transcription start sites, and G/C-rich islands located in the vicinity of transcription initiation, often harboring binding sites for the zinc-finger transcription factors Sp1 or Egr-1. Their expression is usually constitutive but low-level, and may be upregulated under certain stimuli. Housekeeping genes are often ubiquitous, potent gene products, including enzymes such as xanthine dehydrogenase/oxidase (Chow et al., 1994), an enzyme involved in the production of the superoxide radical, or ferrochetolase (Magness et al., 1998), whose low-level expression is ubiquitous but is upregulated during hematopoiesis.

Transcription of the human FGF-2 gene occurs from a single transcription initiation site (Shibata et al., 1991), while the rat FGF-2 promoter contains four identified start sites, which are differentially affected by phorbol ester growth stimulus (Pasumarthi et al., 1997). Deletion studies and promoter analysis of the human gene indicate that the promoter contains negative regulatory elements which suppress expression (Shibata et al., 1991). Elements have also been isolated which confer cell density dependent regulation in astrocytes (Moffett et al., 1996). These elements bind protein at low cell density but not in confluent cells. In contrast, transformed glial cells show binding to these elements at all cell densities, indicating a removal of contact inhibition. Sequences were also

identified that were responsive to growth factors and to PKC and cAMP stimulation (Moffett et al., 1998). Wild-type p53 repressed transcription from the human FGF-2 promoter, while transcription was activated by mutant p53 (Ueba et al., 1994). Finally, the human FGF-2 promoter contains several binding sites for the immediate early transcription factor Egr-1, and Egr-1 was shown to stimulate FGF-2 transcription in astrocytes (Biesiada et al., 1996). The rat FGF-2 promoter is less well studied since it was more recently cloned, but it responds to growth stimuli such as phorbol ester and serum, and deletion analysis pointed to both positive and negative regulatory elements within the 1,058 bp of flanking DNA that was cloned (Pasumarthi et al., 1997).

Table 1. Factors which Increase FGF-2 mRNA Levels in Cardiovascular Cells.

Cell Type	Agent	Reference
Endothelial Cells	AngII, ET-1, FGF-2	Fisher et al., 1997
	scrape injury	Ku and D'Amore, 1995
	shear stress	Malek et al., 1993
	FGF-2, thrombin, phorbol ester	Weich et al., 1991
Vascular Smooth Muscle	FGF-2	Alberts et al., 1994
	HB-EGF	Peifley et al., 1996
	AngII, ET-1	Peifley and Winkles, 1998
Cardiac Myocytes	transplantation (human ventricles)	Ationu and Carter, 1994
	AngII, ET-1, FGF-2, IL-1β	Fisher et al., 1997

Abbreviations: AngII, angiotensin II; ET-1, endothelin-1; HB-EGF, heparin-binding epiderminal growth factor-like growth factor; FGF-2, fibroblast growth factor-2; IL-1β, interleukin-1β.

1.4.3. Regulation of FGF-2 Gene Expression in Cardiac Cells

Promoter work with either the human or rat FGF-2 gene has been carried out in non-cardiac cell types, and mechanisms controlling FGF-2 transcription in cardiac cells is not well understood. However, factors affecting FGF-2 gene expression in cardiac cells have been documented (Table 1). In endothelial cells, FGF-2 mRNA levels are increased by shear stress, non-lethal scrape injury, thrombin, phorbol ester and FGF-2 itself (autoregulation) (Malek et al., 1993; Ku and D'Amore, 1995; Weich et al., 1991; Fisher et al., 1997). Upregulation by sublethal scrape injury was not attributed to autoregulation through release of FGF-2 to the conditioned media (Ku and D'Amore, 1995). However, autoregulation of FGF-2 gene expression was demonstrated in both endothelial cells and adult rat cardiac myocytes (Weich et al., 1991; Fisher et al., 1997), and in human Hep3B cells this autoregulation was shown to be mediated by Egr-1 binding to the FGF-2 promoter (Wang et al., 1997).

In vascular smooth muscle cells (VSMC), FGF-2 mRNA levels were increased with addition of heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Peifley et al., 1996). HB-EGF-induced accumulation of FGF-2 mRNA was blocked with actinomycin D, indicating that transcriptional activation is most likely involved. Measurements of mRNA stability in this same study also indicated that the half life of FGF-2 is not significantly increased with HB-EGF treatment. FGF-2 mRNA accumulation was detected in a similar manner with FGF-2 treatment, indicating that autoregulation occurs in VSMCs (Alberts et al., 1994). Finally, treatment of VSMCs with angiotensin II or endothelin-1 also increased FGF-2 mRNA accumulation (Peifley

and Winkles, 1997). In all cases, the increase in FGF-2 mRNA was blocked with actinomycin D, indicating an increase in transcriptional activity.

In isolated adult rat ventricular myocytes, FGF-2 mRNA could be increased by treatment with angiotenisn II, endothelin-1, FGF-2 itself (autoregulation), and modestly by interleukin-1β (Fisher et al., 1997). Reverse transcriptase polymerase chain reaction was used to detect FGF-2 mRNA in this study, and there was no assessment of mRNA stability. The mechanisms regulating FGF-2 gene expression in cardiac myocytes and in the intact myocardium are not well studied, and no promoter work has been done in these cells to date. However, in addition to the isolated myocyte studies, an increase in FGF-2 mRNA was detected in human ventricles following orthotopic cardiac transplantation (Ationu and Carter, 1994). Thus it is apparent that a wide range of stimuli may increase FGF-2 gene expression, many of these related to injury and repair responses. It is feasible that any or all of these stimuli may act at the level of transcriptional activation.

1.5. Catecholamines in the Heart: The Adrenergic System

1.5.1. Cardiac Catecholamines and Their Receptors

The catecholamines epinephrine and norepinephrine (NE) are well established as mediators of the classic "fight or flight" response in mammals. In the heart, such a response manifests itself as an augmentation of cardiac contractile rate and force, accompanied by changes in peripheral blood pressure. The heart is heavily innervated with sympathetic fibres, and NE is released into the myocardium from the termini of sympathetic neurons, although there is also evidence for an intrinsic adrenergic system in

the heart, where endogenous catecholamines are produced (Huang et al., 1996). Catecholamines exert their effects on inotropy, heart rate and blood pressure through α_1-, α_2- and β-adrenergic receptors (Table 2; Rockman et al., 1997). Cardiac myocytes contain α_1 and β receptors, which act synergistically to control contractility and heart rate through regulating intracellular Ca^{2+} concentrations and phosphorylation cascades (Bylund et al., 1994).

1.5.2. Signal Transduction through Cardiac Adrenergic Receptors

Each adrenergic receptor group (α_1, α_2, and β) is associated with a distinct second messenger system. Within each group, several pharmacological subtypes have been identified based on the selective activity of receptor agonists and antagonists (Bylund et al., 1994; Strosberg, 1993). The subtypes arise from separate genes within a gene family (Rockman et al., 1997). However, the characteristic signal transduction mechanisms appear to apply to all subtypes within a particular receptor group. Pathways originating from the cardiac α_1 and β-adrenergic receptors will be considered here (Figure 3).

All adrenergic receptor (AR) groups have seven membrane-spanning α-helices and are coupled to trimeric guanosine 5′-triphosphate (GTP)-binding proteins (G-proteins). The effectors that are activated depend on the type of α-subunit contained in the G-protein. In cardiac myocytes, α_1-ARs are coupled to several different G proteins, both pertussis toxin sensitive and insensitive (Terzic et al., 1993) This allows the α_1-AR to mediate a variety of cellular responses, including the activation of phospholipases A_2 and D, and increased Na^+/H^+ and Na^+/Ca^{2+} exchange. The "major" signal transduction pathway of

α_1-ARs, however, is pertussis toxin insensitive and is mediated by members of the G_q family (Wu et al., 1992), or by G_h, as demonstrated in failing human hearts (Hwang et al., 1996) (Figure 3). Hydrolysis of GTP by the G-protein leads to activation of phospholipase C (PLC) at the plasma membrane. PLC then catalyzes the conversion of the membrane phospholipid phosphoinositol-4,5-bisphosphate (PIP_2) to the membrane-bound 1,2-diacyl-sn-glycerol (DAG) and the cytosolic compound inositol 1,4,5-triphosphate (IP_3). DAG is a direct activator of protein kinase C, which mediates a wide range of cellular processes. IP_3 increases intracellular Ca^{2+} concentrations through release from intracellular stores. In addition, some α_1-AR subtypes may be linked to a particular G-protein which is directly coupled to voltage-sensitive Ca^{2+} channels in the sarcolemma (Bylund et al., 1994). Thus, stimulation of α_1-ARs in cardiac myocytes leads to activation of PKC and an increase in intracellular Ca^{2+} through both the release of Ca^{2+} from the sarcoplasmic reticulum and the influx of Ca^{2+} across the sarcolemma.

β-Adrenergic receptors are coupled to G_S, which stimulates adenylate cyclase to increase the synthesis of the second messenger cAMP. The cAMP-dependent protein kinase A is then activated to phosphorylate myocardial proteins involved in the positive inotropic and chronotropic responses to β-adrenergic stimulation (Rockman et al., 1997). In addition, in cardiac muscle, there is evidence for direct coupling between G_S and a sarcolemmal voltage-sensitive Ca^{2+} channel (Bylund et al., 1994). The β-induced influx of Ca^{2+} would thus contribute to the increase in contractility and heart rate.

Table 2. Characteristics of Adrenergic Receptor Subtypes.

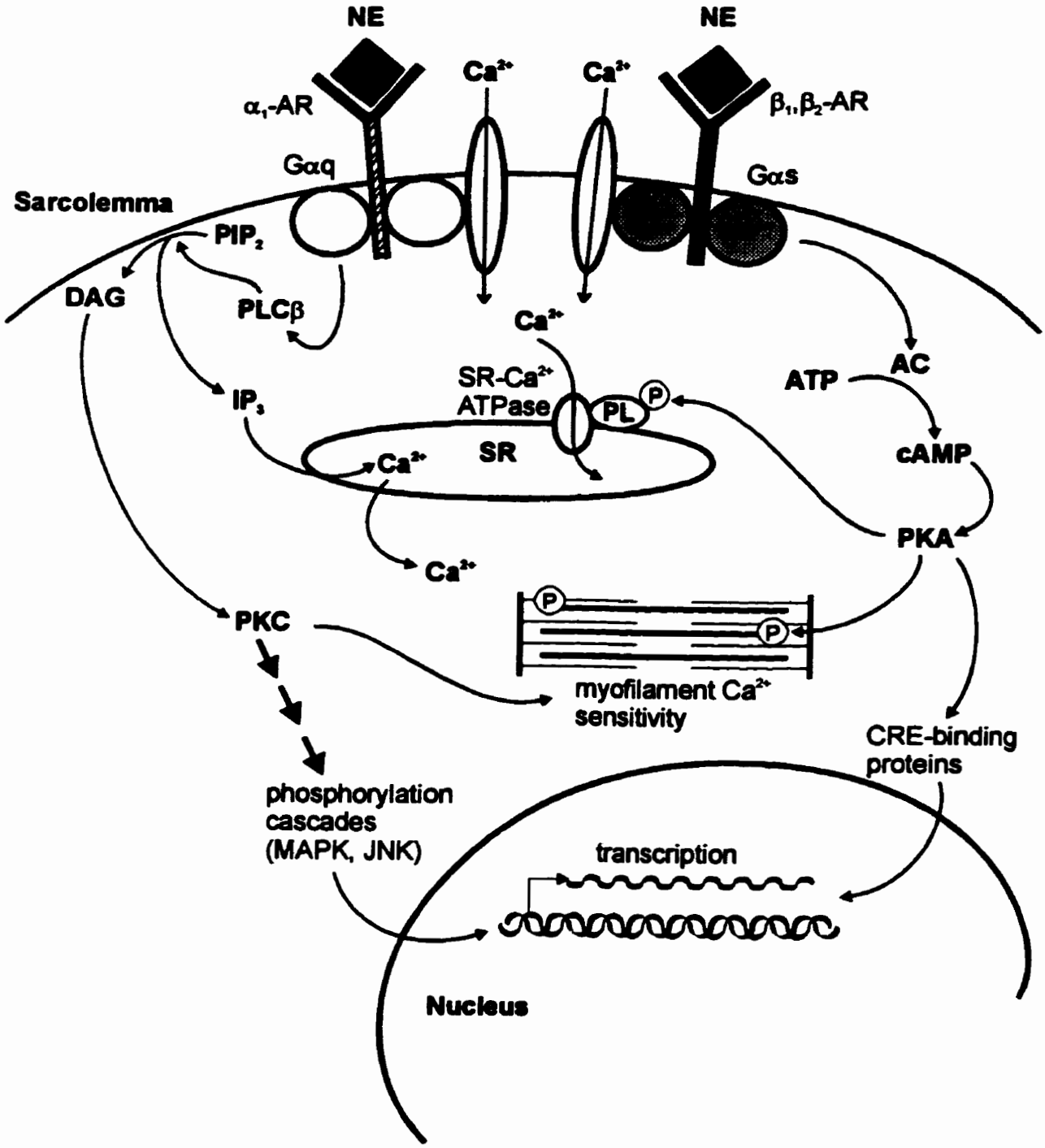
Receptor	G-protein (Example)	Effector (Examples)	Tissue Localization	Physiological Response
$\alpha_{1a/d,b,c}$	G_q, G_h	↑Phospholipases C, D, A_2; DAG, IP_3	Vascular smooth muscle Heart Visceral smooth muscle	Contraction Hypertrophy Contraction
$\alpha_{2A,B,C}$	G_i	↓AC activity; ↑K^+ channels, ↓ L-type Ca^{2+} channels	Nerve terminals Vascular smooth muscle	↓ NE release Contraction
β_1	G_s	↑AC activity; ↑L-type Ca^{2+} channels	Myocardium Cardiac conduction cells	↑Force and rate of contraction ↑Conduction velocity
β_2	G_s	↑AC activity	Myocardium Smooth muscle	↑Force and rate of contraction Relaxation
β_3	G_s	↑AC activity	Adipose tissue	Lipolysis

Adapted from Rockman et al., 1997. AC, adenylate cyclase; DAG, 1,2-diacyl-sn-glycerol; IP_3, inositol-1,4,5-triphosphate

Figure 3.

Signal transduction through adrenergic receptors in cardiac myocytes.

Cardiac myocytes contain α_1- (all subtypes) and β- (β_1 and β_2 subtypes) adrenergic receptors. Stimulation of **α_1-AR** by NE results in the production of diaceylglycerol (**DAG**), a potent protein kinase C (**PKC**) activator, and inositol 1,4,5-triphosphate (**IP_3**). PKC initiates phosphorylation cascades resulting in transcriptional activation in the nucleus (Post and Brown, 1996); as well, it may mediate a positive inotropic effect by increasing myofilament sensitivity to Ca^{2+} (Terzic et al., 1993). IP_3 stimulates the release of Ca^{2+} from the sarcoplasmic reticulum (**SR**), affecting multiple cellular processes and contributing to positive inotropy. Stimulation of **β-AR** by NE results in the activation of protein kinase A (**PKA**) by cAMP. PKA phosphorylates phospholamban (**PL**), increasing the uptake of Ca^{2+} by the **SR-Ca^{2+} ATPase**. PKA can also phosphorylate myofilament proteins and increase their Ca^{2+} sensitivity. Finally, PKA phosphorylates and activates CRE-binding proteins, effecting changes in gene expression. Both α_1- and β-AR may be directly coupled through G-proteins to sarcolemmal voltage-sensitive Ca^{2+} channels, thus increasing Ca^{2+} influx. Details of these processes are given in the text.


NE
NE
Ca2+
Ca2+
α1-AR
β1,β2-AR
Gαq
Gαs
Sarcolemma
PIP2
DAG
PLCβ
Ca2+
SR-Ca2+
ATPase
PL
P
ATP
AC
IP3
SR
Ca2+
cAMP
Ca2+
PKA
PKC
P
P
myofilament Ca2+
sensitivity
CRE-binding
proteins
phosphorylation
cascades
(MAPK, JNK)
transcription
Nucleus

1.5.3. Physiological and Pathophysiological Effects of Adrenergic Receptor Signaling

Catecholamine action on β-ARs represents the primary and rapid way of altering cardiac contractility. Activation of cAMP-dependent protein kinase A results in phosphorylation of phospholamban, a protein on the sarcoplasmic reticulum (SR) which is associated with the SR Ca^{2+}-ATPase. Phosphorylation of phospholamban allows the Ca^{2+}-ATPase to remove intracellular Ca^{2+} released during excitation-contraction coupling from the cytoplasm to the SR lumen. Thus, β-AR stimulation speeds up the relaxation phase of excitation-contraction coupling, shortening the action potential and facilitating an increase in heart rate. In addition, as mentioned above, cardiac β-ARs can be linked directly to sarcolemmal Ca^{2+} channels, increasing the influx of Ca^{2+} into the myocyte and thus, with the phosphorylation of sarcomeric proteins to increase their sensitivity to Ca^{2+}, increase contractility.

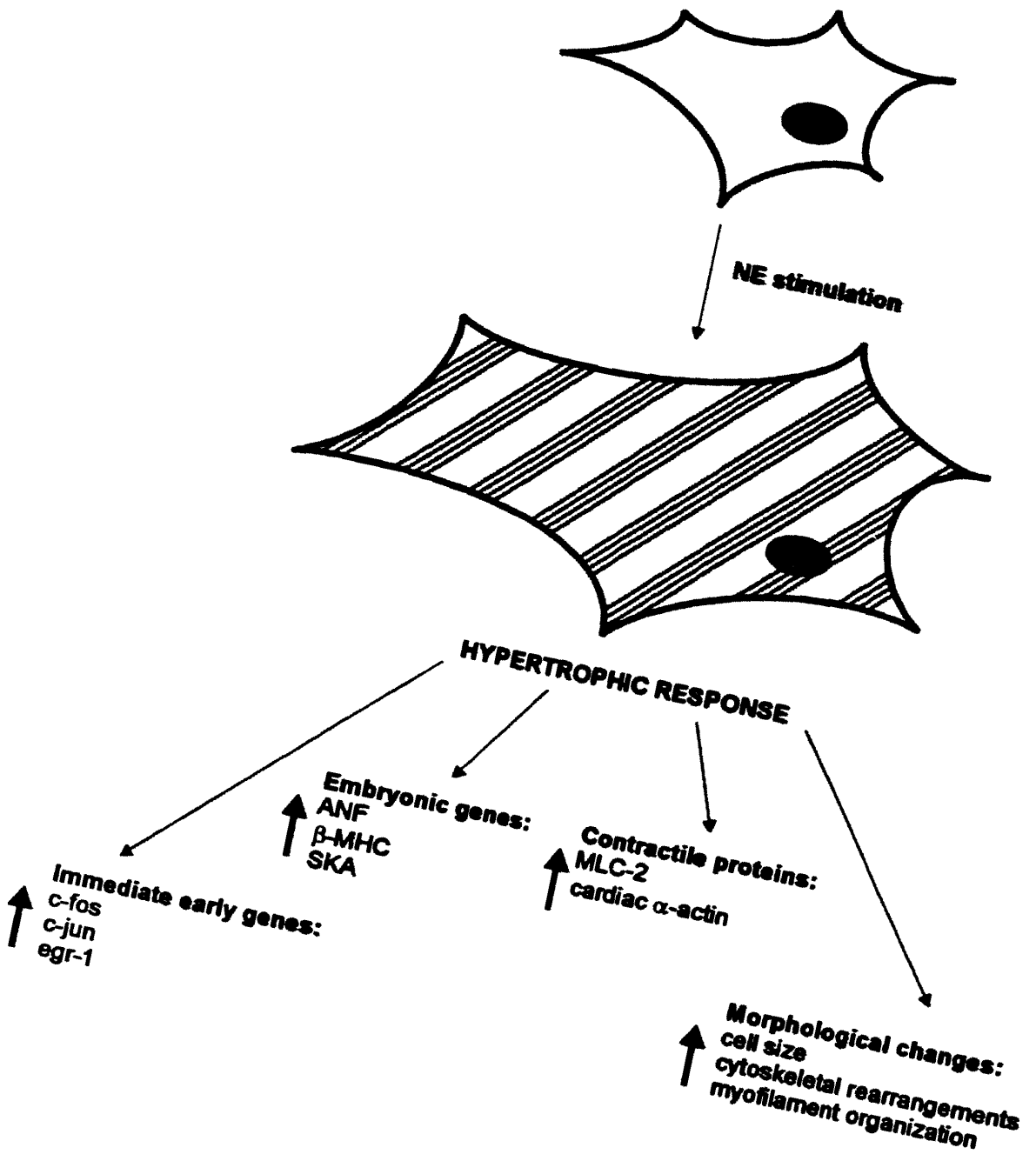
Positive inotropy elicited by NE also appears to have a significant α_1-AR mediated component, which may be augmented in failing hearts (Skomedal et al., 1997). The inotropic effect of α_1-AR differs from that of β-AR in that it is not accompanied by any chronotropic effect, although α_1-AR can play a role in the generation of specific arrhythmias (Terzic et al., 1993). The mechanism underlying the inotropic effect is postulated to involve the phosphorylation of myofilament proteins to increase their sensitivity to Ca^{2+}, combined with prolongation of the action potential through the inhibition of K^+ currents (Terzic et al., 1993).

Norepinephrine also works through both α_1 and β-AR to elicit the hypertrophy response of cardiac myocytes (Yamazaki et al., 1996, and references therein). Cardiac hypertrophy is an adaptive response to an increased load, which can be compensatory, as occurs in exercise, or decompensatory, which occurs in cardiomyopathy associated with hypertension or valvular heart disease. The induction of hypertrophy is associated with a succession of genetic events (Figure 4). First, several immediate-early genes are induced, including c-myc, c-fos, Egr-1, c-jun and jun-B (Terzic et al., 1993; Iwaki et al., 1990). Next, a subset of genes bearing resemblance to a more embryonic pattern of gene expression is also induced, including atrial natriuretic peptide (ANF/ANP), skeletal α-actin (SKA), and β-myosin heavy chain (β-MHC). Finally, there is an upregulation of the constitutive contractile proteins myosin light chain-2 (MLC-2) and cardiac α-actin, and these are incorporated into sarcomeres to increase the cell's content of contractile apparatus. The changes in gene expression may be mediated by the small GTP-binding proteins such as Ras and Raf-1, through a protein kinase cascade involving mitogen-activated protein kinase (MAPK) (LaMorte et al., 1994; Yamazaki et al., 1996). However, other pathways, including those mediated by Rho (Sah et al., 1996; Hines and Thornburn, 1998) and the Janus kinases (JNK) (Ramirez et al., 1997) also seem to play a role in what is an apparently very complex network of signal transduction.

Figure 4.

Events in NE-stimulated cardiac myocyte hypertrophy.

Norepinephrine (NE) functions through both α_1 and β-adrenergic receptors to induce changes in gene expression and cell morphology associated with the hypertrophic response. The order of events, the relative importance of α_1- and β-adrenergic stimulation, and the current knowledge of pathways and *cis*-acting elements involved in the regulation of gene expression are discussed in the text. ANF, atrial natriuretic factor; β-MHC, β-myosin heavy chain; SKA, skeletal α-actin; MLC-2, myosin light chain-2.


NE stimulation
HYPERTROPHIC RESPONSE
Embryonic genes:
ANF
β-MHC
SKA
Contractile proteins:
MLC-2
cardiac α-actin
Immediate early genes:
c-fos
c-jun
egr-1
Morphological changes:
cell size
cytoskeletal rearrangements
myofilament organization

Hypertrophy observed *in vivo* appears to have a very strong α_1-AR-stimulated component, for several reasons: First, β-ARs are quickly downregulated through phosphorylation by PKA, PKC, or a β-adrenergic receptor kinase (β-ARK/GRK) (Rockman et al., 1997; Bristow, 1998). Phosphorylation of a β-AR causes it to bind to β-arrestin, which functionally uncouples it from its G-protein. β-AR downregulation is a hallmark characteristic of heart failure and many types of cardiomyopathy. The "β-blockers" used in the treatment of early stages of heart failure are designed to combat the overstimulation of β-AR by catecholamines released in the heart from sympathetic neural activity (Esler et al., 1997; Will-Shahab and Schubert, 1991). Secondly, while β-AR are rapidly and significantly downregulated in ischemia and heart failure, with eventual changes in receptor density, cardiac α_1-AR density changes little or is even increased (Will-Shahab and Schubert, 1991; Terzic et al., 1993; Zhao et al., 1996) It may be, then, that the stimulation of α-AR under pathological conditions may serve as a reserve mechanism in an attempt to retain responsiveness to catecholamines (Terzic et al., 1993; Skomedal et al., 1997). Finally, it is worth noting that hormones such as angiotensin II and endothelin I activate a similar program of hypertrophy through their own receptors, which, like α_1-ARs, are also coupled to G_q (Post and Brown, 1996). Indeed, overexpression of G_q caused hypertrophy in transgenic mice (D'Angelo et al., 1997; Mende et al., 1998).

1.5.4. Transcriptional Regulation Following Adrenergic Receptor Stimulation

DNA elements in promoters of cardiac genes activated by α_1-adrenergic stimulation have been studied extensively (Table 3). The M-CAT element was shown to be central to the regulation of brain natriuretic peptide (Thuerauf and Glembotski, 1997), β-myosin heavy chain (Kariya et al., 1994), and skeletal α-actin (Karns et al., 1995). The human viral transcription enhancer factor (TEF)-1 was shown to bind to the M-CAT element in the β-MHC and α-SKA promoters (Kariya et al., 1993; Karns et al., 1995), and a related factor, RTEF-1, was later shown to also play a role in the regulation of these genes (Stewart et al., 1998). Unlike for the β-MHC and brain natriuretic peptide (BNP) promoters, for which M-CAT was sufficient for α_1-adrenergic regulation, both CArG (serum response element) and Sp1 elements were necessary in combination with M-CAT for α-adrenergic regulation of the α-SKA promoter (Karns et al., 1995). Multiple elements are also involved in the α-adrenergic regulation of the ANF promoter, including an A/G-rich element (Ardati and Nemer, 1993) and two CArG serum response elements (Sprenkle et al., 1995), as well as a novel upstream A/T rich region (Harris et al., 1997). Stimulation of MLC-2 promoter activity by α-adrenergic activity also requires a 28 bp A/T-rich region, containing overlapping CArG, AP2, and MEF2 elements (Zhu H. et al., 1991). A novel zinc finger protein, HF-1b, was shown to bind to the MEF2 element in this region to confer muscle-specific basal and inducible expression of MLC-2 (Zhu H et al., 1993).

Table 3. α_1-Adrenergic Regulation of Cardiac Gene Expression.

Gene	Element Name	DNA Sequence	Reference
ANF/ANP	A/G-rich	GGGGAGGG	Ardati and Nemer, 1993
	SRE (CArG)	CTTTAAAAGG; CCTTATTTGG	Sprenkle et al., 1995
	A/T-rich	TCTAAAAAAATATAATAGCT	Harris et al., 1997
BNP	M-CAT	AGGAATG	Thuerauf and Glembotski, 1997
β-MHC	M-CAT	TGTGGTATG	Kariya et al., 1994
SKA	M-CAT	CATTCTT	Karns et al., 1995
	CArG	CCAAATATGG	
	Sp1	GGGCGG	
MLC-2	HF-1 (CArG, AP2, MEF2)	GCCAAAAGTGGTCATGGGGTT	Zhu H et al., 1991

ANF/ANP, atrial natriuretic factor/peptide, BNP, brain natriuretic peptide, β-MHC, β-myosin heavy chain; MLC-2, myosin light chain-2; SKA, skeletal α-actin; SRE, serum response element.

The effects of β-adrenergic receptors on gene expression at the promoter level is less well studied. Both α_1- and β-AR stimulation increases the expression of *c-fos* and *c-jun*, which increases skeletal α-actin gene expression through an AP-1 site (Bishopric et al., 1992), and decreases ANP expression independently of AP-1 (McBride et al., 1993). A major distinction between α_1- and β-AR is the increase in cAMP brought about by β-AR stimulation, resulting in the activation of protein kinase A (PKA). PKA phosphorylates and activates a number of transcription factors, including AP-2 and the cAMP response element binding protein (CREB) (Faisst and Meyer, 1992). Several cardiac genes contain a cAMP response element (CRE), including ANP (Harris et al., 1997) and the β_2-AR subtype (Collins et al., 1990). Heart failure is associated with changes in expression of some cardiac genes, and it has been postulated that β-AR stimulation is the source of many of these changes (Müller et al., 1993). CREB is expressed in the human heart (Müller et al,. 1995), as is the CRE modulator protein , CREM (Müller et al., 1998), but the significance of this in the context of heart failure-related changes in gene expression is not yet understood (Müller et al., 1997). There is evidence for transcriptional autoregulation of the β_2-AR promoter via cAMP and a CRE (Collins et al., 1990). Given the complexity of the changes in gene expression which do occur with β-adrenergic stimulation, combined with the tight control exerted over β-AR levels and functional coupling, much remains to be learned about the control of gene expression by β-adrenergic activity.

1.6. Rationale, Hypothesis, and Objectives

Since FGF-2 has a role in cardioprotection, wound healing and stress response, any knowledge of how the FGF-2 gene is regulated would be useful to gain insight into how FGF-2 regulation may be exploited for therapeutic benefit where risk of injury is very high. Indeed, if FGF-2 also plays a role in the maintenance of a healthy myocardium, then understanding how FGF-2 is regulated under conditions of stress and challenge could also lead to regimens for ensuring cardiovascular health in the long term. FGF-2, as we have seen, is regulated at many different levels (Figure 1), and FGF-2 transcription is regulated by a variety of signal transduction pathways, as reflected by changes in mRNA levels (Table 1). As discussed above (section 1.3.3), FGF-2 is released from cardiac myocytes with every contraction of the myocardium (McNeil and Steinhardt, 1997). This release can be increased with adrenergic activity (Clarke et al., 1995). Catecholamines, especially norepinephrine, which acts through both α_1- and β-adrenergic receptors (Zhao et al., 1996) are released in the myocardium under ischemic conditions which lead to infarction (Will-Shahab and Schubert, 1991). Together, these factors suggest the hypothesis that **release of FGF-2 is accompanied by synthesis to replenish intracellular stores, and the stimulation of synthesis, at the level of transcription, can be mediated by adrenergic activity.** Once established, such an idea could lead to studies of other pathways, perhaps related to injury, which link FGF-2 release and synthesis to replenish intracellular stores.

Detection of FGF-2 mRNA at basal levels in most tissues, including cardiac cells, appears to be more difficult than detection of protein (see, for example, Fisher et al.,

1997). This presumably reflects low promoter activity and/or an unstable mRNA. Much of the work caried out with FGF-2 mRNA has used tumour cell lines, which overexpress FGF-2, as the source (Cattini et al., 1998; Murphy et al., 1989). An alternative approach to transcriptional studies is the reporter gene assay. This type of assay can be used to (a) confirm trends observed with mRNA levels, and (b) to isolate and characterize cis-acting elements within a promoter region. Previously, in our laboratory, the 5´-flanking region of the rat FGF-2 gene was cloned (Pasumarthi et al., 1997). Sequencing revealed a promoter region with no TATA or CCAAT boxes, several G/C-rich islands, and multiple transcriptional start sites, typical of many housekeeping genes. The promoter could be regulated by growth stimuli, such as serum and phorbol esters, in a rat glioma cell line (Pasumarthi et al., 1997). Studies using this genomic clone, in conjunction with mRNA studies, both in cultured neonatal rat cardiac myocytes and in transgenic mice, appeared in subsequent published works (Detillieux et al., 1998; Detillieux et al., 1999). Data from these references, as well as additional unpublished work, will be presented in this thesis in answer to the following objectives:

Objective 1. To demonstrate and characterize the regulation of rat FGF-2 gene expression in cardiac myocytes by adrenergic activity (**Chapter 3**).

Specific Aims:

1.1. To demonstrate the relative role of α_1- and β adrenoceptors in the regulation of FGF-2 gene expression in cultured neonatal rat cardiac myocytes.

1.2. To use deletion analysis to determine the region of 5′-flanking DNA responsible for adrenergic regulation of the rat FGF-2 gene.

1.3. To determine the role of Ca^{2+} influx and myocyte contraction in the adrenergic regulation of FGF-2 gene expression.

1.4. To characterize the adrenergic regulation of the rat FGF-2 promoter *in vivo*.

Objective 2. To assess the functional significance of a putative phenylephrine-responsive, A/G-rich element in the rat FGF-2 promoter (**Chapter 4**).

Specific Aims:

2.1. To determine the effect of the A/G-rich region on the activity of a heterologous promoter.

2.2. To characterize the protein-DNA interactions that occur at the A/G-rich region.

2.3. To determine whether structural and functional changes occur at this region upon adrenergic stimulation.

Objective 3. To establish a working model for Langendorff-type perfusion of the isolated mouse heart (**Chapter 5**).

Specific Aims:

3.1 To assemble an apparatus for effective baseline function of an isolated mouse heart undergoing retrograde perfusion at constant pressure.

3.2 To establish conditions for the study of global ischemia and reperfusion using this model.

3.3 To determine the feasibility of the measurement of FGF-2 release from the isolated murine myocardium.

3.4 To measure the activity of the FGF-2 promoter in this system using reporter gene assays of isolated hearts from transgenic mice.

Chapter 2. Materials and Methods

2.1. Cell culture and tissue extracts

Neonatal rat cardiac myocyte cultures were prepared by the Percoll gradient method developed by the laboratory of Dr. Kenneth Chien and modified by the laboratories of Dr. Kardami and Dr. Cattini. Rat pups (Sprague-Dawley, either gender, 1 to 36 hours after birth) were sacrificed by decapitation and ventricles were dissected as quickly as possible into Ham's F10 nutrient mixture (Gibco-BRL) chelated with 1.33 mM EGTA at room temperature. Approximately 36 hearts were used for each myocyte preparation. After dissection, the hearts were transferred to fresh chelated F10 and minced with scissor mincers until the hearts pieces could be taken up with a 10 ml pipette. The pieces were them transferred to a water-jacketed (35°C) spinner flask containing 10 ml of chelated F10 with trypsin (Gibco-BRL, 0.85-1.0 mg/ml) and DNaseI (Sigma-Aldrich, 30U/ml). The cells were thus dissociated with 10 changes of 10 minutes each in this manner. Dissociated cells were collected in fetal bovine serum at room temperature. After completion of this stage, the cells were centrifuged and resuspended in 1x Ads buffer (20 mM HEPES, pH 7.35; 116 mM NaCl; 10 mM $NaH_2PO_4.H_2O$; 5.5 mM glucose; 5.36 mM KCl, 0.8 mM $MgSO_4.7H_2O$), treated with a further 300 U DNaseI for approx. 10 min before they were passed through a Nytex nylon membrane. The cells were than layered on a discontinuous Percoll (Pharmacia) density gradient (1.059 g/ml:1.110 g/ml) and centrifuged at 2500g (PR-7000 centrifuge, swing out bucket rotor, radius 240 mm, 3500 rpm) for 45 minutes at room temperature. The upper non-myocyte layer was then discarded and the enriched myocyte layer was collected and washed twice in 1xAds

buffer. Finally, the cells were resuspended in 30 ml of F10 nutrient mixture with 10% (v/v) FBS (Gibco-BRL), 10% horse serum (Gibco-BRL), antibiotics (Gibco-BRL, 1000 units/ml penicillin, 1 mg/ml streptomycin) and calcium chloride supplemented to 1.05 mM. Cells were counted using a hemocytometer and plated at a density of $1x10^6$ cells per 35 mm plate, or $1.5x10^6$ cells per 60 mm plate. Culture plates (Corning) had been prepared by coating the plates with a layer of collagen (Upstate Biotechnology, rat tail, type I, 0.05%) and leaving the plates to dry under UV radiation overnight.

Rat glioma C6 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, Gibco-BRL) supplemented with 10% (v/v) FBS with antibiotics as above, at 37°C in the presence of 5% CO_2. Plating densities for C6 and U87-MG cells were $0.5x10^6$ and $1.0x10^6$ per 100 mm dish, respectively.

2.2. Plasmids and constructs

The hybrid genes, -1058FGFp.*luc*, -911FGFp.*luc*, and -313FGFp.*luc*, containing fragments of the rat FGF-2 gene fused upstream of a promoterless firefly luciferase gene (-p.*luc*, contained in the vector pXP1) were described previously (Pasumarthi et al., 1997). A 250 base pair fragment of the myosin light chain-2 (MLC-2) promoter cloned upstream of luciferase was described previously (Jin et al., 1995).

The plasmid TKp.*luc* (Nordeen, 1988) contains a portion of the herpes simplex virus thymidine kinase promoter (positions -81 to +52) fused to the firefly luciferase gene. A

37 bp A/G rich (double-stranded) oligonucleotide, corresponding to nucleotide positions -785/-749 (Pasumarthi et al., 1997), and containing the putative AGGG-repeat sequences, 5′-GGGAAAGGGAGGGGGAAGGAAAGGAGGGAGGGAAGGA-3′, was synthesized and inserted upstream of the TK promoter in pT81.*luc* to generate A/G-TKp.*luc*.

The pRL-CMV vector, containing the *Renilla* luciferase gene under the control of the cytomegalovirus promoter, was obtained from Promega Corporation.

2.3. Isolation of RNA from cells and tissue

The day after isolation as described in section 2.1, neonatal rat cardiac myocytes on 60 mm plates were transferred to a serum-free preparation of a 1:1 mixture of DMEM:F12 media (Gibco-BRL), containing a 1x insulin-transferrin-selenium supplement (Redu-Ser II, Upstate Biotechnology), 0.02 mg/ml ascorbic acid and antibiotics. At the same time the media was changed, the plates were divided into experimental groups for stimulation. For experiments with norepinephrine (NE), cells were treated with or without 0.01 mM NE and 0.01 mM prazosin (Research Biochemicals International) for 6 h before harvesting. For experiments with phenylephrine (PE), cells were treated with or without 0.1mM PE (Sigma-Aldrich) for 48 h before harvesting.

RNA isolation was carried out using guanidine isothiocyante (GuanSCN) (Chomczynski and Sacchi, 1987). Each 60 mm plate was washed 3x with PBS-CMF, then 0.6 ml of 4 M GuanSCN (pH 7.0; containing 5 mg/ml N-lauryl sarcosine, 25 mM sodium citrate, and

0.7% β-mercaptoethanol) was added to each plate. The contents of triplicate plates were pooled on ice and each plate was rinsed with 0.6 ml of water-saturated phenol. Once harvesting in this manner was complete, RNA isolation continued according to standard laboratory protocol, based on Maniatis et al. (1982). At the end of isolation, RNA from all plates within a treatment group was pooled, concentration was determined spectrophotometrically, and total RNA was divided into 50 or 100 μg aliquots, precipitated with 95% ethanol and stored at –70°C.

For tissue studies, male CD-1 mice aged 6-8 weeks were injected intraperitoneally with saline or 50 mg/kg PE. After 48h, mice were sacrificed by cervical dislocation and hearts were dissected onto dry ice. Hearts were stored for up to 48h at –70°C before RNA isolation began. Hearts were thawed on ice and homogenized individually in 1 ml 4 M GuanSCN. RNA isolation was then continued according to standard laboratory protocol. At the end of isolation, RNA concentration was determined and total RNA was divided into 100 μg aliquots, precipitated with 95% ethanol and stored at –70°C.

2.4. "Northern" analysis of RNA

2.4.1. Formaldehyde gels for RNA

Fifty or 100 μg aliquots of precipitated total RNA was pelleted, washed, dried and resuspended in sterile distilled water. Once the RNA was fully in solution, a cocktail was added which resulted in the sample being contained in 0.5x MOPS buffer (1x MOPS = 20 mM MOPS, pH7.0, 5 mM sodium acetate, 1 mM EDTA), 2.2 M formaldehyde, 50%

deionized formamide, and 0.02 mg/ml ethidium bromide. The sample was heated at 65°C for 20 min, then 4 µl of a loading dye (10x loading dye = 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol) was added. Ten percent of each sample was resolved on a minigel (1% agarose, 1x MOPS, 2.2 M formaldehyde) in 1x MOPS to test for consistency of loading. The remainder of each sample was then resolved on a larger gel of the same composition overnight. The following day, the gel was photographed and blotted to nitrocellulose membrane using 20x SSC (3 M NaCl, 0.3 M sodium citrate) for 16h. The nitrocellulose membrane was then baked at 80°C for 45-60 min to covalently bind the nucleic acid.

2.4.2. Hybridization and detection of FGF-2 mRNA

Detection of FGF-2 mRNA was achieved with a radiolabeled fragment of the rat FGF-2 cDNA. The ~500 bp fragment corresponds to the entire open reading frame of 18 kDa FGF-2, beginning 18 bp upstream of the AUG start codon and ending ~18 bp downstream of the UGA stop signal. It was obtained by digestion with *Xho*I of a plasmid containing a portion of the rat FGF-2 cDNA which had been depleted of those sequences peculiar to high molecular weight FGF-2 (Pasumarthi et al., 1994). Radiolabeling of 50 ng of this fragment (to a specific activity of >1 x 10^9 cpm/ µg) was achieved using the random priming protocol (Promega Prime-a-Gene kit) according to standard laboratory procedure. After the nitrocellulose blot was incubated with 20 ml prehybridization buffer (50% deionized formamide, 5x SSC, 5x Denhardt's solution, 0.2 M $NaPO_4$ pH 6.5, 0.1% SDS and 1 mg/ml each of salmon sperm DNA and yeast tRNA) overnight at 42°C, the buffer was removed and the probe added to 20 ml of hybridization buffer (50% deionized

formamide, 5x Denhardt's solution, 5x SSC, 0.1M $NaPO_4$ pH 6.5, 0.1 g/ml dextran sulfate) along with 1 mg/ml sonicated salmon sperm DNA and 1 mg/ml yeast tRNA, and incubated with the membrane overnight at 42°C. Following hybridization, the blot was washed 3x for 15 min with prewarmed wash buffer (0.1x SSC, 0.1% SDS), air dried, and exposed to film at –70°C. As an internal control, the blot was stripped with deionized distilled water 3x at 80°C, then reprobed in a similar manner with a 1-kb fragment of the GAPDH cDNA (*Pst I*/*Bgl* I).

2.5. Transient gene transfer

Cardiac myocytes and human and rat glioma cells were all transfected by the calcium phosphate-DNA precipitation method. The media on all cells was changed to DMEM with 10% FBS and antibiotics, prior to transfection.

For the stimulation studies, 60 μg of plasmid was made up to a volume of 1.0 ml in 252 mM $CaCl_2$ and added gradually to an equal volume of aerated 2x HEPES-buffered saline (HEBS) (280 mM NaCl, 50 mM HEPES, pH 7.10, and 1.5 mM Na_2PO_4). Precipitate was allowed to form at room temperature for 30 minutes, and 310 μl was added to each of 6 culture dishes. Following a 16-hour transfection period, the cells were washed thoroughly with calcium- and magnesium-free phosphate-buffered saline. For stimulation, the medium was changed to DMEM-F12 which contained a 1x Insulin-Transferrin-Selenium-A supplement (Redu-Ser II, Upstate Biotechnology), 0.02 mg/ml ascorbic acid, and antibiotics. These "identical" plates of transfected myocytes were then treated with adrenergic agonists or antagonists to ensure a direct comparison of promoter activity in

untreated and treated cells. The adrenergic agents were used at the following concentrations and for the following incubation periods: norepinephrine, 0.01 mM for 6 h; prazosin, 0.01 mM for 6 h; atenolol (Research Biochemicals International), 0.01 mM for 6 h; 2,3-butanedione monoxime, 1.0 M for 6 h; phenylephrine, 0.1 mM for 48 h; nifedipine (Sigma-Aldrich), 1.0 μm for 48 h.

For experiments involving cardiac myocytes or glial cells which were not stimulated, the procedure was modified accordingly: 30 μg of test plasmid (hybrid firefly luciferase gene) and 3 μg of control plasmid (pRL-CMV) was made up to a volume of 0.5 ml in 252 mM $CaCl_2$ and added gradually to an equal volume of aerated 2x HEBS buffer. Precipitate was allowed to form at room temperature for 30 minutes, and 310 μl was added to each of 3 culture dishes of cardiac myocytes (35 mm) or glial tumor cells (100 mm). After 16 hours, the cells were washed thoroughly with PBS-CMF. Cells were fed with DMEM/10% FBS and maintained for 48 hours before processing. Cells were also transfected with -p.*luc* as a control for random transcription initiation. Co-transfection with pRL-CMV was used as a control for DNA uptake and values were used subsequently to normalize the firefly luciferase activity (firefly luciferase/*Renilla* luciferase) from the "test" genes.

2.6. Reporter gene assays

For experiments with adrenergic agents, at the end of the stimulation period, transfected cardiac myocytes were harvested with trypsin-EDTA (Gibco-BRL), pelleted, rinsed with PBS-CMF and lysed on ice in 50-100 μl (depending on pellet size) of 100 mM Tris-HCl,

pH 7.8 containing Triton X-100. After 15 min, insoluble material was removed by centrifugation and the luciferase activity in 20 µl of the supernatant was measured using the Promega "Luciferase Assay System" and a luminometer (ILA900 Luminometer, Tropix Inc.) according to the manufacturer's instructions. Luciferase activity was normalized against lysate protein content as determined by the Bradford Assay (Bio-Rad).

Where stimulation was not required, for both myocytes and glial cells, the "Dual-Luciferase Reporter Assay System" (Promega) was used. According to the manufacturer's instructions, the cells were harvested using active lysis of cells by scraping, and both firefly and *Renilla* luciferase activities were measured in 20 µl of the lysate. The protein content of cell lysates generated in this manner was determined by the bicinchoninic acid protein assay (Smith et al., 1985).

For transgenic mouse tissue assays, frozen tissue was homogenized in 1x Promega Reporter Gene Lysis Buffer. Homogenates were cleared by centrifugation and 20 µl from each of the supernatants, without storage, were assayed for luciferase activity using the Promega "Luciferase Assay System" and a luminometer. Luciferase activity was normalized to protein content as determined by the bicinchoninic acid assay.

2.7. Transgenic mice

All animal experiments were carried out in accordance with protocols approved by the University of Manitoba Animal Care Committee, which conforms to the standards of the

Canadian Council for Animal Care. The microinjection protocol was carried out by Ms. Agnes Fresnoza under the supervision of Dr. Mary Lynn Duckworth at the University of Manitoba's Transgenics Facility. The plasmid -1058FGFp.*luc* was linearized with *PvuI* and *PstI* and introduced into the male pronucleus of single-cell zygotes from CD1 mice. Injected embryos were subsequently transferred to the oviduct of surrogate mothers and brought to term. Tail tips of 3-week-old progeny were removed and genomic DNA was extracted using Proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation.

The DNA was digested with *Xba*1, and electrophoresed on a 1% agarose gel, containing 40 ng/ml ethidium bromide. The DNA in the gel was photographed and then denatured with 2 washes (15 min each) in Solution A (1.5M NaCl, 0.5M NaOH). Following neutralization with 2 washes (15 min each) in Solution B (1.0 M ammonium acetate, 20 mM NaOH), the gel was blotted overnight to nitrocellulose membrane in Solution B using Southern blotting procedure (Maniatis et al., 1982). The presence of the transgene was determined by probing with a 1-kb *Eco*R1 fragment of -1058FGFp.*luc*, which spans the 3′ end of the FGF-2 promoter and the 5′ end of the luciferase cDNA. Hybridization was carried out in a manner similar to that described in section 2.4.2, except that the hybridization was carried out at 37°C for 18-24 h. The fragment was labeled with ^{32}P using the random priming method (Promega "Prime-A-Gene" Kit). With subsequent generations of mice, DNA analysis was done without digestion of the DNA. Instead, DNA was transferred directly to nitrocellulose using a slot blot apparatus, and the same probe was then used, with hybridization at 42°C.

Animals giving positive DNA signals were bred and their neonatal progeny tested for luciferase activity in the heart and brain. Pups were sacrificed by decapitation and their hearts and brain tissues removed and fast frozen on dry ice. Frozen tissues were homogenized in 1x Promega Reporter Lysis Buffer, cleared of insoluble material after 15 min on ice, and 20 µl of supernatant was assayed according to procedures described in section 2.6. This resulted in the establishment of two lines (P300 and P66), which were tested for luciferase activity in the adult heart and for response to phenylephrine. Adult animals (8-10 weeks old, both genders) were divided into two groups and injected intraperitoneally with vehicle (saline) or phenylephrine (50 mg/kg in saline). After 6, 24 and 48 h, the animals were euthanized by cervical dislocation and hearts were dissected, fast-frozen on dry ice, and luciferase activity was determined from tissue homogenates as described above.

2.8. Nuclear protein preparations

Heart tissue was dissected aseptically from neonatal (1-3 days) Sprague Dawley rats of either gender, and fast frozen on dry ice. Frozen tissue was wrapped in weigh paper and foil and ground with a hammer to powder form. The powder was homogenized with a total of 5 tissue volumes (before crushing) of a hypotonic buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF and 2 µg/ml aprotinin), incubated on ice for 10 min, centrifuged at 800g (HN-S centrifuge, swing rotor #215, radius 170 mm) for 10 min at 4°C, and the supernatant removed. The pellet was resuspended in 2 volumes buffer A and homogenized with the "loose" pestle of a Dounce

homogenizer to break open the swollen cells and release the nuclei. The nuclei were then pelleted by centrifugation and resuspended in approx. 0.5 volumes of a high salt buffer C (20 mM HEPES-KOH pH 7.9, 25% v/v glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF) and mixed gently for 30 min to allow high salt extraction to occur. After centrifugation, the supernatant (containing nuclear proteins) was placed in Spectrapor 6-8K dialysis tubing and dialyzed against 100 volumes buffer D (20 mM HEPES-KOH pH 7.9, 20% v/v glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF) at 4°C for 3 hours. Insoluble material was removed with centrifugation at 27,200g (Beckman JA-20 rotor, radius 108 mm,15,000 rpm) for 30 min, and protein concentration was determined by the Bradford Assay (Bio-Rad). To enrich the extract for DNA-binding proteins, total nuclear extract was mixed with a heparin agarose preparation (Sigma-Aldrich) and washed with buffer D/0.1 M KCl before elution with buffer D/0.6 M KCl, with a final dialysis against buffer D/0.1 M KCl. Protein concentration in the enriched preparation was also determined using the Bradford Assay, and the final product was divided into aliquots and stored at –70°C.

A modified, rapid extraction protocol (Andrews and Faller, 1991) was used for isolation of nuclear protein from cultured neonatal rat cardiac ventricular myocytes grown serum-free DMEM:F12 media (as in section 2.3) in the presence or absence of 0.1 mM norepinephrine for 6 hours. Cells from triplicate 60 mm plates were harvested with trypsin/EDTA (Gibco-BRL) and pooled into a single sample. The cells were rinsed with PBS-CMF, pelleted in a benchtop microfuge, and resuspended in 400 µl buffer A (hypotonic) on ice. After allowing the cells to swell on ice for 10 min, the tube was

vortexed for 10 s to break open the cells and the free nuclei were pelleted in the microfuge. The nuclei were then resuspended in 100 µl cold buffer C, left on ice for 20 min for high-salt extraction to occur, and centrifuged for 2 min at 4°C. The protein concentration in the supernatant was determined by using the Bradford Assay. No dialysis was carried out with this micropreparation (Andrews and Faller, 1991).

2.9. Gel mobility shift assay

The 37 bp A/G-rich double stranded oligonucleotide, representing positions -785 to -749 of the rat FGF-2 promoter and having the sequence given in section 2.2 was ^{32}P-end-labeled using T4 polynucleotide kinase. Nuclear protein from neonatal rat hearts or from NE-treated cardiac myocytes was incubated in buffer D with 5 mM $MgCl_2$ and 2 µg of poly-dIdC, for 10 min at room temperature, with cold competitor prior to the addition of radiolabeled A/G-rich oligonucleotide for an additional 20 min. Total reaction volume was 20 µl. Cold competition consisted of 1-100x molar excess of either A/G-rich oligonucleotide or an 23-bp unrelated, non-specific, double-stranded RF-1 element (Lytras and Cattini, 1994), having the sequence 5′-CTCATCAACTTGGTGTGGACGGC-3′.

Following the 30 min total binding incubation, the DNA/protein complexes were resolved by electrophoresis in 0.5x TBE (25 mM Tris, 25 mM boric acid, 0.5 mM EDTA) on a non-denaturing 4% polyacrylamide gel, of 1.5 mm thickness and containing 2.5 % v/v glycerol. Once complete, the gels were dried under vacuum and exposed to film at –70°C.

2.10. Statistical analysis

Data presented in the text and figures are mean values plus or minus standard error of the mean values. Mean values represent data from at least one experiment performed in triplicate (n values are indicated in the figure legends), and all experiments were repeated at least once. Statistical analysis of the results was carried out using the Mann-Whitney (nonparametric) test. In all cases, a value was considered statistically significant if p was determined to be <0.05.

Chapter 3. Results: Adrenergic Stimulation of the Rat FGF-2 Promoter in Cardiac Myocytes

3.1. FGF-2 RNA levels and promoter activity are stimulated by norepinephrine

Neonatal rat cardiac myocytes were isolated and treated without (control) or with 0.01 mM norepinephrine (NE) or NE with an α-adrenergic antagonist (0.01 mM prazosin) for 6 h, to assess any effect on FGF-2 RNA levels. RNA was separated by gel electrophoresis, blotted to nitrocellulose, probed with a radiolabeled fragment of the rat FGF-2 cDNA, and visualized by autoradiography (Figure 5A). The 28S RNA band seen with ethidium bromide staining before transfer to nitrocellulose is also shown to allow a comparison of RNA levels (Figure 5A). Although not as evident because of a discrepancy in loading, the 6.1 kb FGF-2 transcript level was increased after norepinephrine treatment for 6 h. Consistent with this observation, the 6.1 kb FGF-2 transcript was reduced and barely detectable after treatment with norepinephrine and the α_1-specific antagonist, prazosin.

To demonstrate control at the level of transcription, the FGF-2 promoter itself was then tested for adrenergic responsiveness. Neonatal rat cardiac myocytes, transiently transfected with a hybrid firefly luciferase gene directed by 1112 bp (positions -1058 to +54) of FGF-2 5′ flanking DNA (-1058FGFp.*luc*), were treated with 0.01 mM NE in the absence or presence of 0.01 mM prazosin or a β-adrenergic antagonist (0.01 mM atenolol). The results are shown in Figure 5B. Norepinephrine evoked a 2.5-fold increase in -1058FGFp.*luc* activity (expressed per ng protein) after 6 h of stimulation ($p<0.0001$).

This effect was completely abolished in the presence of prazosin. In contrast, a slight, but not significant, decrease in response to norepinephrine treatment was observed in the presence of atenolol.

3.2. A putative phenylephrine responsive element is present in the upstream FGF-2 flanking DNA

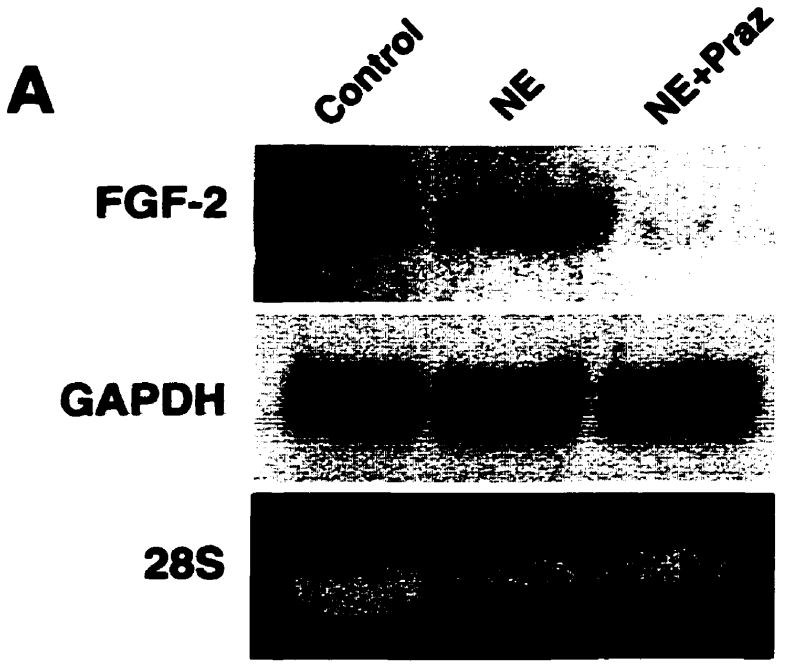
The complete sequence of a 1389 bp genomic fragment containing rat FGF-2 5′ flanking DNA is shown in Figure 6. This corresponds to nucleotide positions -1058 through +331 based on the primary transcription start site (+1) described for the brain (Pasumarthi et al., 1997). Analysis of these sequences revealed two copies, in tandem, of putative phenylephrine (PE) responsive elements (PEREs). These sequences (5′-AGGGAGGG-3′) located at nucleotide positions -780 and -761 were identified based on their high degree of similarity to sequences present in the human skeletal actin (5′-AGGGAGGG-3′) and rat atrial natriuretic factor (ANF) promoters (5′-GGGGAGGG-3′) that have been implicated in the response to α_1-specific adrenergic activation by phenylephrine (Ardati and Nemer, 1993). In the case of the latter, these sequences were shown to bind a specific protein complex and confer phenylephrine responsiveness (Ardati and Nemer, 1993). Consensus binding sites for known transcription factors identified in the FGF-2 sequences are also shown in Figure 6.

Figure 5.

Effect of an α-adrenergic antagonist, prazosin, on the stimulation of FGF-2 RNA levels and promoter activity by norepinephrine.

(A) Cultured neonatal rat cardiac myocytes were treated without (Control) or with 0.01 mM norepinephrine (NE) or NE and 0.01 mM prazosin (NE+Praz) for 6 h. Isolated RNA was electrophoresed, blotted, probed with the radiolabeled rat FGF-2 or glyceraldehyde-3-phosphate (GAPDH) cDNA fragments and visualized by autoradiography. The 6.1 kb FGF-2 and 1.4 kb GAPDH transcripts are indicated. The 28S RNA band for each sample, stained with ethidium bromide and photographed before blotting, is also shown.

(B) Neonatal cardiac myocytes were transfected with -1058FGFp.*luc* and treated with NE, NE+Praz or NE and 0.01 mM atenolol (NE+Atl) for 6 h. Cells were subsequently harvested and luciferase activity and protein concentration were assessed. The promoter activity (luciferase/ng protein) for the -1058FGFp.*luc* gene is shown as the mean from multiple determinations (n=9-15). Bars represent standard error of the mean.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Control
NE
NE+Praz
FGF-2
GAPDH
28S

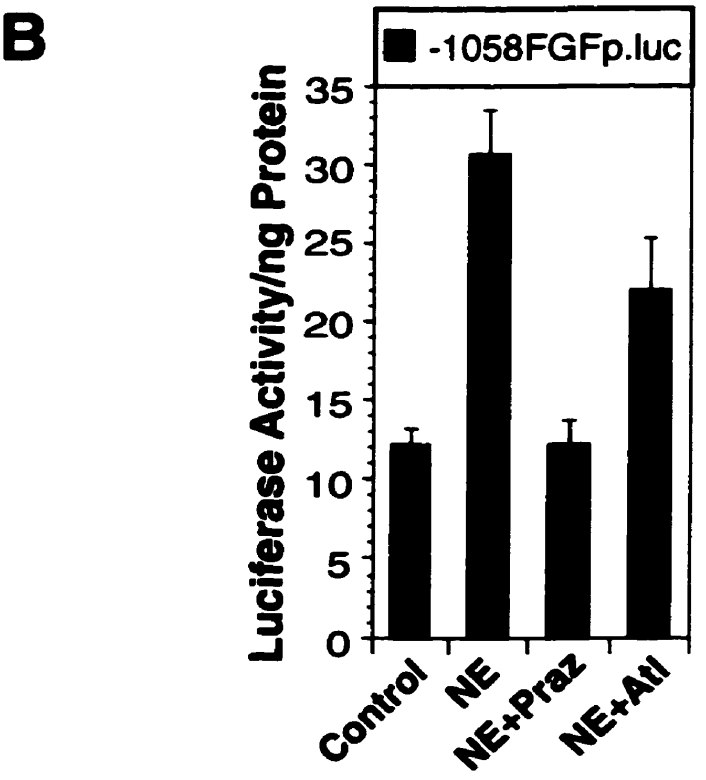
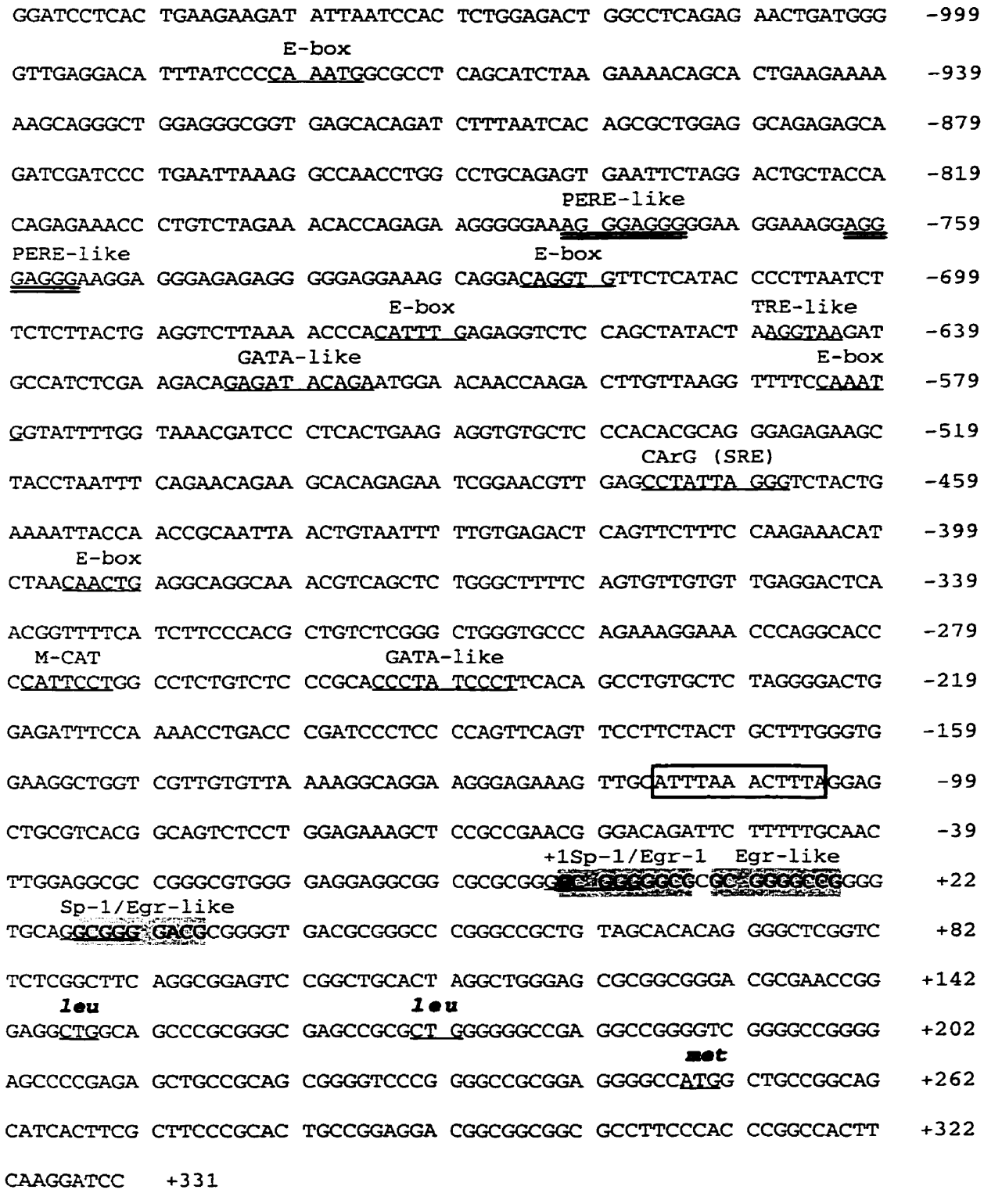

B
-1058FGFp.luc
Luciferase Activity/ng Protein
35
30
25
20
15
10
5
0
Control
NE
NE+Praz
NE+Atl

Figure 6.

Sequence of the 1,389-bp genomic fragment of the rat FGF-2 gene.

The primary transcription start site in the brain (34) is indicated as +1. Consensus binding sites for transcription factors, including Sp-1, are underlined and labeled accordingly, with the exception of the Egr-1 binding site, which is shaded. The putative phenylephrine responsive elements (PEREs) are double underlined. An A/T-rich region conserved between the rat and human FGF-2 promoters is boxed. The two leucine (***leu***) and single methionine (***met***) start codons are indicated. The Genbank accession number: U78079 (Pasumarthi et al., 1997).


GGATCCTCAC TGAAGAAGAT ATTAATCCAC TCTGGAGACT GGCCTCAGAG AACTGATGGG -999
E-box
GTTGAGGACA TTTATCCCCA AATGGCGCCT CAGCATCTAA GAAAACAGCA CTGAAGAAAA -939
AAGCAGGGCT GGAGGGCGGT GAGCACAGAT CTTTAATCAC AGCGCTGGAG GCAGAGAGCA -879
GATCGATCCC TGAATTAAAG GCCAACCTGG CCTGCAGAGT GAATTCTAGG ACTGCTACCA -819
PERE-like
CAGAGAAACC CTGTCTAGAA ACACCAGAGA AGGGGGAAAG GGAGGGGGAA GGAAAGGAGG -759
PERE-like
E-box
GAGGGAAGGA GGGAGAGAGG GGGAGGAAAG CAGGACAGGT GTTCTCATAC CCCTTAATCT -699
E-box
TRE-like
TCTCTTACTG AGGTCTTAAA ACCCACATTT GAGAGGTCTC CAGCTATACT AAGGTAAGAT -639
GATA-like
E-box
GCCATCTCGA AGACAGAGAT ACAGAATGGA ACAACCAAGA CTTGTTAAGG TTTTCCAAAT -579
GGTATTTTGG TAAACGATCC CTCACTGAAG AGGTGTGCTC CCACACGCAG GGAGAGAAGC -519
CArG (SRE)
TACCTAATTT CAGAACAGAA GCACAGAGAA TCGGAACGTT GAGCCTATTA GGGTCTACTG -459
AAAATTACCA ACCGCAATTA ACTGTAATTT TTGTGAGACT CAGTTCTTTC CAAGAAACAT -399
E-box
CTAACAACTG AGGCAGGCAA ACGTCAGCTC TGGGCTTTTC AGTGTTGTGT TGAGGACTCA -339
ACGGTTTTCA TCTTCCCACG CTGTCTCGGG CTGGGTGCCC AGAAAGGAAA CCCAGGCACC -279
M-CAT
GATA-like
CCATTCCTGG CCTCTGTCTC CCGCACCCTA TCCCTTCACA GCCTGTGCTC TAGGGGACTG -219
GAGATTTCCA AAACCTGACC CGATCCCTCC CCAGTTCAGT TCCTTCTACT GCTTTGGGTG -159
GAAGGCTGGT CGTTGTGTTA AAAGGCAGGA AGGGAGAAAG TTGCATTTAA ACTTTAGGAG -99
CTGCGTCACG GCAGTCTCCT GGAGAAAGCT CCGCCGAACG GGACAGATTC TTTTTGCAAC -39
+1Sp-1/Egr-1
Egr-like
TTGGAGGCGC CGGGCGTGGG GAGGAGGCGG CGCGCGGGCG GGGCCGCGCG GGGGCCGGGG +22
Sp-1/Egr-like
TGCAGGCGGG GACGCGGGGT GACGCGGGCC CGGGCCGCTG TAGCACACAG GGGCTCGGTC +82
TCTCGGCTTC AGGCGGAGTC CGGCTGCACT AGGCTGGGAG CGCGGCGGGA CGCGAACCGG +142
leu
leu
GAGGCTGGCA GCCCGCGGGC GAGCCGCGCT GGGGGGCCGA GGCCGGGGTC GGGGCCGGGG +202
met
AGCCCCGAGA GCTGCCGCAG CGGGGTCCCG GGGCCGCGGA GGGGCCATGG CTGCCGGCAG +262
CATCACTTCG CTTCCCGCAC TGCCGGAGGA CGGCGGCGGC GCCTTCCCAC CCGGCCACTT +322
CAAGGATCC +331

3.3. FGF-2 RNA levels and promoter activity are increased by phenylephrine, independently of the putative PEREs

To initiate a characterization of the putative PEREs in the FGF-2 DNA, conditions were established for phenylephrine stimulation of endogenous FGF-2 RNA levels. Neonatal rat cardiac myocytes were isolated and treated with the α_1-adrenergic agonist phenylephrine (PE) for 48 h, and then assessed by RNA blotting. An increase in the 6.1 kb FGF-2 transcript was detected with the FGF-2 cDNA probe after PE treatment for 48 h (Figure 7A). The GAPDH and 28S RNA transcripts were also assessed as controls for RNA loading, and the results are included for comparison.

Transient gene transfer using 5′-deleted hybrid FGF-2/luciferase genes was used to assess the effect of PE on FGF-2 promoter activity, as well as the involvement of the putative PEREs on any response observed. Convenient restriction endonucleases had been previously used to generate 5′-deleted fragments of FGF-2 upstream sequences and produce -1058FGFp.*luc*, -911FGFp.*luc*, and -313FGFp.*luc* (Figure 7B, Pasumarthi et al., 1997). These hybrid genes were used to transiently transfect neonatal rat cardiac myocytes. Cells were also transfected with a promoterless construct (-p.*luc*) to assess levels of random transcription initiation, and with a hybrid myosin light chain-2/luciferase gene (-250MLCp.*luc*) as a positive control for PE responsiveness (Zhu H. et al., 1991). Subsequently these myocytes were treated without (control) or with 0.1 mM PE, and luciferase activity was assessed 48 h later, as previously described for testing the ANF promoter (Ardati and Nemer, 1993). The results (luciferase activity/ng protein) are shown in Figure 7B. All hybrid FGF-2/luciferase genes tested, including -313FGFp.*luc*

which lacks the putative PEREs, showed a significant (~7 fold) increase in promoter activity after PE treatment.

3.4. Adrenergic stimulation of FGF-2 promoter activity was not affected by contraction arrest or Ca^{2+} influx

In an effort to investigate the role of contraction in the response of basal FGF-2 promoter activity to norepinephrine, cardiac myocytes transiently transfected with -1058FGFp.*luc* were treated with 0.01 mM NE in the presence or absence of 50 mM KCl or 1.0 mM 2,3-butanedione monoxime. Stimulation with 0.01 mM NE in the presence of either KCl or 2,3-butanedione monoxime caused a visible arrest of contraction of the cardiac myocytes during the 6-hour incubation period, but did not have a significant effect on NE-stimulated luciferase activity (Figure 8A).

To further assess a role for Ca^{2+} ions in the response of basal FGF-2 promoter activity to α_1-specific adrenergic stimulation, neonatal cardiac myocytes transfected with -1058FGFp.*luc* were treated with 0.1 mM PE for 48 h in the presence or absence of 1.0 μM nifedipine (Figure 8B). Nifedipine arrested contraction, but no significant effect on luciferase activity was detected when compared with cells treated with PE alone.

Figure 7.

Effect of phenylephrine (PE) treatment on endogenous FGF-2 RNA levels and transfected hybrid FGF-2/luciferase gene expression in neonatal rat cardiac myocytes.

(A) Cultured neonatal rat cardiac myocytes were treated without (Cont.) or with 0.1 mM phenylephrine (PE) for 48 hours. Isolated RNA was electrophoresed, blotted, probed with radiolabeled rat FGF-2 or GAPDH cDNA fragments, as indicated, and visualized by autoradiography. The 6.1 kb FGF-2 and 1.4 kb GAPDH transcripts are indicated. The 28S RNA band for each sample, stained with ethidium bromide and photographed before blotting, is also shown.

(B) *Top:* Truncated regions of the rat FGF-2 5′-flanking DNA containing (-1058FGF, -911FGF) or not containing (-313FGF) putative PEREs (hatched box) were inserted upstream of the luciferase coding sequence in the promoterless plasmid (-p.*luc*). Calcium-phosphate/DNA precipitates made for each hybrid gene, was divided between plates of cardiac myocytes to generate "identical" transfected cultures. *Bottom:* These cultures were subsequently treated with or without PE for 48 hours before harvesting and assessment of luciferase activity and protein concentration, to allow a direct assessment of the effect of PE treatment on expression of each hybrid FGF-2 gene. Results are expressed as mean promoter activity (luciferase/ng protein) from multiple determinations (n=13-26). Basal levels for -p.*luc* in the presence or absence of PE were 0.078±0.007 and 0.030±0.004 (n=6), respectively. Bars represent standard error of the mean.

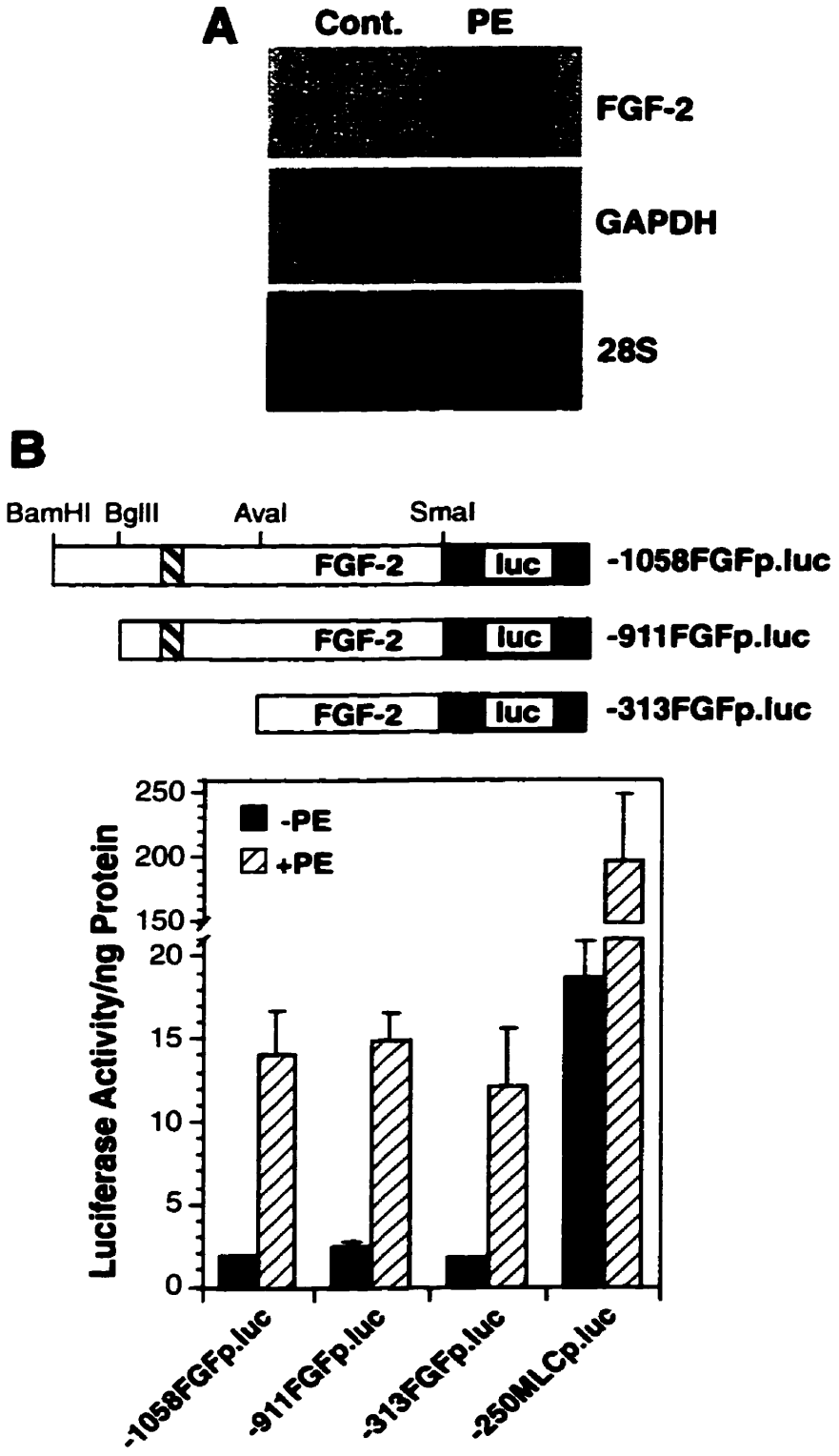

A
Cont.
PE
FGF-2
GAPDH
28S
B
BamHI
BglII
AvaI
SmaI
FGF-2
luc
-1058FGFp.luc
-911FGFp.luc
-313FGFp.luc
Luciferase Activity/ng Protein
250
200
150
20
15
10
5
0
-PE
+PE
-1058FGFp.luc
-911FGFp.luc
-313FGFp.luc
-250MLCp.luc

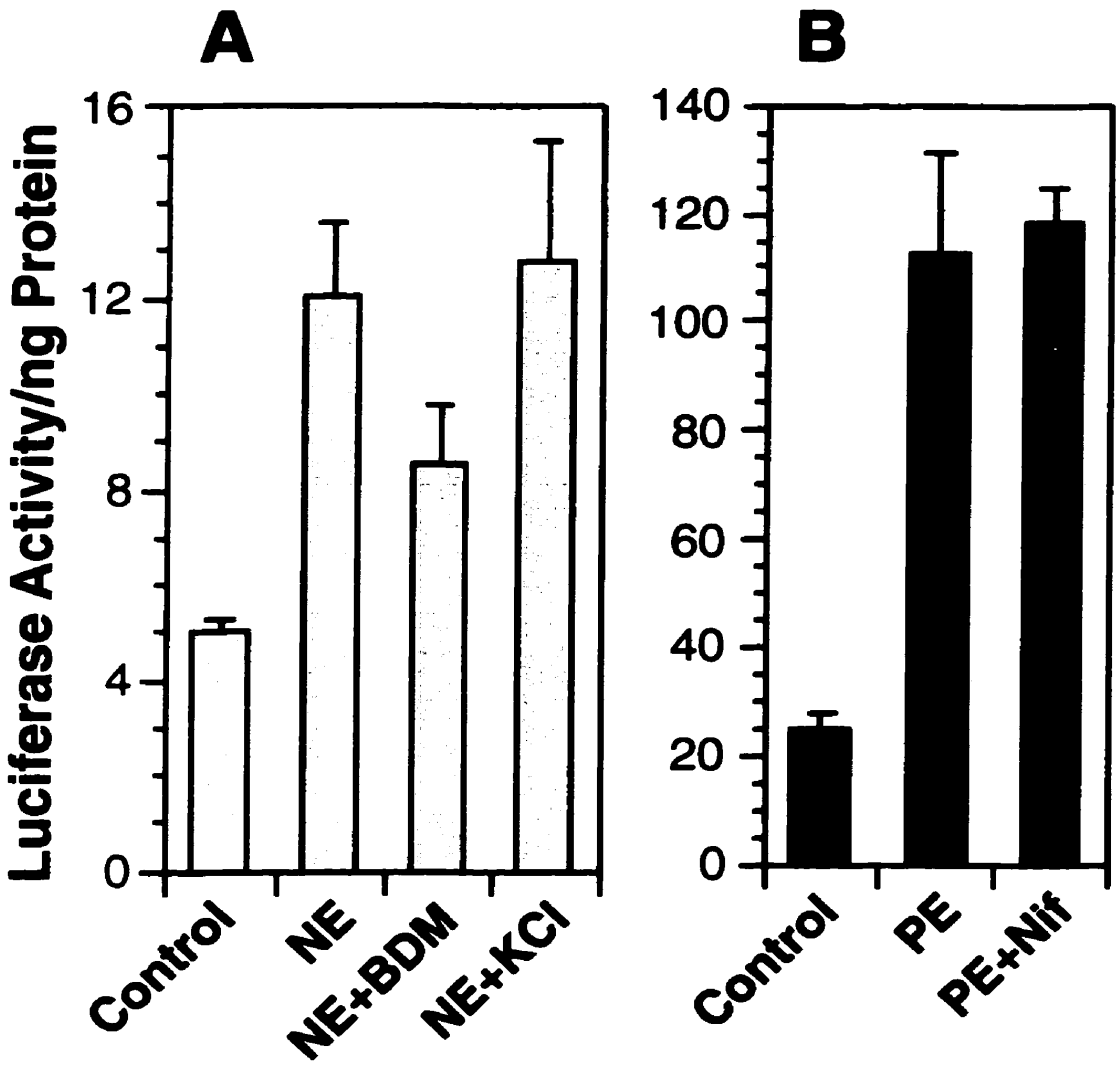
Figure 8.

Effect of myocyte contraction or Ca^{2+} influx on the stimulation of FGF-2 promoter activity by adrenergic stimulation.

(A) Neonatal rat cardiac myocytes were transfected with -1058FGFp.*luc* and treated with 0.01 mM norepinephrine (NE) in the absence or presence of 50 mM KCl (NE+KCl) or 1.0 mM 2,3-butanedione monoxime (NE+BDM) for 6 h.

(B) Neonatal cardiac myocytes were transfected with -1058FGFp.*luc* and treated with 0.01 mM phenylephrine (PE) in the absence or presence of the L-type Ca^{2+} channel blocker nifedipine (PE+Nif) for 48 hours.

For both experiments (A) and (B), cells were subsequently harvested and luciferase activity and protein concentration were assessed. The promoter activities (luciferase/ng protein) for the -1058FGFp.*luc* gene is shown as the mean from multiple determinations (n=3-6). Bars represent standard error of the mean.


A
B
Luciferase Activity/ng Protein
16
12
8
4
0
140
120
100
80
60
40
20
0
Control
NE
NE+BDM
NE+KCl
Control
PE
PE+Nif

3.5. Characterization of basal and PE-induced luciferase activity in -1058FGFp.luc transgenic mouse tissues

3.5.1 Four transgenic mouse lines showed varying patterns of luciferase gene expression

Four independent transgenic mouse lines (P61, P66, P89, and P300) expressing -1058FGFp.*luc* were established. Two of these lines (P300 and P66) showed expression in the postnatal heart, while the other two (P61 and P89) did not (Figure 9A, B and Table 4). For P300 and P66, the relative levels of transgene expression in the brain and heart of non-transgenic and transgenic adult mice were determined, and are expressed as luciferase activity per μg protein in the tissue homogenates (Figure 9A). Interestingly, luciferase activity decreased significantly with postnatal age in both lines (Figure 9B). Line P300 also expressed consistently higher in the heart than did P66, although the levels in P66 adults were still well above background (75 RLU/μg, see also Figure 9A). However, the reverse was true for expression in the brain (Figure 9A). P300 showed luciferase activity in a variety of neural-derived tissues, including brain stem, spinal cord, dorsal root ganglia, and sciatic nerve. Expression for this line in an endocrine organ, the adrenals, was relatively low (Figure 9C). The two remaining lines, P61 and P89, showed expression almost exclusively in neural tissues, with the exception of P61, which also showed significant expression in the testes and ovaries (Table 4). Low levels of expression were also detected in the skeletal muscle of both lines. This may or may not represent residual expression from nerve tissue found within the muscle.

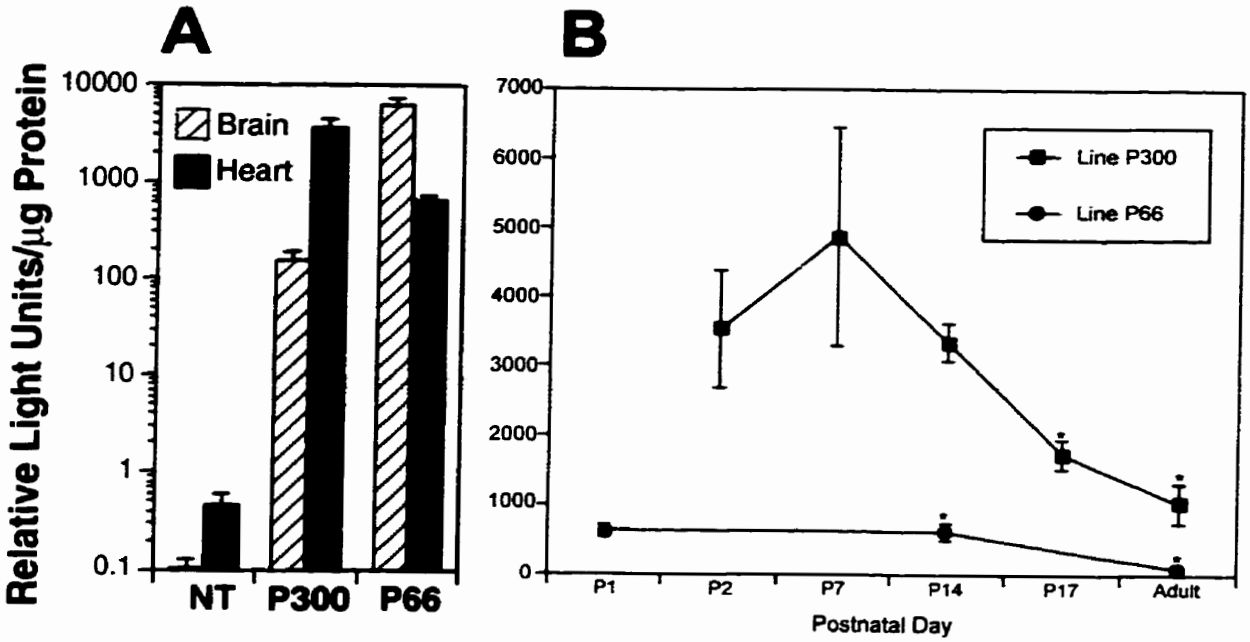
Figure 9.

Luciferase expression in tissues of two independent lines (P300 and P66) of -1058FGFp.luc transgenic mice

(A) Brain and heart from neonatal (2-day old) P300 and P66 mice were dissected, homogenized and assayed for luciferase activity. Non-transgenic (NT, n=5) and transgenic (P300 and P66, n=7) littermate values are shown as mean ● standard error of the mean.

(B) *In vivo* expression of –1058FGFp.*luc* in the postnatal heart. Mice from P300 and P66 were assayed for luciferase activity in the heart at various stages of postnatal development, up to 6-8 weeks of age. Line P300 consistently expressed at higher levels than line P66, but in both lines that was a significant decrease in luciferase activity with age. * = significant at $P<0.05$. Means ± standard error of the mean are shown for n = 3-10.

(C) Expression in neural and endocrine tissues from adult (>3 weeks) P300 mice. BS, brain stem; SC, spinal cord; DRG, dorsal root ganglia; SN, sciatic nerve; AD, adrenal glands. Means ± standard error of the mean are shown for n = 5.


A
B
Relative Light Units/μg Protein
10000
1000
100
10
1
0.1
Brain
Heart
NT
P300
P66
7000
6000
5000
4000
3000
2000
1000
0
Line P300
Line P66
P1
P2
P7
P14
P17
Adult
Postnatal Day

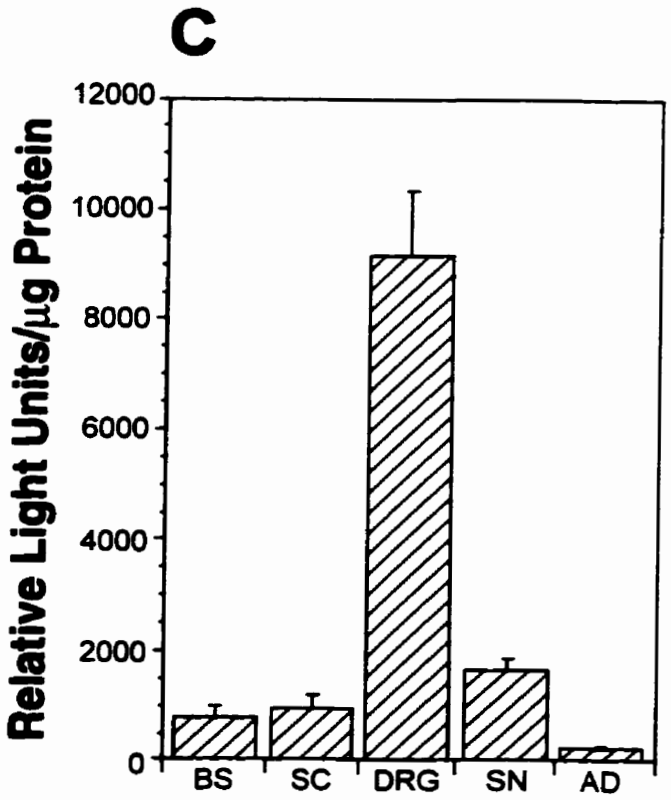

C
Relative Light Units/μg Protein
12000
10000
8000
6000
4000
2000
0
BS
SC
DRG
SN
AD

Table 4. Relative luciferase activity in tissues from lines P61 and P89 of -1058FGFp.luc transgenic mice.

Tissue	P61	P89
Cortex	+++	++++
Brain Stem	++	+++
Spinal Cord	++	+++
Adrenals	–	–
Sciatic Nerve	+/–	++
Heart	–	–
Lung	+/–	–
Kidney	+/–	–
Spleen	–	–
Liver	+/–	–
Skeletal Muscle	+	+
Adipose Tissue	+/–	+/–
Testes	++	+/–
Prostate	+/–	–
Ovaries	+	–

Qualitative values are shown for luciferase activity in 20 µl of tissue homogenate. Symbols: (–), activity did not exceed control (non-transgenic) levels; (+/–), activity at threshold levels; (+) to (++++), relative activity based on orders of magnitude. Based on multiple measurements (n = 1-4).

3.5.2. Phenylephrine treatment increased -1058FGFp.luc transgene expression in the adult heart

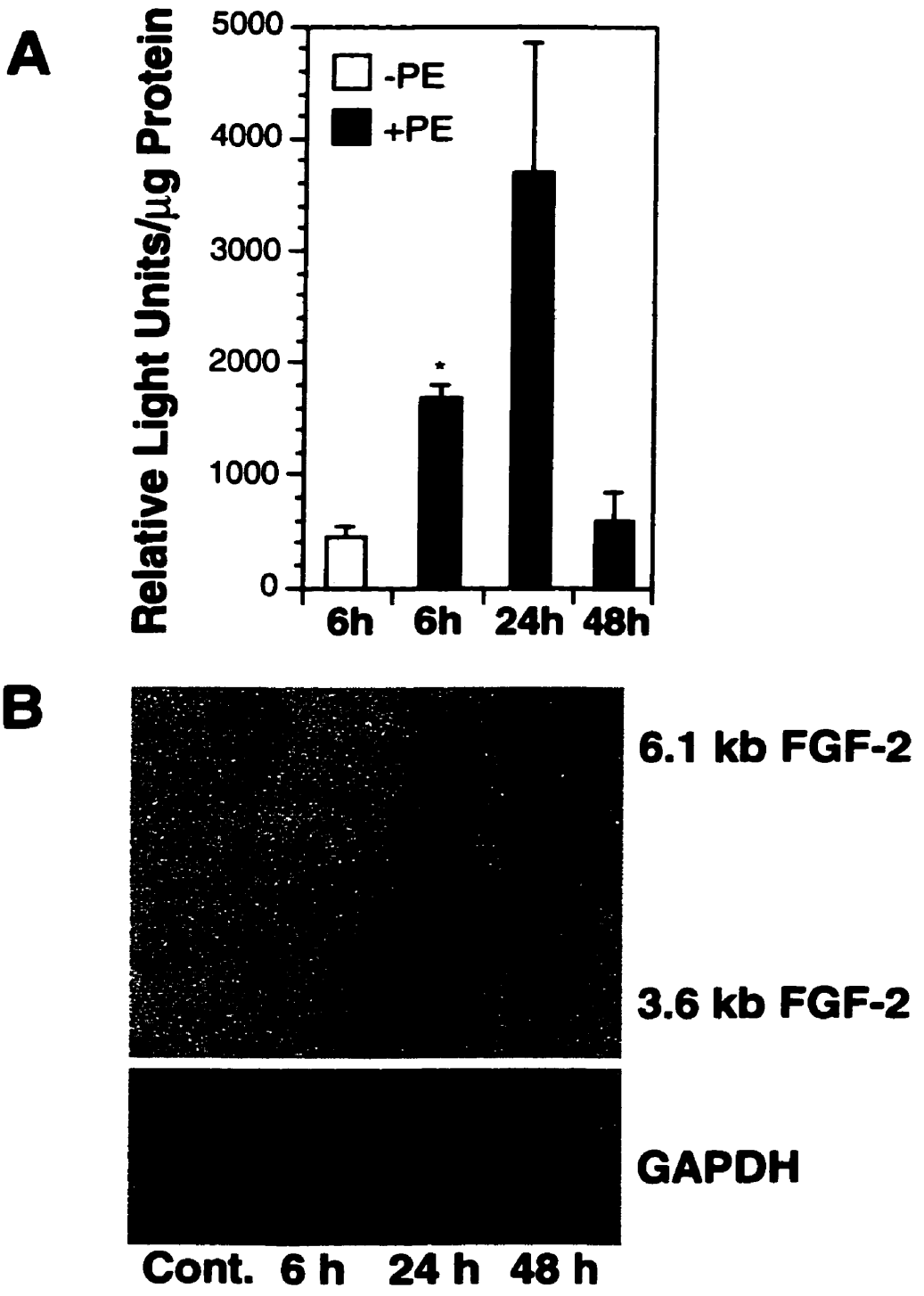
Based on the characterization studies described in section 3.5.1, line P300 was chosen as the primary source of animals to assess whether the FGF-2 promoter responds to α_1-adrenergic stimulation in the heart *in vivo*. Line P66, which expressed consistently in the adult heart but at lower levels relative to P300 adults (Figure 9, A and B) was used as a secondary, independent line. Adult mice (8-10 weeks old) from the P300 line were injected intraperitoneally with 50 mg/kg PE, and then euthanized 6, 24, and 48 h after injection. The hearts were removed and luciferase activity per μg protein was determined (Figure 10A). A significant 3.7 fold increase in luciferase gene expression compared to mice injected with saline alone was observed 6 h after PE treatment ($p<0.0005$, n=6). At 24 h, the difference, although just not significant ($p=0.057$), remained at 3.6 fold, but was lost at 48 h. No significant changes in luciferase activity were observed in saline-injected animals between 6 and 48 h after injection (not shown). The PE response observed was confirmed in the P66 line, where the difference at 6 h after PE administration was 6.9 fold ($p<0.005$, n=4). A parallel assessment of endogenous mouse heart FGF-2 RNA levels at each time point after PE treatment, was done by RNA blotting (Figure 10B). The level of endogenous mouse 6.1 kb FGF-2 transcript was increased at 24 h but was decreased again by 48 h after administration of PE. A second FGF-2 transcript of 3.6 kb was also observed in mouse preparations and showed the same pattern of response as the 6.1 kb mRNA. This 3.6 kb transcript was not seen in rat RNA preparations (Figures 5 and 7).

Figure 10.

Detection of luciferase activity in -1058FGFp.*luc* transgenic mice, and *in vivo* stimulation of FGF-2 gene expression by α_1-adrenoceptor activity in the heart.

(A) Adult P300 mice expressing the -1058FGFp.*luc* gene were injected intraperitoneally with 50 mg/kg phenylephrine (+PE) or saline vehicle (-PE). The mice were euthanized 6, 24 or 48 hours later (6 hours for saline injected animals) and their hearts assayed for luciferase activity. Values for luciferase per μg protein are shown as the mean of multiple measurements (n=3-7), and bars represent the standard error of the mean.

(B) RNA was isolated from the hearts of mice injected intraperitoneally with PE and maintained for 6, 24 and 48 hours. RNA was electrophoresed, blotted, probed with radiolabeled rat FGF-2 or GAPDH cDNA fragments, as indicated, and visualized by autoradiography. The position of the 6.1 kb FGF-2 transcript and a 3.6 kb transcript that was not observed in rat preparations, as well as 1.4 kb GAPDH RNA are indicated.


A
Relative Light Units/µg Protein
5000
4000
3000
2000
1000
0
-PE
+PE
*
6h
6h
24h
48h
B
6.1 kb FGF-2
3.6 kb FGF-2
GAPDH
Cont.
6 h
24 h
48 h

Chapter 4. Results: Functional Role of A/G-rich Sequences from the Rat FGF-2 Promoter

4.1. An A/G-rich region containing an AGGG-repeat stimulates heterologous promoter activity in a tissue- and species-independent manner

The rat FGF-2 promoter sequences (see section 3.2, Figure 6) contain a 74 bp region which is 97% adenine/guanine (A/G)-rich, located between nucleotide positions -793 and -720. Indeed, a 65 bp stretch within this region (-793/-729) contains only A or G residues. Located at the core of this region are two copies of the an AGGG-repeat sequence, 5′-AGGGAGGG-3′. This repeat sequence had been identified as a putative PE-responsive element (section 3.2). The adrenergic responsiveness of the FGF-2 promoter was shown to occur independently of this element (section 3.3). To determine the functional significance of this region, a 37 bp double-stranded A/G-rich oligonucleotide, corresponding to nucleotides -785/-749 of the FGF-2 promoter and containing both copies of 5′-AGGGAGGG-3′, was synthesized (section 2.2).

To assess any effect of the AGGG-repeat sequences on promoter activity, the 37 bp A/G-rich oligonucleotide was inserted upstream of a minimal viral TK gene promoter, which was fused to the firefly luciferase gene (TKp.*luc*) to generate A/G-TKp.*luc*. Both TKp.*luc* and A/G-TKp.*luc* were co-transfected with the hybrid *Renilla* luciferase gene (pRL-CMV) into neonatal rat cardiac myocytes as well as rat C6 and human U87 glial tumor cells, and then tested for activity after 48 hours. The results (firefly luciferase/*Renilla* luciferase activity) are shown in Figure 11A. A significant increase in TK promoter activity was observed in the presence of the A/G-rich sequences in neonatal rat cardiac

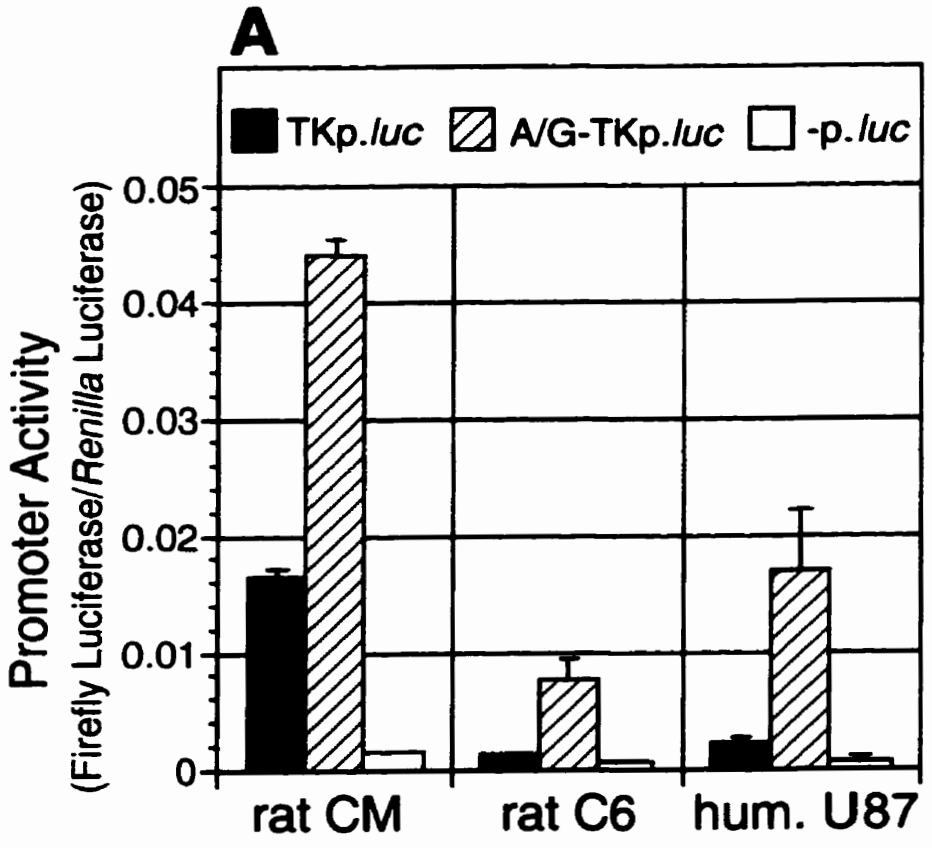
myocytes ($p<0.005$, n=6), rat C6 glioma cells ($p<0.01$, n=5) and human U87-MG astrocytoma cells ($p<0.05$, n=5). However, the level of stimulation was greater in rat or human glial tumor cells (~11 fold) versus primary cardiac myocytes (~3 fold; Figure 11B).

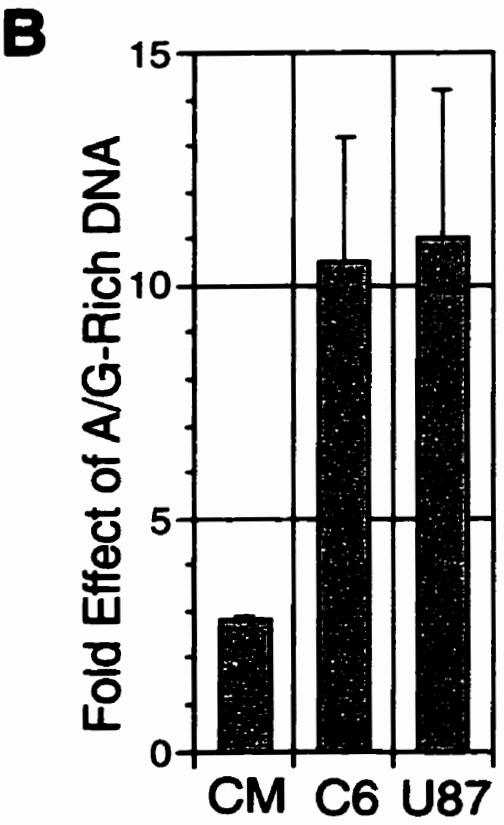
Figure 11.

Enhancer effect of the A/G-rich region on a heterologous promoter

(A) Effect of the 37 bp A/G-rich oligonucleotide on TK promoter activity (firefly luciferase/*Renilla* luciferase) in neonatal rat cardiac myocytes (CM) as well as rat C6 and human U87 glial tumor cells after transient gene transfer. Results are expressed as the mean from at least two independent experiments. The bars represent the standard error of the mean.

(B) The results from (A) are presented to show fold effect of the A/G-rich sequences on TK promoter activity in the various cell types.


A
TKp.luc
A/G-TKp.luc
-p.luc
Promoter Activity
(Firefly Luciferase/Renilla Luciferase)
0.05
0.04
0.03
0.02
0.01
0
rat CM
rat C6
hum. U87


B
Fold Effect of A/G-Rich DNA
15
10
5
0
CM
C6
U87

4.2. Neonatal rat heart nuclear proteins make high affinity/specificity interactions with the A/G-rich sequences

The gel mobility shift assay was used to investigate the presence of specific neonatal rat heart nuclear protein interactions with the 37 bp A/G-rich oligonucleotide. The radiolabelled DNA (0.5 ng) was incubated with 5 μg of neonatal rat heart nuclear protein in the absence or presence of a 25, 50 or 100 fold molar excess of unlabelled A/G-rich oligonucleotide. As a further control, an RF-1 DNA element, containing an unrelated sequence, was also used at a 25, 50 or 100 fold molar excess as a non specific oligonucleotide competitor. Four specific complexes (C1-4) were identified (Figure 12). Both C2 and C4 were competed completely with a 25 fold molar excess of specific (A/G-rich oligonucleotide) but not non specific (RF-1 element) competitor. A slight increase in the amount of C1 and C3 complexes was detected corresponding to the complete competition of C2 and C4 with a 25 (and to a lesser extent with a 50) fold molar excess of specific competitor. The C1 and C3 complexes required a 100 fold molar excess of specific A/G-rich oligonucleotide to be competed efficiently (Figure 12).

To assess the relative affinity of nuclear protein for DNA in the C2 versus C4 complexes, gel mobility shift assays were done using lower doses (1, 2, 5, 10, and 15 fold molar excess) of 37 bp A/G-rich oligonucleotide for competition (Figure 13). Complex C2 represents a very high affinity/specificity interaction since it was competed efficiently with only a 2 fold molar excess of specific A/G-rich oligonucleotide. C4 was competed completely with a 10 fold molar excess of specific competitor. The transient increase in the amount of C1 and C3 with competition of C2 and C4 was also apparent.

Figure 12.

Gel mobility shift assay of neonatal rat heart nuclear proteins and the 37 bp A/G-rich oligonucleotide.

Specificity was determined by competition with "specific" unlabeled A/G-rich or "non-specific" RF-1 element oligonucleotide competitors. The radiolabeled A/G-rich fragment (free probe, FP) was incubated in the (a) absence or (b-h) presence of 5 μg nuclear protein, (b) without or with a (c) 25, (d) 50, or (e)100 fold molar excess of specific, or (f) 25, (g) 50 fold excess of non specific competitor. The positions/mobilities of four specific complexes (C1-C4) are indicated.

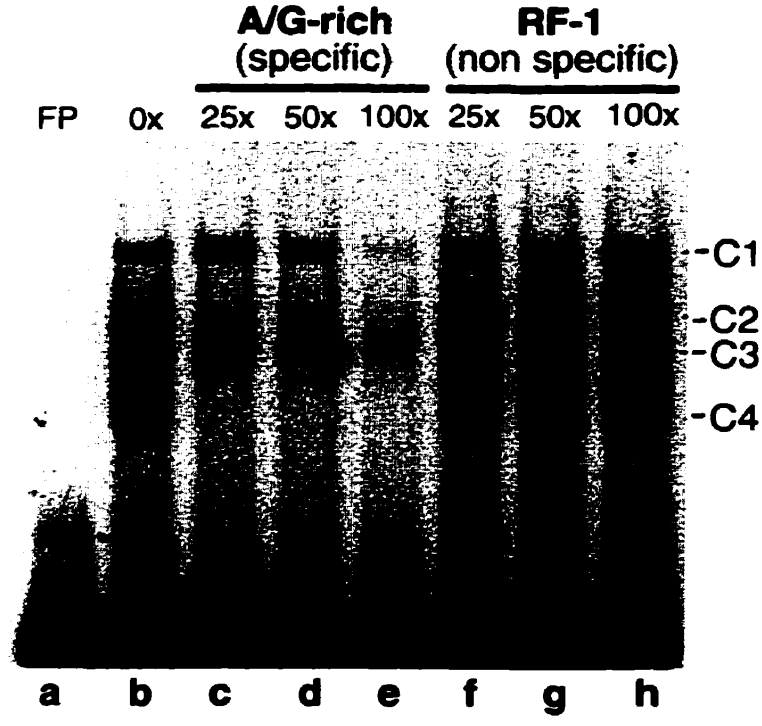
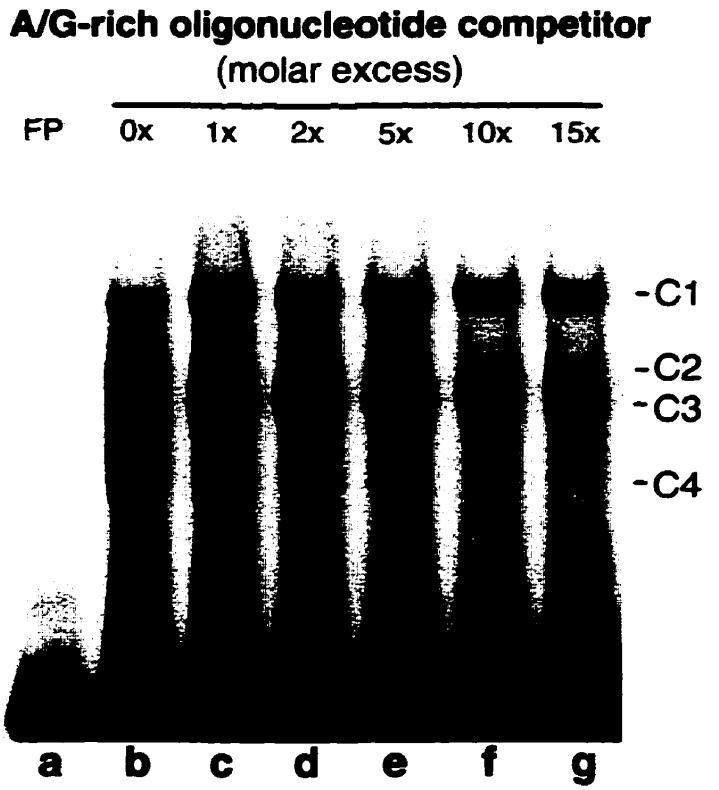

A/G-rich
(specific)
RF-1
(non specific)
FP
0x
25x
50x
100x
25x
50x
100x
C1
C2
C3
C4
a
b
c
d
e
f
g
h

Figure 13.

Detection of very high affinity interactions between neonatal rat heart nuclear protein and the 37 bp A/G-rich DNA fragment.

Affinity was determined by gel mobility shift assay and competition with low amounts of "specific" unlabelled A/G-rich oligonucleotide competitor. The radiolabeled A/G-rich fragment (FP) was incubated in the (a) absence or (b-g) presence of (5 μg) nuclear protein, (b) without or with a (c) 1, (d) 2, (e) 5, (f) 10, or (g) 15 fold molar excess of specific competitor. The positions/mobilities of the four specific complexes (C1-C4) are indicated.


A/G-rich oligonucleotide competitor
(molar excess)
FP
0x
1x
2x
5x
10x
15x
-C1
-C2
-C3
-C4
a
b
c
d
e
f
g

4.3. Effect of phenylephrine treatment on the pattern of interaction between cardiac myocyte nuclear protein and DNA containing the 5′-AGGGAGGG-3′ sequence

To assess the effect of phenylephrine on TK promoter activity in the absence or presence of the 5′-AGGGAGGG-3′ sequences, neonatal rat cardiac myocytes were transfected with TKp.*luc* or A/G-TKp.*luc*. Transfected cells were treated without or with 0.1 mM PE for 48 hours, harvested and firefly luciferase activity per ng protein was determined (Figure 14). PE treatment increased TK promoter activity 4.6±0.8 ($p<0.0001$, n=17) and 6.7±1.1 fold ($p<0.008$, n=5) in the absence and presence of the A/G-rich sequences, respectively. However, although there was an additional ~1.5 fold increase in activity observed in the presence of the 5′-AGGGAGGG-3′ sequence with PE treatment, this was not considered statistically significant ($p=0.19$).

To complement this study, we compared the gel mobility shift assay patterns obtained using the 37 bp A/G-rich oligonucleotide, containing two copies of the 5′-AGGGAGGG-3′ sequence, with nuclear protein isolated from neonatal rat cardiac myocytes grown in the absence versus presence of 0.01 mM NE. Four complexes (C1-4) were observed (Figure 15). This pattern was not altered by norepinephrine stimulation. There was also no difference in the degree of competition with a 50 or 100 fold molar excess of specific A/G-rich oligonucleotide, to suggest a change in affinity of these complexes with adrenergic stimulation (Figure 15).

Figure 14.

Effect of phenylephrine treatment on TK promoter activity in the presence (A/G-TKp.*luc*) or absence (TKp.*luc*) of the 37 bp A/G-rich oligonucleotide in transiently transfected neonatal rat cardiac myocytes.

Results are expressed as mean promoter activity (firefly luciferase/ng protein) for at least two independent experiments performed in triplicate. Basal levels for -p.*luc* in the presence and absence of phenylephrine were 0.078±0.007 and 0.030±0.004, respectively. Bars represent standard error of the mean.

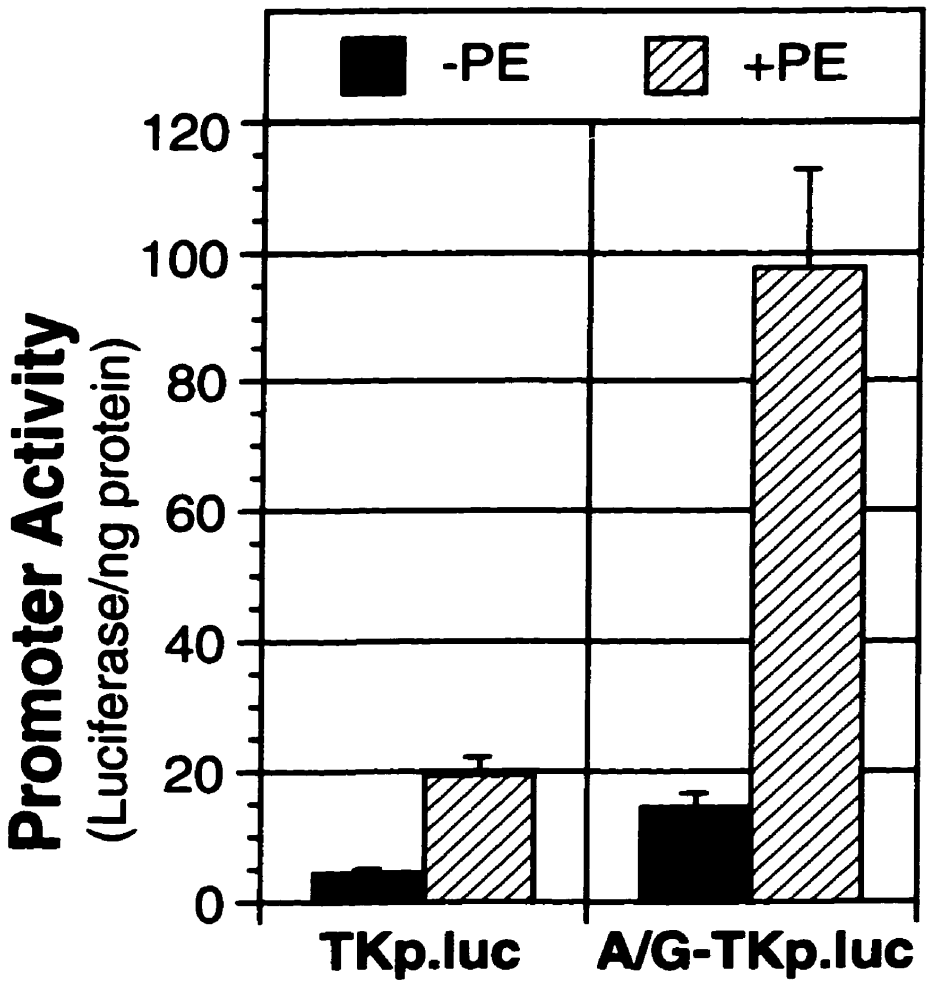
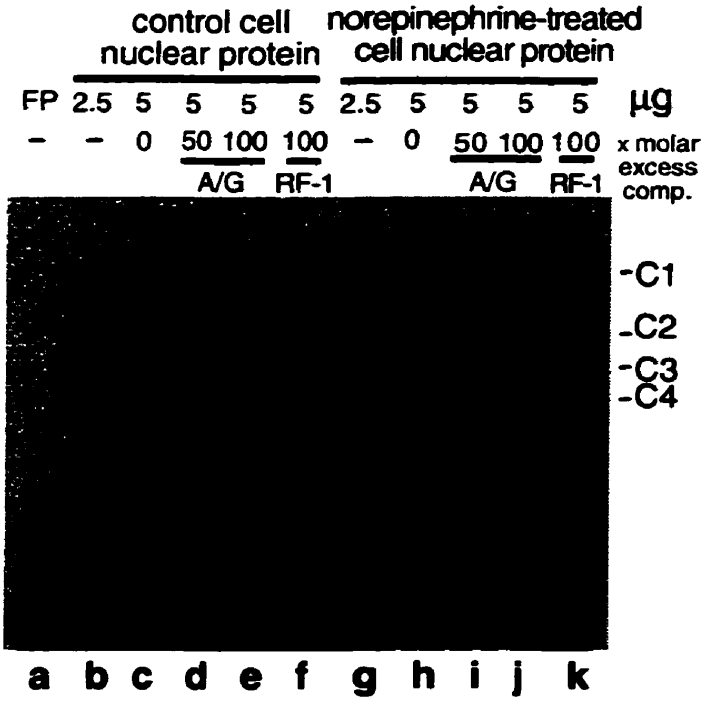

-PE
+PE
120
100
80
60
40
20
0
Promoter Activity
(Luciferase/ng protein)
TKp.luc
A/G-TKp.luc

Figure 15.

Effect of NE on gel mobility shift patterns observed with the 37 bp A/G-rich oligonucleotide.

Comparison of gel mobility shift patterns seen with the 37 bp A/G-rich oligonucleotide and nuclear protein from isolated cardiac myocytes grown in the absence (b-f) or presence (e-k) of norepinephrine. Affinity/specificity was assessed by competition with "specific" unlabelled A/G-rich or "non-specific" RF-1 oligonucleotide competitors. The radiolabeled A/G-rich fragment (FP) was incubated in the (a) absence or (b,g) presence of 2.5 μg or (c-f,h-k) 5 μg of nuclear protein, (c,h) without or (d,i) with a 50, or (e,j) 100 fold molar excess of specific competitor, or (f,k) 100 fold molar excess of non specific competitor. The positions/mobilities of four specific complexes (C1-C4) are indicated.


control cell nuclear protein
norepinephrine-treated cell nuclear protein
FP 2.5 5 5 5 5 2.5 5 5 5 5 µg
– – 0 50 100 100 – 0 50 100 100 x molar excess comp.
A/G RF-1
A/G RF-1
-C1
-C2
-C3
-C4
a b c d e f g h i j k

Chapter 5. Results: Retrograde Perfusion of the Isolated Mouse Heart

5.1. Basal function under constant pressure and a profile of ischemia-reperfusion injury.

In the previous chapters, we have shown that catecholamine action through α_1-adrenergic receptors has a stimulatory effect on FGF-2 transcription as demonstrated by an increase in rat FGF-2 promoter activity. Adrenergic stimulation may represent one of many pathways linking the release of FGF-2 to its synthesis in order to replenish intracellular stores. Release of FGF-2 has been studied in the isolated perfused rat heart (Clarke et al., 1995). Because of our work with transgenic mice (section 3.5), we sought to establish a retrograde (Langendorff-type) perfusion system in which FGF-2 release and synthesis could be studied in murine hearts. Since myocardial infarction has been linked to release and accumulation of FGF-2 at sites of tissue injury (Padua et al., 1993), we also established parameters for global ischemia and reperfusion in these isolated hearts.

As recently as one or two years ago, the reports of isolated mouse hearts were but a handful in the literature. Since then, the numbers have exploded, most likely because of the increasing popularity of the transgenic mouse and thus the increasing appeal of murine systems in which to study cardiovascular physiology. The system described in this chapter was assembled largely under the guidance of Dr. Terje Larsen (laboratory of Dr. D. Severson) of the University of Calgary, and Dr. Raymond Padua (laboratory of Dr. E. Kardami) of the Institute of Cardiovascular Sciences at the University of Manitoba, with additional information from the existing literature.

Because FGF-2 is a heparin-binding growth factor, injectable heparin was not used as an anticoagulant prior to dissection of the heart. Adult mice (8-15 weeks of age, 25-35 g in weight) were euthanized by cervical dislocation and their hearts dissected into ice-cold Krebs-Ringer solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM Mg SO_4, 2.5 mM $CaCl_2$, 10 mM glucose, 24 mM $NaHCO_3$, oxygenated and buffered to physiological pH by aeration with 95% O_2/5% CO_2). The aorta was trimmed and tied with 6-0 suture thread to a 21G stainless steel cannula under a magnifying lens. Perfusion with Krebs-Ringer solution (as above, aerated with 95% O_2/5% CO_2) at 37 °C began within 5 min of dissection of the heart, at 80 cm H_2O (61.5 mmHg). The atria (along with the sinoatrial node) were trimmed from the heart and a thin polyethylene catheter was inserted through the mitral valve into the left ventricle. The mitral valve sealed around the catheter and saline solution was injected into the ventricle to bring the end diastolic pressure to a value between 1 and 10 mmHg. The catheter was connected to a pressure transducer and pressure data was collected using a DigiMed™ Heart Performance Analyzer and the pressure waveform visualized with DigiMed™ System Integrator 200/1 software and a Pentium computer. Platinum electrodes, attached to a Student Stimulator (Harvard Apparatus) were positioned to touch the myocardium and deliver a pacing pulse of width 2 ms, 6.5 Hz (giving a heart rate of ~355 bpm), at a voltage of 2.0-3.5 V (the lowest voltage required to achieve override of the intrinsic rate of the heart). A thermocouple (IT-1E), connected to a Physitemp Thermalert TH-5, was inserted into the right ventricle for real time monitoring of the temperature of the myocardium, and a

water-jacketed glass chamber was raised to enclose the heart in a humid, controlled environment. Photographs of the heart and the apparatus are found in Figure 16A.

After an equilibration period (30 min after dissection), it was determined whether basal function fell within acceptable parameters. Based on existing literature for systems using similar conditions (e.g., Ng et al., 1991), the following parameters were targeted to within these values: developed pressure (maximum pressure less end diastolic pressure), 50-80 mmHg; dP/dt (rate of contraction), >2000 mmHg/sec; coronary flow, 0.7-2.0 g/min. Coronary flow was measured by collecting effluent into a plastic weigh boat over a period of 60 sec and then weighing the effluent. Hearts functioning outside the range of given values at the end of the equilibration period were discarded.

For global ischemia and reperfusion, flow was initially arrested for a period of 30 min, with pacing continuing throughout the entire experiment. It became necessary, however, to adjust the time of ischemia to 20 min and to stop the pacing during this period, in order to allow the heart to achieve an acceptable level of recovery of function during reperfusion. A profile of developed force (shown as percent recovery) after 20 min ischemia and over 60 min reperfusion is shown in Figure 16B. A recovery of ~30% at equilibrium state was achieved, and is comparable to that found by others with a similar experimental protocol (Marber et al., 1995; Sumeray and Yellon, 1998).

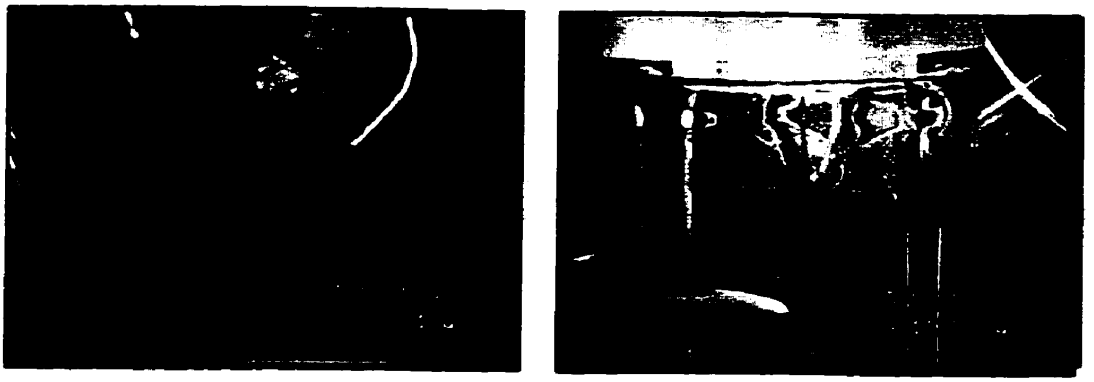
Figure 16.

The isolated perfused mouse heart achieves 30% recovery of function following 20 minutes of global ischemia and reperfusion.

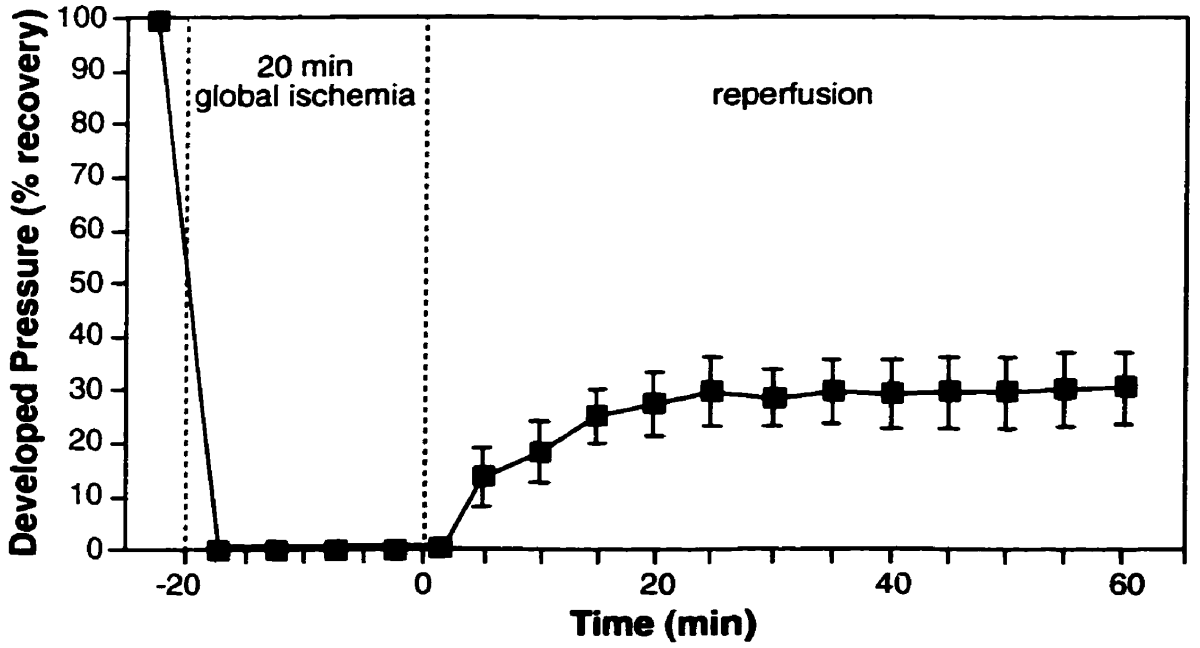
(A) *Left,* Photograph of an isolated perfused mouse heart, shortly following cannulation. The aorta is tied to the cannula with a 6-0 suture thread. A thin polyethylene catheter has been inserted into the left ventricle for pressure measurements, and pacing wires have been attached. The thermocouple, to be inserted into the right ventricle, is seen at the left. *Right,* The thermocouple has been added and the heart has been enclosed in a water-jacketed glass chamber, where it will remain for the duration of the experiment.

(B) A plot of developed pressure (maximum pressure less end diastolic pressure), shown as percent recovery following 20 min of global ischemia. Baseline contractile function has been set to 100%. Mean values ± standard errors of the mean are shown for a group of normal adult mice (n=5).

A



B

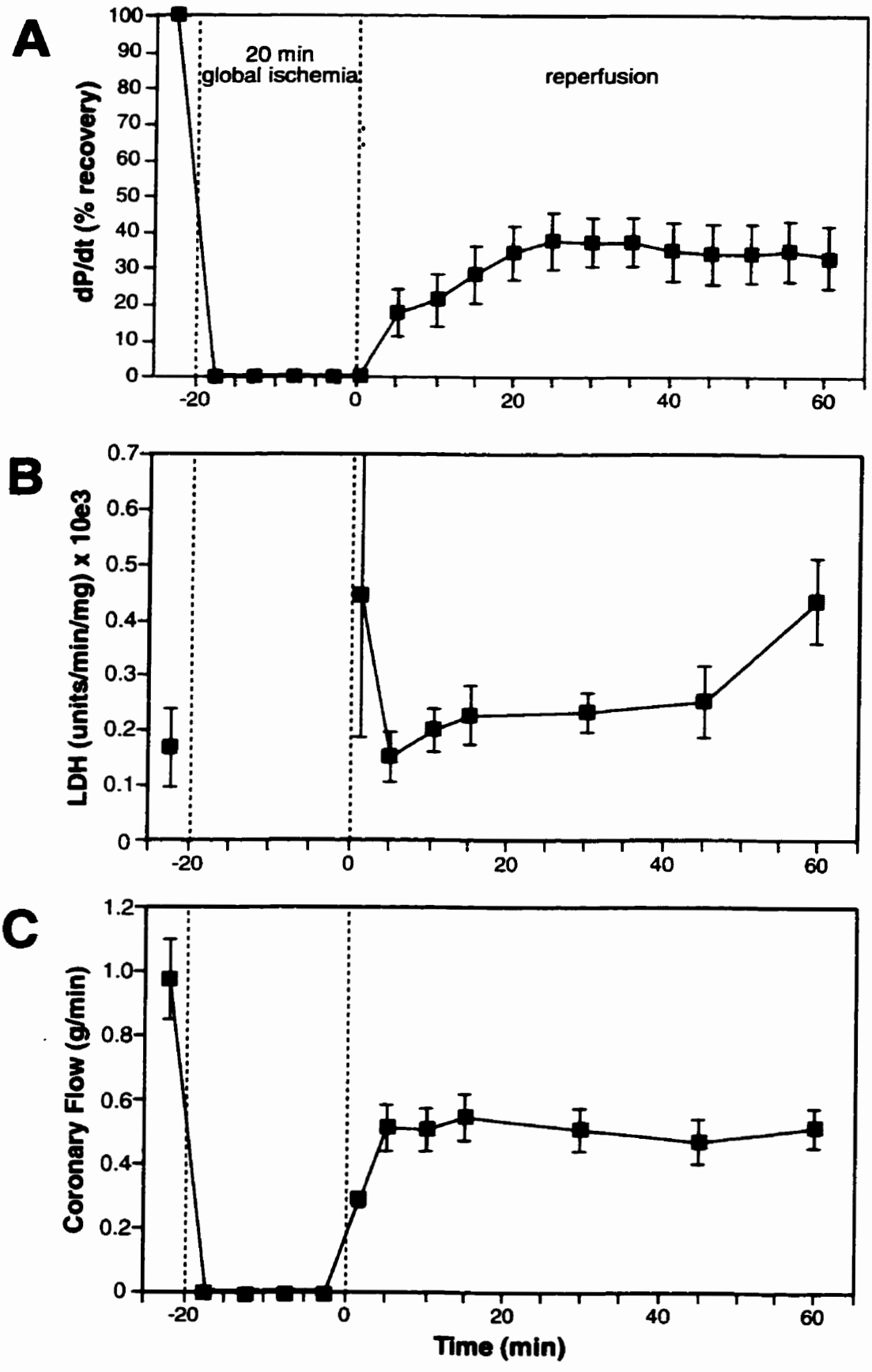

Developed Pressure (% recovery)
100
90
80
70
60
50
40
30
20
10
0
20 min
global ischemia
reperfusion
-20
0
20
40
60
Time (min)

Other parameters were also measured during this type of experiment (Figure 17). The rate of contraction, measured as change in pressure with time (dP/dt), achieved a level of recovery similar to that of developed force, ~30% (Figure 17A). LDH activity was measured in collected effluent using an LDH-optimized spectrophotometric analysis kit (Sigma Diagnostics). Measurement of LDH activity was carried out in effluents that had been stored on ice for <6 h. LDH activity was highly variable, but did increase following ischemia and reperfusion, indicative of tissue damage under these conditions (Figure 17B). Highly variable enzyme release has also been reported for creatine kinase (Marber et al., 1995). Coronary flow also decreased following ischemia and reperfusion under constant pressure, to levels ~50% of baseline (Figure 17C). All of these parameters are useful in determining the extent of injury suffered by an isolated heart under these conditions.

Figure 17.

Rate of contraction, LDH release and coronary flow profiles following 20 min global ischemia and reperfusion of isolated normal mouse hearts.

Rate of contraction (dP/dt) recorded as percentage recovery following 20 minutes of global ischemia **(A)**, LDH release **(B)**, and coronary flow measurements **(C)** from the same experiment as in Figure 16B. Values shown are means ± standard errors of the mean (n=5).


A
20 min
global ischemia
reperfusion
dP/dt (% recovery)
100
90
80
70
60
50
40
30
20
10
0
-20
0
20
40
60
B
LDH (units/min/mg) x 10e3
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-20
0
20
40
60
C
Coronary Flow (g/min)
1.2
1.0
0.8
0.6
0.4
0.2
0
-20
0
20
40
60
Time (min)

5.2. FGF-2 release and synthesis in the isolated mouse heart

5.2.1. FGF-2 is detected in effluent from isolated perfused mouse hearts

FGF-2 has been detected in perfusate from isolated rat hearts that had been passed through a heparin sepharose affinity column (Clarke et al., 1995). For the mouse heart, the volume of perfusate was low enough that we reasoned against the use of an affinity column, and instead, the perfusate was collected on ice and used directly for an FGF-2 immunoassay (Quantikine™ HS, R&D Systems). In these pilot experiments, no ischemia was carried out (Figure 18A). Instead, the heart was cannulated and perfused under constant pressure (80 cm H_2O) as before, and effluent was collected immediately upon commencement of perfusion (time 0), and every 10 min subsequently. The immunoassay was carried out according to the manufacturor's instructions. FGF-2 release was found to be maximal immediately upon perfusion (Figure 18A), decreasing quickly and settling at near-zero levels. The initial burst may reflect a type of injury incurred when the heart is isolated and attached to the Langendorff apparatus.

5.2.2. FGF-2 transcription can be assayed in isolated hearts from –1058FGFp.luc transgenic mice

In addition to FGF-2 release, we were interested to know if FGF-2 synthesis at the level of transcription could be assayed using this system. Hearts from –1058FGFp.*luc* transgenic mice, line P300 (see section 3.5) were dissected, perfused, and subjected to ischemia and reperfusion as described above. In this set of pilot experiments, hearts were not paced and the period of ischemia lasted for 30 min. At various time points during a 30

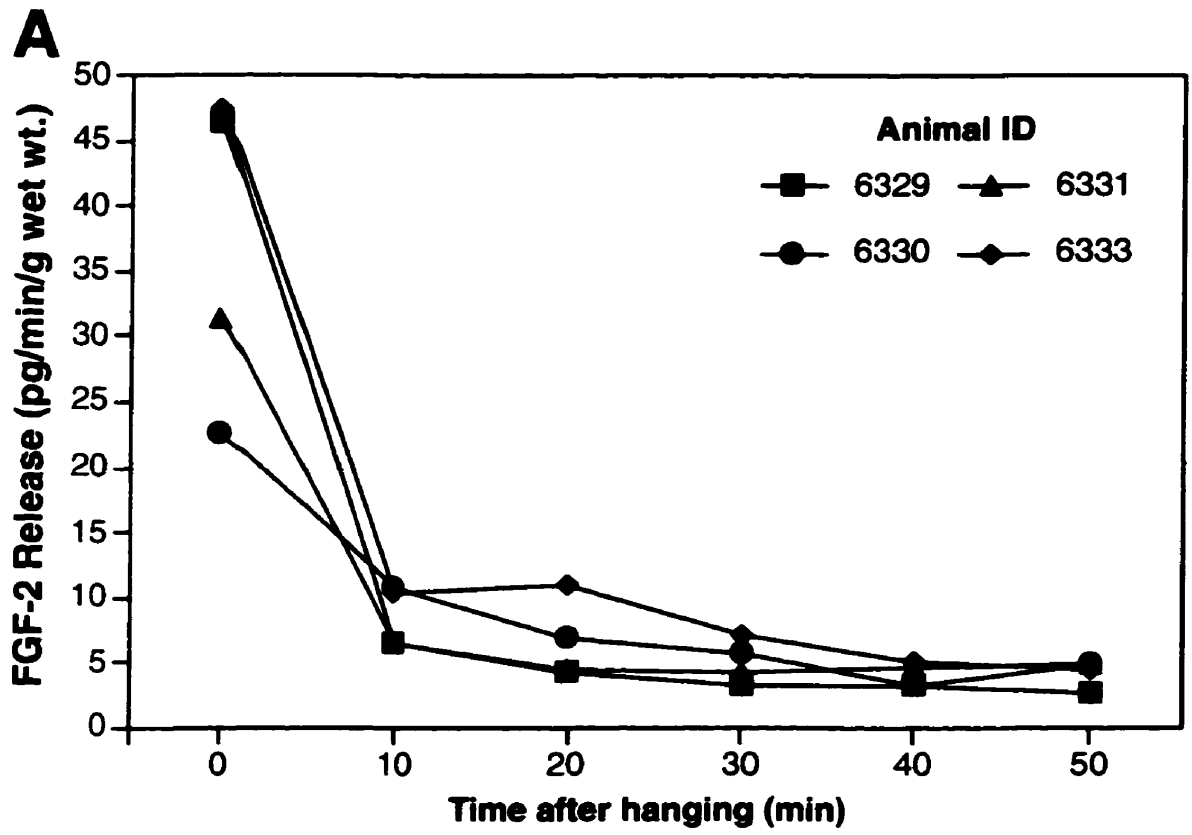
min ischemia/30 min reperfusion protocol, hearts were removed from the apparatus, fast frozen on dry ice and assayed for luciferase activity as described in section 2.6. There was a significant ($P<0.05$, $n=3$) increase in luciferase activity between excision of the heart and the end of the 30-min equilibration period (Figure 18B). There appears to be a decrease in activity during ischemia and reperfusion, although small n-values prevent statistical comparisons. The increase in transcription during the initial equilibration period may reflect a response to depletion of myocardial stores of FGF-2, as indicated by an initial burst of FGF-2 release (Figure 18A). Further experimentation will be necessary to determine whether FGF-2 release and synthesis are linked, either directly or indirectly, in this isolated, denervated heart in which the only sources of catecholamines are intrinsic.

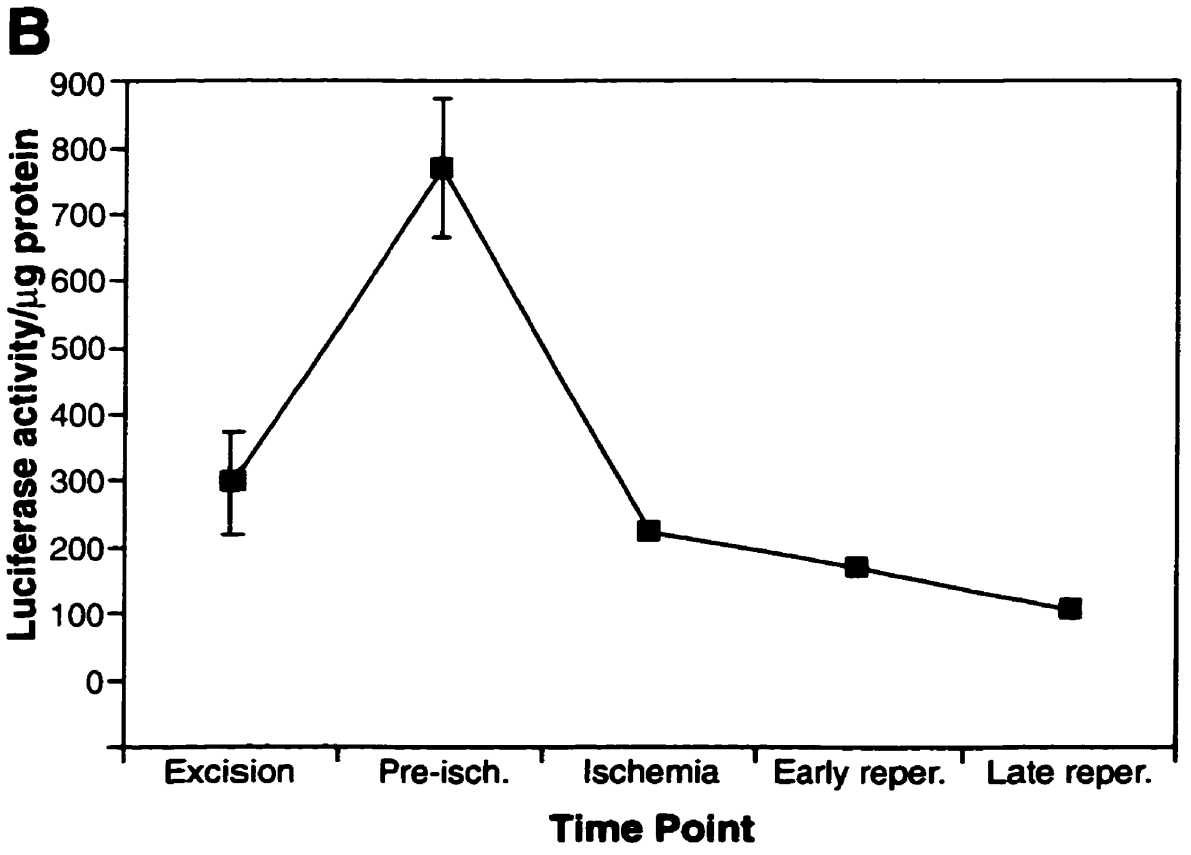
Figure 18.

FGF-2 release and FGF-2 transcription in the isolated mouse heart.

(A) Profile of FGF-2 release from a pilot experiment in which perfusates from four hearts were assayed separately. Two of these hearts (square and circle symbols) were normal mice and the other two (triangle and diamond symbols) were from a transgenic line which, upon subsequent investigation, was also shown to produce normal levels of FGF-2 in the myocardium. Perfusates were collected on ice for 1 min immediately upon starting the flow (time=0), and every 10 min up to 50 min after hanging. FGF-2 was subsequently detected using an immunoassay kit (see text). Values shown are raw data from each heart.

(B) FGF-2 transcription during global ischemia and reperfusion. Hearts from -1058FGFp.*luc* transgenic mice, line P300, were subjected to 30 min global ischemia and 30 min reperfusion as described in the text. At the time points indicated, hearts were removed from the apparatus and assayed for luciferase activity. Mean values (± standard error, where possible) are shown for the following time points: *Excision*, immediately, with no perfusion (n=3); *Pre-isch.*, after a 30-min equilibration period (n=3); *Ischemia*, 20 min into a 30-min period of global ischemia (n=2); *Early reper.*, after 5 min of reflow (n=2); *Late reper.*, after 30 min of reflow (n=1). FGF-2 transcription increased during the equilibration period ($P<0.05$) but appeared to be downregulated during ischemia and reperfusion.


A
FGF-2 Release (pg/min/g wet wt.)
50
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
Time after hanging (min)
Animal ID
6329
6331
6330
6333


B
Luciferase activity/µg protein
900
800
700
600
500
400
300
200
100
0
Excision
Pre-isch.
Ischemia
Early reper.
Late reper.
Time Point

Chapter 6. Discussion and Future Directions

6.1. Adrenergic stimulation of the rat FGF-2 promoter is mediated primarily by α_1-adrenergic receptors.

Previously it was reported that FGF-2 is released from adult rat heart with every contraction of the myocardium (McNeil and Steinhardt, 1997), and both contraction and FGF-2 release can be increased with β-adrenergic (isoproterenol) stimulation (Clarke et al., 1995). We have used RNA blotting and gene transfer to demonstrate that FGF-2 transcription is under positive regulation by norepinephrine (NE) after 6 h (Figure 5). Cardiac myocyte responses to this endogenous catecholamine are mediated by both α_1- and β adrenoceptors (Skomedal et al., 1997; Yamazaki et al., 1996). The response of both endogenous mRNA levels and FGF-2 promoter activity in reporter gene assays to NE was fully blocked with the α_1-specific antagonist, prazosin (Figure 5). Also, endogenous FGF-2 RNA accumulation and transfected FGF-2 promoter activity were stimulated by the α_1-specific agonist, phenylephrine (PE, Figure 7). Although β-adrenergic stimulation was implicated in the regulation of FGF-2 release from cardiac myocytes (Clarke et al., 1995), we were unable to show a direct link between β-adrenergic activity and FGF-2 transcription, since the β-specific antagonist atenolol did not significantly decrease the NE-induced response of FGF-2 promoter activity (Figure 5). Indeed, the β-agonist, isoproterenol, failed to elicit an increase in FGF-2 promoter activity in transient transfection assays (Detillieux, Meij, and Cattini, unpublished results). Of course, this does not rule out the possibility that β-stimulation exerts its effect elsewhere in the synthetic pathway (Figure 1) or that the 1,058 bp of FGF-2 5′-flanking DNA contains

insufficient information to evoke a response. In any case, the data with PE alone (Figure 7) as well as that with NE in the presence of prazosin (Figure 5), strongly implicate the α_1-adrenergic signaling pathway in the regulation of FGF-2 transcription.

6.2. Role of the A/G-rich region in regulating the rat FGF-2 promoter

6.2.1. The A/G-rich region and the phenylephrine response

The rat FGF-2 promoter region contains a 74 bp region which is 97% A/G-rich, located between nucleotide positions -793 and -720 (Figure 6). At the core of this region there is a tandem repeat sequence of an 8-bp element, 5′-AGGGAGGG-3′. This element is nearly identical to an 8 bp region within the ANF promoter, 5′-gGGGAGGG-3′, which was shown to be at least partly responsible for the α_1-inducible expression of ANF (Ardati and Nemer, 1993; Sprenkle et al., 1995). This element was conserved among the human, murine, and bovine ANF genes, and similar elements are also present in other α_1-inducible genes, such as skeletal α-actin and β-myosin heavy chain (Table 5, Ardati and Nemer, 1993). The A/G-rich region in the FGF-2 promoter was thus a candidate for mediating the observed PE response. However, using 5′-deletion analysis of the rat FGF-2 promoter, we showed that the A/G-rich region was not necessary for the PE response to occur (Figure 7). Indeed, the responsive DNA appears to be contained within 313 bp of sequence upstream of the transcription start site (+1, Figure 6). The A/G-rich region, as a 37 bp oligonucleotide, also could not confer any additional PE response on a heterologous (HSV-TK) promoter (Figure 14). Thus, the AGGG repeat elements from the

FGF-2 promoter do not appear to be either necessary or sufficient to mediate a response to α_1-adrenergic activity.

6.2.2. Enhancer effect of the A/G-rich region

Although unable to mediate a response to PE, the A/G-rich region did confer enhancer activity on a heterologous promoter, in both neonatal rat cardiac myocytes and in human and rat glial tumour cell lines (Figure 11). The enhancer effect was stronger in glial cells than in cardiac myocytes, suggesting tissue- or cell-specific regulation by this element. However, although the A/G-rich oligonucleotide was able to stimulate promoter activity in the context of a heterologous promoter and reporter gene (Figure 11), deletion analysis of the rat FGF-2 5′-flanking DNA does not support a major role for the AGGG repeats by themselves in the regulation of the FGF-2 promoter in cardiac myocytes or glial cells (Detillieux, Meyers and Cattini, unpublished observations). This does not rule out the possibility that additional elements and/or factors participate in conjunction with the A/G-rich region, thereby modifying their action and, thus, relative importance.

Table 5. Nucleotide Sequence of the Rat ANF PE Responsive Element

Position	Nucleotide Sequence	Gene
-85	aagtgacagaat **GGGGAGGG** ttcctgctct	rANF
-85	aagtgacagaat **GGGGAGGG** ttctgtctct	hANF
-85	aagtgacagaat **GGGGAGGG** ttctagcccc	mANF
-85	aagtgacagaat **GGGGAGGG** ttccgtccct	bANF
-130	cggccg **AGGGAGGG GG**ctcta	SKA
-125	atacgt gt**GGAGGG GG**ccagtt	βMHC
-316	accagaaa**G GGGGAGGG G**tgggctggc	CarA

rANF, hANF, mANF, and bANF correspond to rat, human, murine, and bovine atrial natriuretic factor, respectively; SKA, human skeletal α-actin; βMHC, rat β-myosin heavy chain; and CarA, human cardiac actin. The conserved A/G-rich motif is in **bold** type. Adapted from Ardati and Nemer, 1993.

6.2.3. DNA-protein interactions at the A/G-rich region

While the exact functional significance of the A/G-rich region remains unclear in the context of FGF-2 transcriptional regulation, the data from gel mobility shift assays indicate multiple protein/DNA interactions. Four complexes (C1-4) between neonatal rat heart nuclear protein and the A/G-rich oligonucleotide were identified (Figures 12 and 13). The same pattern of complexes was also observed with nuclear protein isolated from cultured neonatal rat cardiac myocytes (Figure 15), indicating that these proteins are contained in muscle as well as perhaps non-muscle cells of the heart. All four complexes were specific as they were competed by increasing amounts (25-100 fold molar excess) of unlabeled A/G-rich oligonucleotide, but not by equivalent amounts of the unrelated RF-1 DNA element (Lytras and Cattini, 1994). The complete competition of C2 and C4 with a 25 fold molar excess of cold specific competitor indicates that these complexes possess a higher affinity and/or specificity than do C1 and C3, which required a 100 fold molar excess to be efficiently competed. Interestingly, an increase in C1 and C3 was suggested with complete competition of C2 and C4 in the presence of 25 fold molar excess of competitor (Figure 12). By reducing the amount of competitor, we could observe a difference in the relative affinities of C2 and C4 (Figure 13). C2 and C4 were efficiently competed with a 2 and 10 fold molar excess, respectively, confirming that these represent very high affinity and specificity interactions, where C2>C4. Figure 13 also confirms that there is a transient increase in C1 and C3 binding corresponding to the competition of C2 and C4. This suggests that the protein/DNA interactions represented by C1/C3 and C2/C4 are mutually exclusive, with a preference, under the experimental conditions used, for C2/C4 to form. Thus, when the higher affinity C2/C4 events are

made invisible with low levels of cold competition, there is an increased opportunity for C1 and C3 to bind to labeled probe. The ability to detect all four complexes (C1-4) simultaneously suggests that the proteins involved in C1/C3 are present in excess in the neonatal rat heart. Thus, there is potential for regulation of nuclear protein binding to occur in this region. However, stimulation of cultured cardiac myocytes for 6 h with NE gave no change in the pattern of complexes formed (Figure 15). Thus, any regulation that may occur in this region appears to be independent of adrenergic activity, and may instead be involved in other cellular responses.

6.2.4. Localization of the FGF-2 transcriptional response to phenylephrine

The rat genomic clone containing FGF-2 5′-flanking DNA contains consensus elements which suggest that a wide variety of mechanisms may control FGF-2 transcription (Figure 6). The ability of the -1058FGFp.*luc* gene to respond to PE in the same positive manner as observed with the endogenous FGF-2 gene suggests that the genetic information contained within the region -1,058/+54 of the FGF-2 gene is sufficient for this response. Indeed, the 5′-deletion containing only 313 bp of promoter sequence (-313FGFp.*luc*; Figure 7) also responded to PE, indicating that the sequences necessary for the response of FGF-2 to such treatment are contained proximal to the transcriptional start site, within ~300 bp of position +1. This excludes the A/G-rich region from involvement in the α_1-adrenergic response observed. However, multiple elements, aside from the A/G-rich element (Ardati and Nemer, 1993) have been implicated in the α_1-adrenergic response of ANF as well as other cardiac genes (Table 3). Two CArG serum response elements were shown to play a role in the PE-induced expression of ANF, in addition to the A/G-rich element (Sprenkle et al., 1995). The rat FGF-2 gene contains a

CArG-like element (CCTATTAgGG) at position -475 (Figure 6). However, this element falls outside of the 313 bp of PE-responsive FGF-2 5′-flanking DNA. The M-CAT element (consensus CATNC[c/t][t/a]) which binds TEF-1 and related factors, has been shown to mediate α_1-adrenergic-inducible expression of rat brain natriuretic peptide (Thuerauf and Glembotski, 1997), rat β-myosin heavy chain (Kariya et al., 1994), and murine skeletal α-actin (Karns et al., 1995) (see Table 3). The rat FGF-2 gene contains a consensus M-CAT element at position -277 (CATTCCT, Figure 6), which is exactly identical to the M-CAT element characterized in the BNP promoter (Thuerauf and Glembotski, 1997; see also Table 3). This element falls within the 313 bp of FGF-2 5′-flanking DNA shown to mediate a PE response (Figure 7). In addition, there is an A/T-rich region which is conserved between the rat and human FGF-2 genes, at position -114 of the rat FGF-2 promoter (Figure 6). An A/T-rich region was shown to mediate the α_1-adrenergic inducibility of ANF (Harris et al., 1997; see also Table 3). This is also contained within the 313 bp of PE-responsive FGF-2 5′-flanking DNA. Further deletion and perhaps mutational analysis would be required to elucidate the role of the elements in the FGF-2 PE response.

Though not directly associated with α_1-adrenergic inducibility of cardiac genes, Egr-1 mRNA (determined by RNA blotting) and protein immunofluorescence are upregulated by α_1- but not β-adrenoceptor stimulation (Iwaki et al., 1990). The Egr-1 protein binds to and upregulates the human FGF-2 promoter in astrocytic cells (Biesiada et al., 1996). Egr-1 was also shown to be required for autoregulation of the human FGF-2 gene (Wang et al., 1997). The rat FGF-2 promoter contains one Egr-1 site (spanning the

transcriptional start site [+1]), which is identical to the consensus Egr-1 site (GCGGGGGCG) and two, just downstream, which differ in only one base pair each (Figure 6). Two of these three sites overlap with a consensus Sp1 binding site (GGCGGG, Figure 6). Interplay of Sp1 and Egr-1 at overlapping sites has been demonstrated in the regulation of the human platelet-derived growth factor gene promoter (Khachigian et al., 1995). Egr-1 binds to a 49-bp oligonucleotide which encompasses these sites (Jin, Detillieux, and Cattini, unpublished observations). It remains to be seen whether Egr-1 plays a role in the inducible expression of FGF-2.

6.3. Regulation of FGF-2 gene expression in relation to its release from cardiac myocytes

Although cardiac myocyte contractility can be increased by NE through both α_1- and β-adrenoceptors (see section 1.5.3.), our data indicate that adrenergic stimulation of FGF-2 promoter activity is not dependent on myocyte contraction. Arrest of contraction using high extracellular KCl or 2,3-butanedione monoxime (BDM) did not interfere with the ability of NE to upregulate FGF-2 transcription (Figure 8A). Interestingly, when KCl was used alone, FGF-2 promoter activity increased significantly by 1.5 fold (Detillieux, Meij, and Cattini, unpublished observations). KCl causes a slow, permanent depolarization which results in an increase in intracellular Ca^{2+}. Therefore, it was thought possible that changes in intracellular Ca^{2+} associated with α_1-adrenergic activity (Figure 3, section 1.5.2) would be a component of the FGF-2 response to adrenergic stimulation. However, blocking of the major (L-type) Ca^{2+} channels with nifedipine had no effect on PE-

induced FGF-2 promoter activity (Figure 8B). Even so, this does not rule out the possible contribution of Ca^{2+} via other channel types and intracellular stores.

Knowing the relationship between intracellular Ca^{2+} and FGF-2 gene expression would be very beneficial in light of the "wound hormone" hypothesis (McNeil and Steinhardt, 1997). With every contraction of a cardiac myocyte, molecules may enter as well as exit the cell. It has been suggested that Ca^{2+} may enter a cell this way (McNeil and Steinhardt, 1997). Ca^{2+} entry could then potentially serve as a central mediator for both neurohormonal and mechanical signaling. Such an entry of Ca^{2+} is a candidate signal for regulation of FGF-2 expression. As mentioned above, the failure of nifedipine to block PE-induced FGF-2 expression does not rule out the potential involvement of Ca^{2+} entering the intracellular space through plasma membrane disruptions or from intracellular stores such as the sarcoplasmic reticulum. In order to truly block the action of intracellular Ca^{2+}, regardless of its origin, a membrane-permeable chelating compound, such as BAPTA-AM (Sadoshima et al., 1995), would have to be used. It is also worth noting that a given gene may be regulated differently by hormonal vs. mechanical stimuli, as has been demonstrated for β-myosin heavy chain: The M-CAT element, central to α_1-adrenergic stimulation of β-MHC expression, is not involved in increased expression associated with hypertrophy induced by aortic banding *in vivo* (Hasegawa et al., 1997).

The α_1-adrenergic induced expression of FGF-2 did not disappear in the absence of contraction. If FGF-2 release from cardiac myocytes is dependent on contraction (McNeil

and Steinhardt, 1997), then α_1-adrenergic regulation of FGF-2 transcription may be independent of release of FGF-2. It has been suggested that since FGF-2 autoregulation through FGFR1 does occur, then release of FGF-2 from injured cells may trigger an autocrine response that feeds back on FGF-2 synthesis. However, in endothelial cells, FGF-2 mRNA accumulation triggered by non-lethal scrape injury was found to occur independently of FGF-2 signaling through its receptors, since addition of neutralizing antibodies to FGF-2 did not block the increase in mRNA (Ku and D'Amore, 1995). Thus, it appears that although FGF-2 can regulate its own synthesis at the mRNA level in both endothelial cells (Fisher et al., 1997; Weich et al., 1991) and cardiac myocytes (Fisher et al., 1997), other stimuli such as mechanical (scrape injury) or hormonal (adrenergic activity) operate through mechanisms independent of FGF-2 autoregulation.

6.4. Regulation of FGF-2 transcription in vivo*: -1058FGFp.luc transgenic mice*

Transgenic mice were used to show that FGF-2 synthesis can be regulated at the transcriptional level by α_1-adrenergic stimulation *in vivo* (Figure 10). The rat FGF-2 promoter, like its human counterpart, possesses properties associated with a housekeeping gene, and its product is ubiquitously distributed (Bikfalvi et al., 1997; Kardami and Fandrich, 1989). This was reflected in the detection of luciferase activity in both brain and heart of transgenic mice (Figure 9A). The increase in endogenous mouse FGF-2 mRNA levels observed 24 h after administration of PE is consistent with the stimulation of FGF-2 promoter activity at this time and preceding this event at 6 h (Figure 10, A and B). The loss of increased FGF-2 promoter activity as well as the decrease in FGF-2 RNA levels observed at 48 h likely reflects metabolism and clearance

of the PE and a corresponding reduction in adrenergic stimulation. Regardless, the accumulation of FGF-2 RNA and, more specifically, the stimulation of FGF-2 promoter activity via α_1-adrenoceptors in the transgenic mice indicate a role for this regulatory pathway *in vivo*.

The presence of ubiquitous luciferase activity in the -1058FGFp.*luc* transgenic mice suggests that this region of rat genomic DNA (-1058/+54) contains sufficient genetic information to drive FGF-2 gene expression *in vivo*. This rodent reporter gene model for FGF-2 transcription has the potential to provide insight into FGF-2 transcription in any number of cell or tissue types. The presence of luciferase activity from line P300 has been detected in various neural and endocrine tissues such as spinal cord, dorsal root ganglia, sciatic nerve and adrenal gland (Figure 9C). In addition, luciferase activity is present and can be regulated by adrenergic stimulation in adult cardiac myocytes isolated from line P300 or P66 transgenic mice (Sheikh and Cattini, unpublished observations). Luciferase activity can also be detected in isolated perfused hearts (Figure 18). In this way, the response of FGF-2 promoter activity under conditions of ischemia and reperfusion, or with the infusion of pharmacological agents, could be monitored in whole hearts. A major limitation of the luciferase reporter gene, however, is the difficulty associated with *in situ* localization of expression. Detection of luciferase mRNA by *in situ* hybridization, although it appears in the literature (Magness et al., 1998), has been unsuccessful in our hands (Grothe, Detillieux, and Cattini, unpublished observations). Also, although results using a commercially available anti-luciferase antibody have been published (Agah et al., 1997), reporter genes such as chloramphenicol acetyl transferase

(CAT) or β-galactosidase are more frequently used for *in situ* localization of gene expression (Thomas et al., 1995; Kelly et al., 1995; Rivkees et al., 1999). Therefore, in order to study FGF-2 transcription *in vivo* and its localization relative to, for example, areas of tissue damage, a different reporter gene may prove to be more useful, such as CAT, green fluorescent protein or β-galactosidase.

6.5. FGF-2 transcription: Regulation in context

The question remains, then: how does adrenergic regulation of FGF-2 transcription relate to the role of FGF-2 in normal maintenance and in the injury response? FGF-2 mRNA and protein levels do change in response to a variety of stimuli. In section 1.2, the upregulation of FGF-2 mRNA was discussed in relation to ischemic injury. The release of catecholamines in the heart during ischemia and infarction was discussed in section 1.4. The data presented in Chapter 3 give evidence for stimulation of FGF-2 gene expression through the action of α_1-adrenoceptors. The upregulation of Egr-1 mRNA by α_1-adrenergic activity (Iwaki et al., 1990), and the binding of Egr-1 to the rat FGF-2 promoter (Jin, Detillieux and Cattini, unpublished results) point to this transcription factor as a potential mediator of α_1-induced FGF-2 gene expression. Indeed, Egr-1 is a stress response protein, its synthesis and activity being increased by a range of physiological stresses (in cardiovascular cells as well as in other tissues), including physical/mechanical factors such as shear stress (Schwachtgen et al., 1998), hypoxia (Yan et al., 1998), and cardioplegic arrest and reperfusion (Aebert et al., 1997), or chemical/endocrine signals such as phorbol esters (Cheng et al., 1994; Morita et al., 1995; You et al., 1997), angiotensin II or endothelin 1 (Neyses et al., 1993). Interestingly, many of these stimuli have been observed to upregulate FGF-2 mRNA levels in cardiac

cells (see Table 3), perhaps indicating that Egr-1 can act as a central mediator of the FGF-2 response to several stress-induced stimuli. The identification of *cis*-acting elements in the rat FGF-2 promoter which are responsible for the response to stimuli listed in Table 3 may reveal converging pathways for a generic "stress response" of FGF-2.

If FGF-2 is indeed involved in stress response, then another context in which regulation of FGF-2 gene expression could be explored is in the realm of ischemic preconditioning. This is a clinical phenomenon whereby a short period of ischemia enables the myocardium to build up increased resistance to a subsequent long ischemic period, as would occur during cardiac surgery (Li et al., 1990). Signal transduction in ischemic preconditioning has been shown to be mediated by PKC (Ladilov et al., 1998; Yang et al., 1997). Given the evidence for FGF-2 in cardioprotection (section 1.2), and the regulation of FGF-2 transcription by phorbol esters (Pasumarthi et al., 1997), which are potent activators of PKC, one may wonder if FGF-2 plays a role in ischemic preconditioning. The FGFp.*luc* transgenic mouse may prove to be a useful tool in this context, to study FGF-2 transcription in isolated hearts in a system where ischemic preconditioning can be modeled. Also, if the response of FGF-2 to transcriptional stimuli is delayed, as it appears to be (see discussion immediately following), then the upregulation of FGF-2 transcription could play a role specifically in the "second window" or late phase of preconditioning, about 24 h after the short burst of ischemia (Carroll and Yellon, 1999).

Thus, the relationship between the biological roles of FGF-2 and its regulation by adrenergic activity may be linked by physiological stress. In terms of adrenergic stimulation, however, an important question remains outstanding: The response to norepinephrine and phenylephrine were observed *in vitro* at 6 and 48 h, respectively (Figures 5 and 7). *In vivo*, the response to phenylephrine was observed at 6 h and persisted at 48 h (Figure 10). In contrast, Egr-1 mRNA and binding activity are upregulated much earlier, at 30 min to 2 h following addition of agent (Iwaki et al., 1990; and our unpublished results). Thus, Egr-1 may represent an immediate change which is then followed by a set of events which elicit a more chronic response. Indeed, given the variety of levels at which control over FGF-2 production and activity is exerted (Figure 1), it is quite possible that immediate responses requiring FGF-2 action involve recruitment of active FGF-2 from intracellular stores (release/export), and binding of ligand to receptor, mediated by heparan sulfate proteoglycans in the extracellular matrix. Transcription, then, would represent a process activated quickly (perhaps by Egr-1) but maintained longer-term, as a more chronic response to the depletion of intracellular stores. Further work would be required to elucidate the identity of the players and the *cis*-acting elements in the rat FGF-2 promoter involved.

Finally, it is worth noting that the regulation of FGF-2 in response to injury (section 1.2 and Table 1, section 1.4.3), may be tissue specific. In the heart, FGF-2 accumulates at focal regions of isoproterenol-induced injury (Padua et al., 1993), and FGF-2 expression is upregulated during ischemic injury in rat brain (Lin et al., 1997) and retinal tissue (Miyashiro et al., 1998). However, in skeletal muscle, both FGF-2 protein (Fu et al.,

1995) and mRNA (Fu et al., 1998) are diminished in ischemia and reperfusion. Thus, understanding how various stimuli work to control FGF-2 transcription may point to novel mechanisms for tissue-specific regulation of an otherwise ubiquitously expressed protein.

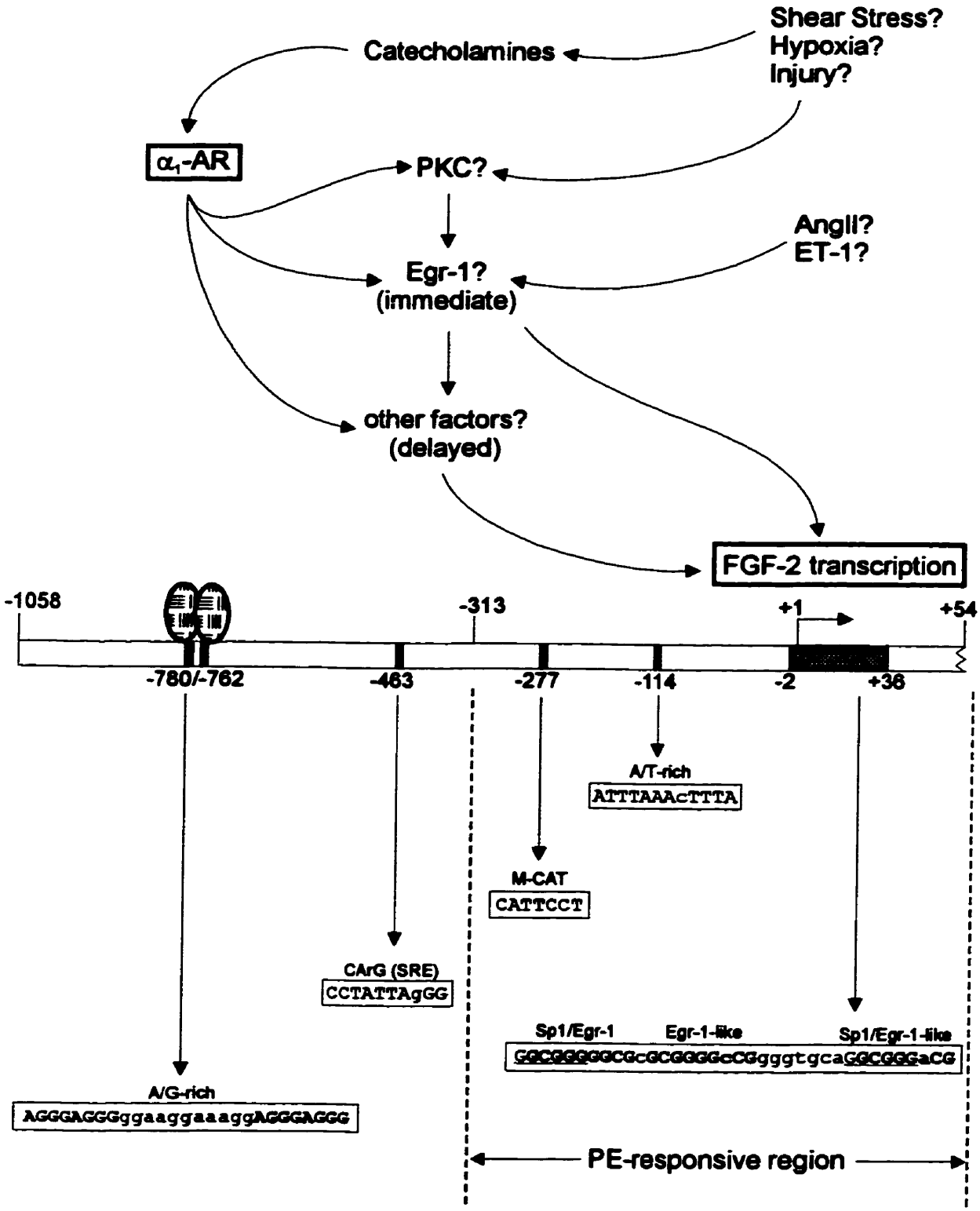
6.6. Concluding Remarks

Figure 19 gives a schematic, overall view of the ideas presented so far in this discussion. To summarize, rat FGF-2 gene expression at the level of transcription is increased by norepinephrine specifically through α_1-adrenergic receptors in cardiac myocytes, and this effect is mediated by regulatory sequences contained within about 300 bp upstream of a primary transcription start site. The α_1-induced increase in transcription occurs independently of two A/G-rich, putative phenylephrine response elements located at positions -780 and -762 in the rat FGF-2 5′-flanking DNA. The α_1-adrenergic regulation of FGF-2 transcription also occurs independently of myocyte contraction or Ca^{2+} entry via L-type Ca^{2+} channels. Potential mediators of this response (protein kinase C, Egr-1), DNA elements playing putative roles, and the relative importance of FGF-2 transcription in the context of injury and stress responses have been discussed.

Figure 19.

A working model for transcriptional regulation of rat FGF-2 gene expression in cardiac myocytes.

This figure summarizes the discussion in sections 5.2.4 and 5.5. Both physical factors, such as shear stress, injury or hypoxia (as would occur in ischemic preconditioning), and endocrine factors, such as angiotensin II (**AngII**), endothelin-1 (**ET-1**), or **catecholamines** such as norepinephrine acting through α_1-adrenergic receptors (α_1-**AR**), act to increase **FGF-2 transcription**, as demonstrated by data in this thesis and elsewhere (see text). Protein kinase C (**PKC**) and the zinc finger transcription factor Egr-1 are likely intermediaries for many of these pathways. While phenylephrine (PE) does invoke an increase in Egr-1 binding to the FGF-2 promoter in the short term, other factors may also be involved in prolonging the increase in transcription. The region of 5′-flanking DNA from the rat FGF-2 gene that is responsive to PE lies between positions –313 and +54, and possible participants in this response are marked: an **M-CAT** element at position -277, a conserved (human vs. rat) **A/T-rich** region at –114, and a G/C-rich island containing a series of overlapping **Sp1** (underlined) and **Egr-1** (**bold type**) binding sites. Outside the PE-responsive region, the **A/G-rich** elements (**bold type**), although not involved in the PE response, bind protein with high affinity and specificity. As well, a **CArG** serum response element (**SRE**) lies outside the PE-responsive region at position -463. DNA-protein interactions outside the PE-responsive region may mediate responses to other physical or endocrine stimuli.


Shear Stress?
Hypoxia?
Injury?
Catecholamines
α1-AR
PKC?
AngII?
ET-1?
Egr-1?
(immediate)
other factors?
(delayed)
FGF-2 transcription
-1058
-313
+1
+54
-780/-762
-463
-277
-114
-2
+36
A/T-rich
ATTTAAAcTTTA
M-CAT
CATTCCT
CArG (SRE)
CCTATTAgGG
Sp1/Egr-1
Egr-1-like
Sp1/Egr-1-like
GGCGGGGGCGcGCGGGGcCGgggtgcaGGCGGGaCG
A/G-rich
AGGGAGGGggaaggaaaggAGGGAGGG
PE-responsive region

In closing, it is worth considering the possibility of furthering the investigation of FGF-2 transcription through the sequencing of rat genomic DNA which lies further upstream than the 1,058 bp of sequence characterized here. In spite of the fact that a phenylephrine response was localized to within 313 bp of 5′-flanking DNA, there are several reasons why it may be a valuable endeavour to clone and characterize additional sequences. First, a 34 kDa, very high molecular weight FGF-2 was recently characterized in human HeLa cells (Arnaud et al., 1999). Translation of this isoform is initiated at a fourth CUG start codon, 86 nt from the FGF-2 mRNA 5′ end. An in-frame CUG codon is contained in the rat 5′-flanking sequence that, if included in protein translation through the FGF-2 open reading frame, would yield a 35 kDa protein. However, the situation is complicated by the fact that (a) two in-frame stop codons lie upstream of the second CUG start codon (yielding 21 kDa FGF-2), and (b) the upstream CUG codon lies more than 200 bp upstream of the major transcription start site. Stop codon read-through has been demonstrated for at least one mammalian gene (Chittum et al., 1998). Also, an alternative promoter and first exon have been described for avian FGF-2 (Borja et al., 1996). The characterization of upstream sequence in the rat genome may lead to information about how additional FGF-2 isoforms may be generated, and the tissue specific regulation of such novel isoforms. It is also interesting to note that the start site for FGF-2 cDNA cloned from gonadotropin-stimulated rat ovaries (Shimasaki et al., 1988) lies at position -284 relative to the major transcription start site indicated for rat brain (Pasumarthi et al., 1997). Thus, tissue-specific regulation of FGF-2 transcription by hormones or other factors may well result in the generation of transcripts from upstream start sites, and the

cloning of additional upstream sequences may reveal regulatory regions involved in these processes.

Finally, we must consider the strength of the FGF-2 response to α_1-adrenergic stimulation relative to other genes which have also been characterized for α_1-adrenergic inducibility. All promoters used in the studies described here responded to some degree to phenylephrine stimulation. This includes viral "control" promoters, such as herpes simplex virus thymidine kinase (Figure 14), as well as Rous sarcoma virus and cytomegalo virus promoters (not shown). Indeed, Rous sarcoma virus was reported to respond positively to stimulation through ras and raf-dependent mechanisms (Cosgaya et al., 1997). Since α_1-adrenergic receptors operate through ras and raf (LaMorte et al., 1994; Yamazaki et al., 1997), it is not surprising that they would elicit such a basal stimulatory response. Since the stimulation of viral promoters was significant (~4 fold, see Figure 14), these promoters were not used as internal controls in the transient transfection assays using FGF-2 promoter. Instead, luciferase activity was normalized against the total protein content of the sample. Even so, the level of stimulation of FGF-2 promoter activity (~7 fold with PE after 48 h) was lower than that of myosin light chain-2 (~10 fold under similar conditions). Other promoters, such as ANF or β-myosin heavy chain, gave 5-10 fold stimulation even after normalization against a viral promoter (Sprenkle et al., 1995; Kariya et al., 1994). Thus, the α_1-induced increase in activity of these promoters seems considerably stronger than that of FGF-2. Two reasons may explain this observation. First, transcriptional regulation of FGF-2 represents a low-level, chronic effect which does not require a huge increase in activity. Results with FGF-2

mRNA, however, indicate that the induction is large (Figure 7). Other agents, such as angiotensin II or endothelin-1, invoke an increase in FGF-2 mRNA as high as 47 fold (Peifley and Winkles, 1998). Thus, it is possible that the 1,058 bp of FGF-2 5′-flanking DNA is enough to evoke a basal response, but that additional upstream sequences may contain regulatory information that would enable an even greater magnitude of response.

The 1058FGFp.*luc* transgenic mice also support this idea. Although the 1,058 bp of 5′-flanking DNA was sufficient to drive luciferase activity ubiquitously in two independent lines (P300 and P66), the relative levels among tissues differed in these two lines (Figure 9, A and C), indicating that the expression of the transgene is being influenced by surrounding DNA at the site of insertion. Indeed, two other independent lines (P61 and P89) showed expression almost exclusively in neural and neural-derived tissues (Table 4). It would appear, then, that the –1,058/+54 rat FGF-2 promoter is "gullible", in the sense that it is easily influenced by sequences which are in close proximity. For this reason, the cloning and characterization of additional sequence upstream of –1,058 may reveal enhancers or suppressors which regulate FGF-2 transcription *in situ*.

In any case, with the available 5′-flanking DNA we were able to establish and characterize the α_1-inducible expression of the rat FGF-2 promoter. FGF-2 transcription is part of a vast array of regulatory processes governing FGF-2 production and bioactivity. From the data presented here, we can conclude that FGF-2 transcription can respond to cellular changes which would demand the replenishing of FGF-2 intracellular stores following a physiological stress.

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