Foreign Gene Regulation and Adenoviral Mediated Gene Transfer in Models of Myocardial Ischaemia

A thesis submitted for the degree of

Doctor of Philosophy

at the

University of Glasgow

by

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December 1997

The research reported in this thesis is my own and original work, except where otherwise stated and has not been submitted for any other degree.

This thesis is dedicated with love and thanks

to Mum and Dad.

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Abbreviations

A1AR - A1 adenosine receptor

AHR - arylhydrocarbon receptor

ARNT - arylhydrocarbon-receptor nuclear translocator

bHLH - basic helix-loop-helix

CAct - cardiac α-actin

CAT - chloramphenicol acetyl transferase

CHO - chinese hamster ovary

cTnC - cardiac troponin C

EPO - erythropoietin

ET-1 - endothelin-1

FGF - fibroblast growth factor

flt-1 - fms-like tyrosine kinase-1

GFP - green fluorescent protein

HIF-1 - hypoxia inducible factor-1

HRE - hypoxia response element

HSF1 - heat-shock transcription factor-1

HSP - heat-shock protein

HSV - herpes simplex virus

HVJ - haemagglutinating virus of Japan

IL-2 - interleukin-2

IR - ischaemia followed by reperfusion

JNK1 - c-jun N-terminal kinase

KDR - kinase domain region

MHC - myosin heavy chain

MLC - myosin light chain

Mn-SOD - manganese superoxide dismutase

PA - plasminogen activators

PCR - polymerase chain reaction

PDGF - platelet-derived growth factor

SkAct - human skeletal α-actin

SRE - serum response element

SRF - serum response factor

SV40 - simian virus 40

TGFβ - transforming growth factor β

TNF-α - tumour necrosis factor α

VEGF - vascular endothelial growth factor

Acknowledgements

Firstly, I must thank my supervisor Howard Prentice, the Division of Molecular Genetics and the BBSRC for allowing me to do this work. I would also like to thank our collaborators, Drs Keith Webster and Nanette Bishopric in Miami and Dr Martin Hicks at Glasgow Royal Infirmary.

Very special thanks to everyone in the lab for all your help, support and most of all, friendship. To Yvonne for being simply fantastic, to Jill for being an absolute star and being able to screw up experiments even more spectacularly than me !! and to Patsy for taking science even less seriously than I do. To Obaid for all your stock solutions - even when you resorted to labelling them in foreign squiggles, to Karen for making sequencing look far too easy and more recently to Simon for being even more sarcastic than me.

Thanks to the rest of the BPU, especially the 'poker boys' for allowing me to fleece them on a regular occaision. I think the phrase says something about 'candy' and 'children' !! Thanks to everyone at Genetics with whom I leave the legacy of SNoG. Also thanks to Martin and everyone at the Royal Infirmary who are all fantastic and where I never once arrived on time !

Thanks to my eternal drinking partner Dave for introducing me to the hypnotic charms of the kilt and to everyone at Woodlands Drive, past and present. Life in Woodlands Drive was never dull - neither was it ever sober !!!!

Thanks to my family, Mum, Dad, Karen, Shona and Vicki for all their love and support. And finally to Ali, all my love and thanks - you're the best distraction in the world !!

Abstract

Myocardial ischaemia is characterised by a reduction in blood flow sufficient to cause a pathological change in myocardial function. One of the myocardial adaptive responses to ischaemic stress involves a change in gene expression which is regulated by the interaction of DNA binding proteins with specific DNA sequences. Identifying intracellular signalling pathways that link the changes in the extracellular environment during ischaemia to alterations in gene expression may provide important information on how the myocardium adapts to ischaemic stress. Specific regulatory sequences that interact with DNA binding proteins in response to ischaemic stress may be used as part of a gene therapy protocol to regulate foreign gene expression in the ischaemic myocardium.

One of the aims of this study was to identify control regions that may play a role in the myocardial response to ischaemic stress. Promoter elements have been characterised with respect to their ability to respond to ischaemic stress in animal models of ischaemia with reperfusion. A hybrid promoter, containing myosin heavy chain basal regulatory sequences plus four copies of the erythropoietin HIF-1 binding site, conferred inducible expression of a luciferase reporter gene in response to 15 minutes of ischaemia followed by reperfusion. This induction was rapid and reversible upon reperfusion of the ischaemic myocardium. The human skeletal α-actin (SkAct) promoter was also investigated but showed no significant activation in response to 15 minutes of ischaemia followed by reperfusion in rat and rabbit myocardium at the time points examined. The SkAct promoter was further characterised by analysing the relative expression of mutant and wild-type SkAct promoters in the rat heart after direct DNA injection. This study indicated possible regulatory sequences that may be involved in the cardiac specific regulation of the SkAct gene and indicated for some promoter constructs a possible discordance between *in vivo* and *in vitro* data.

The extent and severity of the pathological loss of function caused by myocardial ischaemia is critically dependent on the collateral circulation perfusing the ischaemic region. Stimulating angiogenesis in the ischaemic heart by adenovirus mediated overexpression of angiogenic factors may offer the possibility of increasing collateral perfusion to ischaemic areas, improving myocardial function. Adenoviral vectors are capable of delivering foreign genes to the myocardium with high efficiency and low toxicity. With a view to limiting the extent of myocardial ischaemia and preventing further expansion of the ischaemic areas, the present study also aimed to develop a potentially therapeutic adenoviral vector expressing an angiogenic factor. A replication deficient recombinant adenovirus expressing the highly specific and potent angiogenic factor, vascular endothelial growth factor (VEGF), was constructed. Using this viral vector for infection of cell lines in culture, evidence was obtained of foreign expression of the recombinant gene product.

Chapter 1

INTRODUCTION

1.1 Myocardial Ischaemia

Myocardial ischaemia is one of the most common cause of mortality in developed societies (Mangano, 1990). The condition is characterised by a pathological alteration of cardiac contractile, electrical or biochemical function caused by a reduction of coronary blood flow (Hearse, 1994). The inadequate supply of substrates, particularly oxygen and glucose, leads to a switch from aerobic to anaerobic metabolism which in turn results in a depletion of high energy phosphates and accumulation of ADP, AMP and inorganic phosphate (Elliott et al., 1992). The inadequate removal of metabolites, particularly H^+ and lactate leads to acidosis and production of highly reactive free radicals (Elliott et al., 1992; Pierce and Czubryt, 1995). Furthermore, accumulation of potassium (K^+) in the extracellular space leads to depolarisation of the myocytes and plays a crucial role in slowing conduction and the production of arrhythmias (Harris et al., 1954).

Within minutes, myocardial ischaemia leads to a complete loss of contractile function (Elliott et al., 1992). The loss of contractile function is accompanied by a fall in intracellular phosphocreatine levels and a rise in the intracellular inorganic phosphate concentration. ATP levels decline more gradually and as ATP degrades, adenosine accumulates in the extracellular space. The metabolic changes are accompanied by an intracellular acidosis as protons accumulate and intracellular pH falls (Elliott et al., 1992).

Clinically, three distinct injuries can arise as a result of an episode of ischaemia: reversible contractile dysfunction with no cell death - often referred to as stunning, irreversible contractile dysfunction with cell death - often referred to as infarction, and arrhythmias (Ytrehus and Downey, 1993). Contractile dysfunction leads to congestive heart failure and arrhythmias lead to non-synchronous electrical conduction in the heart, either of which can be fatal.

The tightly regulated ionic balance of the heart is disrupted during ischaemia and subsequent reperfusion and evidence suggests that there are a number of possible mechanisms by which this may happen (Poole-Wilson, 1985; Jennings and Steenbergen, Jr. 1985; Pierce and Czubryt, 1995; Allen and Orchard, 1987). Nevertheless, calcium (Ca^{2+}), sodium (Na^{+}), protons (H^{+}) and potassium (K^{+}) all appear to play central roles. One possible mechanism of ion redistribution during ischaemia and reperfusion (Pierce and Czubryt, 1995) proposes that protons, generated by anaerobic metabolism, accumulate during ischaemia and equilibrate with the extracellular fluid. Reperfusion flushes out the extracellular protons leaving a large concentration gradient across the plasma membrane. This gradient drives the sodium ion/proton exchanger, which extrudes intracellular H^{+} in exchange for extracellular sodium ions (Na^{+}). Consequently, intracellular Na^{+} levels rise. The Na^{+}/Ca^{2+} exchanger expels intracellular Na^{+} in exchange for extracellular Ca^{2+} leading to a build up of intracellular Ca^{2+}. Alternatively, intracellular Ca^{2+} levels may rise as a result of extracellular Na^{+} entering the cell through Ca^{2+} channels that become highly permeable to Na^{+} during ischaemia (Hess and Tsien, 1984). When extracellular Ca^{2+} levels return during reperfusion, the Na^{+}/Ca^{2+} exchanger expels intracellular Na^{+} in exchange for extracellular Ca^{2+} leading to Ca^{2+} accumulation. High levels of intracellular Ca^{2+} are toxic to the myocyte, inhibiting oxidative phosphorylation (Kusuoka et al., 1988), activating proteases that destroy cytoskeletal proteins, such as vinculin (Steenbergen et al., 1987) and triggering arrhythmias (Marban et al., 1986).

Ischaemia followed by reperfusion results in the formation of highly reactive, oxygen free radicals, including superoxide ($O_2^{-}\cdot$), singlet oxygen ($^1O_2$), the hydroxyl radical ($OH\cdot$) and their intermediary, hydrogen peroxide (H_2O_2) (Shlafer et al., 1987; Kloner et al., 1989). Potential sources of these reactive oxygen species include the action of xanthine oxidase on xanthine to produce $O_2^{-}\cdot$ (McCord, 1985) and the mitochondrial reduction of oxygen by NADH dehydrogenase leading to the formation of $O_2^{-}\cdot$ and H_2O_2 (Turrens and Boveris, 1980). The auto-oxidation of catecholamines (Singal et al., 1980) and the

metabolism of arachidonic acid (Kontos et al., 1980) are further sources of reactive oxygen species. Neutrophils, activated by the complement system, also generate and release highly reactive and cytotoxic oxygen derivatives that are destructive to the vascular endothelium and to the cardiac myocytes (McCord and Day, 1978). Membrane phospholipids, proteins and nucleic acids can all react with free radicals, leading to functional alterations and structural changes which may contribute to reversible or irreversible cell damage. Membrane characteristics are altered due to the peroxidation of cell membrane lipids (Coetzee and Lochner, 1993) and free radical mediated oxidation of membrane proteins, including ion channels, leads to alterations in membrane characteristics. Low levels of oxidative stress results in transcriptional activation but significantly higher levels of oxidative stress will induce DNA damage, especially DNA strand breaks (Das et al., 1995).

Prolonged periods of ischaemia lead to cell death and the formation of an infarct (Fishbein et al., 1978; Braunwald, 1987; Reimer et al., 1977). The non-contracting myocardial infarct increases the workload for the remaining myocardium (Fishbein et al., 1978). Since myocytes lose the ability to divide soon after birth, myocytes must adapt to this increased workload by cell enlargement rather than cell division (Claycomb, 1975). This increase in cellular mass is a significant component of myocardial hypertrophy. Myocardial hypertrophy is characterised by increased protein synthesis and both qualitative and quantitative changes in gene expression (Schiaffino et al., 1989; Takala, 1981; Komuro and Yazaki, 1993; Schwartz et al., 1993). The two major contractile proteins of the cardiac sarcomere, the myosin heavy chains and α-actins, are selectively induced during myocardial hypertrophy. β-Myosin heavy chain RNA levels are elevated during hypertrophy at the expense of the α-isoform. This transition from the α-isoform to the β-isoform improves the efficiency of contraction for an equivalent amount of work (Alpert and Mulieri, 1982). Skeletal α-actin, which is normally only expressed during fetal development, is re-expressed during cardiac hypertrophy to a level equivalent to the cardiac α-actin isoform (Schwartz et al., 1986). The primary stimulus for myocardial

hypertrophy is unclear, however, catecholamines (Bishopric and Kedes, 1991; Bishopric et al., 1987), growth factors (Parker et al., 1990) and hormones (Nadal-Ginard and Mahdavi, 1989) are all capable of eliciting a hypertrophic response as well as mechanical stress, such as stretch (Komuro et al., 1991; Mann et al., 1989) or pressure overload (Izumo et al., 1988; Schwartz et al., 1986).

1.2 Gene Expression in the Ischaemic Myocardium

Prolonged, severe ischaemia leads to cell death and the subsequent formation of an infarct, characterised by a large area of necrosis and non-contracting scar tissue (Reimer et al., 1977; Braunwald, 1987; Fishbein et al., 1978). Moderate levels of transient ischaemia, however, lead to a variety of cellular adaptions including changes in gene expression (Brand et al., 1992; Das et al., 1995). Indeed, one or more episodes of brief ischaemia, each interrupted by brief reperfusion, have been found to paradoxically protect the heart from subsequent sustained coronary artery occlusion (Reimer et al., 1990; Reimer et al., 1986). This phenomenon has been referred to as ischaemic preconditioning.

The myocardial adaptive response to ischaemic stress includes the induction of the immediate early genes, c-fos, Egr-1, c-jun and jun-B (Brand et al., 1992). The induced expression of these gene products is known to be involved in the trans-activation of genes which underlie the repair processes of the reversibly damaged myocardium. Repeated ischaemia and reperfusion episodes will induce several stress related genes including the heat-shock protein (HSP) 27,70, and 89 genes as well as the heat-shock transcription factor 1 (HSF1); the principal factor involved in regulating the transcription of heat shock genes (Nishizawa et al., 1996). The antioxidant genes, Manganese-Superoxide dismutase (Mn-SOD) and peroxisomal catalase genes (Das et al., 1993) are also induced by

ischaemia and reperfusion and with successive ischaemic and reperfusion episodes the enzyme activities of these antioxidative enzymes are also increased (Das et al., 1993).

β-adrenergic receptors play an important role in the myocardium and stimulation by catecholamines leads to an increase in cardiac contractility (Czyzyk-Krzeska et al., 1994). Myocardial ischaemia leads to a rapid increase in the number of β-adrenergic receptors in the plasma membrane, despite the release of large, desensitising levels of catecholamines. This increase is due, in part, to the translocation of intracellular receptors to the cell surface. However transcription, specifically of the β_1-adrenergic receptor, is also increased (IhlVahl et al., 1995). The angiogenesis cascade is also initiated by ischaemia through the rapid increase in expression of the potent, endothelial specific angiogenic factor, vascular endothelial growth factor (VEGF) (Hashimoto et al., 1994; Banai et al., 1994). The expression of the genes encoding transforming growth factor beta (TGFβ1), a growth factor involved in the modulation of cardiac growth and differentiation (Herskowitz et al., 1995) and the cytokines, tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-2 (IL-2) (Herskowitz et al., 1995) are also induced by ischaemia with reperfusion.

Gene expression changes in the ischaemic myocardium also involve genes which further exacerbate the deterioration of myocardial function. Endothelin-1 (ET-1), a potent vasoconstrictor peptide, has been implicated in the pathogenesis of myocardial ischaemia (Pernow and Wang, 1997). Ischaemia and reperfusion leads to an increase in the synthesis and release of ET-1 and an enhanced coronary constrictor response to ET-1. Accordingly, ET-1 antagonists have been shown to reduce ischaemia/reperfusion injury, improving myocardial performance and coronary blood flow (Pernow and Wang, 1997).

1.3 Gene Regulation in Myocardial Ischaemia

By virtue of the restricted tissue perfusion , ischaemia involves both inadequate supply of substrates, notably oxygen and glucose, and inadequate removal of products of metabolism, notably lactate, H^+ and K^+. Individual aspects of ischaemia therefore include hypoxia, hypoglycaemia and an altered redox status of the cell. There is evidence that each of these components may be involved in triggering the transcriptional activation of specific genes involved in the myocardial adaptive response to ischaemic stress.

1.3.1 Hypoxia

Hypoxia constitutes an intrinsic component of ischaemia and is known to result in the specific up-regulation of a range of genes including transcription factors, metabolic enzymes and growth factors (Semenza and Wang, 1992; Goldberg and Schneider, 1994; Semenza et al., 1994; Goldberg et al., 1988). The transcriptional activation of the gene encoding erythropoietin (EPO), a glycoprotein growth factor, by hypoxia stimulation is mediated through cis-acting DNA sequences in the 3' flanking region (Semenza and Wang, 1992). A hypoxia responsive enhancer element was identified in this region and found to bind the trans-acting factor hypoxia-inducible factor 1 (HIF-1) (Semenza and Wang, 1992). HIF-1 binding activity is induced by hypoxia in many cell types that have been tested, including mouse C_2C_{12} myoblasts, rat fibroblasts and human HeLa cervical carcinoma cells (Wang and Semenza, 1993b). In addition, the EPO enhancer that binds HIF-1 is capable of mediating hypoxia-inducible reporter gene expression in non-EPO-producing cells (Maxwell et al., 1993; Wang and Semenza, 1993). Several hypoxia inducible genes contain HIF-1 binding sites, including glycolytic enzymes (Semenza et al., 1994) and the angiogenic factor VEGF (Goldberg and Schneider, 1994). Desferrioxamine and $CoCl_2$ will also induce HIF-1 DNA binding activity with similar kinetics to hypoxia, suggesting the involvement of haem proteins in the O_2 sensing mechanism of HIF-1 (Semenza, 1994). HIF-1 therefore appears to play a central role in

a common hypoxia signal transduction pathway through the transcriptional regulation of hypoxia-responsive genes.

1.3.2 Tissue Hypoglycaemia

By virtue of the restricted tissue perfusion, low glucose or regional tissue hypoglycaemia is another intrinsic component of ischaemia. HIF-1 has also been implicated in transcriptional activation in response to low glucose levels. The HIF-1 transcriptional activity comprises a heterodimer composed of a 120-kDa HIF-1α subunit complexed with a 91- to 94-kDa arylhydrocarbon-receptor nuclear translocator (ARNT) subunit, also known as HIF-1β(Wang et al., 1995). Both HIF-1 subunits belong to the basic helix-loop-helix (bHLH)-per-arnt-sim (PAS) protein family (Wang et al., 1995). Other members of the bHLH-PAS family of heterodimeric transcription factors include the arylhydrocarbon receptor (AHR) and the *Drosophila* proteins period (Per) and single-minded (Sim) (Nambu et al., 1991; Hoffman et al., 1991; Crews et al., 1988; Wang et al., 1995). Genetically modified mouse lines with a targeted disruption of HIF-1β are incapable of responding to low oxygen or to a decrease in glucose concentration (Maltepe et al., 1997). HIF-1, therefore appears to mediate responses to hypoglycaemia, as well as to hypoxia.

Hypoxia and hypoglycaemia also work co-ordinately in the induction of the immediate early gene, c-jun, in the human squamous carcinoma cell line (SiHa) (Ausserer et al., 1994). C-jun is a major component of the inducible transcription regulation complex AP-1 (Bohmann et al., 1987). In these cells hypoxic exposure produced increased levels of c-jun mRNA resulting from message stabilisation in addition to transcriptional activation. However, simultaneous deprivation of oxygen and glucose resulted in a superinduction of c-jun message (Ausserer et al., 1994). The c-jun transcription factor is inactive until tyrosine residues on its N-terminal are phosphorylated by c-jun N-terminal kinase (JNK1). JNK1 activity has also been found to respond to ischaemia and reperfusion.

JNK1 translocates to the nucleus during ischaemia but does not phosphorylate c-jun. Upon reperfusion, further translocation of JNK1 is inhibited but JNK1 activity becomes significantly increased (Mizukami et al., 1997).

The exact role of c-jun in the myocardial adaptive response to ischaemic stress is unclear. However, a number of genes involved in the response of the myocardium to ischaemia contain recognition sites for AP-1, the fos/jun complex, including the angiogenic factor VEGF (Tischer et al., 1991), endothelin-1 (Kawana et al., 1995), β_1-adrenergic receptor (Tseng et al., 1995) and TGFβ (Kim et al., 1989).

1.3.3 Redox Status

Ischeamia with reperfusion is known to lead to changes in the redox state of the cell and exerts part of its influence on altered gene expression through modification of transcription factors. Redox regulation of DNA-binding proteins has been found to occur for a range of transcription factors through the reversible oxidation of key cysteine sulfhydryl groups. Such factors include AP-1 (Abate et al., 1990), Sp-1 (Ammendola et al., 1994), Egr-1 (Huang and Adamson, 1993), p53 (Hainaut and Milner, 1993) and USF (Pognonec et al., 1992). The reactive cysteines have been proposed to constitute redox-sulfhydryl switches which directly regulate gene expression (Pognonec et al., 1992; Hentze et al., 1989). In particular the binding of the transcription factors c-fos and c-jun to the AP-1 binding site, through a leucine zipper domain, is inhibited by the sulfhydryl oxidising agent diamide (Abate et al., 1990). Likewise, the binding of Sp-1, a member of the zinc finger DNA-binding proteins, to its GC-rich DNA binding domain, is repressed by oxidising agents (Wu et al., 1996).

The ability of transcription factors in particular, to respond directly to hypoxia, tissue hypoglycaemia or redox changes in the cell is likely to represent an important adaptive response to ischaemic stress. These responses demonstrate the presence of an

intracellular signalling pathway that can link changes in the extracellular environment directly to alterations in gene expression.

1.4 Models for Investigating Myocardial Ischaemia

The nature of ischaemic injury depends on the duration of the ischaemic period and the degree of reduction in coronary flow (Applegate et al., 1990). Reperfusion of an ischaemic area of myocardium does not necessarily restore cardiac function to pre-ischaemic levels (Pierce and Czubryt, 1995). Ischaemia is a multifactorial process that cannot readily be studied in the clinical setting and because of the complexity, no single animal model will appropriately mimic the pathophysiology of human ischaemia. A number of *in vivo* models have therefore been developed to study individual aspects of myocardial ischaemia. These models fall into three general categories - permanent ischaemia (infarct model), transient ischaemia (reperfusion model) and chronic ischaemia.

1.4.1 Infarct Model

Infarct models involve permanent occlusion of a coronary artery branch, leading to ischaemia of the myocardium downstream of the occlusion. This model closely simulates the clinical model of acute myocardial infarction (Ytrehus and Downey, 1993). Depending on the extent of collateral blood flow to the ischaemic area, different areas may be perfused to varying degrees. Areas with no collateral blood flow become necrotic, while regions with low collateral perfusion become permanently ischaemic. The necrotic areas of the myocardium are known to have a detrimental effect on the function of the surrounding myocardium, compromising both contraction and impulse conduction (Ytrehus and Downey, 1993). The contractile dysfunction seen in this model

allow studies into the compensatory mechanisms the heart employs to overcome the non-contracting infarct tissue. A number of studies using the infarct model have demonstrated that the overall composition, architecture and functioning of the heart change by a process referred to as myocardial remodelling (Whittaker and Kloner, 1991; Pfeffer and Braunwald, 1990; McKay et al., 1986). One of the major components of myocardial remodelling is hypertrophy. Myocytes are terminally differentiated and as such cannot replicate to compensate for pathological insults such as infarction. In order to adapt to the increased workload, myocytes increase in cell size by a process referred to as hypertrophy. Infarct models have allowed investigations into the qualitative and quantitative changes in gene expression that are characteristic of myocardial hypertrophy (Schiaffino et al., 1989; Takala, 1981; Komuro and Yazaki, 1993; Schwartz et al., 1993).

1.4.2 Reperfusion Model

Transient models of ischaemia involve a temporary interruption of coronary blood flow typically of 15 minutes ischaemia to greater than 1 hour, followed by reperfusion. Models with 15 minutes of ischaemia or less do not result in myocyte death or necrosis (Jennings and Reimer, 1983; Reimer et al., 1983; Kloner and Braunwald, 1980). Cardiac function may become seriously compromised during the early stages of reperfusion and may never fully return to pre-ischaemic levels. Periods of ischaemia followed by reperfusion can also arise during clinical conditions such as angina, coronary by-pass surgery, angioplasty and thrombolytic drug therapy (Pierce and Czubryt, 1995). Transient models of ischaemia are particularly useful for identifying the myocardial processes involved in adapting to ischaemic stress, including changes in gene expression levels (Brand et al., 1992; Das et al., 1995). Studies using transient occlusion of the coronary artery followed by a prolonged occlusion lead to the identification of a phenomenon known as ischaemic preconditioning. Ischaemic preconditioning dramatically reduces infarct size after sustained ischaemia followed by reperfusion (Reimer et al., 1990; Reimer et al., 1986). However, the clinical significance of this phenomenon is unclear.

1.4.3 Chronic Ischaemia Model

Chronic ischaemia involves reducing the perfusion of the myocardium to a level at which myocytes are still viable but function is compromised. Clinically this model relates to partial occlusion of a coronary artery by either an atherosclerotic plaque or blood clot resulting in insufficient perfusion to meet the metabolic demands of the myocardium. Chronic ischaemia models have demonstrated that as a consequence of the reduced myocardial perfusion, oxygen consumption and contractility become depressed. However, despite the reduced oxygen consumption and contractility, the myocardium adapts to the subsequent metabolic demands (Fedele et al., 1988; Pantely et al., 1990).

1.4.4 Cell Culture Model

Primary cultures of isolated myocytes have also provided a valuable insight into the myocardial adaptive response to ischaemic stress. Both neo-natal and adult myocytes can be studied in culture, although adult myocytes are prone to dedifferentiation depending upon the culture conditions (Ytrehus and Downey, 1993). Techniques such as patch clamp, flow cytometry and immunofluorescence staining can all be easily applied to isolated myocyte systems. A number of different ischaemia syndromes can be represented using isolated cardiac myocytes depending on the experimental conditions. Infarct, transient ischaemia and chronic models can all be paralleled using isolated myocytes, providing further information regarding biochemical, metabolic and gene expression adaptions to the varying ischaemic stimuli (Laderoute and Webster, 1997; Webster et al., 1994; Webster and Bishopric, 1992).

Despite the variety of models available, a number of fundamental questions remain unanswered particularly with respect to the molecular pathways involved in the myocardial adaptive response to ischaemic stress *in vivo*. Characterising the role of an individual molecular component of a signalling pathway *in vivo* requires, in many cases,

the expensive and time-consuming construction of multiple transgenic lines. Gene transfer technology allows single components of a signalling pathway to be manipulated in specific tissues and at specific time points. Importantly, gene transfer technology may also be directly applicable to the clinical setting as the emergence of gene therapy for the myocardium becomes a reality.

1.5 Gene Transfer and Gene Therapy in the Myocardium

Current therapeutic strategies for the treatment of myocardial ischaemia are limited to drug therapy and in severe cases, surgery. With the advances in gene transfer technology, a novel therapeutic approach may be available. Whereas drug therapy is aimed at treating the consequences of myocardial ischaemia, gene therapy may be aimed at treating both the consequences and underlying pathogenesis of myocardial ischaemia.

In order to successfully treat myocardial ischaemia using a gene transfer strategy, three important criteria must be met. A safe and efficient gene transfer system to deliver the therapeutic construct must be used. Appropriate gene(s) must be identified as targets for manipulation and crucially, the therapeutic gene(s) must be accurately regulated and controlled. In the case of myocardial ischaemia candidate genes should be accurately and efficiently delivered to the myocardium and only expressed during periods of ischaemia.

1.5.1 Gene Transfer Vectors

Delivering a therapeutic construct to the appropriate target cell, safely and efficiently, is the first critical step in gene therapy. A variety of viral and non-viral methods have been tested in the myocardium with varying degrees of success. Non-viral methods of gene

transfer are characterised by low transfection efficiency but with little or no immune response. Viral mediated gene transfer is characterised by high transduction efficiency but in many cases, significant host immune reactions. The genome of the recombinant viral vector is modified such that it cannot replicate in the host cell and contains the expression cassette carrying the cDNA of interest.

1.5.1a Non-Viral Mediated Gene Transfer

Direct DNA Injection

Direct injection of uncomplexed plasmid DNA into both skeletal muscle and the myocardium results in the reproducible expression of significant levels of functionally active recombinant protein (Buttrick et al., 1992; Lin et al., 1990; Wolff et al., 1990). Circular DNA has greater uptake efficiency than linearised DNA although only 60 -100 myocytes successfully express directly injected DNA (Wolff et al., 1990; Buttrick et al., 1992). The reason why circular DNA has greater uptake efficiency than linearised DNA is unclear. Transfected DNA is not integrated into the host genome but remains episomal and reporter gene expression has been reported up to 19 months after direct injection into mouse muscle (Wolff et al., 1990). Muscle tissue in general and the myocardium in particular appears to be very receptive to direct DNA injection (Wolff et al., 1990). The reason for this is also unclear, although it has been proposed that it may be due to the transverse tubule system which is unique to skeletal and heart muscle (Kitsis et al., 1991; Wolff et al., 1990).

Direct DNA injection has provided significant insights into cardiac-specific gene regulation and a number of cardiac specific enhancer regions have been identified (Prentice et al., 1994; Kitsis et al., 1991). Promoter inducibility by thyroid hormone and the antibiotic, tetracycline, has also been demonstrated *in vivo* using direct DNA injection (Fishman et al., 1994; Kitsis et al., 1991). Recently, Tsurumi et al (1996) demonstrated

improved collateral vessel development and tissue perfusion in an animal model of hindlimb ischaemia using direct injection of DNA encoding the angiogenic factor VEGF.

Liposome mediated Gene Transfer

Liposome mediated gene transfer systems are non-immunogenic, have low toxicity and are biodegradable. Repeated doses can therefore be administered without increasing toxicity. Liposome DNA complexes are characterised by low levels of transfection efficiency with transient expression and have therefore limited use in the heart. However, low levels of transgene expression can be detected in the heart after systemic administration of DNA complexed with cationic liposomes and protamine sulphate (Li and Huang, 1997).

Combining the DNA-liposome complex with the inactivated fusogenic virus, Haemeagglutinating Virus of Japan (HVJ), significantly increases gene transfer efficiency in the myocardium using either direct injection or coronary infusion (Aoki et al., 1997). Compared to direct injection of plasmid DNA, direct injection of the HVJ liposome complex exhibits significantly higher levels of reporter gene expression in the myocardium (Aoki et al., 1997).

1.5.1b Viral Mediated Gene Transfer

Herpes Simplex Virus

The herpes simplex virus (HSV) is a large DNA virus (152 kb), capable of infecting a variety of cell types with high efficiency (Huard et al., 1995; Coffin et al., 1996). The HSV genome remains episomal but forms a stable DNA structure making it suitable for long term expression (Coffin et al., 1996). HSV vectors have been proposed as the vector of choice for gene delivery into the nervous system as they can produce long term latent

infection of neurons (Coffin and Latchman, 1996). Nevertheless, direct injection of HSV vectors into the myocardium results in efficient gene delivery around the site of injection (Coffin et al., 1996).

Retrovirus

Retroviruses are RNA viruses that bind to specific receptors on the cell surface of replicating cells through glycoproteins on the surface of the retrovirus. The viral core is released into the cytoplasm following fusion of the cellular and virion membranes. The viral reverse transcriptase makes a double stranded DNA copy of the virion RNA, which is then randomly incorporated into the host genome (Gordon and Anderson, 1994). Retroviral integration can only occur in replicating cells. Cardiac myocytes, however, are terminally differentiated cells with little or no replicative potential and as such retroviral integration into the chromosome cannot occur (Watanabe et al., 1986). Nevertheless, infarcted myocardium contains replicating cardiac fibroblasts which have been successfully transduced using retroviral gene transfer methods (Prentice et al., 1996). Although retroviral integration provides stable long-term expression, the site of integration in the genome is essentially random. Accurate regulation of foreign gene products is critical if a true therapeutic benefit is to be achieved. With random integration, accurate, reproducible levels of foreign gene expression are not possible since different areas of the genome exert a different chromatin context on the inserted transcription unit. Likewise, random integration near an oncogene may result in the oncogene being inappropriately expressed and result in a malignant cell.

Adenovirus

Adenovirus is a 36 kb double stranded DNA virus and is only a minor pathogen in man (reviewed in (Horwitz, 1990; Ring, 1997)). It has a broad host range and can infect and direct high levels of protein expression in both proliferating and quiescent cells. The adenovirus genome is relatively easy to manipulate and can be grown to high titers (10^{11}-

10^{12} virus particles/ml) using current recombinant DNA techniques. Unlike retroviruses, the adenovirus genome remains episomal and is not integrated into the host genome. Random integration near an oncogene may result in the oncogene being inappropriately expressed and result in a malignant cell.

Adenoviral particles gain access to the cell by receptor-mediated endocytosis (Pastan et al., 1986; Greber et al., 1993). Fiber proteins on the surface of the adenovirus bind to an as yet uncharacterised cell surface receptor, become engulfed into clathrin-coated pits and internalised into an endosome. Acidification of endosomes containing adenovirus particles results in endosome disruption (Defer et al., 1990). Endosome disruption is a property of the viral capsid proteins and does not depend on viral gene expression (Pastan et al., 1986). Once released, virions proceed to the nucleus to complete their life cycle.

The adenovirus life cycle operates a cascade system of gene expression. Following infection, the pre-early gene E1a is expressed. The E1a gene products are regulatory proteins that provide functions required for the subsequent expression of the early genes, E1b, E2a, E2b, E3 and E4. The expression of the early genes occurs prior to DNA replication. Late gene expression occurs after DNA replication and is driven by the major late promoter, early activation of which is also controlled by E1a. The life cycle of adenovirus therefore depends on the E1a pre-early gene. Viruses with a deletion of their E1 sequence are unable to replicate in all cell types except those that provide E1 expression in trans. The E3 region encodes proteins that are not involved in viral replication (Kelly and Lewis, 1973). Deletion of both E1 and E3 regions of the adenovirus genome has allowed insertion of up to 8kb of a foreign DNA sequence. *In vivo*, foreign gene expression from adenoviral recombinants with intact E3 sequences lasts significantly longer than foreign gene expression from adenoviral recombinants with E3 sequences deleted (Poller et al., 1996). The E3 region is therefore thought to play a role in defending the vector-infected cells from host immune responses. Retaining the E3 region may therefore be beneficial for a gene therapy regime.

The ability to infect a wide range of cell types and direct high levels of protein expression in non-proliferating cells whilst remaining episomal makes adenovirus an ideal candidate for gene transfer in the myocardium. Indeed Barr et al (1994) reported high levels of recombinant gene expression in adult rabbits up to two weeks after intracoronary infusion. Functionally, coronary infusions of recombinant adenovirus encoding the angiogenic factor, FGF-5, significantly improves myocardial blood flow and function in a pig model of stress-induced myocardial ischaemia (Giordano et al., 1996).

Adenoviral vectors are not, however, without drawbacks. Many gene transfer protocols require long term expression of the transgene. Adenovirus directed transgene expression, however, is transient, lasting only for a few weeks (Gilgenkrantz et al., 1995). E1 deleted recombinants cannot replicate in non-permissive cell lines. However, co-infection of a non-permissive cell line with a wild type adenovirus would render the E1 deficient recombinant replication competent by 'donating' E1 functions to the E1 deleted recombinant. This would allow further infection of surrounding tissue and spread of the transgene. The use of adenoviral vectors is also limited by the antigenicity against viral proteins from both humoral and cytotoxic T-lymphocytes and possible toxicity at high doses. The host mediated immune response is also thought to be responsible for the transient expression of transgenes (Gilgenkrantz et al., 1995; Quinones et al., 1996). However, Muhlhauser et al (1996) studied the safety and efficacy of adenoviral gene transfer in the porcine heart and reported no significant immune response or ventricular dysfunction. Although adenovirus is only a minor pathogen in man, wild-type adenovirus infections have been associated with more serious diseases including myocarditis and pneumonitis (Henson and Mufson, 1971; Karjalainen et al., 1983).

Adeno-Associated Virus

Adeno-associated virus (AAV) is a non-pathogenic DNA virus with a number of unique characteristics that may make it useful as a gene transfer vector (Flotte and Carter, 1995; During, 1997). AAV has no known pathogenicity and a broad host cell range. It can

infect non-dividing cells with high efficiency and low immunogenicity. Wild-type AAV frequently integrate into chromosome 19 in a site specific manner and can persist in infected cells for at least 6 months. Rep-deleted AAV vectors integrate into the host genome in a less specific manner and may also exist in an episomal state. AAV vectors either injected into the wall of rat hearts or infused through a catheter in pig hearts results in significant foreign gene expression which persists for at least 6 months with no apparent toxicity or inflammation (Kaplitt et al., 1996).

In summary, a number of gene transfer approaches have been investigated in the heart. Adenovirus, retrovirus, herpes simplex virus, HVJ-liposome/DNA complexes and direct plasmid DNA injection have all demonstrated varying degrees of gene transfer efficiency. Of the gene transfer approaches used, adenovirus mediated gene transfer has shown significant promise. Adenovirus infects a wide range of non-dividing and dividing cell types with very high efficiency. The adenovirus genome is relatively easy to manipulate and can carry up to 8kb of foreign DNA. From a safety standpoint, adenovirus is only a minor pathogen in man and the adenovirus genome remains entirely episomal with very low probability of random integration into the host genome.

1.5.2 Molecular Targets for Therapeutic Intervention

Identifying appropriate genes as targets for manipulation is essential if a true therapeutic benefit is to be achieved. Knowledge of the key molecular pathways involved in ischaemia reperfusion injury is therefore crucial if the appropriate genes are to be identified. Within the ischaemic myocardial region there is a population of cells that are reversibly injured. Reperfusion within a specified period of time is capable of restoring the majority of the jeopardised cells to a normal status. This region of cells at risk of either reversible or irreversible damage can be described as the area at risk. Early reperfusion must be established if the number of cells progressing from being reversibly injured to irreversibly injured is to be reduced. However, reperfusion subjects the myocardium to oxidative stress from highly reactive oxygen free radicals that accumulated during the ischaemic episode (Shlafer et al., 1987; Kloner et al., 1989). This can lead to further cell damage and death. It is therefore also important to limit the cellular injury sustained during reperfusion.

1.5.2a Improving Vascular Supply to the Ischaemic Myocardium

Myocardial ischaemia occurs as a result of reduced coronary blood flow and leads to a pathological alteration of cardiac contractile, electrical or biochemical function (Hearse, 1994). Collateral circulation in the heart plays a critical role in determining the extent and severity of myocardial ischaemia (Cohen, 1995). Stimulating the growth of collateral blood vessels perfusing ischaemic regions of the heart offers the possibility of reducing the area at risk and limiting the extent of irreversible cellular damage that would otherwise occur.

Angiogenesis

Collateral vessel formation in the myocardium is controlled by a process termed angiogenesis. Angiogenesis is a cascade process beginning with enzymatic degradation of the basement membrane, followed by migration and proliferation of endothelial cells toward the angiogenic stimulus and cumulates in capillary morphogenesis and maturation (Folkman and Klagsbrun, 1987). A number of factors including, transforming growth factor-α (Schreiber et al., 1986), epidermal growth factor(Schreiber et al., 1986), angiogenin (Fett et al., 1985) and prostaglandin E_2 (Ziche et al., 1997), are able to promote angiogenesis *in vivo* but have no direct mitogenic effect on endothelial cells (Ferrara et al., 1992). In contrast, evidence has shown that platelet derived growth factor (PDGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF) and more recently vascular growth factor (VEGF) are able to stimulate both angiogenesis *in vivo* and endothelial growth *in vitro* and are referred to as directly acting angiogenic factors (Thomas et al., 1985; Miyazono et al., 1987; Ishikawa et al., 1989; Gospodarowicz et al., 1987; Ferrara et al., 1992)

VEGF as a candidate gene for gene transfer in the ischaemic myocardium

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a heat stable, 45 kDa homodimeric protein with structural similarity to PlGF (~ 53% amino acid identity) and more distant homology to the PDGF family (~18% to 20%, including all eight cysteines) (Maglione et al., 1991; Keck et al., 1989; Leung et al., 1989). VEGF expression *in vivo* has been detected in many cell types tested including, cardiac myocytes, smooth muscle cells and endothelial cells (Berse et al., 1992; Levy et al., 1995; Namiki et al., 1995; Stavri et al., 1995). VEGF has two crucial advantages over the other directly acting angiogenic factors that make it an ideal choice for revascularising the ischaemic heart. Unlike PDGF and the FGFs, which lack a signal peptide required for extracellular transport according to classic secretory pathways (von Heijne, 1986; Walter

and Blobel, 1981), the N-terminal sequence of VEGF is preceded by 26 amino acids corresponding to a typical signal sequence (Keck et al., 1989; Leung et al., 1989). Furthermore, PDGF and FGF are mitogenic for a variety of cell types, other than endothelial cells (Ross et al., 1986; Folkman and Klagsbrun, 1987) whereas VEGF is a potent mitogen (ED_{50} 2-10pM) for vascular endothelial cells but is devoid of mitogenic activity for most other cell types (Ferrara et al., 1992), the A375 M melanoma cell line being the only known exception (Liu et al., 1995).

The expression of the serine proteases, urokinase-type and tissue-type plasminogen activators (PA) are induced by VEGF in cultured bovine microvascular endothelial cells (Pepper et al., 1991). VEGF also induces the expression of metalloproteinase interstitial collagenase in human umbilical vein endothelial cells (Unemori et al., 1992). By co-inducing plasminogen activators and collagenase, VEGF promotes a prodegradative environment that facilitates migration of endothelial cells toward the angiogenic stimulus.

VEGF, as well as bFGF, induce coronary microvascular relaxations in a dose-dependent fashion. Indeed microvascular relaxations to VEGF and bFGF were significantly greater in vessels harvested from ischaemic myocardium that is dependent on collateral perfusion than the normal myocardium (Sellke et al., 1996). Conversly PDGF induces concentration-dependent contraction of strips of rat aorta (Berk et al., 1986).

Molecular and Biological Properties of VEGF

VEGF exists as one of at least four different molecular species, having 121, 165, 189 and 206 amino acids respectively. ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) (reviewed in (Houck et al., 1991; Ferrara et al., 1992)). The four isoforms arise through alternative splicing of RNA rather than transcription from separate genes. The organisation of the human VEGF gene is shown in figure 1.1. The VEGF gene consists of eight exons and

its coding region has been predicted to be approximately 14 kb. VEGF is secreted from a number of cell types, including cardiac myocytes, smooth muscle cells and a variety of tumour derived cell lines. $VEGF_{165}$ is the predominant isoform secreted, while $VEGF_{206}$ has only been detected in human fetal liver cDNA libraries.

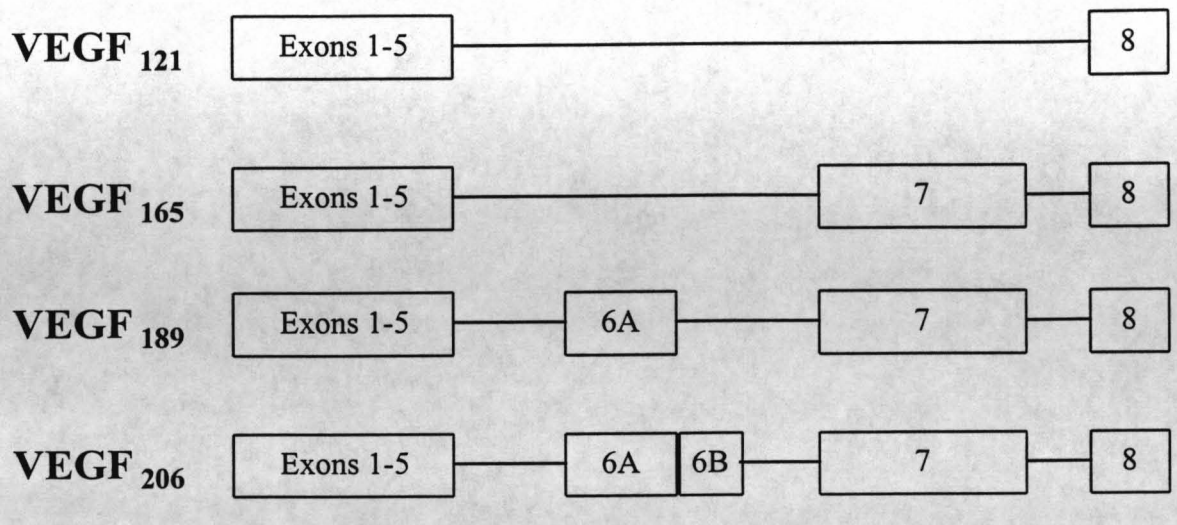

Figure 1.1 Exon-Intron splicing pattern of VEGF.

The VEGF isoforms have similar bioactivities but markedly different bioavailabilities. $VEGF_{121}$ is a weakly acidic protein that does not bind to heparin and is secreted as a freely soluble protein. $VEGF_{165}$ is a weakly basic protein with heparin binding properties that is also secreted but with up to 80% remaining bound to heparin sulphate containing proteoglycans in the extracellular matrix. $VEGF_{189}$ and $VEGF_{206}$ are highly basic proteins that bind heparin with high affinity due to the inclusion of exon 6A (see figure 1.1) which is highly enriched in basic residues. This results in these isoforms being almost completely sequestered by heparin sulphate containing proteoglycans in the extracellular matrix. Despite being tightly bound to heparin sulphate containing proteoglycans in the extracellular matrix, the longer VEGF isoforms, $VEGF_{189}$ and $VEGF_{206}$, can be released through protease activation and cleavage. VEGF is therefore

available to endothelial cells as either a freely soluble protein ($VEGF_{121}$ and $VEGF_{165}$) or following protease action ($VEGF_{189}$ and $VEGF_{206}$).

VEGF Receptors

Two tyrosine kinase receptors, fms-like tyrosine kinase-1 (flt-1) and kinase domain region (KDR) bind VEGF with high affinity (Kaipainen et al., 1993; Quinn et al., 1993; Terman et al., 1992). Flt-1 has slightly higher affinity (K_D ~10-20pM) for VEGF than KDR (K_D ~75pM). Both receptors, flt-1 and KDR, have a single hydrophobic leader peptide, a single transmembrane domain, seven immunoglobulin-like domains in the extracellular domain and a consensus tyrosine kinase sequence that is interrupted by a kinase insert domain. Analysis of flt-1 and KDR receptor expression *in vivo* indicates that expression is tightly restricted to endothelial cells (Jakeman et al., 1992; Vaisman et al., 1990). An alternatively spliced form of flt-1 was identified in human umbilical vein endothelial cells and is a secreted, soluble protein, lacking the seventh immunoglobulin domain, the cytoplasmic domain and the transmembrane sequence. This soluble flt-1 protein has been proposed as a physiological negative regulator of VEGF action, 'sequestering' free VEGF (Kendall and Thomas, 1993).

VEGF Regulation

The principal form of regulation of VEGF is hypoxia. VEGF expression is induced by hypoxia *in vitro* and by ischaemia *in vivo* (Hashimoto et al., 1994; Goldberg and Schneider, 1994; Banai et al., 1994; Banai et al., 1994; Shweiki et al., 1992). The hypoxic induction of VEGF involves both transcriptional activation and an increase in mRNA stability. Hypoxia induces VEGF steady state mRNA 25.0 ± 11.4 fold in rat primary cardiac myocytes and 12.0 ± 0.6 fold in rat pheochromocytoma PC12 cells (Levy et al., 1995b). The 5' promoter region of VEGF contains a 28bp element that has sequence and protein binding similarities to the HIF-1 binding site within the EPO 3'

enhancer element (Levy et al., 1995b). However, hypoxia only increases the rate of transcription of VEGF in PC12 cells 3.1 ± 0.4 fold, as demonstrated by nuclear run-off transcription assays (Levy et al., 1995b). Such a discrepancy suggests the role of post-transcriptional mechanisms in the hypoxic induction of VEGF expression. Measurements of VEGF mRNA half-life indicated a 2.5 ± 0.4 fold increase in mRNA stability in PC12 cells during hypoxia (Levy et al., 1996). The increase in VEGF steady state levels is therefore due to a combination of increased transcriptional activation and an increase in mRNA half-life.

Therapeutic Angiogenesis using VEGF

The rabbit ischaemic hind limb model is designed to simulate the ischaemia characteristics of patients with lower extremity arterial occlusive disease. Using this model, VEGF administered as recombinant protein significantly augments revascularisation and muscular function in the ischaemic limb (Walder et al., 1996). Recombinant VEGF infused in a porcine model of chronic ischaemia increased collateral flow to ischaemic areas of the myocardium, thereby reducing the area at risk from irreversible injury and improving cardiac function (Pearlman et al., 1995). These studies demonstrate that the angiogenic activity of VEGF is sufficiently potent to achieve a therapeutic benefit. The rabbit ischaemic hind limb model was also used by Tsurumi et al (1996) to show that direct intramuscular gene transfer of plasmid DNA encoding VEGF into the ischaemic hind limb successfully augments tissue reperfusion and collateral vessel development. Importantly, this study demonstrates the feasibility of using a VEGF expressing construct to elicit a therapeutic angiogenic response in ischaemic tissue. VEGF expression from a replication deficient recombinant adenovirus has also been demonstrated (Muhlhauser et al., 1995). Matrigel plugs containing recombinant VEGF adenovirus injected subcutaneously into mice elicit a potent angiogenic response and neovascularisation of surrounding tissue (Muhlhauser et al., 1995). The localised expression of VEGF recombinant protein from either direct DNA

injection or adenovirus-mediated gene transfer may limit serious side effects that may be associated with systemically administered VEGF protein. In particular, systemic VEGF administration may promote the growth and development of latent tumours (Plate et al., 1992; Kim et al., 1993) and proliferative retinopathy in diabetic patients (Aiello et al., 1994).

Initial studies in man using a VEGF based therapy for the treatment of a patient with an ischaemic right leg gave encouraging results (Isner et al., 1996). 2000μg plasmid DNA encoding human VEGF was applied to the hydrogel polymer coating of an angioplasty balloon and the balloon advanced into the ischaemic limb. The balloon was inflated and the plasmid DNA transferred to the distal popliteal artery. 12 weeks after the administration of plasmid DNA encoding human VEGF, there was an increase in the number of collateral vessels and increased resting and maximum blood flow to the ischaemic limb, demonstrating the therapeutic potential of VEGF based therapies in man.

1.5.2b Reducing cellular injury sustained during Ischaemia and Reperfusion

Early reperfusion of an ischaemic region is essential if the area at risk from irreversible cellular injury is to be reduced. However, the ischaemic region is still at risk during the reperfusion phase from a number of cellular processes resulting from the re-introduction of molecular oxygen and other circulating elements in the blood.

Antioxidant Enzymes

One such source of cellular injury arises from free radical mediated oxidation of membrane phospholipids, proteins and nucleic acids leading to functional alterations and structural changes within the myocardium. The myocardium has a number of antioxidant systems that are designed to metabolise free radicals. However, under conditions of

excessive free radical production, such as ischaemia/reperfusion, these systems become saturated and leave the myocardium exposed to free radical mediated oxidation. Hearts removed from transgenic lines overexpressing the antioxidant enzymes, superoxide dismutase or glutathione peroxidase, showed significant improvement in recovery after a short period of ischaemia, 7 minutes and 30 minutes respectively (Chen et al., 1996; Yoshida et al., 1996), indicating the potential of antioxidant enzymes at reducing ischaemia/reperfusion injury.

Heat Shock Proteins

A variety of cellular stresses, including ischaemia/reperfusion, result in the induction of a family of proteins called heat shock proteins (Lindquist, 1986; Ananthan et al., 1986; Plumier et al., 1996). This 'heat shock response' is highly conserved across species suggesting that these proteins serve a vital function within the cell (Donnelly et al., 1992; Currie et al., 1993). The HSP70 family consist of both inducible and constitutively expressed isoforms and function by binding to nascent and denatured proteins (Mestril and Dillmann, 1995). Hearts removed from transgenic mice overexpressing the inducible heat shock protein HSP70 show improved recovery of contractile force and contraction rate after a 30 minute period of global ischaemia and release lower levels of creatine kinase, an indicator of cellular injury (Marber et al., 1995; Plumier et al., 1995).

Adenosine

Adenosine has been proposed to act as a cardioprotectant in the ischaemic heart (Ely and Berne, 1992) through activation of the A_1 adenosine receptor (A1AR), in particular (Mizumura et al., 1996). A1AR activation reduces myocardial injury during global ischaemia and improves bioenergetic and mechanical recovery in reperfused myocardium (Zhi Qing Zha et al., 1993; Angello et al., 1991; Lasley et al., 1990). Evidence suggests, however, that the cardioprotectant effect of adenosine in the ischaemic myocardium may be limited by the number of A1ARs present (Rudolphi et al., 1989; Matherne et al.,

1997). Overexpression of the A1AR in transgenic mice significantly improves functional recovery during reperfusion after a period of global ischaemia (Matherne et al., 1997). A1AR overexpression using a gene transfer strategy may therefore be an ideal approach to increase the tolerance of the myocardium to the injury sustained during ischaemia and reperfusion.

Anti-Apoptotic Genes

Programmed cell death, or apoptosis, accounts for a significant proportion of myocyte cell death during ischaemia, and more importantly, reperfusion (Saraste et al., 1997; Gottlieb et al., 1994; Fliss and Gattinger, 1996). Bcl-2 is an oncogenic protein that acts by inhibiting programmed cell death through mechanisms that may involve its ability to function both as an ion channel and as an adaptor or docking protein (Reed, 1997). Overexpression of the anti-apoptotic gene Bcl-2 in the ischaemia/reperfused myocardium may therefore afford significant protection from the cell death associated with ischaemia reperfusion events.

In summary, a number of genes have been identified as possible targets for manipulation. Early reperfusion of the ischaemic myocardium is crucial if a true therapeutic benefit is to be achieved. One attractive possibility is to stimulate the growth of new collateral vessels in the heart, in order to improve collateral perfusion of an ischaemic area. Several studies have shown that the angiogenic activity of VEGF is potent enough to stimulate new blood vessel growth and functionally improve perfusion of ischaemic muscle (Tsurumi et al., 1996; Walder et al., 1996; Pearlman et al., 1995). A number of molecular targets have also been identified that may reduce the cellular injury sustained during ischaemia and reperfusion. In particular overexpression of genes encoding antioxidant enzymes, heat shock proteins, anti-apoptotic proteins and adenosine receptors may provide a significant defence against the myocardial damage sustained during ischaemia/reperfusion events.

Nevertheless, identifying appropriate molecular targets and designing a safe, efficient gene transfer system is not sufficient to ensure a true therapeutic benefit. Regulation of potential therapeutic genes is absolutely essential if potentially serious side effects are to be avoided. In the case of VEGF, unregulated production of such a potent angiogenic factor may lead to the growth and development of latent tumours and proliferative retinopathy in diabetic patients (Aiello et al., 1994; Plate et al., 1992; Kim et al., 1993).

1.5.3 Transgene Regulation

Accurate regulation of transferred genes is central to the success of any gene therapy protocol. Expressing transferred genes in the wrong context will have unknown but finite potential side effects. Furthermore, ischaemic heart disease does not involve a single ischaemic episode followed by reperfusion but often involves regional cycles of ischaemia punctuated by normal cardiac function. Optimal treatment would therefore require the expression of the cardioprotective agent(s) to be restricted to the ischaemic region, during the ischaemic episode and to remain silent at other times.

The use of tissue specific promoters, such as α-myosin heavy chain (Molkentin et al., 1994), cardiac actin (Biben et al., 1996; Ching Yi Chen et al., 1996) or troponin C (Prentice et al., 1994), would provide an important degree of regulation. However, these promoters direct constitutive expression that is independent of the pathophysiological environment. Including externally regulatable promoter elements within a tissue specific promoter would convey further control of the transferred gene(s). The rat α-myosin heavy chain promoter is cardiac specific and is positively regulated by thyroid hormone

in vivo (Kitsis et al., 1991). Using this promoter construct, the expression of transferred genes can be directly modulated by varying the thyroid hormone levels. In a clinical situation, however, altering thyroid hormone levels, may have detrimental side effects.

A possible alternative is to regulate foreign gene expression by modulating the concentration of an 'activator' compound or drug in the blood. The antibiotic tetracycline has shown potential in this area. Intra-cardiac injections of luciferase constructs under the control of a tetracycline responsive promoter have been shown to be rapidly and reversibly controlled by manipulating antibiotic administration (Gossen and Bujard, 1992; Fishman et al., 1994). This system has the advantage over hormonal modulation of transgenes in that tetracycline is a commonly used antibiotic with relatively minor side effects. Transgene expression can also be regulated using metal-responsive enhancers. The expression of a TGFβ-1 transgene in an intracardiac graft, driven by an inducible metallothionein promoter, was shown to be modulated by altering dietary zinc in mice (Gou Young Koh et al., 1995). Manipulating foreign gene expression by altering circulating levels of antibiotic or metal ion concentration allow foreign genes to be independently and reversibly regulated. However, in the clinical situation, a pathological state can often only be treated once the clinical symptoms appear. The progress of the pathological state would also have to be carefully monitored to allow antibiotic or metal ion levels to be altered if necessary.

Ideally, enhancer elements incorporated into tissue-specific promoters should respond directly to the pathophysiological state that the therapeutic construct is targeted to and before clinical symptoms appear. In the case of myocardial ischaemia, therapeutic genes driven by tissue-specific promoters with enhancer elements responsive to one or more components of ischaemia would be the ultimate goal. In this respect promoters responsive to hypoxia, hypoglycaemia, redox stress or components of a hypertrophic response, show promise for incorporation into regulated therapeutic vectors.

Finally, inducible promoter systems must be have no or very low basal activity under normal physiological conditions. Promoter systems that are highly induced but also have relatively high basal activity may potentially lead to serious side effects. It is also important that inducible promoter systems can return to very low basal activity as quickly as they can be activated. Prolonged expression of a foreign gene long after the pathological state has receded may also lead to potentially serious side effects.

1.6 Specific Aims

The long term goal of this work is to design a safe efficient gene transfer system to deliver an accurately regulated foreign gene targeted to the ischaemic myocardium. The specific aims of this project are firstly to investigate the ability of promoter enhancer constructs to respond to a transient period of myocardial ischaemia and secondly to construct an adenoviral recombinant expressing the angiogenic factor, VEGF.

The ability of a hybrid promoter, containing tissue specific basal regulatory sequences plus four copies of the erythropoietin HIF-1 binding site, to respond to 15 minutes of ischaemia followed by reperfusion will be investigated. The transcriptional regulation of the human skeletal α-actin gene will also be investigated with a view to identifying regulatory domains within the promoter that respond to 15 minutes of ischaemia followed by reperfusion. Finally, a functional replication-deficient adenoviral recombinant, expressing the angiogenic factor VEGF, will be constructed.

Chapter 2

MATERIALS & METHODS

2.1 Manipulation of Bacterial cultures

Preparation of Competent Cells

Glycerol stocks of the *E. Coli* strain DH5α were used to inoculate 10ml L-broth (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 171mM NaCl) and incubated overnight at 37°C with orbital shaking. 1ml of this overnight culture was used to inoculate 50ml of L-broth and incubated at 37°C for 2 hours with orbital shaking. When the optical density of the inoculated L-broth increased to 0.3, at a wavelength of 600nm, the culture was placed on ice for 10 minutes. The cells were then pelleted by centrifugation at 3000rpm for 15 minutes at 4°C. The cell pellet was gently resuspended in 10ml transformation buffer (TFB) (30mM KAc, 50mM $MnCl_2$, 100mM RbCl, 15% glycerol, 10mM $CaCl_2$) and incubated on ice for 20 minutes. The cells were pelleted as described above and gently resuspended in 2ml TFB. The cells were then aliquoted and snap frozen on dry ice and stored at -70°C until required for transformations.

Streaking Agar Plates

L-Agar (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 171mM NaCl, 1.5% w/v bacto-agar, 950ml water) plates were prepared by warming the agar until molten then allowed to cool and ampicillin added to a final concentration of 10mg/ml. Molten agar containing 10μg/ml ampicillin, for selection of clones containing plasmids with ampicillin resistance genes, was then poured into 100mm petri dishes and allowed to set. Sterile inoculating loops were used to take a sample of the bacterial culture and then streaked across the agar. Loops were re-sterilised by flaming, cooled and used to streak the previously streaked bacterial culture perpendicular to the original direction in order that single colonies were clearly visible. This process was repeated once more. Plates were then incubated overnight at 37°C.

2.2 Nucleic acids

2.2.1 DNA Isolation

Small Scale DNA Purification

Wizard miniprep DNA purification system (Promega, USA) or Qiagen plasmid mini-kits (Qiagen, USA) were used to isolate small quantities (3-5μg) of plasmid DNA. Kits were used as manufacturer's recommendations.

Briefly, 1.5ml bacterial cultures were pelleted and lysed by alkaline lysis procedures. Protein and genomic DNA precipitate was pelleted by centrifugation and the supernatant added to a spin filter containing silica gel matrix. The spin filter was centrifuged for 1 minute and the flow through discarded. Plasmid DNA was eluted off the silica gel matrix by addition of water or TE and the DNA recovered by centrifugation.

Large Scale DNA Purification

- Caesium Chloride Gradients

Large scale DNA isolation using caesium gradients was performed based on the method described by Sambrook, Fritsch and Maniatis (1989).

5ml LB broth containing 100μg/ml ampicillin was inoculated using a single colony picked from a freshly streaked plate and incubated overnight, with shaking, at 37°C. The saturated 5ml culture was then used to inoculate 600ml LB broth, containing 100μl/ml ampicillin, and incubated overnight, with shaking, at 37°C. Saturated cultures were pelleted by centrifugation at 5000g for 5 minutes.

Alkaline lysis was carried out as follows. The pellet was resuspended in 18ml solution 1 (50mM glucose, 10mM EDTA, 25mM Tris-HCl (pH 8)). The cells were lysed by adding 2ml freshly prepared 10mg/ml lysosyme in 10mM Tris-HCl (pH 8) to the resuspended pellet and mixed thoroughly. The cells were then denatured with 40ml solution 2 (0.2M NaOH, 1% SDS), the mixture gently shaken, and placed on ice for 10 minutes. DNA was precipitated and the solutions pH neutralised by adding 30ml ice cold solution 3 (3M potassium acetate, 5M acetic acid, pH 5.4). The mixture was then gently shaken, and placed on ice for 15 minutes. Precipitated proteins and genomic DNA was pelleted by centrifugation at 10000rpm for 15 minutes without braking at 4°C.

The DNA was precipitated by the following procedure. The supernatant was carefully poured through sterile gauze into a fresh centrifuge flask and the DNA precipitated by adding 75ml (0.7-1.0 x volume) isopropanol. The DNA was allowed to precipitate for 15-30 minutes at room temperature and then pelleted by centrifugation at 10000g for 20 minutes. The supernatant was carefully poured off and the pellet rinsed with 70% ethanol and centrifuged at 10000g for 5 minutes. The 70% ethanol was carefully removed and the pellet allowed to dry for 5-10 minutes. The DNA pellet was then resuspended in 6ml sterile water and once resuspended the volume was made up to exactly 7ml. 7.3g caesium chloride was added and placed in a 30°C water bath to aid dissolving. 560µL of 10mg/ml ethidium bromide was then added and the entire solution transferred to quick seal tubes (Beckman, USA). Tubes were topped up using mineral oil, sealed and centrifuged at 49000 rpm for 16 hours at 20°C in a Beckman L8-55 ultracentrifuge and Ti 70 fixed angle rotor.

Caesium bands were removed as follows. The sealed tubes were clamped in a clamp stand, above a UV transluminator, to allow visualisation of the DNA bands. A 21 gauge needle was inserted into the top of the tube and then an 18 gauge needle inserted immediately below the upper DNA band (nicked circular or linear DNA). A syringe was attached to the 18 gauge needle and the upper DNA band drawn off. A second 18 gauge

needle was then inserted immediately below the lower DNA band (closed circular plasmid DNA), a syringe attached, and the lower DNA band drawn off.

Ethidium bromide and caesium chloride were removed as follows. To the solution of DNA, an equal volume of water saturated butan-1-ol was added and the two phases mixed by vortexing. The two phases were allowed to settle and then the upper pink layer containing ethidium bromide was removed and discarded. This extraction procedure was repeated until all the pink colour had completely disappeared from both the aqueous and organic layers. DNA was precipitated out from the caesium chloride solution by firstly diluting the DNA/caesium solution with 3 volumes of water and then precipitating the DNA by adding a further 2 volumes of 100% ethanol for 15 minutes at 4°C followed by centrifugation at 12000g for 20 minutes at 4°C. The DNA pellet was rinsed with 70% ethanol and centrifuged at 12000g for 15 minutes at 4°C. The 70% ethanol was carefully decanted off and the pellet allowed to air-dry at room temperature for 10-15 minutes. The DNA pellet was resuspended in TE buffer (10mM Tris, 1mM EDTA, pH 7.5-8.0).

To ensure complete removal of trace levels of caesium, the DNA solution was subjected to dialysis. The DNA was placed into a dialysis bag(Sartorius, USA), suspended in a beaker containing 5 litres of TE (10mM Tris, 1mM EDTA) at 4°C, with constant stirring. The TE was replaced with fresh TE every hour for 5-6 hours. The DNA was then precipitated with one tenth volume of 3M sodium acetate (pH 5.5) and 2.5-3 volumes of ice cold 100% ethanol. The DNA was pelleted by centrifugation at 15000 rpm for 15 minutes at 4°C. The pellet was rinsed with 70% ethanol and re-centrifuged for 15 minutes at 15000 rpm. The 70% ethanol was carefully removed and the pellet allowed to air-dry for 10-15 minutes. The DNA pellet was then resuspended in phosphate buffered saline (PBS) (Gibco, USA) for use in *in vivo* injection experiments.

Plasmid quality and degree of supercoiling was confirmed by 1% agarose gel electrophoreisis and the DNA concentration calculated using OD_{260} measurements.

- Qiagen Maxi Kits

Large scale DNA isolation (300-500μg plasmid DNA) was performed using Qiagen maxi DNA purification kits (Qiagen, USA) and used according to manufacturer's instructions.

Briefly, bacterial cultures were lysed using alkaline lysis procedures. Protein and genomic DNA precipitate was pelleted by centrifugation and the supernatant carefully decanted into an equilibrated Qiagen filter. The supernatant solution was allowed to pass through the filter and the flow through discarded. Plasmid DNA was eluted off the filter and collected in a fresh tube. Purified, supercoiled plasmid DNA was then precipitated out of solution, pelleted by centrifugation and resuspended in PBS buffer for use in *in vivo* injection experiments.

2.2.2 DNA Manipulation

Ethanol Precipitation

DNA solutions were precipitated by adding one-tenth volume 3M sodium acetate (pH 5.4) and 2.5-3 volumes ice cold 100% ethanol. Precipitated DNA was pelleted by centrifugation at 12000g for 15 minutes. Pellets were carefully rinsed with 70% ethanol, and re-centrifuged at 12000g for 5 minutes. The 70% ethanol was carefully removed and the DNA pellet allowed to air dry for 5-10 minutes before resuspension in an appropriate volume of water or TE.

Nucleic Acid Quantitation

DNA was diluted 1 in 200 or 1 in 500 in an appropriate buffer. The optical density measured at 260nm and 280nm. A concentration of 50μg/ml of double stranded DNA corresponds to a 260nm absorbance of 1. The purity of the DNA solution in terms of protein contamination was estimated by calculating the 260 : 280nm absorbance ratio. Highly purified DNA has a ratio of 1.8 (Sambrook et al., 1989).

RNA solutions were diluted in either DEPC-treated water or TE buffer made using DEPC-treated water and the optical density measured at 260nm and 280nm. A RNA concentration of 40μg/ml of RNA corresponds to a 260nm absorbance of 1. The purity of the RNA solution in terms of protein contamination was estimated by calculating the 260 : 280nm absorbance ratio. Pure RNA has a ratio of 2.0 (Sambrook et al., 1989).

DNA Purification

Amicon Microcon filters

Plasmid and cDNA was purified from contaminating proteins and oligonucleotides using microcon-EZ (Amicon, USA) spin filters as recommended by manufacturer's guidelines. Briefly, samples were loaded into the sample loading well and centrifuged at 12000g for 10 minutes at room temperature. The flow through was discarded and the filter unit inverted and placed in a fresh tube. The DNA was recovered by centrifugation at 12000g for 30 seconds to 1 minute.

Phenol/Chloroform Extraction

Phenol/chloroform extractions were performed to remove contaminating proteins and high molecular weight DNA. An equal volume of 1:1 phenol/chloroform:IAA(24:1) was

added to DNA solutions, vortexed and the organic and aqueous phases separated by centrifugation at 12000g for 5 minutes. The upper aqueous phase containing the DNA was carefully drawn off and placed in a fresh tube.

Agarose Gel Electrophoreisis

0.6-2% agarose gels were prepared for nucleic acid separation according to size. Small molecular weight fragments (<400bp) were separated on high percentage gels, whereas high molecular weight fragments (>3kb) were separated on low percentage gels. All DNA agarose gels were run in TBE buffer (89mM Tris, 89mM Boric acid, 2mM EDTA) at 25-100V. Agarose gels contained ethidium bromide at a concentration of 0.5μg/ml. One tenth volume of sample loading buffer, consisting of 0.25% bromophenol blue, 40% (w/v) sucrose in water (10x buffer), was added to DNA solutions. 1kb size markers from Gibco-BRL were included in all gels.

2.2.3 Polymerase Chain Reaction (PCR)

Unless otherwise indicated, PCR experiments were carried out using a Hybaid 'Omnigene' thermal cycler. Reaction mixtures were made to a final volume of 50μl and contained amplification buffer (50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 15mM $MgCl_2$), 200μM of each dNTP, 1μM of each oligonucleotide primer, 0.5-1 unit Taq DNA polymerase (Gibco-BRL) and 10-50ng of DNA template.

Unless otherwise stated, all oligonucleotides (purchased from Genosys) were deprotected and desalted and, upon arrival, resuspended in sterile water to the required concentration

2.2.4 DNA Cloning

Restriction Digests

Restriction digests were carried out using Gibco-BRL restriction enzymes and appropriate REACT buffers. 1μg DNA, 2μl 10x REACT buffer, 5-10 units restriction enzyme(s). Reactions were made up to 20μl using sterile water, mixed carefully and incubated at 37°C for 1-2 hours. Volumes were scaled up for large scale digests.

DNA Ligations

Unless otherwise stated, ligation reactions were set up as a 3:1 molar ratio of insert to vector in order to favour insertion of the insert fragment into the vector. 10μl reactions were set up containing 1μl 10X ligase buffer (500mM Tris-HCl pH 7.6, 100mM $MgCl_2$, 100mM DTT, 500mg/ml BSA) and 2-3 units T4 ligase. Reactions were incubated at 16°C overnight. Control ligations were set up using linearised vector alone.

T-vector cloning (Novagen) was carried out according to manufacturer's instructions. The pT7Blue vector system is designed to take advantage of the single 3' A-nucleotide overhang that polymerases, such as Taq polymerase, leave on the PCR product (Clark, 1988). These products can be directly ligated to a vector containing compatible single T-nucleotide overhangs (Marchuk et al., 1991). The pT7Blue vector is prepared for T-cloning by digestion with EcoRV followed by the addition of single 3' dT residues at each end and is supplied in this form ready to ligate with any DNA amplified by Taq.

Transformation of bacterial cells

50-100 ng plasmid DNA or 5-10μl DNA ligation mixture was added to 50μl freshly thawed DH5α competent cells and left on ice for 15-30 minutes. Cells were heat shocked at 42°C for 1.5-2 minutes and immediately returned to ice. 500μl L-broth was added and the reaction incubated at 37°C for 30-60 minutes. Cells were then pelleted by centrifugation at 6000g for 2 minutes and the supernatant carefully removed. The pelleted cells were resuspended in 100μl L-broth, spread onto agar plates containing 10μg/ml ampicillin, inverted and incubated overnight at 37°C. Colonies were visible the following morning.

Single Colony PCR Analysis

Single colony PCR analysis was used to screen for putative ligation recombinants. Single colonies picked from agar plates were used to innoculate 30μl sterile water. The solution was heated to 99°C in a heating block for 5 minutes to lyse the cells, then immediately placed on ice. Cell debris was pelleted by centrifugation at 12000g for 2 minutes. 10μl of supernatant was used as template for PCR reactions. The PCR reactions were performed using primer sequences that were insert specific.

2.2.5 DNA Sequencing

Cycle Sequencing Reactions

All sequencing was performed using Thermo Sequenase (Amersham) cycle sequencing kit and used as the manufacturer recommends. Briefly, a reaction mixture was prepared containing 2μl reaction buffer, 50-500ng DNA, 0.5-2.5 pmol primer and 8 units thermo sequenase DNA polymerase and made up to 20μl with sterile water. 4 termination

reactions were prepared containing 2µl termination master mix and 0.5µl of each labeled ddNTP. The two reaction mixtures were carefully mixed together and subjected to 30-60 cycles of : denaturation, 95°C for 30 seconds, annealing, 55°C for 30 seconds and extension, 72°C for 1 minute. 4µl stop solution was then added to terminate the reaction.

Samples were denatured to 70°C for 5 minutes immediately before loading onto a vertical 6% acrylamide (19:1 acrylamide:bis-acrylamide), 7M urea, 1 x TBE, gel cast in a Bio-Rad sequencing apparatus. Sequencing gels were run at 1600V, 50W and the temperature maintained at 50°C. Gels were transferred onto Whatmann 3MM paper, wrapped in cling film, and dried in a vacuum drier. Dried gels were then exposed to film overnight and developed in an X-OGRAPH Compact X2 film developer.

2.2.6 RNA Isolation

All glassware for handling RNA was treated with diethylamine pyrocarbonate (DEPC) as a 0.1% v/v solution to minimise degradation of RNA due to endogenous or contaminating RNases, then autoclaved to remove traces of DEPC. Gloves were worn and freshly sterilised tips and polypropylene tubes were used at all times to avoid RNase contamination.

RNA isolation was carried out using RNAzol B according to manufacturer's guidelines.

Tissue, snap frozen in liquid nitrogen, was ground up to a fine powder using a mortar and pestel, sitting on dry ice. The mortar and pestel were removed from the dry ice and 2ml RNAzol B was added per 100mg of tissue. The homogenate was then transfered to a sterile eppendorf and 0.2ml chloroform added per 2ml of homogenate.

RNA isolation from cells in culture involved removing culture media then adding 1ml RNAzol B per 10^6 cells, as recommended by manufacturer. The lysate was then transferred to a sterile eppendorf and 0.1ml chloroform added per 1ml of lysate.

Tubes containing RNA isolated from either tissue or cells in culture were shaken vigorously for 15 seconds, put on ice for 5 minutes, and then centrifuged at 12 000g for 15 minutes at 4°C. The upper aqueous phase was then transferred to a fresh tube and an equal volume of isopropanol added. The samples were stored on ice for a further 15 minutes then centrifuged at 12 000g for 15 minutes at 4°C. The supernatant was removed and the RNA pellet was washed once with 0.8ml 75% ethanol by vortexing and subsequent centrifugation for 8 minutes at 7500g at 4°C. The 75% ethanol was carefully removed and the pellet allowed to dry for 5-10 minutes before resuspending the RNA pellet in a suitable volume of DEPC-treated water. Aliquots were removed for optical density measurements and agarose gel electrophoresis. Yields of total cellular RNA were about 20μg per 2 x 10^6 cells, quantified as described in section 2.2.2.

In order to determine the integrity of the RNA isolated, ethidium bromide staining of the RNA separated electrophoretically on an agarose gel revealed the ratio of 28s and 18s RNAs was approximately constant (Dolnick and Pink, 1983)

2.3 Gene Expression Analysis

2.3.1 cDNA Synthesis for Reverse Transcription-PCR

cDNA synthesis was performed using a kit (Clontech Advantage RT-for-PCR). Manufacturer's recommendations were followed throughout.

Briefly, 1μg total RNA isolated, from rat neonatal isolated cardiac myocytes or rat neonatal hearts, was made up to 12.5μl using DEPC-treated water. 1 μl oligo dT primer was added and the mixture denatured to 70°C for 2 minutes, then quenched rapidly on ice. 4μl 5X reaction buffer, 1μl dNTP mix (10mM each), 0.5μl RNAse inhibitor and 1μl MMLV reverse transcriptase were added to the RNA/primer mixture. The reaction was incubated at 42°C for 1 hour and then heated to 94°C for 5 minutes to stop the reaction. The reaction was then diluted to 100μl by adding 80μl DEPC-treated water. 10μl of the diluted cDNA was typically used as template for each 50μl PCR reaction.

2.3.2 CAT assay

400μl rat or rabbit heart homogenate supernatant was incubated at 65°C for 10 minutes to inactivate endogenous deacetylase activity. The cell debris was pelleted by centrifugation of samples at 12000g for 2 minutes. 300μl supernatant was removed and 49μl 250mM Tris-HCl (pH 8), 15μl 5mg/ml Acetyl Coenzyme A and 9μl ^{14}C-chloramphenicol (activity 0.925 MBq) added. Negative controls were prepared by replacing the heart homogenate supernatant with sterile water. Positive controls were prepared by replacing the heart homogenate supernatant with sterile water containing 0.01 units chloramphenicol acetyl transferase enzyme (Promega). All samples were incubated overnight at 37°C.

300µl of mixed xylenes was added to each sample to terminate the reaction and the samples vortexed for 30 seconds. Samples were then centrifuged at 12000g for 2 minutes and the upper xylene phase carefully removed and transferred to a fresh tube. 100µl 250mM Tris-HCl (pH 8) was added to each sample which was then vortexed and centrifuged at 12000g for 2 minutes. 200µl of the xylene upper phase was carefully removed and added to 5ml scintillant fluid for analysis of ^{14}C-chloramphenicol conversion on a Packard Tri-Carb 2100TR scintillation counter for 5 minutes. CAT activity was measured as counts per minute (cpm) above background, representing the conversion of ^{14}C-chloramphenicol to its acetylated product by chloramphenicol acetyl transferase.

2.3.3 Luciferase assay

10µl triton X-100 (10%) was added to 90µl of heart homogenate supernatant and mixed by vortexing. A 20µl aliquot was removed and 100µl luciferase assay substrate added (Promega). Promega's luciferase assay system allows for greater enzymatic turnover of luciferase, resulting in greater light intensity that is nearly constant for measurements of up to several minutes. Luciferase enzyme activity was measured by counting on a luminometer (Turner Designs). Luciferase activity was measured in relative light units (RLU), representing the oxidation of luciferin by luciferase with concomitant production of a photon.

2.3.4 Western Blot Analysis of Proteins

Preparation of Protein Lysates from Cell Cultures

Media was removed and cells washed twice with phosphate buffered saline (PBS). Cells were then scraped into 4ml PBS, pelleted by centrifugation at 1000g for 10 minutes and resuspended in 750µl water. 6.25µl 2% (w/v) deoxycholic acid and 250µl 24% (w/v) trichloroacetic acid was added to the resuspended cells and incubated on ice for 15 minutes. Samples were centrifuged at 12000g for 5 minutes, the supernatant removed and the pellet resuspended in 75µl 1M Tris base. 15µl was removed and the protein concentration determined using a BioRad protein assay kit as manufacturer's recommendations. To the remaining volume of sample, an equal volume of SDS-sample buffer (5.6ml water, 138µl 2-mercaptoethanol, 1ml glycerol, 250µl 1M Tris-HCl pH 6.8, 1ml 2% (w/v) bromophenol blue, 2ml 10% (w/v) SDS) was added. Samples were boiled for 2 minutes and then placed on ice immediately prior to loading onto an SDS-PAGE gel.

SDS-PAGE gel electrophoreisis (stacking gel method)

The SDS-PAGE gel consisted of a 10% resolving gel (pH 8.8) and a 5% stacking gel (pH 6.8). The 10% resolving gel consisted of 8.5ml buffer 1 (18.17g Tris base, 4ml 10% SDS in 100ml, pH 8.8), 14.4ml water, 8.5ml 40% acrylamide, 2.26ml 50% glycerol. 254µl 10% ammonium persulphate and 23µl TEMED were added last. The 5% stacking gel consisted of 5.6ml buffer 2 (6g Tris base, 4ml 10% SDS in 100ml, pH 6.8), 15ml water, 1.7ml 40% acrylamide. Finally 223µl 10% ammonium persulphate and 12µl TEMED were added last.

An aliquot of protein sample containing 10µg protein in 15-35µl loading buffer was loaded onto the gel and run in running buffer (3g Tris base, 14.4g glycine, 10ml 10%

SDS made up to 1 litre with water) at 40V overnight at room temperature. Once the dye front had run off the bottom of the gel, the apparatus was dismantled and the gel carefully removed for blotting.

Western Blotting and Protein Detection

After the protein samples were separated on an SDS-PAGE gel, the proteins on the gel were carefully transferred onto probind 45 membrane roll (Pharmacia Biotech cat no. 80-1247-86) at 1 amp per hour in transfer buffer (72g glycine, 15g Tris base, 1 litre methanol, made up to 5 litres with water) using a Hoeffer electroblotter at 4°C. The apparatus was carefully dismantled and the membrane 'blocked' to aid reduction of background signal by submerging in TBS (29.22g NaCl, 10ml 2M Tris pH 7.5, made up to 1 litre with water) containing 5% non fat milk (NFM) for 2 hours at room temperature on a rocking platform. The membrane was then removed and washed three times with TBS. 100µl primary antibody (rabbit anti-human VEGF with rat VEGF cross reactivity) was added to 10ml TBS containing 1% NFM and incubated with the membrane overnight at 4°C on a rocking platform. The membrane was removed, washed three times in TBS/0.1% tween and then three times in TBS. 5µl Goat anti-rabbit horseradish peroxidase conjugate secondary antibody in 25ml 1% NFM (1:5000 dilution) was then incubated with the membrane for 1 hour at room temp. The membrane was again removed, washed three times with TBS/0.1% tween and six times with TBS.

Proteins bound to antibody were detected using the ECL detection kit (Amersham) according to manufacturer's instructions. Equal volumes of 'detection reagents' 1 and 2 were mixed and carefully added to a container containing the nitrocellulose membrane. Horse radish peroxidase catalysed oxidation of luminol results in the emission of light which subsequent exposure of the membrane to autoradiography film highlights the appropriate band. After exposure, the membranes were then wrapped in cling film and exposed to photographic film for varying times.

2.4 Tissue Culture

2.4.1 Maintenance of 293 cell line

293 cells were grown in 75ml plastic falcon flasks (Nunc), maintained in modified eagles medium (MEM) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine and penicillin/streptomycin (100μg/ml-100IU/ml) and incubated at 37°C with 5% CO_2.

Cells were maintained in sub-confluent conditions (10^6 cells/25ml media) by sub-culturing every 3 days. For subculturing, growth medium was removed and the cells washed twice with citric saline (134mM potassium chloride, 150mM sodium citrate in sterile tissue culture grade water). Cells were incubated at 37°C for 5 minutes or until the cells freely detached from the surface of the culture vessel upon knocking the vessel against the palm of the hand. Cells were then resuspended in an appropriate volume of growth medium and transferred to fresh culture vessels.

2.4.2 Maintenance of NIH 3T3 cell line

The NIH 3T3 cell line is a murine fibroblast cell line which is noted for its ease of transfection. NIH 3T3 cells were maintained in MEM supplemented with 5% FCS, 2mM L-glutamine and penicillin/streptomycin (100μg/ml-100IU/ml) and incubated at 37°C with 5%CO_2.

At 90% confluency, cells were passaged. Growth medium was removed, cells washed twice with PBS (serum in the media inhibits the action of trypsin) and trypsin solution (0.25% trypsin, 0.002% EDTA in Dulbeccos buffered saline) gently layered over the cells. Cells were incubated for 5 minutes at 37°C with 5% CO_2. 4ml PBS was then added and the cells scraped into the PBS. Cells were pelleted by centrifugation on a bench centrifuge at 1000g for 10 minutes. The supernatant was removed, the pelleted

cells resuspended in fresh NIH 3T3 media and decanted into the required number of fresh dishes.

2.4.3 Calcium Phosphate Transfection

5μg pJM17 adenoviral genome plasmid and 5μg pCA13/VEGF shuttle plasmid prepared as described in section 2.2.1, were mixed and the volume raised to 225μl with sterile tissue culture grade water. 25μl 2.5M $CaCl_2$ was added and mixed carefully. 250μl, 2 x HBS (42mM HEPES, 0.274mM NaCl, 10mM KCl, 1.4mM Na_2HPO_4, 11mM glucose, pH 7.1) was then added to the DNA/$CaCl_2$ solution with low speed vortexing and the calcium phosphate / DNA precipitate allowed to form at room temperature for 45 minutes. The DNA solution was then carefully added to 293 cells (~80% confluent) in a 60mm petri dish containing 5ml MEM supplemented with 5% FCS, 1mM glutamine and penicillin/streptomycin (100μg/ml-100IU/ml). The cells were incubated with the calcium phosphate transfection mix for 6-16 hours at 37°C with 5% CO_2.

2.4.4 Agar Overlay

Agar overlays were performed when pure populations of adenoviral recombinants were required. Culture media containing the calcium phosphate transfection mix was completely removed. 2.2% w/v low melting point agarose (Sigma CAT. A-4018) was melted in a microwave then allowed to cool until hand hot. 10ml 2 x BME (Gibco CAT. 21017), 1ml FCS and 200μl penicillin/streptomycin (100μg/ml-100IU/ml) were then mixed, warmed to 37°C, and added to 10ml warm agarose. 4ml of agarose/media mix was then overlayed per 60mm dish. Further agarose overlays were performed 4, 8 and 12 days after transfection to provide the cells with fresh culture media.

2.4.5 Plaque isolation and Amplification

12-15 days after transfection, areas of cells exhibiting a cytopathic effect (cpe) (a hole in the cell sheet bordered with cells with a rounded morphology) were identified. A plugged pasteur pipette was used to isolate the virus released by lysed cells within the vicinity of the cpe, and agar immediately above the cells. This was then placed in 500µl PBS and vortexed.

Media was removed from 293 cells, ~80% confluent, and 100µl of the above supernatant added evenly to the surface of the cells. Viral particles were allowed to infect 293 cells for 45 minutes at 37°C with 5% CO_2. Fresh 293 media (see section 2.3.1) was then added and the cells incubated at 37°C with 5% CO_2 for 4-5 days. Cells infected with a functional recombinant adenovirus showed signs of a cpe after 3-4 days.

2.4.6 Viral Lysate isolation

293 cells showing evidence of a cpe were harvested and the viral lysates isolated as follows. Cells were scraped into the culture media and then pelleted by centrifugation at 1000g for 10 minutes in a bench top centrifuge. Pelleted cells were then resuspended in 800µl tris-saline and subjected to three freeze thaw cycles. Freeze thaw cycles involved placing the eppendorf containing the resuspended solution in dry ice for 10 minutes followed by a 37°C water bath for 10 minutes. Cell debris was pelleted by centrifugation at 7000g for 5 minutes, and the supernatant carefully removed and placed in a fresh eppendorf tube. The supernatant was then available to be used to infect cells.

2.4.7 Isolation of Viral DNA

A 340µl aliquot of supernatant was removed, 20µl proteinase K (20mg/ml), 20µl 10% SDS and 20µl EDTA (100mM) added and incubated at 37°C to digest the protein coat of

viral particles. This solution was then phenol chloroform extracted and the viral DNA ethanol precipitated as described in section 2.2.2. Viral DNA was resuspended in 39µl TE buffer with 1µl RNAse (10µg/µl) and stored until required.

2.4.8 Identification of Viral recombinants

Viral DNA was analysed by restriction endonuclease digestion with HindIII and PCR analysis using VEGF-cDNA sequence specific primers (table 5.1), as described in sections 2.2.4 and 2.2.3 respectively.

2.4.9 Titration of Recombinant Adenovirus

293 cells were grown in 60mm dishes to 80% confluence, at which stage the medium was removed. 100µl of a range of adenoviral lysate dilutions (10^{-6}-10^{-12}) were added to the dishes and allowed to infect for 45 minutes at 37°C with 5% CO_2. Cells were then overlayed with 3ml of agar media containing 19ml MEM medium, 1ml FCS and 5ml 3% nobel agar. The cells were fed by repeating this procedure again at 4 days later when the plates were examined for plaque formation. The number of plaques on dishes at each 'dilution' enabled the titre of the original lysate to be calculated. Titre determination was performed according to the formula : [mean no. of plaques] x 10 x [dilution factor] = titre (pfu/ml).

2.4.10 Infection of NIH 3T3 cells

Media was removed from the NIH 3T3 cells, 100µl of adenoviral lysate, as prepared in section 2.4.4 was gently dropped evenly onto the cells. Viral particles were allowed to infect cells for 45 minutes before fresh media was added. Cells were incubated for 48 hours at 37°C with 5% CO_2. Cells were harvested and protein lysates prepared as described in section 2.3.5.

2.5 Animal Models

All DNA employed in animal models was prepared as described in section 2.2.2 and resuspended in PBS with the concentration adjusted to 1μg/μl. The identity of the DNA isolated was confirmed by restriction digest analysis as described in section 2.2.4. All surgical procedures were carried out by Dr Martin Hicks, Glasgow Royal Infirmary.

2.5.1 Rat Model of Direct DNA Injection

Anaesthesia was induced in male Sprague Dawley rats (350-450g) by intra-peritoneal injection of a mixture of midazolam (0.075 ml of 0.5 mg/ml hypnovel (Roche) and hypnorm 0.05ml/100g body weight) intra peritoneal. Animals were maintained under anaesthesia with an inhaled mixture of equal concentrations of nitrous oxide and oxygen plus 1% halothane with a 2.25ml stroke volume at 70 strokes/minute. The chest was opened by a left thoracotamy and the pericardium removed for DNA injection. 100μg DNA (1μg/μl) in PBS was introduced by injection into the apex of the heart using a 27 gauge needle. Postoperative analgesia was administered (0.2mg/kg buprenorphine intramuscular) at 30 minutes and 16 hours after the procedure.

2.5.2 Rat Model of Ischaemia with Reperfusion

Anaesthesia was induced in male Sprague Dawley rats (350-450g) with a mixture of midazolam (0.075 ml of 0.5 mg/ml hypnovel (Roche) and hypnorm 0.05ml/100g body weight) intra peritoneal. Animals were maintained under anaesthesia with an inhaled mixture of equal concentrations of nitrous oxide and oxygen plus 1% halothane with a 2.25ml stroke volume at 70 strokes/minute. The chest was opened by a left thoracotamy and the pericardium removed. The major coronary artery supplying the apex of the heart was identified and ligated by insertion of a suture. The presence of an area of ischaemia

was confirmed by visualisation of tissue decolourisation and by ECG. Immediately before removing the suture, 100μg DNA (1μg/μl) in PBS was introduced by injection into the apex of the heart using a 27 guage needle. 15 minutes after the onset of ischaemia the suture was removed to allow the apex to be reperfused. Sham operated animals were subjected to the same procedures but without coronary ligation. Postoperative analgesia was administered (0.2mg/kg buprenorphine im) at 30 minutes and 16 hours after the procedure.

2.5.3 Rabbit Model of Ischaemia with Reperfusion

Male New Zealand White rabbits (2.5-3.0 kg) were premedicated with intramuscular Hypnorm (0.3 mg/kg; fluanisone (10mg/ml), fentanyl citrate (0.315mg/ml)) and treated with intravenous midazolam (0.25-0.5mg/kg) to permit endotracheal intubation (size 3-4). Animals were ventilated (0.3-0.4 l/min/kg) on a small animal ventilator with 1-2cm H_2O of positive end-expiratory pressure. Animals were maintained under anaesthesia with an inhaled mixture of equal concentrations of nitrous oxide and oxygen plus 1% halothane at a flow rate of 2 litres/min. The chest was opened by a left thoracotamy and the pericardium removed for DNA injection. 100μg DNA (1μg/μl) in PBS was introduced by injection using a 27 guage needle, firstly into the cardiac apex (three injections) and secondly into the proximal left ventricular wall (three injections). Postoperative analgesia was administered (0.2 mg/kg buprenorphine im) at 30 minutes and 6-8 hours after the procedure.

Seven days after DNA injection, rabbits were subjected to a second thoracotamy for implementation of transient myocardial ischaemia. Premedication, ventilation and anaesthesia were performed as described above. After thoracotamy, the first obtuse marginal artery was ligated by insertion of a suture at the midpoint between the atrioventricular groove and the cardiac apex. The presence of an area of ischaemia was

confirmed by visualisation of tissue decolourisation and by ECG. After 15 minutes of ischaemia the suture was removed to allow the heart to be reperfused. Intravenous quinidine was administered 5 minutes before ligation to decrease the likelihood of arrhythmias in this procedure. Ventricular arrythmias during surgery were treated with DC shock using a defibrillator equipped with small paddles. Postoperative analgesia was administered as described above. One to eight hours after surgery animals were sacrificed with a lethal dose of pentobarbitol sodium and the heart excised for assays of reporter gene expression.

2.5.4 Preparation and Analysis of Heart Homogenates

Heart tissue samples from rats and rabbits were finely chopped into $2mm^3$ pieces using a scalpel blade and forceps, and placed in a 50ml round bottomed tube. 1ml homogenate buffer (25mM gly-gly, 15mM $MgSO_4$, 4mM EGTA, 1mM dithiothreitol) per 0.2g tissue was added and the samples homogenised using a polytron homogeniser (Kinematica) until all large debris had been homogenised (approximately 1 minute). Homogenised samples were centrifuged at 12000g for 10 minutes to pellet cell debris. The supernatant was carefully decanted into polypropylene tubes and stored at -70°C for subsequent use in CAT and luciferase assays, as described in sections 2.3.2 and 2.3.3.

Chapter 3

RESULTS 1

3.1 Introduction

Myocardial ischaemia has been defined as a condition characterised by a pathological alteration of cardiac contractile, electrical or biochemical function resulting from a reduction of coronary blood flow (Hearse, 1994). The reduced blood supply deprives the myocardium of substrates, notably oxygen and glucose, and allows metabolites to accumulate. The hypoxia that results from the reduction in oxygen tension, leads to a cascade of cellular events designed to limit the energy usage of the cell by shutting down non-essential cell functions (Hochachka et al., 1996).

3.1.1 Hypoxia

Hypoxia is not only a signal for energy conservation but also a signal for the upregulation of a number of genes including transcription factors, metabolic enzymes and growth factors (Semenza and Wang, 1992; Goldberg and Schneider, 1994; Semenza et al., 1994; Goldberg et al., 1988). One of the first genes found to be upregulated by hypoxia was the gene encoding the glycoprotein growth factor erythropoietin (EPO) (Goldberg et al., 1988). EPO expression was found to be induced by divalent metals such as cobalt, which can mimic the effect of ferrous iron, and by iron-chelating agents such as desferrioxamine but not by inhibitors of mitochondrial respiration such as cycloheximide or 2-aminopurine (Semenza et al., 1994; Wang and Semenza, 1993; Wang and Semenza, 1993; Goldberg et al., 1988). These results suggest the involvement of a haem-containing protein as part of an oxygen-sensing pathway (Goldberg et al., 1988). Treatment with the translational inhibitor, cycloheximide, blocks the hypoxic induction of EPO, indicating the requirement of de novo protein synthesis (Goldberg et al., 1988). Analysis of the 3' flanking region of the EPO gene identified a 50-nucleotide enhancer element that conferred hypoxic induction and was found to bind the transcription factor, hypoxia-inducible factor 1 (HIF-1) (Semenza and Wang, 1992). HIF-1 binding activity is specifically induced by hypoxia and the induction requires de novo protein synthesis

(Semenza and Wang, 1992). HIF-1 is a heterodimeric transcription factor consisting of two basic helix-loop-helix subunits, HIF-1α and HIF-1β (also known as arylhydrocarbon nuclear translocator (ARNT)) (Wang et al., 1995). HIF-1β protein levels remain constant under conditions of hypoxia (Kallio et al., 1997; Huang et al., 1996), however, HIF-1α protein levels and correspondingly HIF-1 DNA binding activity is markedly induced by hypoxia (Kallio et al., 1997; Huang et al., 1996). HIF-1 is induced by hypoxia in all cell types tested (Wang and Semenza, 1993b) and the EPO enhancer element that binds HIF-1 is capable of mediating hypoxia-inducible reporter gene transcription in non-EPO producing cell lines (Wang and Semenza, 1993b). Several other hypoxia inducible genes, including glycolytic enzymes (Semenza et al., 1994) and the angiogenic factor VEGF (Goldberg and Schneider, 1994), contain HIF-1 binding sites. HIF-1, therefore, appears to play a central role in a general oxygen sensing pathway.

3.1.2 Foreign Gene Regulation

The ability to accurately regulate foreign gene expression in the ischaemic myocardium would be crucial for a true therapeutic benefit of gene transfer is to be achieved. The hypoxia response element (HRE) that binds HIF-1 has been shown to confer hypoxia-inducibility when cloned 3' to a SV40/CAT reporter gene construct in chinese hamster ovary (CHO) cells (Wang and Semenza, 1993b). Hypoxia-regulatable foreign gene expression may allow potentially therapeutic genes to be expressed in the heart exclusively during periods of hypoxia and possibly ischaemia. Incorporating hypoxia inducible elements into a basal muscle specific promoter, such as that for α-myosin heavy chain (MHC), cardiac α-actin (CA) or cardiac troponin C (cTnC), to create a hybrid promoter construct, could provide a further degree of regulation.

3.1.3 Direct DNA injection

Direct injection of naked plasmid DNA has been applied to a variety of systems, including skeletal and cardiac muscle (Buttrick et al., 1992; Lin et al., 1990; Wolff et al., 1990). Direct DNA injection has been successfully employed to demonstrate tissue specificity, hormonal induction and antibiotic regulated foreign gene expression (Fishman et al., 1994; Kitsis et al., 1991; Prentice et al., 1994). The promoter element of the immediate early gene, c-fos, that responds to left ventricular pressure overload was identified using direct DNA injection (Aoyagi and Izumo, 1993), demonstrating the feasibility of a directly injected promoter/reporter gene construct to respond to physiological stimuli resulting from the cellular environment.

3.2 Results

This study was designed to investigate the possibility that hypoxia responsive enhancer elements incorporated into muscle specific promoters can be used to regulate foreign gene expression in response to myocardial ischaemia.

Four copies of the hypoxia responsive enhancer element (HRE), isolated from the 3'-flanking sequence of the human erythropoietin gene (Semenza and Wang, 1992), were cloned 5' to variety of promoter constructs and tested for hypoxia inducibility in isolated cardiac myocytes, by Prof Keith Webster's group, University of Miami. HREs were cloned 5' to the promoters, α-myosin heavy chain (MHC), cardiac α-actin (CA) and SV40. The purpose of the present study was to assess the ability of these constructs to confer inducible expression *in vivo* of the reporter gene luciferase in response to a 15 minute period of myocardial ischaemia.

Initially, the rat model of ischaemia with reperfusion was employed, as described in section 2.5.2. Briefly, male Sprague Dawley rats were anaesthetised and artificially ventilated. The hearts were exposed and subjected to 15 minutes ischaemia followed by reperfusion (IR) or to sham operation only. DNA promoter/reporter gene constructs were introduced by direct injection into the cardiac apex. Hearts were removed 24 hours later and assayed for reporter gene expression as described in section 2.5.4.

In order to take account of varying uptake efficiencies between injections, a ubiquitously expressing construct is co-injected with the promoter construct of interest. Results are expressed as a ratio of the promoter construct to the ubiquitously expressing construct. In the following experiments a CAT reporter gene, under the control of a SV40 promoter, is used to normalise for DNA uptake.

Table 3.1 Effect of 15 mins ischaemia with reperfusion (IR) on the MHC-HRE promoter at 24 hours in the rat heart.

Protocol : **rat heart, 15 mins ischaemia with reperfusion**

Time point : **24 hours**

Luciferase construct : **MHC-HRE / luciferase**

CAT construct : **SV40 / CAT**

Experiment no.	Procedure	CAT(SV40)	LUC (HRE)	Ratio	
1	SHAM	1753	4888	**2.8**	
2	SHAM	3513	7224	**2.1**	
3	SHAM	2597	5302	**2.0**	
4	SHAM	2507	4618	**1.8**	***Mean***
5	SHAM	1248	4968	**4.0**	**2.26 ± 0.43**
6	SHAM	1293	1071	**0.8**	
7	IR	397	2047	**5.2**	
8	IR	2044	6950	**3.4**	
9	IR	2229	1701	**0.8**	***Mean***
10	IR	252	1620	**6.4**	**3.56 ± 1.02**
11	IR	3336	6792	**2.0**	

Promoter activities from 11 individual experiments are shown. Hearts were injected with the plasmid DNA constructs MHC-HRE / Luciferase and SV40 / CAT in equal ratios. Animals were then subjected to 15 minutes of ischaemia with reperfusion (IR) or to Sham operation only (SHAM), as described in section 2.5.2. Hearts were excised and assayed for reporter gene expression 24 hours post ischaemia. CAT activities, under the control of a SV40 promoter, are shown in column 3. CAT activity is presented as counts per minute (cpm) above background, representing the conversion of ^{14}C-chloramphenicol to its acetylated product by the reporter gene product chloramphenicol acetyl transferase. Luciferase activities, under the control of the MHC-HRE promoter, are shown in column 4. Luciferase activity is presented as relative light units (RLU) multiplied by 1000, representing the oxidation of luciferin by luciferase with concomitant production of a photon. Ratio values in column 5 represent the Luciferase data point divided by the CAT data point for each experiment. Ratio values allow the test promoter (MHC-HRE) to be normalised by reference to the internal control (SV40). The mean of the ratios ± standard error for the Sham or IR procedures are shown in column 6. Statistical analysis was performed using the Students t-test for unpaired samples.

Table 3.2 Effect of 15 mins ischaemia with reperfusion (IR) on the SV40-HRE promoter at 24 hours in the rat heart.

Protocol : **rat heart, 15 mins ischaemia with reperfusion**

Time point : **24 hours**

Luciferase construct : **SV40-HRE / luciferase**

CAT construct : **SV40 / CAT**

Experiment no.	Procedure	CAT(SV40)	LUC (HRE)	Ratio	
1	SHAM	3897	5402	**1.39**	
2	SHAM	3185	44	**0.01**	***Mean***
3	SHAM	266	656	**2.47**	**1.29 ± 0.71**
4	IR	3660	13760	**3.76**	
5	IR	3152	5470	**1.74**	
6	IR	3291	3317	**1.01**	
7	IR	1093	419	**0.38**	***Mean***
8	IR	533	439	**0.82**	**1.84 ± 0.57**

Promoter activities from 8 individual experiments are shown. Hearts were injected with the plasmid DNA constructs SV40-HRE / Luciferase and SV40 / CAT in equal ratios. Animals were then subjected to 15 minutes of ischaemia with reperfusion (IR) or to sham operation only (SHAM), as described in section 2.5.2. Hearts were excised and assayed for reporter gene expression 24 hours post ischaemia. The format and layout of this table is as table 3.1

Table 3.3 Effect of 15 mins ischaemia with reperfusion (IR) on the CA-HRE promoter at 24 hours in the rat heart.

Protocol : **rat heart, 15 mins ischaemia with reperfusion**

Time point : **24 hours**

Luciferase construct : **CA-HRE / luciferase**

CAT construct : **SV40 / CAT**

Experiment no.	Procedure	CAT(SV40)	LUC (HRE)	Ratio	
1	SHAM	1616	455	**0.28**	
2	SHAM	534	83	**0.16**	
3	SHAM	900	54	**0.06**	
4	SHAM	8639	2362	**0.27**	***Mean***
5	SHAM	1318	731	**0.55**	**0.27 ± 0.08**
6	IR	730	180	**0.25**	
7	IR	1910	433	**0.23**	***Mean***
8	IR	382	31	**0.08**	**0.18 ± 0.05**

Promoter activities from 8 individual experiments are shown. Hearts were injected with the plasmid DNA constructs CA-HRE / Luciferase and SV40 / CAT in equal ratios. Animals were then subjected to 15 minutes of ischaemia with reperfusion (IR) or to sham operation only (SHAM), as described in section 2.5.2. Hearts were excised and assayed for reporter gene expression 24 hours post ischaemia. The format and layout of this table is as table 3.1

Levels of Activity from MHC-HRE, SV40-HRE and CA-HRE Promoter Constructs at 24 hours in Sham and IR Rat Myocardium

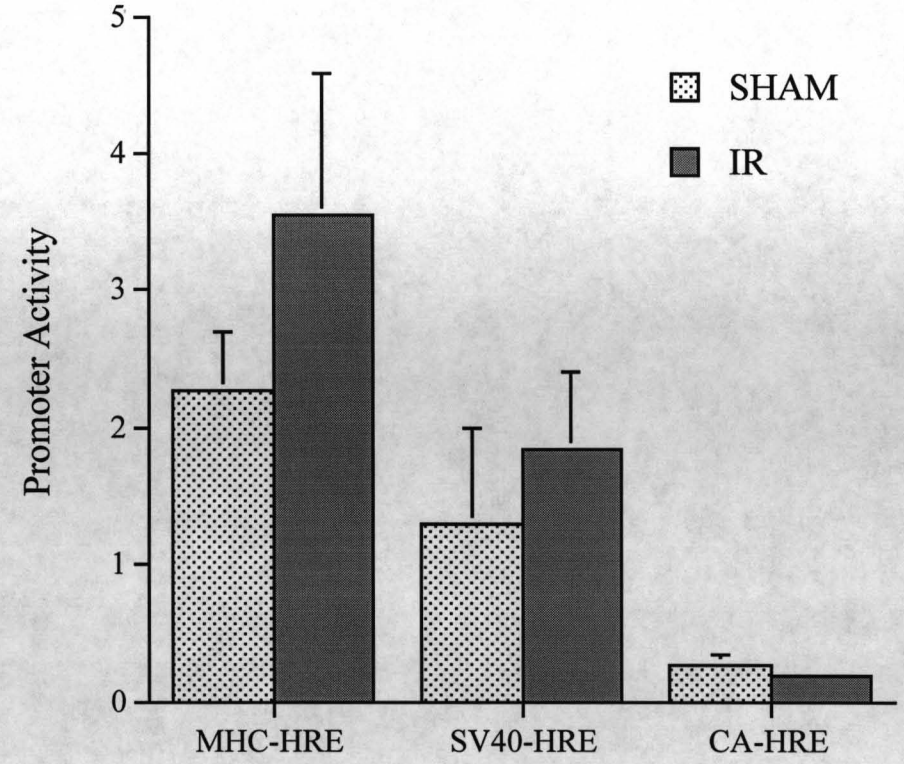

Figure 3.1 The surgical procedures, injections and assays are described in section 2.5.2. Hearts were injected with either MHC-HRE, SV40-HRE or CA-HRE promoter constructs and subjected to IR or sham procedures. Hearts were excised and reporter gene activities assayed 24 hours after surgery. This figure summarises the data presented in tables 3.1, 3.2 and 3.3. Promoter activity is the ratio of average levels of reporter gene expression driven by the HRE promoter to average levels of reporter gene expression driven by the SV40, ubiquitously expressing, promoter, with standard errors indicated.

MHC-HRE, CA-HRE and SV40-HRE promoter activities in IR and sham operated animals are shown in figure 3.1. The MHC-HRE promoter activity at 24 hours in sham operated animals was 2.26 ± 0.43 and IR animals 3.56 ± 1.02. SV40-HRE promoter activities at 24 hours for sham and IR operated animals was 1.29 ± 0.71 and 1.84 ± 0.57 respectively. The CA-HRE promoter activity at 24 hours was 0.27 ± 0.08 in sham operated animals and 0.18 ± 0.05 in IR operated animals. The inducibility of the SV40 basal promoter, used as a control for uptake efficiency, has been previously investigated using this model at 1 day and shows no significant induction (H Prentice, personal communication) No significant difference was observed for any promoter at 24 hours between sham and IR operated animals in the rat model of ischaemia with reperfusion.

Further experiments could potentially have revealed significant differences between the sham and IR groups. However, the data obtained indicated no significant change in response to IR at 24 hours and further experiments were deemed likely to be unproductive. Promoter activation in response to IR at 12 hours was investigated. Despite significant luciferase activity above background, CAT activity levels were not reproducibly significant (table 3.4). The luciferase reporter gene sytem is 10 to 100-fold more sensitive than the CAT assay system and may explain the presence of luciferase activity despite the absence of CAT activity at these short time points.

Protocol	CAT (SV40)	LUC (HRE)
SHAM	66	23
IR	0	248
IR	107	445
IR	0	1634
IR	37	969

Table 3.4 CAT (CAT) and luciferase (LUC) promoter activities in rat myocardium 12 hours after DNA injection. The surgical procedures, injections and assays are described in section 2.5. Rat hearts were injected with 50μg SV40/CAT plasmid and 50μg MHC-HRE/luciferase plasmid. Hearts were subjected to sham or IR procedures, excised and assayed as in table 3.1.

It was decided that the rat model of ischaemia with reperfusion was unsuitable for investigating the inducibility of promoters containing HRE elements in response to ischaemia at time points below 24 hours. The sequence of events from DNA uptake through to reporter gene expression may take several hours. Any activation of cellular pathways by the ischaemic stimulus may therefore be complete by the time the promoter construct is able to respond to them. Indeed, several lines of *in vitro* evidence suggest that HIF-1 binding to the HRE elements during hypoxia is rapid with maximal binding after 4 hours (Kallio et al., 1997; Huang et al., 1996; Wang et al., 1995; Wang and Semenza, 1993). Upon re-oxygenation HIF-1 dissociation is equally rapid (Kallio et al., 1997; Huang et al., 1996; Wang et al., 1995). A 24 hour time point may not, therefore, be appropriate. It was clear that because of the low levels of foreign gene expression, DNA constructs would need to be present before the onset of ischaemia to investigate time points less than 24 hours. However, this approach would require two surgical procedures; one to inject the DNA and one to induce ischaemia. In view of the requirement for two surgical procedures on the same animal it was decided to use a larger species. The rabbit model of ischaemia with reperfusion was developed employing two thoracotamies as distinct surgical interventions.

3.2.1 Rabbit Model of Ischaemia with Reperfusion

This model has no restriction on the time points assayed because the DNA is present in the myocardium at the onset of ischaemia. Time points from 0 minutes to days or weeks can therefore be investigated.

The rabbit model of ischaemia with reperfusion is described in detail in section 2.5.3. Briefly, rabbits were anaesthetised and artificially ventilated. The heart was exposed by a left thoracotamy and DNA injected firstly into the cardiac apex and secondly into the proximal left ventricular wall. Seven days after DNA injection, rabbits were subjected to a second thoracotamy and the first obtuse marginal artery ligated immediately above the

cardiac apex. Ischaemia was confirmed by visualisation of tissue decolourisation. After 15 minutes of ischaemia, the suture was removed to allow reperfusion of the apex. Hearts were excised and assayed 1-8 hours post ischaemia.

This model has two major advantages over the previous rat model. Firstly, there is no restriction on the time points that can be assayed. Secondly, because the rabbit heart is physically larger than the rat heart, both sham and IR experiments can be performed in the one heart. DNA injected into the cardiac apex is subjected to IR whereas DNA injected into the proximal left ventricular wall remains well perfused.

In vitro results demonstrated that MHC-HRE promoter construct expression was the most responsive to hypoxia (K. Webster, personal communication). The effect of IR on the MHC-HRE promoter construct activity was therefore investigated using the rabbit model of ischaemia with reperfusion at varying time points. The effect of IR on SV40 promoter construct activity, used as a control for uptake efficiency, was also investigated.

Table 3.5 Effect of 15 mins ischaemia with 1 hour of reperfusion on the MHC-HRE promoter in the rabbit heart.

Protocol : **rabbit heart, 15 mins ischaemia with reperfusion**

Time point : **1 hour**

Luciferase construct : **MHC-HRE / luciferase**

CAT construct : **SV40 / CAT**

Experiment no.	Procedure	CAT(SV40)	LUC (HRE)	Ratio	
1	SHAM	7833	330	**0.4**	
2	SHAM	4708	399	**0.8**	
3	SHAM	1941	0.0	**0**	***Mean***
4	SHAM	4616	946	**2.0**	**0.76 ± 0.34**
5	SHAM	2063	127	**0.6**	
6	IR	1473	122	**0.8**	
7	IR	10100	1916	**1.9**	
8	IR	1393	81	**0.6**	***Mean***
9	IR	3590	2283	**6.4**	**3.08 ± 1.24**
10	IR	960	546	**5.7**	

Observed Induction of Mean Activity : 4.1 ± 1.0 ($p < 0.05$)

Promoter activities from 10 experiments are shown. Hearts were injected in the apical and proximal lateral wall regions with the plasmid DNA constructs MHC-HRE / Luciferase and SV40 / CAT in equal ratios. 7 days after the DNA injection, a second thoracotamy was performed and the apical region of the heart subjected to 15 minutes of ischaemia by left marginal artery ligation followed by 1 hour of reperfusion (IR), as described in section 2.5.3. The proximal lateral wall remained perfused throughout (SHAM). CAT activities, under the control of a SV40 promoter, are shown in column 3. CAT activity is presented as counts per minute (cpm) above background, representing the conversion of ^{14}C-chloramphenicol to its acetylated product by the reporter gene product chloramphenicol acetyl transferase. Luciferase activities, under the control of the MHC-HRE promoter, are shown in column 4. Luciferase activity is presented as relative light units (RLU) multiplied by 1000, representing the oxidation of luciferin by luciferase with concomitant production of a photon. Ratio values in column 5 represent the Luciferase data point divided by the CAT data point for each experiment, multiplied by 10. The mean of the ratios ± standard error for the Sham or IR procedures are shown in column 6. Ratio values allow the test promoter (MHC-HRE) to be

normalised by reference to the internal control (SV40). Statistical analysis was performed using a students t-test, comparing related samples.

Table 3.6 Effect of 15 mins ischaemia with 4 hours of reperfusion on the MHC-HRE promoter in the rabbit heart.

Protocol : **rabbit heart, 15 mins ischaemia with reperfusion**

Time point : **4 hours**

Luciferase construct : **MHC-HRE / luciferase**

CAT construct : **SV40 / CAT**

Experiment no.	Procedure	CAT(SV40)	LUC (HRE)	Ratio	
1	SHAM	10587	401	**0.38**	
2	SHAM	12603	1165	**0.92**	
3	SHAM	10953	1982	**1.81**	
4	SHAM	2790	545	**1.95**	
5	SHAM	6633	127	**0.19**	
6	SHAM	3457	50	**0.14**	
7	SHAM	8587	177	**0.21**	
8	SHAM	872	51	**0.58**	***Mean***
9	SHAM	2122	29	**0.14**	**0.64 ± 0.22**
10	SHAM	1753	5	**0.03**	
11	IR	2246	544	**2.42**	
12	IR	14760	12350	**8.37**	
13	IR	11514	2693	**2.34**	
14	IR	7903	518	**0.65**	
15	IR	4374	1978	**4.52**	
16	IR	2436	1169	**4.80**	
17	IR	8398	3097	**3.69**	
18	IR	2563	482	**1.88**	***Mean***
19	IR	911	55	**0.60**	**2.96 ± 0.78**
20	IR	839	28	**0.33**	

Observed Induction of Mean Activity : 4.7 ± 0.7 ($p < 0.001$)

Promoter activities from 20 experiments are shown. Hearts were injected in the apical and proximal lateral wall regions with the plasmid DNA constructs MHC-HRE / Luciferase and SV40 / CAT in equal ratios. 7 days after the DNA injection, a second thoracotamy was performed and the apical region of the heart subjected to 15 minutes of ischaemia by left marginal artery ligation followed by 4 hours of reperfusion

(IR), as described in section 2.5.3. The proximal lateral wall remained perfused throughout (SHAM). The format and layout of this table is as table 3.5

Table 3.7 Effect of 15 mins ischaemia with 8 hours of reperfusion on the MHC-HRE promoter in the rabbit heart.

Protocol : **rabbit heart, 15 mins ischaemia with reperfusion**

Time point : **8 hours**

Luciferase construct : **MHC-HRE / luciferase**

CAT construct : **SV40 / CAT**

Experiment no.	Procedure	CAT(SV40)	LUC (HRE)	Ratio	
1	SHAM	1163	1276	**11.0**	
2	SHAM	1306	392	**3.0**	***Mean***
3	SHAM	137	10	**0.7**	**4.9 ± 3.1**
4	IR	138	94	**6.8**	
5	IR	449	109	**2.4**	***Mean***
6	IR	717	13	**0.2**	**3.1 ± 1.9**

No Significant Alteration in the Mean Activity Observed

Promoter activities from 6 experiments are shown. Hearts were injected in the apical and proximal lateral wall regions with the plasmid DNA constructs MHC-HRE / Luciferase and SV40 / CAT in equal ratios. 7 days after the DNA injection, a second thoracotamy was performed and the apical region of the heart subjected to 15 minutes of ischaemia by left marginal artery ligation followed by 8 hours of reperfusion (IR), as described in section 2.5.3. The proximal lateral wall remained perfused throughout (SHAM). The format and layout of this table is as table 3.5

Table 3.8 Effect of 15 mins ischaemia with 4 hours of reperfusion on the MHC(-86bp) promoter in the rabbit heart.

Protocol : **rabbit heart, 15 mins ischaemia with reperfusion**

Time point : **4 hours**

Luciferase construct : **MHC(-86bp) / luciferase**

CAT construct : **SV40 / CAT**

Experiment no.	Procedure	CAT(SV40)	LUC (HRE)	Ratio
1	SHAM	12117	0	**0**
2	SHAM	1601	0	**0**
3	SHAM	6778	4	**0.01**
4	SHAM	927	0	**0**
5	IR	14046	28	**0.02**
6	IR	37091	57	**0.02**
7	IR	7326	0	**0**
8	IR	8603	6	**0.01**

In this experiment all samples displayed an absence of luciferase promoter activity

Promoter activities from 8 experiments are shown. Hearts were injected in the apical and proximal lateral wall regions with the plasmid DNA constructs MHC (-86bp) / Luciferase and SV40 / CAT in equal ratios. 7 days after the DNA injection, a second thoracotamy was performed and the apical region of the heart subjected to 15 minutes of ischaemia by left marginal artery ligation followed by 4 hours of reperfusion (IR), as described in section 2.5.3. The proximal lateral wall remained perfused throughout (SHAM). The format and layout of this table is as table 3.5

Table 3.9 Effect of 15 mins ischaemia with 4 hours of reperfusion on the SV40 promoter in the rabbit heart.

Protocol : **rabbit heart, 15 mins ischaemia with reperfusion**

Time point : **4 hours**

Luciferase construct : **RSV / luciferase**

CAT construct : **SV40 / CAT**

Experiment no.	Procedure	CAT(SV40)	LUC (HRE)	Ratio	
1	SHAM	1734	273.9	**6.3**	
2	SHAM	2419	374.1	**6.5**	
3	SHAM	386	72.28	**5.3**	***Mean***
4	SHAM	1467	155.5	**9.4**	**7.6 ± 1.0**
5	SHAM	5594	536.1	**10.4**	
6	IR	2296	231	**9.9**	
7	IR	1594	299.6	**5.3**	
8	IR	158	22.57	**7.0**	***Mean***
9	IR	982	107.2	**9.2**	**8.9 ± 1.4**
10	IR	5046	380.2	**13.3**	

No Significant Alteration in the Mean Activity Observed

Promoter activities from 10 experiments are shown. Hearts were injected in the apical and proximal lateral wall regions with the plasmid DNA constructs RSV / Luciferase and SV40 / CAT in equal ratios. 7 days after the DNA injection, a second thoracotamy was performed and the apical region of the heart subjected to 15 minutes of ischaemia by left marginal artery ligation followed by 4 hours of reperfusion (IR), as described in section 2.5.3. The proximal lateral wall remained perfused throughout (SHAM). The format and layout of this table is as table 3.5

Levels of Activity from MHC-HRE Promoter Construct at 1, 4 and 8 hours in Sham and IR Rat Myocardium

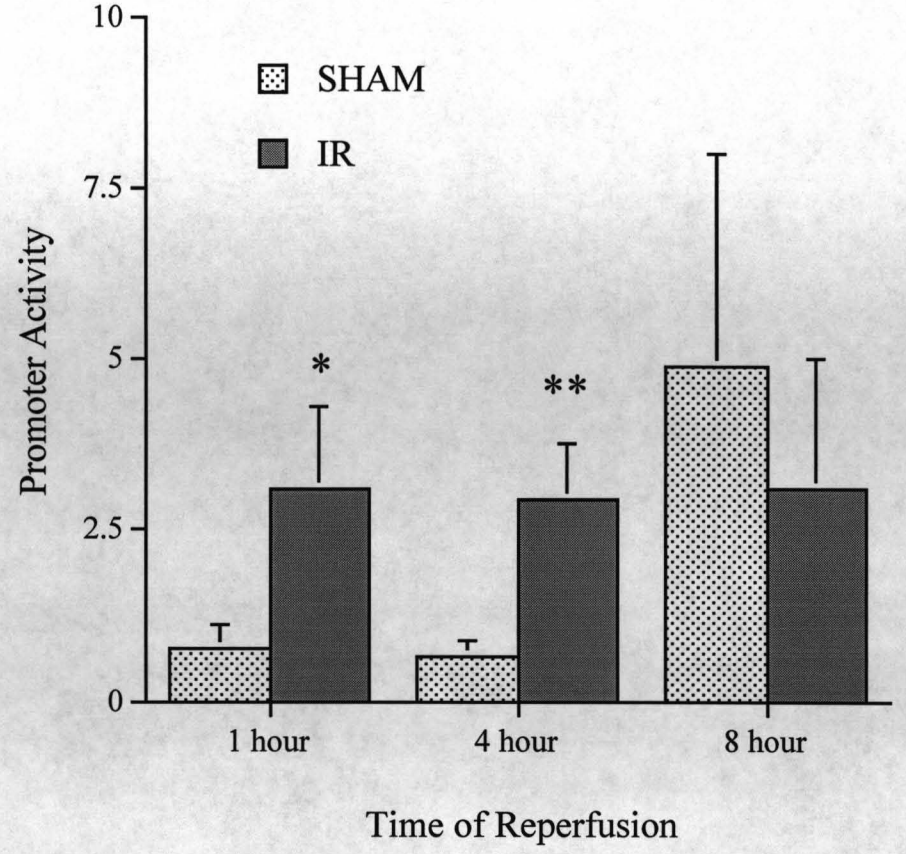

Figure 3.2 The surgical procedures, injections and assays are described in section 2.5.3. Hearts were injected with the MHC-HRE promoter construct. 7 days after DNA injection, a second thoracotamy was performed and the apical region of the heart subjected to 15 minutes of ischaemia followed by 1, 4 or 8 hours of reperfusion as indicated on the graph. Hearts were excised and reporter gene activities assayed. This figure summarises the data presented in tables 3.5, 3.6 and 3.7. Promoter activity is the ratio of average levels of reporter gene expression driven by the HRE promoter to average levels of reporter gene expression driven by the SV40, ubiquitously expressing, promoter, injected simultaneously with the MHC-HRE construct. Induction of luciferase gene expression by ischaemia was significantly different from shams at 1 hour ($p<0.05$ (*)) and 4 hours ($p<0.001$ (**)) by t-test, comparing related samples.

Figure 3.2 illustrates the transient induction of MHC-HRE promoter activity by IR. Luciferase activity, 1 hour after IR was increased 4.1 ± 1.0 fold compared with the sham. 4 hours after IR, luciferase activity was increased 4.7 ± 0.7 fold compared to sham. Levels of luciferase activity was not significantly different 8 hours after IR than the sham operated tissue. There was no difference in the expression of an MHC construct lacking HREs or of the SV40-CAT construct between sham and ischaemic hearts at 4 hours. In 'sham/sham' control experiments, reporter gene expression levels did not differ between the apical and base regions of the heart in the absence of the ischaemic insult.

3.3 Discussion

The results demonstrate that four copies of the human EPO hypoxia responsive enhancer element (HRE) linked to a basal muscle specific α-MHC promoter convey inducible expression of a luciferase reporter gene in response to 15 minutes of ischaemia followed by reperfusion in the rabbit heart. This response was dependent on the presence of the HRE's as the α-MHC promoter alone demonstrated no such induction in response to the same ischaemic stimulus. Promoter activation, in response to ischaemia, was rapid and transient (figure 3.2). Near maximal induction was reached 1 hour after ischaemia and enhanced expression remained for at least 4 hours. Expression levels returned to sham levels after 8 hours.

In vitro MHC-HRE was induced 9.2-fold after 16 hours of hypoxia in cultured neonatal cardiac myocytes. Glucose levels in the culture media were maintained at 3mM which corresponds to a low normal physiologic level. Expression from the MHC-HRE promoter construct *in vitro* was also demonstrated to be significantly higher in C_2C_{12} myocytes and neonatal cardiac myocytes than in HeLa cells, indicating that the -86 MHC basal regulatory elements retain their tissue specificity (Prentice et al., 1997).

The magnitude and transient nature of the response induced by ischaemia is related to the levels and binding activity of the HRE- binding transcription factor HIF-1. HIF-1 is a heterodimeric transcription factor consisting of two basic helix-loop-helix subunits, HIF-1α and HIF-1β (also known as arylhydrocarbon nuclear translocator (ARNT)) (Wang et al., 1995). HIF-1α and HIF-1β mRNA levels remain constant and show no significant induction with hypoxia (Gradin et al., 1996; Huang et al., 1996). HIF-1β protein levels also remain constant under conditions of hypoxia (Kallio et al., 1997; Huang et al., 1996). However, HIF-1α protein levels and correspondingly HIF-1 DNA binding activity is markedly induced by hypoxia (Kallio et al., 1997; Huang et al., 1996). The re-introduction of oxygen to hypoxic cells leads to a rapid decline in both HIF-1α protein

levels and HIF-1 DNA binding activity (Wang and Semenza, 1993a; Huang et al., 1996a). Hypoxia induced HIF-1 binding is also abolished by short pre-exposure to hydrogen peroxide, which generates reactive oxygen intermediates, via blocking accumulation of HIF-1α protein(Huang et al., 1996). HIF-1α stability therefore depends on the oxidative state of the cell and is rapidly destabilised in the presence of oxygen. HIF-1 DNA binding in hypoxic cell extracts is reversibly blocked by sulfhydryl oxidation and enhanced by addition of reduced thioredoxin (Huang et al., 1996), possibly indicating that HIF-1 DNA binding may be dependent on the reduction of critical cysteine sulfhydryls as is the case with AP-1, Egr-1, USF and Sp-1 (Pognonec et al., 1992; Huang and Adamson, 1993; Ammendola et al., 1994; Abate et al., 1990).

HIF-1α is intrinsically unstable due to the presence of two PEST-rich sequences in the C-terminal domain of the protein (Wang et al., 1995). PEST-rich sequences are amino acid sequences rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues (Rogers et al., 1986). PEST regions are commonly found in proteins that have very short half-lifes (< 2 hours). However a direct link between the redox status of the cell and PEST-rich sequences has yet to be identified. HIF-1α is also stabilised upon dimerisation with HIF-1β (Kallio et al., 1997). Correlated with activation of DNA binding activity, HIF-1α undergoes a conformational change that renders it more resistant to proteolytic digestion *in vitro* (Kallio et al., 1997). This effect is not purely a consequence of dimerisation since truncated HIF-1β mutants fail to induce such a change (Kallio et al., 1997).

In summary, activation of HIF-1 binding is critically dependent on the relative abundance of its α subunit whose levels are determined through redox-stabilisation of the HIF-1α protein (Huang et al., 1996). HIF-1β levels, however, are also critically important. Hypoxia induced levels of HIF-1α exceed those of the constitutively expressed HIF-1β, in both HeLa and HepG2 cells (Kallio et al., 1997), thereby implicating HIF-1β as the limiting factor in the magnitude of response to hypoxic stimulation. HIF-1α may

therefore be responsible for the timing of the response but HIF-1β is responsible for the magnitude of response.

The response of the HRE-MHC promoter construct to hypoxia compared to ischaemia observed in this study is striking. 16 hours of hypoxia *in vitro* leads to a 9.2 fold induction yet only 15 minutes of ischaemia leads to a 4.7 fold induction. These results suggest that either the *in vivo* response to hypoxia is extremely sensitive or other components of ischaemia may also be acting either directly or indirectly on the HIF-1 mediated induction. A reduction of coronary blood flow leads to an inadequate supply of nutrients, particularly oxygen and glucose, and an inadequate removal of metabolites. The ischaemic myocardium is therefore subjected to hypoxia, tissue hypoglycaemia, acidosis, and an altered redox status. Redox regulation of HIF-1 activation has already been established (see above). Embryonic stem cells, isolated from mouse lines with a targeted disruption of the HIF-1β subunit fail to respond to a decrease in glucose concentration as well as to a decrease in oxygen tension (Maltepe et al., 1997). It may be possible, therefore, that under conditions of ischaemia, the HRE-MHC promoter construct, is responding to both hypoxia and tissue hypoglycaemia. Co-ordinate regulation by hypoxia and hypoglycaemia has previously been demonstrated with the immediate early gene, c-jun, in human squamous carcinoma cells (SiHa) (Ausserer et al., 1994). Hypoxia alone produced approximately a 5-fold c-jun mRNA induction in the presence of glucose (Ausserer et al., 1994). Glucose deprivation in conjunction with hypoxia produced greater than a 30-fold induction of c-jun message (Ausserer et al., 1994). It is therefore possible that the 'super-induction' of the HRE-MHC promoter construct by ischaemia, compared to hypoxia alone, is due to the combined effect of low glucose and low oxygen that occurs during ischaemia.

The activation of promoter activity in response to ischaemia, demonstrated by these results, was dependent on the presence of the HRE enhancer elements. Expression of the α-MHC promoter alone was barely detectable indicating that the HRE elements not only

confer inducibility but also increase basal levels of luciferase expression. An increase in basal levels of expression may be as a result of independent transcription factors interacting with the transcription complex, activating transcription.

The time course of promoter induction observed in the rabbit model may explain why no induction was observed in the rat model. In the rabbit model, promoter activity returned to baseline (sham) levels after 8 hours. The shortest time point at which expression could be detected in the rat model was 24 hours. Any induction, therefore, that may have occurred in the rat model had either receded by 24 hours or was unable to be detected by the model system employed.

The magnitude of induction is calculated by comparing the levels of promoter activity in the sham versus ischaemic areas of the heart. Visual signals (i.e. tissue decolourisation) were used to confirm ischaemia in the apical region of the heart, previously injected with DNA. However this method for establishing a region of ischaemia is not 100% accurate and a greater degree of variability in promoter activity was often observed within ischaemic areas by comparison to the variability within sham areas of the same heart (tables 3.5, 3.6). Variability of promoter activity in ischaemic regions is to be expected because ischaemia is not an 'all or nothing' event. Depending on the extent of collateral blood flow to the ischaemic region, transfected cells on the periphery of the ischaemic region may only be exposed to a slight reduction of blood flow or indeed no reduction at all, whereas transfected cells in the central areas may be exposed to a complete cessation of blood flow. Promoter activities would vary accordingly. The contribution of promoter activities from ischaemic regions of the heart which may have only received a slight reduction in blood flow, or none at all, would lead to greater degree of variability and a decrease in the overall level of induction.

It was not possible to ensure that DNA injected into sham areas of the heart were at no time exposed to brief interruptions in coronary flow. Indeed, the physical manipulation of the heart required to interrupt coronary flow to the apical region of the heart would

inevitably have led to brief alterations in coronary flow to the sham areas (Kloner et al., 1995). Furthermore difficulty in anaesthetising the animals may have lead to periods of general hypoxia and the physical damage caused by intramuscular injection may lead to small local areas of reduced perfusion. It is therefore possible that the promoter activity in the sham areas of the heart may have been increased by such stimuli. Increased promoter activity in the sham areas compared to the IR areas, may result in the calculated induction of promoter activity by IR to be lower than the true value. HRE-MHC promoter activity at 1 and 4 hours may therefore be induced to a greater degree than 4.1 and 4.7-fold, respectively, recorded relative to sham areas.

The work presented here demonstrates a 4.65 fold induction of reporter gene expression in response to ischaemia. It may be possible, however, to further manipulate and optimise the response since *in vivo* erythropoietin expression is induced 1000 fold in response to hypoxia or anaemia (Jelkmann, 1992). Incorporating HRE elements 5' and 3' to the target gene may enhance the transcriptional activation. It may also be beneficial to overexpress the transcription factor subtypes HIF-1α and/or HIF-1β in conjunction with the therapeutic construct that is driven by a promoter containing HIF-1 binding sites. Overexpressing the HIF-1β subtype, in particular, may enhance the magnitude of induction since HIF-1β levels are the limiting factor in the activation of HIF-1 DNA binding activity (Kallio et al., 1997).

Alternatively, gene product levels may be enhanced by increasing the stability of the mRNA message. Hypoxia increases the stability of tyrosine hydroxylase mRNA three fold in the pheochromocytoma-derived PC12 clonal cell line (Czyzyk-Krzeska et al., 1994). This effect is specific to tyrosine hydroxylase as neither β-actin, nor GAPDH mRNAs are stabilised under the same conditions (Czyzyk-Krzeska et al., 1994). The observed increase in mRNA half-life correlates with enhanced binding of a 66-kDa protein to a 27 base pyrimidine-rich tract in the 3' untranslated region of the tyrosine hydroxylase mRNA (Czyzyk-Krzeska and Beresh, 1996). A number of other genes,

including erythropoietin, inducible nitric oxide synthase, tumour necrosis factor α, myoglobin and tryptophan hydroxylase also contain sequences similar to the 27 base pyrimidine rich tract found in the 3' untranslated region of tyrosine hydroxylase (Czyzyk-Krzeska and Beresh, 1996). Incorporating this hypoxia inducible binding site in the 3' untranslated region of the therapeutic gene of choice would not only increase the stability of the mRNA message during periods of hypoxia, thereby increasing the levels of the gene product, but also confer a further degree of hypoxia regulation to the therapeutic gene. Accurate regulation of foreign genes is crucial in any gene transfer approach. The ability to regulate gene product levels at both the transcriptional level and the translational level would be crucially important.

These results demonstrate for the first time that the human EPO hypoxia responsive enhancer element (HRE) linked to a basal muscle specific α-MHC promoter confers inducible expression of a luciferase reporter gene in response to myocardial ischaemia. It may therefore be possible to regulate a therapeutic gene such that its expression is directly controlled by the pathological state it is designed to treat. The reversible nature of the reporter gene activation in response to ischaemia also implies that the tissue levels of a therapeutic gene product would fluctuate in accordance with the duration, frequency and severity of the ischaemic episode. Thus the ability to control foreign gene expression in a tissue specific and ischaemia responsive manner represents an important step forward in gene therapy models of heart disease.

Chapter 4

RESULTS 2

4.1 Introduction

The myocardial adaptive response to ischaemic stress involves changes in the transcription of a variety of genes including immediate early genes (Brand et al., 1992), antioxidant enzymes (Das et al., 1993), growth factors (Herskowitz et al., 1995; Hashimoto et al., 1994; Banai et al., 1994), cytokines (Herskowitz et al., 1995) and receptors (IhlVahl et al., 1995). The cellular pathways responsible for changes in gene expression are likely to involve components responsive to hypoxia, hypoglycaemia or the redox status of the cell. To further elucidate potential cellular pathways that underly responses to ischaemia with reperfusion we investigated the transcriptional response of the skeletal α-actin promoter in an experimental model of ischaemia with reperfusion.

4.1.1 The Skeletal α-actin Gene

Human skeletal α-actin (SkAct) is a member of the highly conserved actin multigene family, consisting of six major isoforms (Vandekerckhove and Weber, 1979). The two striated muscle isoforms, skeletal α-actin and cardiac α-actin (CAct) and the two smooth muscle isoforms, α-smooth and γ-smooth are found in the contractile apparatus of muscle fibres (Vandekerckhove and Weber, 1979; Vandekerckhove and Weber, 1984). The cytoskeletal isoforms, β and γ are found in the cytoskeleton of muscle and non-muscle cells (Vandekerckhove and Weber, 1978). Each isoform is encoded by a separate gene (Schwartz et al., 1993) and their expression is temporally and tissue specifically regulated (McHugh et al., 1991).

SkAct and CAct mRNA levels accumulate during the early development of cardiac muscle in foetal and neonatal rodent hearts (Vandekerckhove et al., 1986; Mayer et al., 1984). In the adult heart, CAct expression remains high whereas SkAct expression is very low or absent (Schwartz et al., 1986; Mayer et al., 1984; Vandekerckhove et al., 1986). However, during cardiac hypertrophy, SkAct is re-expressed at levels equivalent

to the CAct isoform (Schiaffino et al., 1989; Schwartz et al., 1986). This reversion to the foetal programme of gene expression during cardiac hypertrophy is also seen with the myosin heavy chains (MHC), where the β-isoform is re-expressed at the expense of the α-isoform (Swynghedauw, 1986; Schiaffino et al., 1989; Schwartz et al., 1981). Human ventricular myosin light chain (vMLC) is expressed in foetal and adult ventricular muscle but not in either foetal or adult atrial muscle. However, vMLC is induced in human atrial muscle by pressure overload which plays a role in the onset of cardiac hypertrophy (Kurabayashi et al., 1988). This pattern of expression of different isoforms of contractile proteins such as SkAct, β-MHC and vMLC is part of an adaptive response by the heart to an increased haemodynamic load. Other adaptive mechanisms include an increased number of small mitochondria and an overall increase in the rate of protein synthesis and accumulation resulting in cardiac hypertrophy (Swynghedauw and Delcayre, 1982; Korecky and Rakusan, 1978).

The SkAct promoter has been extensively characterised with respect to its activation during cardiac hypertrophy (MacLellan et al., 1994; Parker et al., 1992; Bishopric et al., 1992; Bishopric et al., 1987). α_1-Adrenergic stimulation of isolated cardiac myocytes induces myocardial hypertrophy, increasing cell size as measured by cell protein content and surface area (Bishopric et al., 1987). α_1-Adrenergic stimulation also leads to a specific up-regulation of human SkAct mRNA expression (Bishopric et al., 1987). It was subsequently shown that specific upstream promoter sequences between full length (-2000) and 5' deleted (-1300) are required for the up-regulation of SkAct expression during α_1-adrenergic stimulated myocardial hypertrophy (Bishopric and Kedes, 1991).

β-Adrenergic stimulation of isolated cardiac myocytes leads to a specific up-regulation of SkAct which is preceded by an induction of the immediate early genes, c-fos and c-jun (Bishopric et al., 1992). Overexpression of c-jun, or c-fos and c-jun in cardiac myocytes transactivates the SkAct promoter by about 5-fold. The transactivation of the SkAct promoter by c-fos and c-jun was identified to be mediated via sequences from -153 to -

36, and c-fos and c-jun protein was shown to bind specifically within this region (Bishopric et al., 1992). Mutations or deletions of base pairs -153 to -36 also prevented the functional interaction with the AP-1 complex.

Mechanisms underlying the muscle restricted transcription of the chicken SkAct (cSkAct) gene reveal further regulatory elements (MacLellan et al., 1994; Lee et al., 1991; Lee et al., 1992). However, differences exist between the patterns of regulation of the human SkAct and chicken SkAct promoters. The muscle specific expression of the cSkAct gene is determined by the competitive binding of a positive regulator, serum response factor (SRF), and a negative regulator, YY1, to the proximal serum response element (SRE) (Lee et al., 1992; Lee et al., 1991). SRF interacts with the cSkAct promoter through a high affinity proximal SRE and a distal SRE, whereas YY1 binds solely to the proximal SRE (Lee et al., 1991). The co-operative promoter binding of SRF, however, displaces prebound YY1 and activates the cSkAct promoter (Lee et al., 1991). cSkAct promoter activity can also be induced by transforming growth factor β (TGFβ) and basic fibroblast growth factor (bFGF) and conversely suppressed by acidic growth factor (aFGF) (MacLellan et al., 1994; Parker et al., 1992).

SkAct mRNA levels are selectively activated by hypoxia/reoxygenation cycles (Webster et al., 1993) and catecholamine stimulation (Meggs et al., 1992; Bishopric et al., 1987), both of which are integral components of ischaemia. The immediate early genes c-fos and c-jun, which are both induced by ischaemia (Brand et al., 1992), will positively regulate SkAct, through proximal AP-1 binding sites (Bishopric et al., 1992). Indeed, the response of the SkAct promoter to a variety of growth factors, hormones and proto-oncogenes have been extensively characterised and a number of specific regulatory domains subsequently identified (Parker et al., 1992; MacLellan et al., 1994; Bishopric et al., 1992; Karns et al., 1995).

4.1.2 Direct DNA injection

Direct DNA injection into both skeletal and cardiac muscle results in the reproducible expression of functionally active recombinant protein (Buttrick et al., 1992; Lin et al., 1990; Wolff et al., 1990). Using the direct injection technique, a variety of regulatory elements responsible for tissue specificity have been identified (Kitsis et al., 1991; Boveris and Cadenas, 1975). Direct DNA injection has also been employed to demonstrate the hormonal modulation of the rat α-myosin heavy chain promoter (Kitsis et al., 1991), illustrating the potential of direct DNA injection for identifying regulatory elements of genes expressed in the myocardium. Direct injection of DNA into myocardium subjected to 15 minutes of ischaemia followed by reperfusion results in reporter gene expression levels comparable to that of non-ischaemic myocardium (Prentice et al., 1996). Evidence also shows that the tissue specificity of directly injected promoter constructs is retained after direct DNA injection into the myocardium (Prentice et al., 1994).

4.2 Experimental Design

4.2.1 Alterations in Skeletal α-actin promoter activity following ischaemia with reperfusion

This study was designed to further elucidate potential cellular pathways that underly responses to ischaemia with reperfusion. The skeletal α-actin promoter (SkAct) was chosen because although it is known to be a marker of hypertrophy, it is capable of responding to components of ischaemia, namely hypoxia and catecholamine stimulation. Furthermore, the SkAct promoter has been extensively characterised, and extensive *in vitro* data on both wild type and mutated SkAct promoter sequences was available through a collaboration with Drs Keith Webster and Nannette Bishopric. The access to deleted and mutated SkAct promoter constructs may provide valuable information about SkAct promoter regulation in the adult and allow comparisons between *in vivo* and *in vitro* responses. These constructs may also potentially allow domains of the SkAct promoter responsive to ischaemia with reperfusion to be mapped. Such domains could then be incorporated into synthetic hybrid promoters for construction of gene delivery vectors.

4.2.2 Transactivation of the SkAct promoter by AP-1

Cotransfection assays in cardiac myocytes demonstrated that overexpression of c-jun, or of c-c-fos plus c-jun, transactivated the skeletal actin promoter by up to 5 fold (Bishopric et al., 1992). Skeletal actin sequences between -153 and -36 were required for maximal transactivation by c-fos/c-jun, and purified c-fos and c-jun were bound specifically within this region. A direct physiological role was therefore suggested for the AP-1 transcription factor complex in regulating skeletal actin gene expression (Bishopric et al., 1992).

4.2.3 Regulation of SkAct promoter activity in the heart by GATA-4

SkAct expression in the heart involves the complex interaction of a number of transcription factors. GATA-4 is a member of the GATA DNA binding family of zinc finger proteins that interact with DNA sequence elements containing the conserved motif 5'-GATA-3' (Arceci et al., 1993). This conserved motif was recently identified in the promoter sequence of the human SkAct gene (N. Bishopric, personal communication).

GATA-4 is expressed early in development and its expression is restricted to the heart and endodermally-derived tissues (Arceci et al., 1993). The α-myosin heavy chain (MHC) gene is the major structural protein in the heart and contains two putative GATA-binding sites in the proximal enhancer region of the gene (Molkentin et al., 1994). GATA-4 interacts with these sites, activating cardiac-specific expression (Molkentin et al., 1994). A number of other cardiac-specific structural genes, including β-myosin heavy chain, atrial and ventricular myosin light chain and cardiac α-actin, also contain putative GATA-4 binding sites, implicating GATA-4 as a major regulator of cardiac-specific gene expression (Molkentin et al., 1994).

4.3 Results

4.3.1 Alterations in Skeletal α-actin promoter activity following ischaemia with reperfusion

In order to assess the inducibility of the SkAct promoter in response to ischaemia with reperfusion (IR), the rat model of ischaemia with reperfusion was initially employed, as described in section 2.5.2. Briefly, male Sprague Dawley rats were anaesthetised and artificially ventilated. The hearts were exposed and subjected to 15 minutes ischaemia followed by reperfusion (IR) or to sham operation which involves an identical procedure but without the coronary ligation. DNA promoter/reporter gene constructs were introduced by direct injection into the cardiac apex. Hearts were removed and assayed for reporter gene expression one and seven days later, as described in section 2.5.4.

To take into account varying uptake efficiencies between injections, a ubiquitously expressing SV40 construct is co-injected with the SkAct promoter construct. Results are expressed as a ratio of the SkAct construct to the ubiquitously expressing SV40 construct.

Two SkAct promoter constructs, both driving the expression of a luciferase reporter gene, were used. Firstly, the full length -2000 human SkAct promoter and secondly, the shorter length -1282 human SkAct promoter.

Table 4.1 Levels of activity from the -2000 skeletal actin promoter at 7 days in sham and IR rat myocardium.

Protocol : **rat heart, 15 mins ischaemia with reperfusion**

Time point : **7 days**

CAT construct : **SV40 / CAT (50μg)**

Luciferase construct : **-2000 Skeletal actin / luciferase (50μg)**

Experiment no.	Protocol	CAT(SV40)	LUC(-2000)	Ratio	
1	SHAM	19717	770	**0.04**	
2	SHAM	57267	20100	**0.35**	
3	SHAM	53235	15160	**0.28**	
4	SHAM	41690	12420	**0.30**	
5	SHAM	9099	1150	**0.13**	
6	SHAM	28520	1830	**0.06**	***Mean***
7	SHAM	7413	1560	**0.21**	**0.19 ± 0.04**
8	SHAM	24767	3140	**0.13**	
9	IR	4414	660	**0.15**	
10	IR	4165	280	**0.07**	
11	IR	15299	580	**0.04**	
12	IR	67069	2930	**0.04**	***Mean***
13	IR	9683	2750	**0.28**	**0.13 ± 0.04**
14	IR	6028	1280	**0.21**	

Promoter activities from 14 individual experiments are shown. Hearts were injected with the plasmid DNA constructs -2000 Skeletal actin / Luciferase and SV40 / CAT in equal ratios. Animals were then subjected to 15 minutes of ischaemia with reperfusion (IR) or to Sham (SHAM) operation only, as described in section 2.5.2. Hearts were excised and assayed for reporter gene expression 7 days post ischaemia as described in section 2.5.4. CAT activities, under the control of a SV40 promoter, are shown in column 3. CAT activity is presented as counts per minute (cpm) above background, representing the conversion of ^{14}C-chloramphenicol to its acetylated product by the reporter gene product chloramphenicol acetyl transferase. Luciferase activities, under the control of the -2000 Skeletal actin promoter, are shown in column 4. Luciferase activity is presented as relative light units (RLU) multiplied by 1000, representing the oxidation of luciferin by luciferase with concomitant production of a photon. Ratio values in column 5 represent the luciferase data point divided by the CAT data point for each experiment. Ratio values allow the test promoter (-2000 Skeletal actin) to be normalised by reference to the internal control (SV40). The

mean of the ratios ± standard error for the Sham or IR procedures are shown in column 6. Statistical analysis was performed using the Students t-test for unpaired samples.

Table 4.2 Levels of activity from the -1282 skeletal actin promoter at 7 days in sham and IR rat myocardium.

Protocol : **rat heart, 15 mins ischaemia with reperfusion**

Time point : **7 days**

CAT construct : **SV40 / CAT (50μg)**

Luciferase construct : **-1282 Skeletal actin / luciferase (50μg)**

Experiment no.	Protocol	CAT(SV40)	LUC(-1282)	Ratio	
1	SHAM	77772	11260	**0.14**	
2	SHAM	71525	24020	**0.34**	
3	SHAM	14989	13880	**0.93**	***Mean***
4	SHAM	28812	14330	**0.50**	**0.52 ± 0.14**
5	SHAM	97240	68050	**0.70**	
6	IR	25931	1818	**0.07**	
7	IR	42925	19550	**0.46**	
8	IR	36589	8291	**0.23**	
9	IR	55521	31680	**0.57**	***Mean***
10	IR	68890	43390	**0.63**	**0.40 ± 0.09**
11	IR	56299	26810	**0.48**	

Promoter activities from 11 individual experiments are shown. Hearts were injected with the plasmid DNA constructs -1282 Skeletal actin / Luciferase and SV40 / CAT in equal ratios, as described in section. Animals were then subjected to 15 minutes of ischaemia with reperfusion (IR) or to Sham (SHAM) operation only, as described in section 2.5.2. Hearts were excised and assayed for reporter gene expression 7 days post ischaemia. The format and layout of this table is as table 4.1.

Levels of Activity from the -2000 and -1282 Skeletal α-actin Promoter at 7 days in sham and IR Rat Myocardium

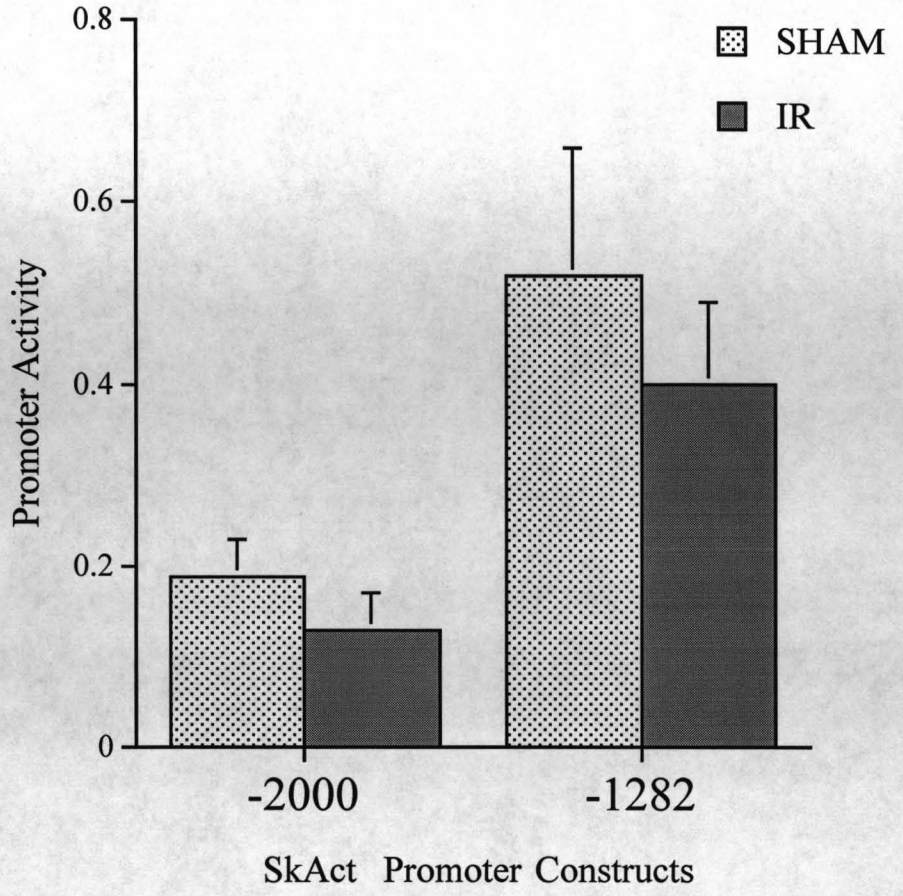

Figure 4.1 The surgical procedures, injections and assays are described in section 2.5.2. Hearts were injected with either -2000 SkAct (-2000) or -1282 SkAct (-1282) promoter constructs and subjected to IR or sham procedures. Hearts were excised and reporter gene activities assayed 7 days after surgery. This figure summarises the data presented in tables 4.1 and 4.2. Promoter activity is the ratio of average levels of reporter gene expression driven by the SkAct promoter to average levels of reporter gene expression driven by the SV40, ubiquitously expressing, promoter, with standard errors indicated.

A comparison of skeletal actin promoter activities in IR and sham operated animals at 7 days are shown in figure 4.1. No difference was observed between the mean of the ratios for the -2000 SkAct promoter activity in the sham experiments (0.19 ± 0.04) compared to the IR experiments (0.13 ± 0.04). The mean of the ratios for the -1282 SkAct promoter activity was also similar in the two groups, being 0.52 ± 0.14 in the sham experiments and 0.40 ± 0.09 in the IR experiments. The inducibility of the SV40 basal promoter has been previously investigated using this model at 7 days and is not induced (H. Prentice, personal communication). IR has therefore no detectable effect on the activity of the skeletal actin promoter at 7 days.

There are two possible explanations for this data. Either, IR has no effect on the activity of the skeletal actin promoter, or the effects of IR on the activity of the skeletal actin promoter have receded by 7 days. Ischaemia leads to a variety of responses, including an increase in the production and release of catecholamines. Evidence shows that α_1-adrenergic stimulation of cultured neonatal myocytes leads to a specific up-regulation, over ten fold, of the mRNA for the skeletal actin gene (Bishopric et al., 1987). Indeed, increased sympathetic nervous system activity, induced by deoxycorticosterone acetate (DOCA) salt hypertension, up-regulates skeletal actin mRNA levels (Meggs et al., 1992). Furthermore, hypoxia, an integral part of ischaemia, induces skeletal actin mRNA levels in cultured neonatal myocytes (K. Webster, personal communication). *In vivo*, skeletal actin mRNA levels are up-regulated in myocardial infarction-induced left ventricular failure (Meggs et al., 1990) and aortic stenosis leads to an accumulation of skeletal actin mRNA levels within four hours (Schiaffino et al., 1989).

Therefore in order to determine if the effects of IR on the activity of the skeletal actin promoter occur at an earlier stage, the study was repeated using a one day time point.

Table 4.3 Effect of IR on the induction of the -2000 skeletal actin promoter at 1 day in the rat.

Protocol : **rat heart, 15 mins ischaemia with reperfusion**

Time point : **1 day**

CAT construct : **SV40 / CAT (50μg)**

Luciferase construct : **-2000 Skeletal actin / luciferase (50μg)**

Experiment no.	Protocol	CAT(SV40)	LUC(-2000)	Ratio	
1	SHAM	839	1199	**1.4**	
2	SHAM	523	4086	**7.8**	
3	SHAM	1645	41650	**25.3**	
4	SHAM	655	11300	**17.3**	***Mean***
5	SHAM	283	3929	**13.9**	**11.5 ± 3.7**
6	SHAM	1884	6700	**3.6**	
7	IR	277	4371	**15.8**	
8	IR	449	4379	**9.8**	
9	IR	580	5684	**9.8**	
10	IR	403	11270	**28.0**	***Mean***
11	IR	1488	5686	**3.8**	**12.2 ± 3.6**
12	IR	721	4336	**6.0**	

Promoter activities from 12 individual experiments are shown. Hearts were injected with the plasmid DNA constructs -2000 Skeletal actin / Luciferase and SV40 / CAT in equal ratios, as described in section. Animals were then subjected to 15 minutes of ischaemia with reperfusion (IR) or to Sham (SHAM) operation only, as described in section 2.5.2. Hearts were excised and assayed for reporter gene expression 1 day post ischaemia. The format and layout of this table is as table 4.1.

Table 4.4 Effect of IR on the induction of the -1282 skeletal actin promoter at 1 day in the rat.

Protocol : **rat heart, 15 mins ischaemia with reperfusion**

Time point : **1 day**

CAT construct : **SV40 / CAT (50μg)**

Luciferase construct : **-1282 Skeletal actin / luciferase (50μg)**

Experiment no.	Protocol	CAT(SV40)	LUC(-1282)	Ratio	
1	SHAM	335	2500	**7.5**	
2	SHAM	1922	58200	**30.3**	
3	SHAM	2284	42900	**18.8**	***Mean***
4	SHAM	1393	22000	**15.8**	**20.8 ± 4.5**
5	SHAM	363	11400	**31.5**	
6	IR	2340	85000	**36.3**	
7	IR	2953	88200	**29.9**	***Mean***
8	IR	1455	45100	**31.0**	**41.3 ± 9.0**
9	IR	601	40800	**67.8**	

Promoter activities from 9 individual experiments are shown. Hearts were injected with the plasmid DNA constructs -1282 Skeletal actin / Luciferase and SV40 / CAT in equal ratios, as described in section. Animals were then subjected to 15 minutes of ischaemia with reperfusion (IR) or to Sham (SHAM) operation only, as described in section 2.5.2. Hearts were excised and assayed for reporter gene expression 1 day post ischaemia. The format and layout of this table is as table 4.1.

Levels of Activity from the -2000 and -1282 Skeletal α-actin Promoter at 1 day in sham and IR Rat Myocardium

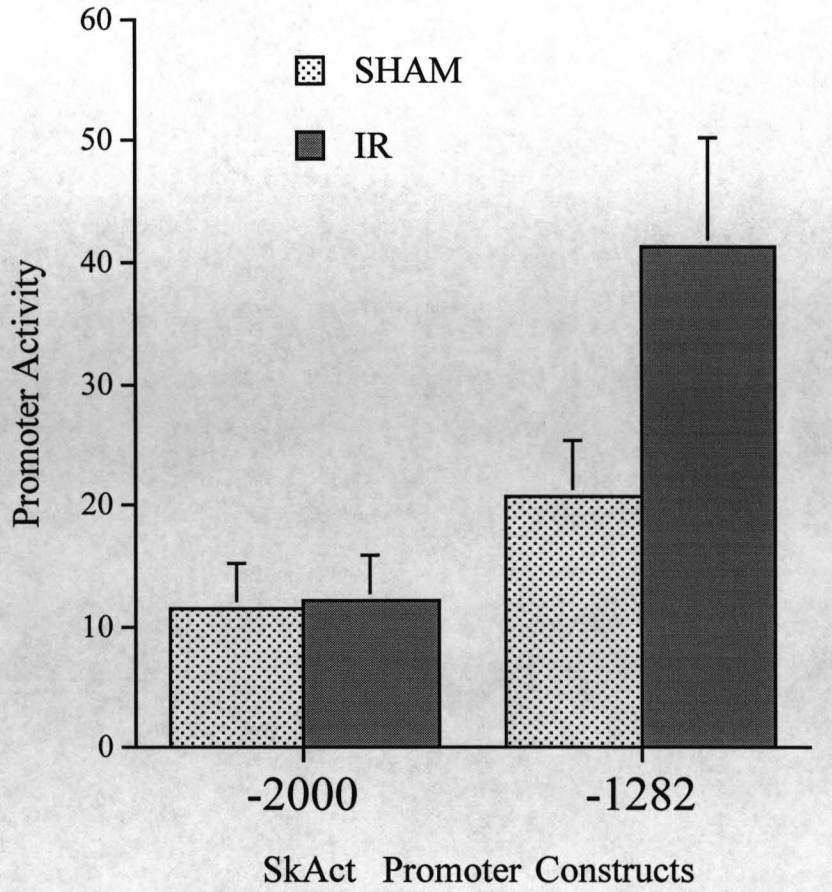

Figure 4.2 The surgical procedures, injections and assays are described in section 2.5.2. Hearts were injected with either -2000 SkAct (-2000) or -1282 SkAct (-1282) promoter constructs and subjected to IR or sham procedures. Hearts were excised and reporter gene activities assayed 1 day after surgery. This figure summarises the data presented in tables 4.3 and 4.4. Promoter activity is the ratio of average levels of reporter gene expression driven by the SkAct promoter to average levels of reporter gene expression driven by the SV40, ubiquitously expressing, promoter, with standard errors indicated.

Figure 4.2 shows a comparison of SkAct promoter activities in IR and sham operated animals. The mean of the ratios for the -2000 SkAct promoter activity was 11.5 ± 3.7 for the sham experiments and 12.2 ± 3.6 for the IR experiments. The -1282 SkAct promoter activity in the sham experiment was 20.8 ± 4.5 compared to 41.3 ± 9.0 following IR. The inducibility of the SV40 basal promoter has been previously investigated using this model at 1 day and is not induced (H. Prentice, personal communication). The -2000 SkAct promoter activity shows no difference between sham and IR animals. The -1282 SkAct promoter activity shows a non-significant trend possibly suggesting an effect of IR.

From the 9 samples tested, no clear conclusions can be drawn about the activation, if any, of the -1282 SkAct promoter in response to IR. Any activation of the -1282 SkAct promoter that may be present at 1 day might represent an earlier induction that is now fading. To determine if this non-significant trend was indicative of an earlier response or merely an experimental artefact, time points less than 1 day were investigated.

Time Point	Mean SV40/CAT activity
3 hours	0 (n=2)
8 hours	52 (n=1)
12 hours	70 (n=3)

Table 4.5 Mean CAT reporter gene activity at 3, 8 and 12 hour time points. Rat hearts were injected with 50μg SV40/CAT plasmid as described in section 2.5.1. At the time points indicated in column 1, hearts were excised and assayed for CAT reporter gene activity. CAT activity is presented in column 2 as counts per minute (cpm) above background, representing the conversion of ^{14}C-chloramphenicol to its acetylated product by the reporter gene product chloramphenicol acetyl transferase.

As table 4.5 illustrates, significant levels of reporter gene activity could not be detected at time points less than 1 day using the rat model of ischaemia with reperfusion (see table

4.5). Therefore, in order to overcome this problem alternative models were considered. The rabbit model of ischaemia with reperfusion was developed and described below.

Rabbit model of Ischaemia with Reperfusion

This model has been discussed previously in section 3.2.1. Briefly, rabbits were anaesthetised and artificially ventilated. The heart was exposed by a left thoracotamy and DNA injected firstly into the cardiac apex and secondly into the proximal left ventricular wall. Seven days after DNA injection, rabbits were subjected to a second thoracotamy and the first obtuse marginal artery ligated immediately above the cardiac apex. Ischaemia was confirmed by visualisation of tissue decolourisation. After 15 minutes of ischaemia, the suture was removed to allow reperfusion of the apex. Hearts were excised and assayed 1 hour post ischaemia.

As discussed in section 3.2.1, this model ensures that the injected DNA is present 7 days before the ischaemic insult, there is no restriction on the time points that can be assayed and both sham and IR experiments can be performed in the one heart.

Table 4.6a Effect of 15 mins ischaemia with 1 hour of reperfusion on the -1282 Skeletal actin promoter in the rabbit heart.

Protocol : **rabbit heart, 15 mins ischaemia with reperfusion**

Time point : **1 hour**

Luciferase construct : **-1282 Skeletal actin / luciferase (50μg)**

CAT construct : **SV40 / CAT (50μg)**

Experiment no.	Region	CAT(SV40)	LUC(-1282)	Ratio	
A	SHAM	6155	19	**3.1**	***Mean***
B	SHAM	572	0.9	**1.5**	**2.3**
C	IR	27547	236	**8.6**	***Mean***
D	IR	10662	88	**8.2**	**8.4**

Promoter activities from 4 experiments are shown. Hearts were injected in the apical and proximal lateral wall regions with the plasmid DNA constructs -1282 Skeletal actin / Luciferase and SV40 / CAT in equal ratios. 7 days after the DNA injection, a second thoracotamy was performed and the apical region of the heart subjected to 15 minutes of ischaemia by left marginal artery ligation followed by 1 hour of reperfusion (IR), as described in section 2.5.3. The proximal lateral wall remained perfused throughout (SHAM). CAT activities, under the control of a SV40 promoter, are shown in column 3. CAT activity is presented as counts per minute (cpm) above background, representing the conversion of ^{14}C-chloramphenicol to its acetylated product by the reporter gene product chloramphenicol acetyl transferase. Luciferase activities, under the control of the -1282 Skeletal actin promoter, are shown in column 4. Luciferase activity is presented as relative light units (RLU), representing the oxidation of luciferin by luciferase with concomitant production of a photon. Ratio values in column 5 represent the luciferase data point divided by the CAT data point for each experiment, multiplied by 1000. Ratio values allow the test promoter (-1282 Skeletal actin) to be normalised by reference to the internal control (SV40). The mean of the ratios ± standard error for the Sham or IR regions are shown in column 6. Statistical analysis was performed using the Students t-test for paired samples.

Further experiments using this protocol failed to give any activity from either the CAT or luciferase expressing constructs. The absence of reporter gene expression was identified to have occurred at the same time as a fresh preparation of plasmid DNA was used. Further batches of DNA were prepared using the caesium gradient method (described in section 2.2.1) but as before they also failed to give any activity of either the CAT or luciferase expressing constructs when injected into the rabbit myocardium. The reason for the inactivity is unclear but may be due to contamination from the caesium gradient purification protocol. This is discussed later. An alternative method of preparing the DNA for injection was used. DNA prepared using Qiagen filters (described in section 2.2.1) gave reproducible levels of both CAT and luciferase reporter gene activity when injected into the rabbit myocardium. Qiagen DNA was therefore used for the following experiments.

Table 4.6b Effect of 15 mins ischaemia with 1 hour of reperfusion on the -1282 Skeletal actin promoter in the rabbit heart.

Protocol : **rabbit heart, 15 mins ischaemia with reperfusion**

Time point : **1 hour**

Luciferase construct : **-1282 Skeletal actin / luciferase (50μg)**

CAT construct : **SV40 / CAT (50μg)**

Experiment no.	Region	CAT(SV40)	LUC(-1282)	Ratio	
1	SHAM	26781	296	**11.1**	
2	SHAM	25094	276	**11.0**	
3	SHAM	22726	234	**10.3**	
4	SHAM	43479	414	**9.5**	***Mean***
5	SHAM	30327	393	**13.0**	**10.3 ± 0.8**
6	SHAM	6739	47.2	**7.0**	
7	IR	27239	197	**7.2**	
8	IR	25598	241	**9.4**	
9	IR	19022	247	**13.0**	
10	IR	24502	620	**25.3**	***Mean***
11	IR	22208	225	**10.1**	**11.7 ± 2.9**
12	IR	24846	124	**5.0**	

Promoter activities from 12 experiments are shown. Hearts were injected in the apical and proximal lateral wall regions with the plasmid DNA constructs -1282 Skeletal actin / Luciferase and SV40 / CAT in equal ratios. 7 days after the DNA injection, a second thoracotamy was performed and the apical region of the heart subjected to 15 minutes of ischaemia by left marginal artery ligation followed by 1 hour of reperfusion (IR), as described in section 2.5.3. The proximal lateral wall remained perfused throughout (SHAM). The format and layout of this table is as table 4.6a.

Table 4.7 Effect of 15 mins ischaemia with 1 hour of reperfusion on the -2000 Skeletal actin promoter in the rabbit heart.

Protocol : **rabbit heart, 15 mins ischaemia with reperfusion**

Time point : **1 hour**

Luciferase construct : **-2000 Skeletal actin / luciferase (50μg)**

CAT construct : **SV40 / CAT (50μg)**

Experiment no.	Region	CAT(SV40)	LUC(-1282)	Ratio	
1	SHAM	2220	54	**24.3**	
2	SHAM	5385	331	**61.5**	
3	SHAM	6300	236	**37.4**	
4	SHAM	19128	935	**48.9**	***Mean***
5	SHAM	14200	670	**47.2**	**49.8 ± 7.8**
6	SHAM	19191	1527	**79.6**	
7	IR	13871	454	**32.7**	
8	IR	6449	309	**47.9**	
9	IR	4573	71	**15.5**	
10	IR	13620	341	**25.0**	***Mean***
11	IR	15584	838	**53.8**	**36.1 ± 5.9**
12	IR	17647	732	**41.5**	

Promoter activities from 12 experiments are shown. Hearts were injected in the apical and proximal lateral wall regions with the plasmid DNA constructs -2000 Skeletal actin / Luciferase and SV40 / CAT in equal ratios. 7 days after the DNA injection, a second thoracotamy was performed and the apical region of the heart subjected to 15 minutes of ischaemia by left marginal artery ligation followed by 1 hour of reperfusion (IR), as described in section 2.5.3. The proximal lateral wall remained perfused throughout (SHAM). The format and layout of this table is as table 4.6a.

Levels of Activity from the -2000 and -1282 Skeletal α-actin Promoter at 1 hour in sham and IR Rabbit Myocardium

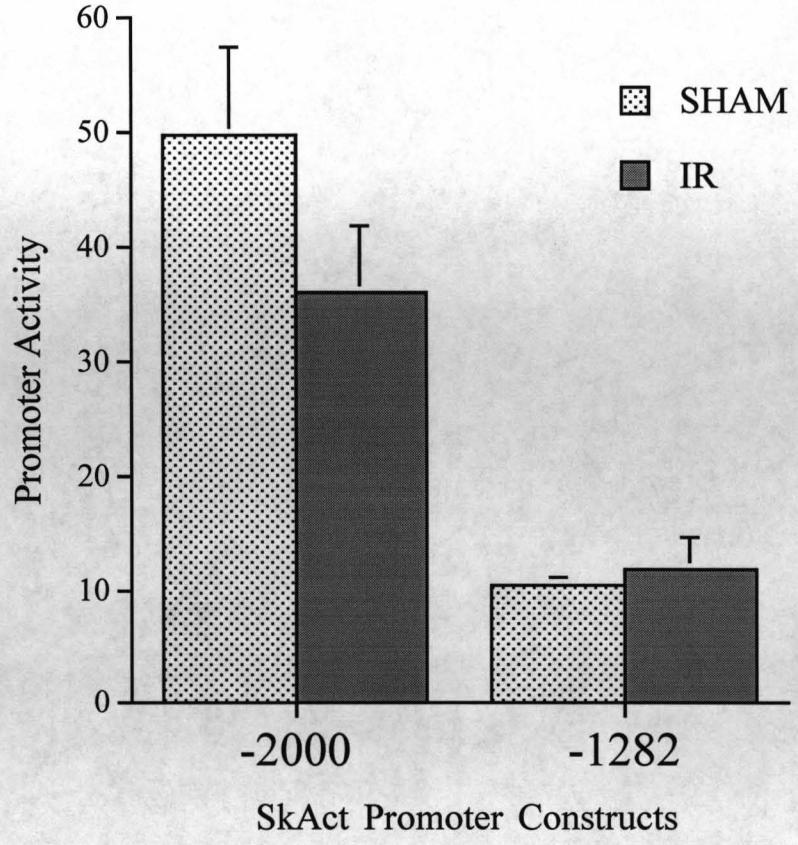

Figure 4.3 The surgical procedures, injections and assays are described in section 2.5.3. Hearts were injected with either -2000 SkAct (-2000) or -1282 SkAct (-1282) promoter constructs and subjected to IR or sham procedures. Hearts were excised and reporter gene activities assayed 1 hour after IR. This figure summarises the data presented in tables 4.6b and 4.7. Promoter activity is the ratio of average levels of reporter gene expression driven by the SkAct promoter to average levels of reporter gene expression driven by the SV40, ubiquitously expressing, promoter, with standard errors indicated.

-1282 SkAct promoter activities from caesium purified DNA in sham and IR areas of the heart at 1 hour are shown in table 4.6a. Mean -1282 SkAct promoter activity was approximately three and a half times higher in IR areas of the heart than in sham areas of the heart. From the four samples tested no clear conclusions can be drawn, however, the induction of SkAct promoter activity in IR areas of the heart shows a non-significant trend. Further experiments could not be performed because DNA batches prepared using the caesium gradient method no longer gave reporter gene expression when injected into the rabbit myocardium. DNA prepared using Qiagen filters did, however, give reporter gene expression. Qiagen purified DNA was therefore used for the remainder of the study.

-1282 SkAct and -2000 SkAct promoter activities using Qiagen purified DNA in sham and IR areas of the heart at 1 hour are shown in figure 4.3. The mean -2000 SkAct promoter activity in sham areas of the heart was 49.8 ± 7.8 compared to 36.1 ± 5.9 in IR areas after 1 hour of reperfusion. The mean -1282 SkAct promoter activity in sham areas of the heart was 10.3 ± 0.8 compared to 11.7 ± 2.9 in IR operated areas after 1 hour of reperfusion. The inducibility of the SV40 basal promoter, used as an internal control, has been previously investigated using this model at 4 hours and was not induced (see table 3.9). Promoter activities for both -2000 SkAct and -1282 SkAct were therefore not significantly different in tissue subjected to 15 minutes of ischaemia followed by 1 hour of reperfusion than in sham operated tissue.

The rabbit model of ischaemia with reperfusion has previously been used successfully at 1 hour to demonstrate the induction of a hybrid promoter containing myosin heavy chain basal regulatory sequences plus four copies of the erythropoietin HIF-1 binding site (see Chapter 3). It is therefore unlikely that this model is incapable of detecting changes in promoter activity at 1 hour in response to 15 minutes ischaemia followed by reperfusion. Further time points are necessary, particularly four and eight hour time points, to fully characterise the activation characteristics of the SkAct promoter following 15 minutes of ischaemia followed by reperfusion. The work presented here is, however, part of an

ongoing research project and the response of the SkAct promoters four and eight hours after IR in the rabbit heart, will be characterised by other investigators.

4.3.2 Transactivation of the SkAct promoter by AP-1

Transactivation of the SkAct promoter by c-fos and c-jun was investigated *in vivo* using the rat model of direct DNA injection, as described in section 2.5.1. Expression vectors encoding c-fos and c-jun, the main constituents of transcriptional activator protein AP-1 were co-injected with the -1282 skeletal actin luciferase construct and SV40 CAT construct. Sham experiments consisted of -1282 skeletal actin, SV40 and non-expressing carrier DNA. The -1282 skeletal actin luciferase construct was chosen to investigate the transactivation of the SkAct promoter. SV40 CAT expression controls for uptake efficiency, as with the ischaemia/reperfusion experiments.

Table 4.8 Regulation of skeletal actin promoter activity by c-fos and c-jun in the rat heart.

Protocol : **rat heart, direct DNA injection**

Time point : **2 days**

DNA injected : **-1282 Skeletal actin / luciferase (50μg)**

SV40 / CAT (25μg)

+ CMV / c-Jun (12.5μg), CMV / c-Fos (12.5μg)

or carrier DNA (25μg)

Experiment no.	Protocol	CAT(SV40)	LUC(-1282)	Ratio	
1	SA	6642	7690	**1.2**	
2	SA	8377	2730	**0.3**	
3	SA	6935	4030	**0.6**	
4	SA	12236	9550	**0.8**	***Mean***
5	SA	17696	19520	**1.1**	**0.79 ± 0.16**
6	SA + F/J	2913	1960	**0.7**	
7	SA + F/J	4327	3740	**0.9**	
8	SA + F/J	1608	1830	**1.1**	
9	SA + F/J	3713	3280	**0.9**	***Mean***
10	SA + F/J	5014	5820	**1.2**	**0.94 ± 0.09**

Promoter activities from 10 individual experiments are shown. Sham hearts were injected with 50μg -1282 Skeletal actin / Luciferase, 25μg SV40 / CAT and 25μg non-expressing cloning vector (SA). c-fos/c-jun hearts were injected with 50μg -1282 Skeletal actin / Luciferase, 25μg SV40 / CAT, 12.5μg CMV / c-jun and 12.5 μg CMV / c-Fos (SA+F/J), as described in section 2.5.1. Hearts were excised and assayed for reporter gene expression 2 days after DNA injection. CAT activities, under the control of a SV40 promoter, are shown in column 3. CAT activity is presented as counts per minute (cpm) above background, representing the conversion of ^{14}C-chloramphenicol to its acetylated product by the reporter gene product chloramphenicol acetyl transferase. Luciferase activities, under the control of the -1282 Skeletal actin promoter, are shown in column 4. Luciferase activity is presented as relative light units (RLU) multiplied by 1000, representing the oxidation of luciferin by luciferase with concomitant production of a photon. Ratio values in column 5 represent the Luciferase data point divided by the CAT data point for each experiment. Ratio values allow the test promoter to be normalised by reference to the internal control (SV40). The mean of the ratios ± standard error for the Sham or IR procedures are shown

in column 6. Statistical analysis was performed using the Students t-test for unpaired samples.

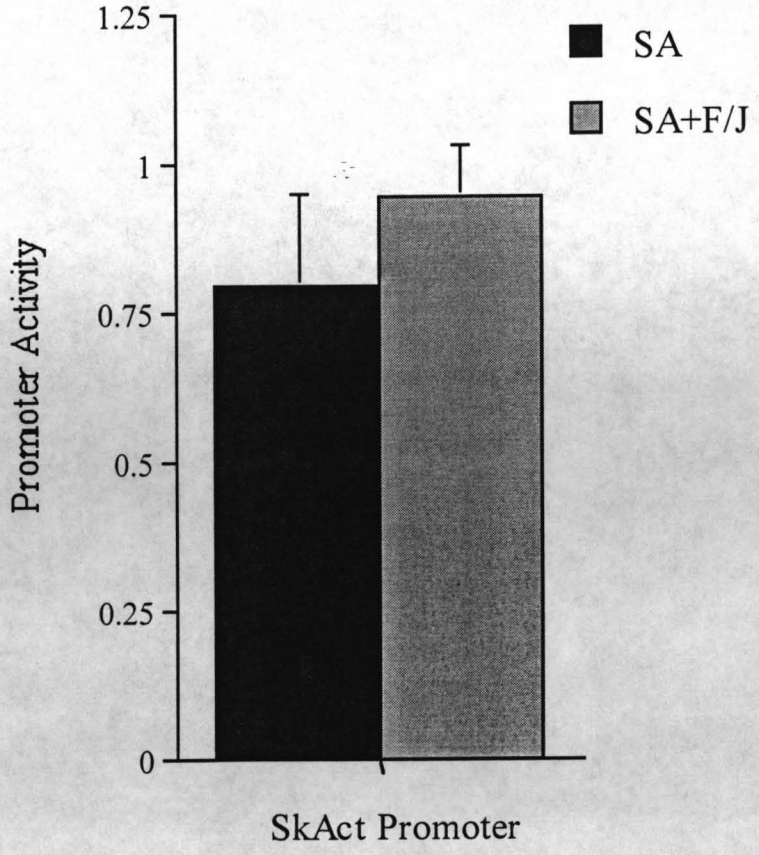

Figure 4.4 The surgical procedures, injections and assays are described in section 2.5.1. Hearts were injected with -1282 SkAct promoter construct and either c-fos and c-jun expressing constructs (SA + F/J) or carrier DNA (SA). Hearts were excised and reporter gene activities assayed 2 days after injection. This figure summarises the data presented in table 4.8. Promoter activity is the ratio of average levels of reporter gene expression driven by the SkAct promoter to average levels of reporter gene expression driven by the SV40, ubiquitously expressing, promoter, with standard errors indicated.

Transactivation of the -1282 SkAct promoter by c-fos and c-jun expressing plasmids is shown in figure 4.4. Mean -1282 SkAct promoter activity in the rat heart 2 days after injection was 0.79 ± 0.16. Mean activity of the -1282 SkAct promoter co-injected with c-fos and c-jun expressing constructs was 0.94 ± 0.09 in rat hearts, 2 days after injection. In this *in vivo* model of direct DNA injection into the rat heart, the -1282 SkAct promoter is not transactivated by c-fos and c-jun overexpression.

4.3.3 Regulation of SkAct promoter activity in the heart by GATA-4

In order to determine the influence of GATA-4 on the regulation of SkAct expression in the heart, full length (-2000) SkAct constructs with a mutation in the GATA-4 DNA binding domain, were injected into the rat myocardium, as described in section 2.5.1. Relative expression levels were compared to non-mutated full length SkAct promoter constructs injected in the same manner. Ubiquitously expressing SV40/CAT constructs were co-injected with either GATA mutant full length SkAct / luciferase constructs (GATA) or non-mutated full length SkAct / luciferase constructs (Wt) to normalise for variations in uptake efficiency.

Table 4.9 Regulation of skeletal actin promoter activity by GATA-4 in the rat heart.

Protocol : **rat heart, DNA injection**

Time point : **7 days**

CAT construct : **SV40 / CAT (25μg)**

Luciferase construct : **-2000 Skeletal actin / luciferase (50μg)**

or -2000 Skeletal actin GATA mutant / luciferase (50μg)

Experiment no.	Protocol	CAT(SV40)	LUC	Ratio	
1	WT	50563	1650	**0.03**	
2	WT	14690	696	**0.05**	***Mean***
3	WT	78818	2940	**0.04**	**0.04 ± 0.01**
4	GATA	33989	13920	**0.41**	
5	GATA	14131	13200	**0.93**	
6	GATA	102107	45510	**0.45**	
7	GATA	136064	28870	**0.21**	***Mean***
8	GATA	107327	46140	**0.43**	**0.46 ± 0.10**
9	GATA	122649	40120	**0.33**	

Promoter activities from 9 individual experiments are shown. Sham hearts were injected with 50μg -2000 Skeletal actin / Luciferase and 25μg SV40 / CAT (WT). GATA hearts were injected with 50μg -2000 Skeletal actin GATA mutant / Luciferase and 25μg SV40 / CAT (GATA), as described in section 2.5.1. Hearts were excised and assayed for reporter gene expression 7 days after DNA injection. CAT activities, under the control of a SV40 promoter, are shown in column 3. CAT activity is presented as counts per minute (cpm) above background, representing the conversion of ^{14}C-chloramphenicol to its acetylated product by the reporter gene product chloramphenicol acetyl transferase. Luciferase activities, under the control of either the -2000 Skeletal actin promoter or -2000 Skeletal actin GATA mutant promoter, are shown in column 4. Luciferase activity is presented as relative light units (RLU) multiplied by 1000, representing the oxidation of luciferin by luciferase with concomitant production of a photon. Ratio values in column 5 represent the Luciferase data point divided by the CAT data point for each experiment. Ratio values allow the test promoter to be normalised by reference to the internal control (SV40). The mean of the ratios ± standard error for the Sham or IR procedures are shown in column 6. Statistical analysis was performed using the Students t-test for unpaired samples.

Involvement of GATA-4 Binding Domain on Skeletal α-actin Promoter Activity in the Rat Myocardium

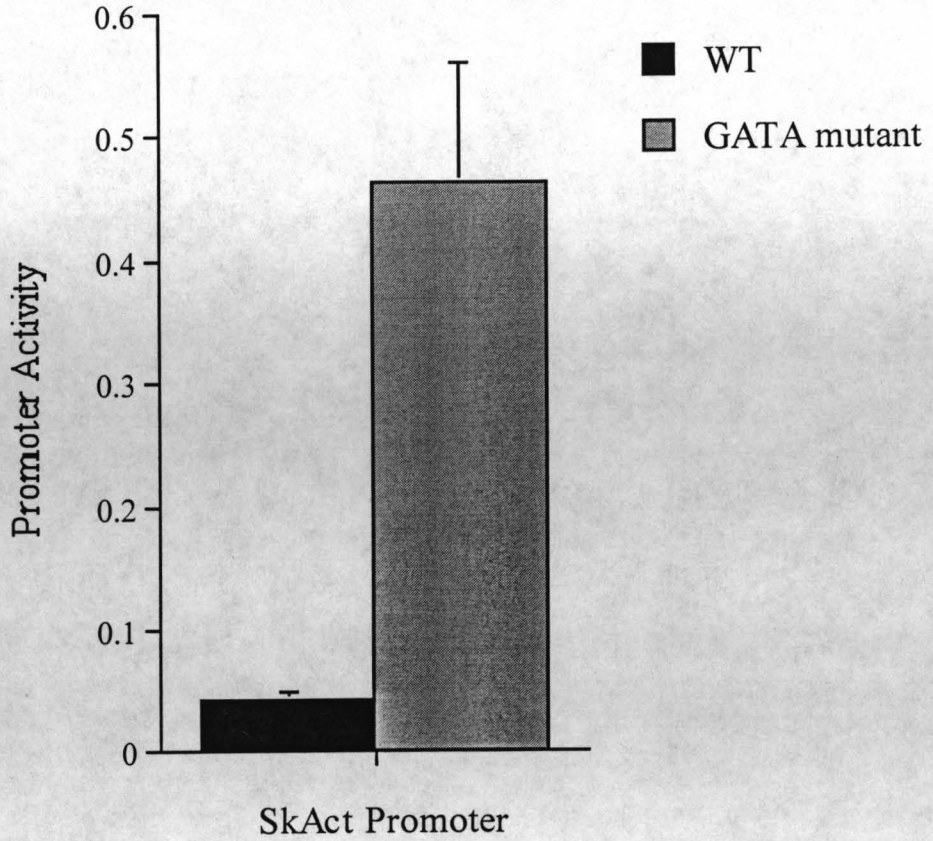

Figure 4.5 The surgical procedures, injections and assays are described in section 2.5.1. Hearts were injected with the full length SkAct promoter (WT) or the full length SkAct promoter with a mutation in its GATA-4 binding domain (GATA mutant). Hearts were excised and reporter gene activities assayed 7 days after injection. This figure summarises the data presented in table 4.9. Promoter activity is the ratio of average levels of reporter gene expression driven by the SkAct promoter to average levels of reporter gene expression driven by the SV40, ubiquitously expressing, promoter, with standard errors indicated.

Regulation of skeletal actin promoter activity by GATA-4 in the rat heart is shown in figure 4.5. Mean full length SkAct promoter activity was 0.04 ± 0.01 7 days after intra-cardiac injection in the rat. Mean activity from the full length SkAct promoter with a mutation in the GATA-4 binding domain was 0.46 ± 0.10. The GATA mutant SkAct promoter, therefore expressed > 10 fold higher than the non-mutated SkAct promoter.

4.4 Discussion

4.4.1 Induction of the Skeletal α-actin promoter by ischaemia with reperfusion

Transcription of the SkAct gene is known to respond to certain components of ischaemia, specifically hypoxia/reoxygenation cycles *in vitro* (Webster et al., 1993) and catecholamine stimulation *in vitro* and *in vivo* (Bishopric et al., 1992; Meggs et al., 1992; Bishopric et al., 1987). However, the models of ischaemia with reperfusion used in this study demonstrate no significant activation of the SkAct promoter in response to a 15 minute ischaemic episode followed by 1 hour of reperfusion in the rabbit heart. Likewise, the SkAct promoter was not activated in response to 15 minutes of ischaemia followed by reperfusion for 1 or 7 days in the rat heart. A number of possible conclusions can therefore be drawn. Firstly, the SkAct promoter may not be responsive to ischaemia at the time points assayed. Alternatively, either the model system employed may have been incapable of detecting any effect, or a transient 15 minute ischaemic episode may not be sufficient to activate the SkAct promoter. The local tissue trauma experienced during the direct DNA injection may have resulted in the instigation of local cellular events including inflammation, remodelling and hypertrophy which is known to be a stimulus for SkAct activation (Schwartz et al., 1986; Schiaffino et al., 1989). It is therefore possible that the SkAct promoter was maximally activated before the IR episode.

Preliminary data from the first 1 hour time point rabbit heart assayed, indicated an induction of -1282 SkAct promoter activity in the IR tissue (table 4.6a). However, subsequent experiments using fresh batches of DNA, prepared using caesium gradients according to the same protocol (see section 2.2.1), failed to give either CAT or luciferase reporter gene activity. Further experiments indicated that the same batch of DNA that failed to give reporter gene activity in the rabbit, gave significant levels of reporter gene activity when injected into the rat myocardium.

When injected DNA was prepared using Qiagen filters, as described in section 2.2.1, instead of caesium gradients, reporter gene activity in the rabbit myocardium was observed. The degree of supercoiling which is known to be potentially important for expression, was similar between the two methods. The absence of reporter gene activity in later experiments with DNA isolated using caesium gradients could be due to a variety of factors including ethidium bromide contamination. Ethidium bromide is an integral reagent in the caesium gradient protocol but not of the Qiagen filter protocol. Ethidium bromide intercalates DNA and is used to locate the supercoiled DNA in the tube containing the caesium gradient, as described in section 2.2.1. Ethidium bromide contamination may lead to extensive DNA intercalation, which may prevent the DNA from expressing in the cell.

The study of the effect of IR on SkAct promoter activity in the rabbit myocardium at 1 hour was subsequently examined using DNA prepared with Qiagen filters. Injection of Qiagen DNA resulted in activities that differed from the preliminary data and showed no induction of SkAct promoter activity in IR tissue at 1 hour (see figure 4.3).

DNA injection into the myocardium is associated with tissue injury and inflammation (Buttrick et al., 1992; Lin et al., 1990). The presence of fibrous tissue is characteristic of the site of DNA injection and represents local inflammation and tissue trauma caused by the DNA injection. Trace contaminants, carried over from preparing the DNA may be responsible for this inflammatory response. Promoter activities in an environment of local inflammation and trauma may no longer be representative of endogenous patterns of transcriptional activity. Any such inflammatory responses that do occur would happen in both sham and IR tissue and any induction observed between sham and IR areas would be controlled for and therefore still stand. However, it is possible that inflammatory responses may lead to maximal activation of the promoter, preventing any potential induction to be observed. The presence of fibrous tissue causes additional stress to adjacent tissues and the surrounding myocardium. SkAct expression is known to be

activated by a number of stress-related stimuli including stretch or pressure overload and catecholamine stimulation (Bishopric and Kedes, 1991; Mann et al., 1989; Schwartz et al., 1986). It is possible, therefore, that the SkAct promoter may have become maximally activated in both sham and IR tissue by contaminants in the Qiagen prepared DNA, resulting in the apparent lack of effect of IR. Unfortunately a comparison of the possible differences between Qiagen prepared and caesium gradient prepared DNA could not be examined because no expression could be detected from DNA prepared using caesium gradients.

4.4.2 Transactivation of the SkAct promoter by AP-1

No transactivation of the -1282 SkAct promoter was observed when c-fos and c-jun expressing constructs were co-injected with the SkAct promoter construct. This is in contrast with the published *in vitro* data which reports a consistent 4-5 fold induction of a series of SkAct promoter deletion fragments, including the -1282 SkAct promoter (Bishopric et al., 1992). The ratio of the SkAct reporter plasmid to the c-fos and c-jun expressing plasmids used in this study was identical to that used in the cell culture experiments (Bishopric et al., 1992).

Following transfection in the cell culture experiments, cells were maintained in serum-free media (Bishopric et al., 1992). The human SkAct promoter region contains serum response factor binding sequences (Bishopric et al., 1992). The activity of the SkAct promoter *in vivo* may therefore be markedly different from data obtained from *in vitro* cell culture models. Furthermore, serum rapidly and transiently induces both c-fos and c-jun levels in 3T3 fibroblasts (Herschman, 1991). Subsequently c-fos and c-jun levels will also be substantially higher *in vivo* than *in vitro*. It is therefore possible that the SkAct promoter activity is activated by the presence of serum and by the higher *in vivo* levels of c-fos and c-jun. This may explain the discordance between the *in vivo* and *in vitro* observations.

4.4.3 Regulation of SkAct promoter activity in the heart by GATA-4

A full length (-2000) SkAct promoter with a mutation in the GATA-4 binding site expressed >10-fold higher in the rat myocardium than the full length (-2000) SkAct promoter with no mutation. *In vitro*, no difference in expression levels was observed between the mutated full length (-2000) SkAct promoter and the non-mutated full length (-2000) SkAct promoter (N. Bishopric, personal communication). GATA-4 has been proposed as a major regulator of cardiac-specific gene expression (Molkentin et al., 1994; Arceci et al., 1993). The present study demonstrates that mutations in the GATA-4 binding domain significantly increase the activity of the SkAct promoter. GATA-4 binding to the SkAct promoter, therefore appears to be involved in the repression of SkAct promoter activity in the adult rat heart *in vivo*. In contrast, GATA-4 is known to interact with the α-myosin heavy chain (α-MHC) GATA sites to stimulate cardiac muscle-specific expression (Molkentin et al., 1994).

It is possible, therefore, that GATA-4 may play a role in the developmental regulation of SkAct expression in the heart. GATA-4 is expressed in the embryonic mouse heart tube at day seven postcoitum and remains high throughout development (Molkentin et al., 1994). In foetal and neonatal rodent hearts, SkAct mRNA levels accumulate during the early development of cardiac muscle (Vandekerckhove et al., 1986; Mayer et al., 1984). However, in the adult heart SkAct expression is very low or absent (Vandekerckhove et al., 1986; Mayer et al., 1984; Schwartz et al., 1986). The *in vitro* analysis of the levels of expression of the SkAct promoter with and without a mutation in the GATA-4 binding domain are performed using isolated rat neonatal cardiac myocytes, when, developmentally, SkAct expression levels are high. These experiments do not show any effect of GATA-4. The *in vivo* experiments presented here were performed in adult rat hearts, when SkAct expression in the myocardium is low or absent. These experiments appear to implicate GATA-4 in the suppression of SkAct promoter activity. The

developmental suppression of SkAct expression in the adult heart may therefore be due, in part, to GATA-4 binding to the SkAct promoter.

4.4.4 General Observations

As reported by other investigators, this study demonstrates that uncomplexed plasmid DNA injected into the myocardium reproducibly expresses significant levels of functionally active recombinant protein (Buttrick et al., 1992; Lin et al., 1990; Wolff et al., 1990). Direct DNA injection allows the *in vivo* analysis of gene regulatory elements without the cost and time involved in generating multiple transgenic cell lines. Expression was only observed in myocardium immediately surrounding the injection site, however, this expression was reliably detected as early as one day and remained stable for at least seven days after injection. This work also agrees with previous work that both ischaemic and non-ischaemic tissue are capable of expressing injected DNA (Prentice et al., 1996). Furthermore, both viral and cellular promoters were capable of driving high levels of reporter gene expression. The cellular distribution of injected DNA was not determined, however other studies using similar protocols have shown that cardiac myocytes are the major recipients of the transferred DNA (Buttrick et al., 1992; Prentice et al., 1996; Prentice et al., 1994; Lin et al., 1990). The mechanism by which injected DNA is taken up by the myocardium is unclear. However, the lower efficiency of uptake in non-muscle cell types (Wolff et al., 1990) suggests that structural features of the muscle itself may be involved.

During early development in the rat, SkAct expression in the heart is high (Vandekerckhove et al., 1986; Mayer et al., 1984). However, this expression is down-regulated in the adult myocardium (Schwartz et al., 1986). Nevertheless, this work demonstrates that a luciferase reporter gene under the control of a human SkAct promoter reliably expresses significant levels of functionally active luciferase in the adult rat heart.

Significant, reproducible levels of expression were detected from reporter gene constructs driven by either a viral SV40 promoter or a cellular SkAct promoter as early as 1 day after injection in the rats. However, the SV40 / CAT construct, but not the SkAct / luciferase construct, expressed approximately ten-fold higher 7 days after injection than it did 1 day after injection in both sham and ischaemic tissue (tables 4.1 to 4.4). Early experiments conducted using SkAct / CAT constructs, as opposed to the SkAct / luciferase constructs presented here, demonstrated a similar increase in expression levels from day 1 to day 7 as that seen with the SV40 / CAT constructs (H Prentice, personal communication). It therefore appears that constructs containing CAT reporter genes express higher 7 days after injection than they do 1 day after injection. The mechanism underlying this observation is unclear. It may relate to the secondary structure of the promoter domains contained within these episomally expressed plasmids. Alternatively, it may be explained in terms of reporter protein content of the transfected cells, however, because the half-life of CAT enzyme is less than 16 hours (Gorman, 1977) this is unlikely. Given that the mechanism of uptake of directly injected DNA and plasmid status of the episomal DNA is not fully characterised, it may be difficult to explain this observation definitively.

In the GATA mutant SkAct promoter study (section 4.3.3), only 25μg of SV40/CAT plasmid was injected compared to 50μg SV40/CAT plasmid in earlier IR experiments (section 4.3.1). However, CAT activity, 7 days after injection did not appear to change, despite half the quantity of DNA injected. Injecting higher quantities of DNA may lead to more inflammatory responses. Injecting 25μg of DNA may therefore lead to lower levels of inflammation than 50μg. Lower levels of inflammation may allow higher levels of foreign gene expression per μg.

The levels of expression of the full length (-2000) SkAct promoter compared to the shorter length (-1282) SkAct promoter were different in the rat myocardium than in the rabbit myocardium. Relative to SV40 CAT expression in sham hearts, the shorter length

(-1282) SkAct promoter expressed 2-3 times higher than the full length (-2000) SkAct promoter in the rat myocardium (see figures 4.1 and 4.2). However, in the sham areas of the rabbit myocardium, the full length (-2000) SkAct promoter expressed 6 times higher than the shorter length (-1282) SkAct promoter (see figure 4.3). The expression levels of -2000 SkAct compared to -1282 SkAct in cell culture is similar to that observed in the rat myocardium (N Bishopric, personal communication). The difference in relative expression levels between -2000 and -1282 in the two *in vivo* models is probably due to a species difference as all other injection parameters were identical.

This study highlights a number of examples where the *in vivo* data differs from the *in vitro* data and warns against extrapolating *in vitro* data to the *in vivo* situation, particularly when the two model systems employed involve cells at different developmental stages. The SkAct gene is positively regulated by c-fos and c-jun *in vitro* (Bishopric et al., 1992) yet the *in vivo* model used in this study does not demonstrate any such regulation. Furthermore, mutating the GATA-4 binding domain in the SkAct promoter has no effect on expression levels *in vitro* (N. Bishopric, personal communication) but produces more than a 10 fold increase in reporter gene activity *in vivo*. Discordance between *in vivo* and *in vitro* results in the heart has previously been reported for α-myosin heavy chain (α-MHC) (Buttrick et al., 1993). The α-MHC gene and 5' deletions thereof do not express in the absence of thyroid hormone in neonatal cardiac myocytes (Tsika et al., 1990), however, all the constructs injected into the adult rat heart expressed in hypothyroid states (Buttrick et al., 1993). Furthermore, α-MHC constructs containing short upstream sequences are reversibly responsive to thyroid hormone in cell culture but remain active *in vivo* even in the absence of thyroid hormone (Buttrick et al., 1993).

In summary, reporter gene expressing constructs under the control of the viral SV40 promoter or either full-length (-2000) or 5' deleted versions (-1282) of the human skeletal α-actin promoter were injected into the myocardium of both rats and rabbits. Significant, reproducible levels of reporter gene expression could be detected as early as 24 hours

after injection. At the time points assayed in the rat and rabbit models, 15 minutes of ischaemia followed by reperfusion did not affect the activity of either the -2000 or -1282 SkAct promoter constructs. The -1282 SkAct promoter was not activated by overexpression of c-fos and c-jun expressing constructs as had been previously reported *in vitro* (Bishopric et al., 1992). A mutation in the GATA-4 binding domain of the SkAct promoter increased the activity of the promoter at least ten-fold in the rat myocardium. This finding is in direct contrast to the cell culture models where a mutation in the GATA-4 binding domain does not affect the activity of the SkAct promoter (N. Bishopric, personal communication).

Chapter 5

RESULTS 3

5.1 Introduction

Myocardial ischaemia is characterised by a reduction in blood flow sufficient to cause a pathological change in myocardial function (Hearse, 1994). The extent and severity of the pathological loss of function is critically dependent on the collateral circulation perfusing the ischaemic region (Cohen, 1995). Indeed, guinea pigs have such a well-developed collateral circulation that occlusion of a branch of the coronary artery cannot induce a level of ischaemia sufficient to produce an infarct big enough to be lethal (Schaper, 1984). Angiogenesis is defined as the growth of new blood vessels from an established vascular network (Klagsbrun and D'Amore, 1991; Folkman and Klagsbrun, 1987). Stimulating angiogenesis in the ischaemic heart may offer the possibility of increasing the collateral perfusion of ischaemic areas, improving myocardial function.

5.1.1 Angiogenesis

Angiogenesis is known to be a cascade process that begins with enzymatic degradation of the basement membrane, followed by migration and proliferation of endothelial cells toward the angiogenic stimulus and culminates in capillary morphogenesis and maturation (Folkman and Klagsbrun, 1987). A number of factors, of both peptide and non-peptide nature, have been identified. Epidermal growth factor, transforming growth factor-α (TGF-α), TGF-β, tumour necrosis factor-α (TNF-α), angiogenin, prostaglandin E_2, and monobutyrin are all able to induce neovascularisation *in vivo* but have little or no mitogenic effects on cultured endothelial cells. TGF-β and TNF-α paradoxically inhibit cultured endothelial cell growth (Ziche et al., 1997; Fett et al., 1985; Schreiber et al., 1986). The ability of these factors to elicit an angiogenic response *in vivo* is thought to be mediated via the paracrine release of directly acting angiogenic factors. Directly acting angiogenic factors include platelet-derived growth factor (PDGF), acidic and basic fibroblast growth factor (aFGF, bFGF) and the highly specific regulator of angiogenesis, vascular endothelial growth factor (VEGF) (Thomas et al., 1985; Miyazono et al., 1987;

Ishikawa et al., 1989; Gospodarowicz et al., 1987; Ferrara et al., 1992). These factors induce neovascularisation *in vivo* and are mitogenic for endothelial cells *in vitro*.

5.1.2 Vascular Endothelial Growth Factor

VEGF is a 45-kDa heparin-binding dimeric glycoprotein that is mitogenic for endothelial cells (Ferrara et al., 1992; Keck et al., 1989; Leung et al., 1989; Tischer et al., 1991; Ferrara et al., 1991). As a directly acting angiogenic factor, VEGF has a number of crucial advantages over other directly acting angiogenic factors that make it an ideal choice for a strategy directed towards revascularising the ischaemic heart. The NH_2-terminal amino acid sequence of VEGF is preceded by 26 amino acids which correspond to a classical signal sequence (Perlman and Halvorson, 1983). Cleavage of the signal sequence generates the mature VEGF protein which is then secreted by the cell (Ferrara et al., 1992; Leung et al., 1989). VEGF binds to the tyrosine kinase receptors, fms-like tyrosine kinase-1 (flt-1) and kinase domain region (KDR) with high affinity (Vaisman et al., 1990). Expression of the flt-1 and KDR receptors is restricted to endothelial cells (Vaisman et al., 1990). VEGF exists as one of at least four isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (Ferrara et al., 1992; Houck et al., 1991). $VEGF_{165}$, and in particular, $VEGF_{121}$ are diffusible after secretion, allowing their mitogenic activity to be exerted at sites distant to their release (Ferrara et al., 1992; Houck et al., 1992). After secretion, $VEGF_{189}$ and $VEGF_{206}$ are immediately sequestered by heparin-containing proteoglycans in the extracellular matrix (Ferrara et al., 1992).

The therapeutic potential of VEGF for revascularisation of ischaemic muscle through stimulation of the development of collateral vessels has been demonstrated in animal models of peripheral and myocardial ischaemia (Takeshita et al., 1995; Bauters et al., 1995; Takeshita et al., 1994; Pearlman et al., 1995). In a rabbit model of hindlimb ischaemia, direct intramuscular injection of plasmid DNA encoding VEGF into the ischaemic hindlimb muscle significantly augmented tissue reperfusion and collateral vessel development in the ischaemic muscle (Tsurumi et al., 1996). This study

demonstrates that despite the low transfection efficiency observed with direct injection of plasmid DNA (Wolff et al., 1990) the angiogenic potential of VEGF is sufficient to elicit a therapeutic angiogenic response in ischaemic muscle. The ischaemic muscle itself is thought to have contributed to the therapeutic actions of VEGF (Tsurumi et al., 1996). The VEGF receptor, KDR, is known to be upregulated 13-fold in endothelial cells exposed to media conditioned by hypoxic myoblasts (Brogi et al., 1996). The ischaemic muscle itself may therefore already have increased VEGF receptor numbers, amplifying the impact of any given concentration of VEGF and possibly accounting for the localised nature of the angiogenic response. The angiogenic potential of VEGF expressed from replication deficient adenovirus has also been demonstrated using matrigel plugs containing the recombinant adenovirus injected subcutaneously in mice (Muhlhauser et al., 1995). Areas immediately surrounding the injection site show extensive neovascularisation and the matrigel plugs employed for viral transfer show an increased haemoglobin content in VEGF adenovirus injected mice.

From a safety standpoint, localised expression of VEGF using a gene transfer approach, as opposed to systemic administration of recombinant VEGF protein, may limit the potential detrimental effects of VEGF on proliferative retinopathy in diabetic patients and growth and development of latent tumours (Plate et al., 1992; Kim et al., 1993).

Direct DNA injection of a VEGF expressing construct into the myocardium may, perhaps surprisingly, have a direct clinical application. Creating transmural channels in rat hearts using a 400-μm-diameter syringe needle protects the heart from a subsequent sustained coronary artery occlusion (Whittaker et al., 1996). It is thought that the protective effect of creating transmural channels in the heart may due to the direct access of blood flow from the ventricular cavity to the ischaemic tissue. Injecting plasmid DNA constructs expressing VEGF, whilst creating transmural channels, may not require much extensive modification of the transmural channel protocol. Significant therapeutic benefit may be gained if the therapeutic angiogenic response, observed after direct DNA injection of a

VEGF expressing construct in ischaemic skeletal muscle (Tsurumi et al., 1996), can be repeated in cardiac muscle. Nevertheless, such procedures involve extensive surgical intervention.

A number of gene transfer approaches have demonstrated high levels of foreign gene expression in the myocardium. These include the delivery of viruses: adenovirus (Barr et al., 1994), retrovirus (Prentice et al., 1996), herpes simplex virus (Coffin et al., 1996) or adeno-associated virus (AAV) (Kaplitt et al., 1996)), delivery of liposome based vehicles: haemagglutinating Virus of Japan (HVJ) / cationic liposome-DNA complexes (Goldberg et al., 1988)) as well as gene transfer by direct plasmid DNA injection (Buttrick et al., 1992; Lin et al., 1990).

Coronary infusion via a catheter advanced from an accessible major artery is a relatively non-invasive procedure that provides direct access to the coronary circulation. Adenoviral vectors delivered to the heart from a catheter placed in the coronary artery have directed high levels of recombinant protein expression in the myocardium (Barr et al., 1994). Gene transfer approaches using adenoviral vectors may therefore provide a safe, non-invasive method of directing foreign gene expression in the heart.

5.1.3 Adenovirus

Adenovirus is a 36 kb double stranded DNA virus that is capable of infecting both dividing and non-dividing cell types. Adenoviral particles enter the cell by receptor-mediated endocytosis (Pastan et al., 1986; Greber et al., 1993). Fiber proteins on the surface of the adenovirus bind to an as yet uncharacterised cell surface receptor, become engulfed into clathrin-coated pits and internalised into an endosome. Once internalised, acidification of the endosomes containing the adenovirus particles results in endosome disruption, allowing virions to proceed to the nucleus where the adenoviral genome remains episomal (Defer et al., 1990). The adenovirus system has a number of attractive

features which make it ideal for *in vivo* applications. The adenovirus genome is relatively easy to manipulate and can be grown to high titers (10^{11}-10^{12} virus particles/ml). It has a broad host range and can direct high levels of protein expression in both proliferating and quiescent cells. From a safety point, adenovirus is only a minor pathogen and because the genome remains episomal there is a low probability of random integration into the host genome. Random integration near an oncogene may result in the oncogene being inappropriately expressed and result in a malignant cell.

The principles of recombinant construction are based on the adenovirus replication cycle. There are 47 serotypes of adenovirus in man alone, however, the majority of adenoviral recombinants are based on the Adenovirus type 5 (Ad5) genome (Ring, 1997). The genomic sequence of Ad5 is known and the genetic organisation and protein functions are well characterised (Chroboczek et al., 1992). The adenovirus life cycle operates a cascade system of gene expression, with separate prereplicative (early) and postreplicative (late) stages, reviewed in (Horwitz, 1990; Ring, 1997). Early genes are expressed before viral replication and late genes are expressed after viral replication. The E1A pre-early gene is the first early gene to be transcribed and translated using the host cell machinery. E1A is a trans-acting transcriptional regulatory factor that is necessary for the transcriptional activation of the other early genes, E1B, E2, E3 and E4. E1A does not bind to the DNA directly but binds to the transcription factors that are bound to the adenoviral DNA regulatory sequences, modulating their activity. Viral DNA replication occurs in the nucleus where at least three viral gene products (encoded by E2A and E2B) and four host cell gene products are required for viral replication. The expression of the early genes leads to the rapid shut down of the host cells protein and DNA synthesis, however, the mechanism by which this occurs is unclear. Immediately after replication the transcription pattern changes and the late genes are expressed under the control of the major late promoter (MLP). Late genes expressed under the control of the MLP encode viral coat proteins and other proteins required to complete the viral life cycle.

The entire life cycle of adenovirus is therefore critically dependent on the gene products of the pre-early gene, E1A. Accordingly, adenoviruses lacking the E1 region are unable to replicate. Viruses containing deletions in the E1 region have been employed in methodologies directed at generating replication-deficient recombinant adenoviral vectors. The E3 region encodes proteins that are not involved in viral replication (Kelly and Lewis, 1973). Deletion of both E1 and E3 regions of the adenovirus genome has allowed insertion of up to 8kb of a foreign DNA sequence. *In vivo*, foreign gene expression from adenoviral recombinants with intact E3 sequences lasts significantly longer than foreign gene expression from adenoviral recombinants with E3 sequences deleted (Poller et al., 1996). The E3 region is therefore thought to play a role in defending the vector-infected cells from host immune responses.

In conventional approaches to adenovirus recombinant generation, E1 deleted replication deficient adenoviruses maybe propogated in the embryonic kidney cell line, 293, whose genome constituitively expresses E1 gene products. The E1 gene products are therefore provided in trans by the 293 cell line, allowing efficient replication of E1 deleted recombinant adenoviruses.

5.2 Experimental Aim

It has been shown previously that VEGF can improve ischaemic muscle perfusion and function in models of peripheral and myocardial ischaemia (Takeshita et al., 1995; Bauters et al., 1995; Takeshita et al., 1994; Pearlman et al., 1995). The abilities of adenoviral vectors to infect a wide range of cell types, including the myocardium, and to direct high levels of protein expression in non-proliferating cells may make these vectors suitable candidates for gene transfer in the myocardium (Horwitz, 1990; Ring, 1997; Barr et al., 1994). The long term goal of this work is to demonstrate improved cardiac function in models of myocardial ischaemia. To do this we aim to use a replication

deficient recombinant adenovirus expressing VEGF to stimulate angiogenesis in ischaemic areas of the myocardium in order to improve perfusion and prevent further expansion of ischaemic regions. The objectives of this project are to :

1. Isolate and clone VEGF isoforms
2. Generate VEGF / Adenovirus shuttle vectors
3. Construct VEGF adenoviral recombinant
4. Successfully infect cells with the VEGF recombinant adenovirus
5. Demonstrate expression of VEGF protein in infected cells

Having successfully demonstrated VEGF protein expression from a recombinant adenovirus in this project, further collaborative studies will aim to fully characterise the angiogenic potential of VEGF with a view to improving cardiac function in models of myocardial ischaemia.

primers designed to contain EcoR1 and BglII restriction sites that could be used later for cloning fragments into plasmid vectors (table 5.1).

Name	Size	position relative to transcription start site	Sequence (5' - 3')
V1 sense	34 nt	-4 to 14	*AGCAAGCTGT***GAATTC**AACCATG AACTTTCTGCT
V2 antisense	34 nt	570 to 552	*TAGGAATTGA***AGATCT**TCACCGC CTTGGCTTGTC
V3 seq (R)	22 nt	180 to 158	GATGTCCACCAGGGTCTCAATT
V4 seq (F)	21 nt	355 to 376	ATGAGCTTCCTGCAGCATAGC
V5 seq (F)	21 nt	103 to 124	AAAGCCCATGAAGTGGTGAAG

Table 5.1 Primers used for PCR amplification and sequencing

Sequence in italics represents non-specific sequence. Sequence in bold represents restriction sites, V1 sense - EcoRI, V2 antisense - BglII. F, forward and R, reverse indicate the orientation of the primers with respect to the VEGF sequence

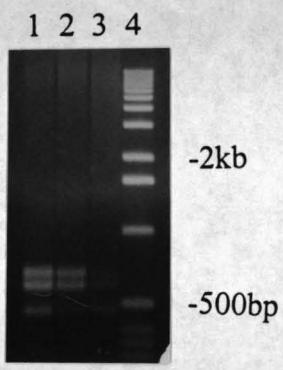

Figure 5.2 VEGF specific PCR of cDNA synthesised from total RNA

PCR amplification using VEGF specific primers of cDNA synthesised from rat neo-nate heart, isolated rat neo-nate cardiac myocytes and human placenta RNA, as described in section 2.2.3, was run on a 1% agarose gel. Lane 1 - isolated rat neo-nate cardiac myocytes, lane 2 - isolated rat heart, lane 3 - human placenta, lane 4 - 1kb size marker.

VEGF cloning

PCR products, containing all three bands, from the isolated rat neo-nate cardiac myocytes RT-PCR reaction (figure 5.2) were cloned into the pT7Blue cloning vector (Novagen) as described in section 2.2.4. The pT7Blue vector system is designed to take advantage of the single 3' A-nucleotide overhang that polymerases, such as Taq polymerase, leave on the PCR product (Clark, 1988). These products can be ligated directly to a vector containing compatible single T-nucleotide overhangs (T-cloning) (Marchuk et al., 1991). The pT7Blue vector is prepared for T-cloning by digestion with EcoRV followed by the addition of single 3' dT residues at each end and is supplied in this form ready to ligate with any DNA amplified by Taq.

Positive white colonies were picked and screened for the presence and size of insert by single colony PCR using the VEGF specific V1 and V2 primers (table 5.1) used for the initial PCR, as described in section 2.2.3 and 2.2.4. As all three DNA fragment sizes were cloned in the one reaction, each individual clone had to be identified not only for the presence of the fragment but also the size of the fragment. An alternative approach would have been to purify DNA from each band first and then clone individual DNA fragment sizes. However, because of the proximity of each band to the other, it was decided to 'shotgun' clone all three bands and subsequently identify the size of fragment present when screening the colonies. 100 colonies were screened. Figure 5.3 shows an example of a single colony PCR screen of 8 colonies.

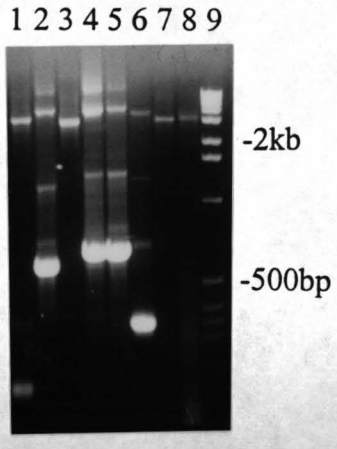

Figure 5.3 Single colony PCR screen of potential T-vector clones

Single colony PCR of colonies arising from the cloning procedure described above using VEGF specific primers. Lanes 1-8 - putative VEGF, lane 9 - 1kb ladder.

Figure 5.3 indicates that lanes 2, 4, 5 and 6 contain VEGF clones. Clones identified by single colony PCR screening, such as those in lanes 2, 4, 5 and 6, were confirmed by restriction digestion analysis using small scale plasmid DNA amplification (mini-preps) and restriction digest protocols, as described in section 2.2.4. EcoRI and BglII restriction enzymes were used to removed the VEGF fragment from the plasmid vector at the specific restriction sites incorporated into the primers used for VEGF fragment amplification (table 5.1).

VEGF Sequencing

T-vector clones containing VEGF cDNA inserts were sequenced using the ThermoSequenase cycle sequencing kit (Amersham). 3 primers were designed from published rat VEGF sequence in order to sequence the entire VEGF cDNA.

Clones containing cDNA inserts that corresponded in size to the PCR products obtained from RT-PCR (figure 5.2) were sequenced to determine the identity of the cDNA sequence. The smallest band (<500bp) was sequenced and with reference to published sequence (Tischer et al., 1991; Conn et al., 1990), identified to be $VEGF_{121}$. Likewise, the middle and largest bands were sequenced and identified to be $VEGF_{165}$ and $VEGF_{189}$, respectively.

Point mutations were discovered in a number of VEGF clones. An example of one such point mutation is shown below (figure 5.4).

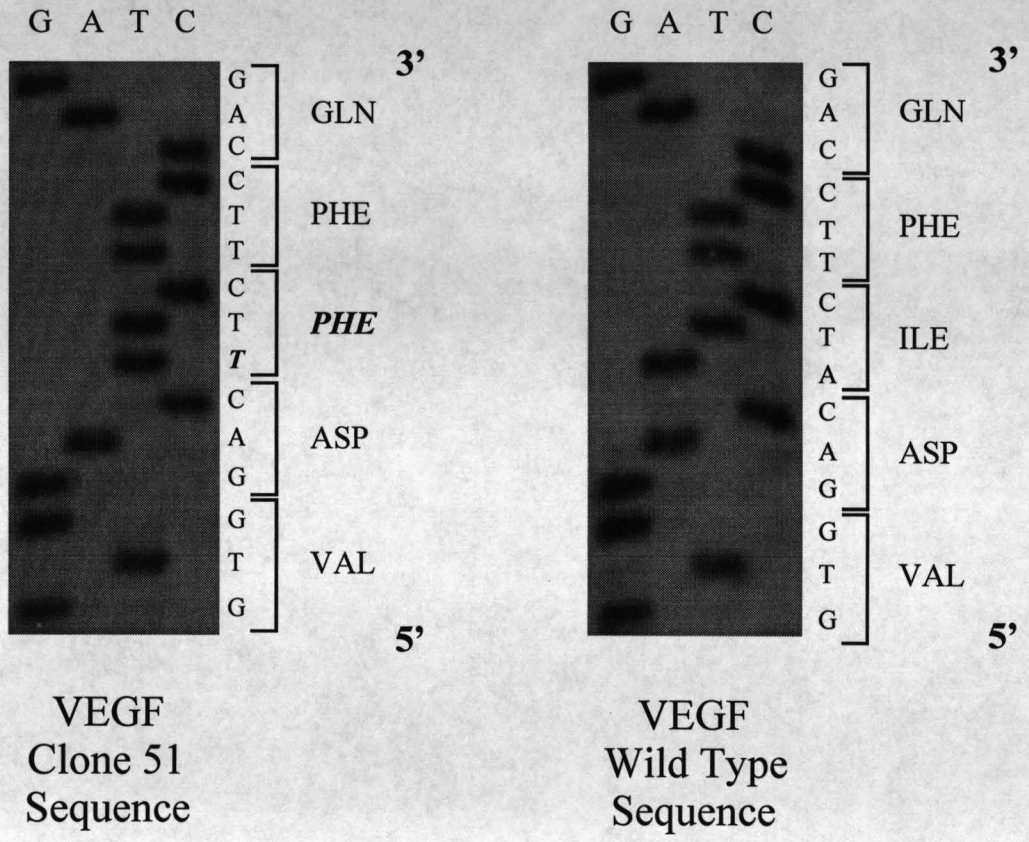

Figure 5.4 VEGF sequence showing point mutations in VEGF encoding cDNA.
Putative VEGF clones were sequenced as described in section 2.2.5 and run on a 6% sequencing gel. Sequence analysis indicates a A→T point mutation at base 178 in clone 51, as compared to wild type VEGF sequence, results in a isoleucine to phenyl alanine amino acid change

Sequence analysis of one particular clone, clone no. 92, revealed DNA sequence corresponding to what may potentially be a novel splice variant of VEGF. The sequence of this clone indicated that it contained exons 1-6 and exon 8 of VEGF sequence and would potentially encode a mature protein with 145 amino-acids. The sequence of this clone and comparison with other reported VEGF sequences is shown below (figures 5.5 + 5.6).

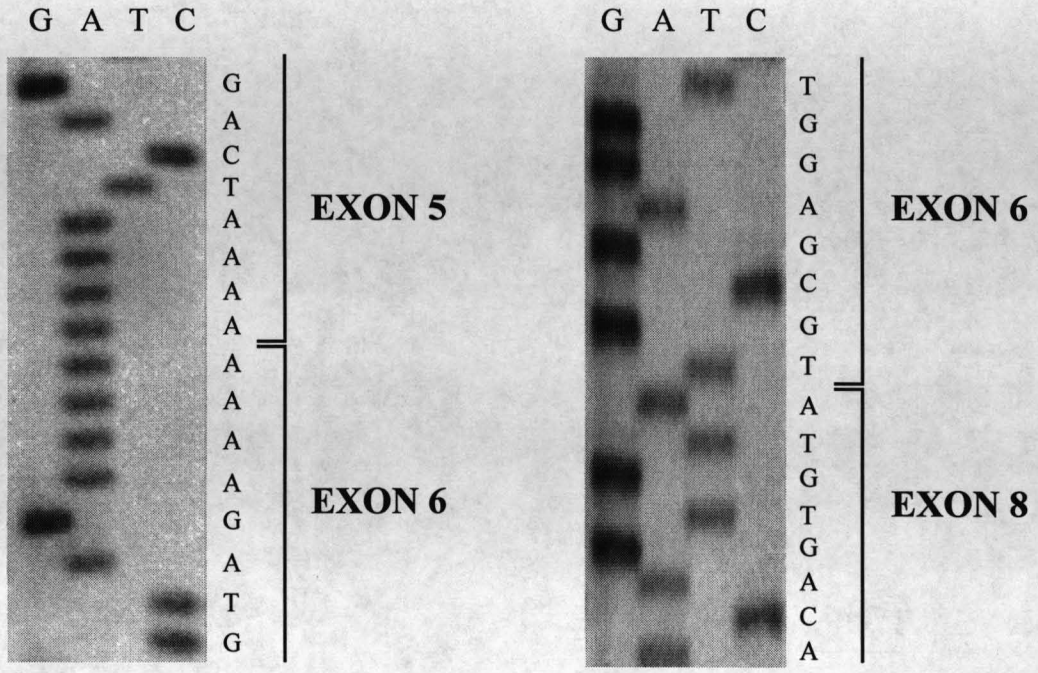

Figure 5.5 Novel splicing pattern of VEGF clone 92

Sequence analysis of VEGF clone no. 92 indicated sequence containing a novel exon splicing pattern of VEGF exons 1-6 and 8, which may potentially correspond to a 145 amino acid protein

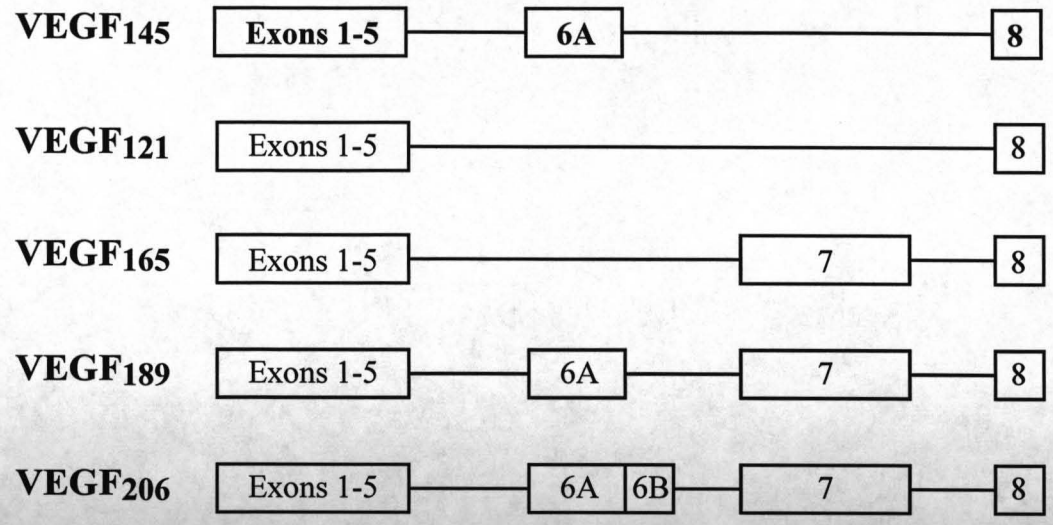

Figure 5.6 Comparison of splicing patterns of different VEGF isoforms

VEGF subcloning into adenoviral shuttle vector

$VEGF_{165}$ is the most abundant isoform of VEGF in the body (Ferrara et al., 1992) and was therefore chosen as the isoform to be incorporated into the adenoviral recombinant. A $VEGF_{165}$ clone, free of mutations in its amino acid sequence was chosen and cloned into the adenoviral shuttle vector pCA13 (microbix). pCA13 contains an expression cassette consisting of a CMV promoter, multiple cloning site and an SV40 poly adenylation sequence. This cassette is flanked by adenoviral sequence allowing homologous recombination between the adenoviral shuttle plasmid and the adenovirus genome.

$VEGF_{165}$ clone no. 52 was sub-cloned from the pT7Blue vector and ligated into the pCA13 adenoviral shuttle vector using standard cloning procedures, as described in section 2.2.4. The $VEGF_{165}$ cDNA was removed from the pT7Blue vector as an EcoRI, XbaI fragment and cloned directly into the same sites of previously digested pCA13 plasmid. Putative clones were confirmed by restriction digestion analysis using small scale plasmid DNA amplification (mini-preps) and restriction digest protocols, as described in section 2.2.1 and 2.2.4. The ligated ends of the VEGF

cDNA were sequenced to ensure there were no mutations or insertions at the join sites. A diagram of the resulting pCA13/VEGF expression plasmid is shown in figure 5.7.

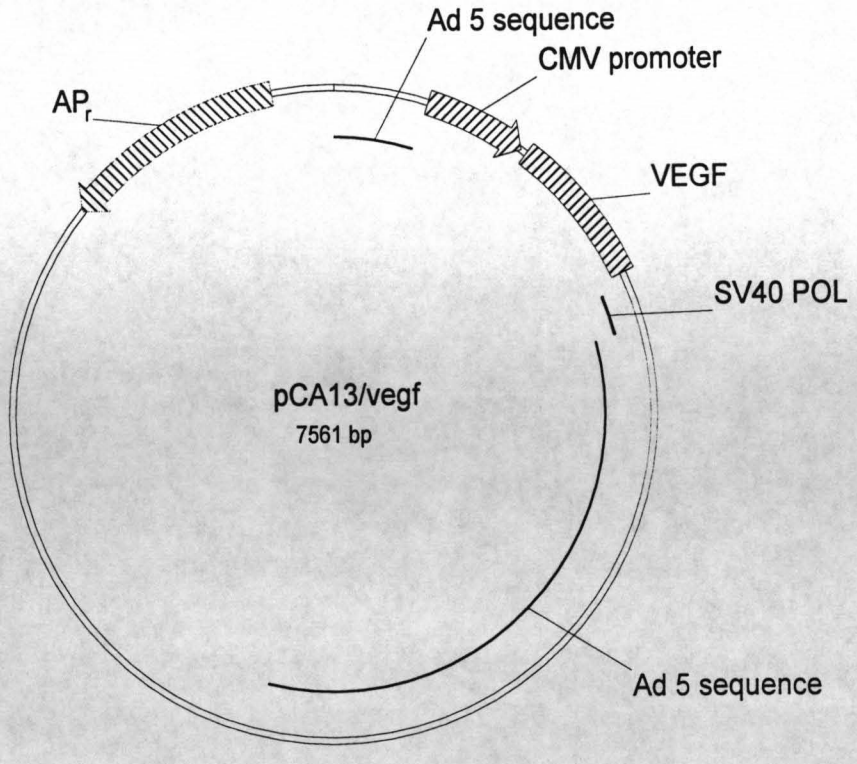

Figure 5.7 pCA13/VEGF expression plasmid

5.3.2 Recombinant Adenovirus construction - Overview

The experimental protocol for recombinant adenovirus construction is described in detail in section 2.4. The method for adenoviral construction described in this section is based on a protocol provided by Microbix and modified by Obaid Khan (personal communication). The system relies on homologous recombination between the shuttle plasmid, containing the gene of interest flanked by adenovirus sequence, with sequence contained in the adenovirus genome.

The adenovirus genome is contained in a large (~ 40kb) plasmid (pJM17) and is designed to give low or no background of non-recombinant infectious progeny upon co-transfection. This is due to the insertion of pBR322 DNA into the E1 region which results in the size of the adenovirus genome exceeding the packaging constraints of the adenovirus. Using pJM17, up to 5.4kb of foreign DNA can be packaged, however recombinant viruses that contain inserts that approach the upper limit of packaging are less stable than smaller inserts (Bett et al., 1993).

In order to transfer the gene of interest from the shuttle plasmid to an infectious recombinant adenovirus, 293 cells are co-transfected with the circular adenovirus genome plasmid, pJM17, and the shuttle plasmid containing the gene of interest flanked by E1 adenovirus sequence. Homologous recombination takes place between the adenovirus flanking sequence of the shuttle plasmid and the adenovirus genome plasmid (see figure 5.8, below). Successful recombination results in the gene of interest incorporated into the adenovirus genome and this results in replacement of the entire pBR322 bacterial sequence. This recombination event results in the adenovirus genome reducing in size such that it can now be packaged. The recombinant adenovirus, having overcome the packaging constraints, can now efficiently propagate in the E1-complementing 293 cell line. After amplification in 293 cells, the virus can be isolated and recombination confirmed by restriction analysis.

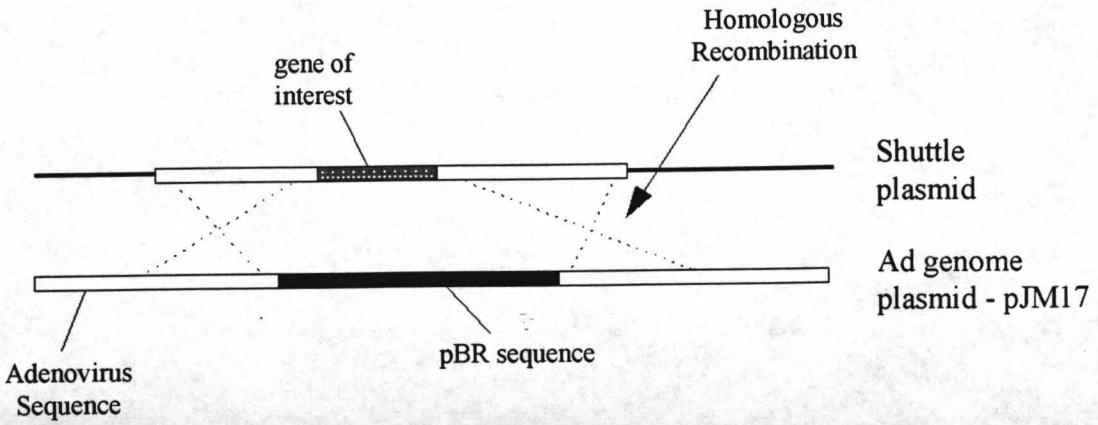

Figure 5.8 Linear representation of the plasmids involved in homologous recombination to generate a recombinant adenovirus. The dotted lines indicate the regions of homology between the two plasmids that enables the pBR sequence to be replaced by the gene of interest.

5.3.3 $VEGF_{165}$ Recombinant Adenovirus Construction

Co-transfection of the adenoviral genome plasmid, pJM17, and the pCA13/ $VEGF_{165}$ shuttle plasmid into 293 cells is described in section 2.4.1. Briefly, plasmid DNA was purified by caesium chloride gradients and RNA and protein contamination removed by RNAse treatment, phenol chloroform extraction and ethanol precipitation steps, as described in section 2.2.2. 293 cells were set up at 70% confluency in minimal essential medium containing 5% fetal calf serum. DNA was transfected using calcium/phosphate transfection methods described in section 2.4.1. After transfection, 293 cells were overlayed with agar and incubated at 37°C with 5% CO_2. Successful recombination events form plaques in the agar overlayed 293 cell monolayer. Plaques are populations of 293 cells formed in soft agar overlay that are undergoing a cytopathic effect due to the presence of infectious viral particles. Fresh agar, containing media, was added to the cells every 3-4 days. 12-15 days after transfection plaques were picked and further amplified in 293 cells as described in section 2.4.3. Viral DNA was then isolated from the amplified viral population and recombinant adenovirus confirmed by restriction and PCR analysis as described below.

Identification of $VEGF_{165}$ recombinant adenovirus

After co-transfection, three plaques were picked and further amplification by re-infecting a 60mm petri dish containing 293 cells with each plaque was performed. Viral DNA was then isolated from the three viral colonies and digested with HindIII, as described in section 2.4.6. Digested DNA was run overnight on a 1% agarose gel as shown in figure 5.9.

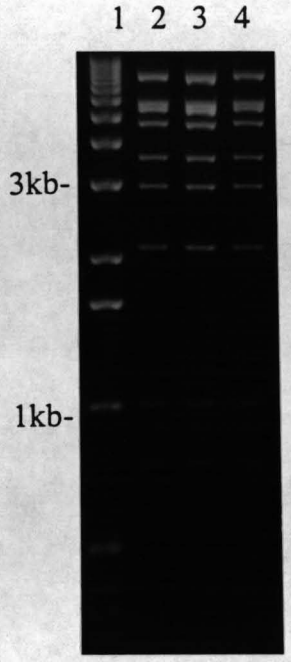

Figure 5.9 HindIII digest of isolated viral DNA

Putative adenoviral recombinants were amplified in 293 cells and viral DNA isolated. Viral DNA was digested with HindIII and run on a 1% agarose gel, as described in section 2.2.4. Lane 1 - 1kb ladder, lanes 2-4 - putative VEGF recombinant adenovirus

The presence of a bands at 757bp and the absence of 2 bands at approximately 3kb in figure 5.9 confirms that all three viral particles isolated have undergone homologous recombination with the shuttle plasmid. The viral DNA, isolated from the three viral colonies, was further analysed for the presence of VEGF and the absence of adenoviral E1 sequence using PCR techniques described in section 2.2.3. A positive PCR for VEGF

confirms the presence of VEGF in the adenoviral recombinant. The absence of amplified product using E1 primers confirms that the homologous recombination event has replaced the adenovirus E1 region which is essential for the initiation of the adenovirus life cycle. VEGF specific and E1 specific primers were used to demonstrate the presence of VEGF and absence of E1 respectively. PCR products were run on a 1% agarose gel as shown in figure 5.10 below.

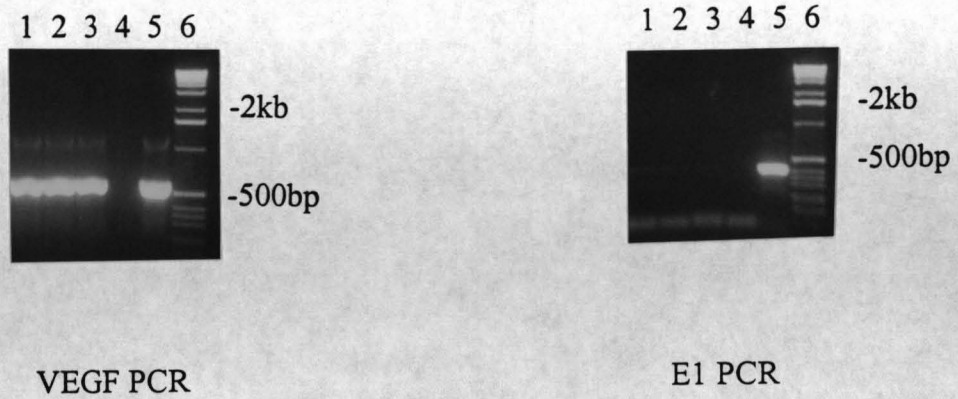

Figure 5.10 PCR analysis of VEGF recombinant adenovirus.

Viral DNA isolated from putative adenoviral recombinants was used as template for VEGF specific and E1 specific PCR amplification. Lanes 1-3 - putative adenoviral recombinants, lane 4 - -ve PCR control, lane 5 - VEGF plasmid, lane 6 - 1kb ladder. Lanes A-C - putative adenoviral recombinants, lane D - -ve PCR control, lane E - wild type adenoviral DNA, lane F - 1kb ladder.

5.3.4 Expression analysis of $VEGF_{165}$ recombinant adenovirus

Recombinant protein expression from a VEGF recombinant adenovirus was confirmed by western blot analysis. The mouse fibroblast cell line NIH3T3 was chosen as an easily transfected cell line that was available in the laboratory. NIH3T3 cells were set up at 50% confluency and allowed to reach exponential growth phase for 24 hours. 100µl of either Ad $VEGF_{165}$ or a recombinant adenovirus expressing green fluorescent protein (Ad

GFP) at approximately 1-5 x 10^8 plaque forming units(pfu)/ml was allowed to adsorb to the NIH3T3 cells for 45 minutes. Fresh media was added and the cells incubated for 48 hours before harvesting, as described in section 2.4.8.

Protein lysates were isolated from un-infected NIH3T3 cells, Ad $VEGF_{165}$ infected and Ad GFP infected NIH3T3 cells. The concentration of total protein in each lysate was determined using a Biorad protein assay kit. Equal quantities (10μg) of protein lysate were separated out according to size on an SDS-PAGE gel. The separated protein species were then transferred onto nitrocellulose membrane and probed with a monoclonal anti-VEGF antibody, as described in section 2.3.4. The results of the western blot are shown in figure 5.11.

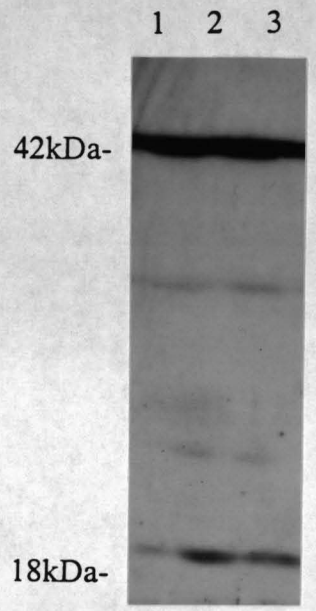

Figure 5.11 Western analysis of $VEGF_{165}$ recombinant adenovirus expression.
Protein lysates run on a SDS-PAGE gel and blotted onto a nitrocellulose membrane were probed with an anti-VEGF antibody. Lane 1 - un-infected NIH3T3 cells, lane 2 - Ad $VEGF_{165}$ infected NIH3T3 cells, lane 3 - Ad GFP infected NIH3T3 cells. VEGF monomers are 18-20kDa and VEGF dimers are 42-45kDa.

5.4 Discussion

VEGF cDNA was successfully generated, cloned and sequenced by ^{33}P-thermo sequenase cycle sequencing (Amersham) from cardiac myocyte mRNA isolated from rat neonates. This cDNA was then subcloned into an adenoviral shuttle vector and used to construct a $VEGF_{165}$ recombinant adenovirus. The recombinant was confirmed by restriction analysis and PCR. Expression from the recombinant was confirmed by western blot analysis. The techniques employed here have resulted in the construction of an infectious, replication-deficient, recombinant adenovirus containing a $VEGF_{165}$ cDNA under the control of a CMV promoter. The recombinant adenovirus has been further demonstrated to express recombinant $VEGF_{165}$ protein in the fibroblast cell line NIH3T3.

5.4.1 VEGF cloning

PCR amplification of cDNA synthesised from RNA isolated from rat neo-nate heart, isolated neo-nate cardiac myocytes and human placenta, resulted in three bands being amplified. Cloning and sequencing of the bands amplified from isolated rat neo-nate cardiac myocytes subsequently identified these bands to represent the three VEGF isoforms, $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$. The inability to clone a $VEGF_{206}$ isoform is consistent with published observations that indicate that $VEGF_{206}$ is only known to be significantly expressed in human fetal liver (Houck et al., 1991).

Point mutations were observed in some of the VEGF clones. The protocol used in this study involved the use of Taq polymerase to amplify reverse transcribed VEGF mRNA. Taq polymerase is thought to incorrectly incorporate one base in every thousand (Gibco-BRL, personal communication). The VEGF fragments amplified were approximately 500bp. The low fidelity of Taq polymerase may therefore be a possible source of point mutations observed in some of the VEGF clones. Mutations may also have been

introduced as a result of poor quality template cDNA. cDNA was synthesised from total RNA isolated from neonatal tissue. Synthesising cDNA from a pure mRNA population may have improved the quality of template cDNA. Recently, polymerase enzymes that possess proof-reading activities have become widely available and would probably be the polymerase of choice for future cloning strategies. Nevertheless, VEGF clones were successfully isolated with wild type amino acid sequences.

Sequence analysis of clone no. 92 indicated that this clone encoded VEGF sequences corresponding to what may prove to be a novel splice variant of VEGF. This clone contained exons 1-6 and 8 of the VEGF gene and is predicted to encode a VEGF isoform of 145 amino acids ($VEGF_{145}$). Shortly after the discovery of this novel splice variant, Poltorak et al (1997) reported the discovery of a novel $VEGF_{145}$ isoform, isolated from cell lines derived from carcinomas of the female reproductive system. Characterisation of $VEGF_{145}$ indicated that it induced the proliferation of endothelial cells and promoted angiogenesis *in vivo*. Poltorak et al further demonstrated that $VEGF_{145}$ possessed a unique combination of biological properties distinct from those of previously characterised VEGF species. However, Poltorak et al discussed that, to date, $VEGF_{145}$ protein expression has only been reported in the female reproductive system. The isolation of a clone from rat neonatal heart containing sequences that correspond to $VEGF_{145}$ requires further characterisation to determine whether or not this VEGF sequence corresponds to mRNA species that are translated into functionally active VEGF protein. The isolation of this clone from rat neonatal heart may prove to be the first evidence of $VEGF_{145}$ expression outside the female reproductive system.

Of the three VEGF isoforms cloned, $VEGF_{165}$ was chosen as the isoform to be expressed from a recombinant adenovirus because it is the predominant isoform in the body and freely diffusable after secretion from the cell. The ability of $VEGF_{165}$ to diffuse away from the point of secretion allows it to exert its angiogenic potential at sites distant to its release. With respect to myocardial ischaemia this may allow VEGF released from non-ischaemic, healthy cardiac myocytes to elicit an angiogenic response in ischaemic areas

of the myocardium. $VEGF_{121}$ is also a diffusable isoform and may therefore be appropriate for incorporation into adenoviral vectors, however with the exception of the placenta, $VEGF_{121}$ transcript levels are lower than $VEGF_{165}$ (Ferrara et al., 1992). $VEGF_{189}$ and $VEGF_{206}$ remain cell associated once secreted from the cell and are not diffusable (Ferrara et al., 1992).

5.4.2 Ad $VEGF_{165}$ construction

The method used for recombinant adenovirus construction was based on a protocol supplied by Microbix and modified by Obaid Khan (personal communication). Transfection efficiency is of crucial importance when generating adenoviral recombinants. The DNA used for transfection has to be of optimum quality and the 293 cells have to be at an optimal density and in an exponential growth phase. A number of problems were encountered amplifying the large 40kb adenoviral genome plasmid, JM17. JM17 DNA does not amplify very efficiently and is prone to recombination events resulting in deleted, 5-10kb, fragments being isolated. The large size also makes the plasmid susceptible to shearing and loss of the supercoiled structure. Great care had therefore to be taken when storing and pipetting this plasmid. The plating density and growth phase of the 293 cells was also of great importance. Several dishes of cells were often passaged and only dishes of cells containing healthy cells at exactly the right plating density were used. The growth rate of the 293 cells was also important. Slow growing cells, supplemented with only 5% fetal calf serum (FCS) were the most efficient at producing adenoviral recombinants. Cells maintained in 10% FCS tended to become overconfluent and show signs of toxicity. Toxicity was also a major problem with the calcium phosphate transfections. In order to form a calcium phosphate-DNA complex, the method used to mix each reaction mixture component and the pH of the solutions were of critical importance. Transfections into 293 cells using DOTAP transfection mix (Boehringer Manheim), despite being less toxic, did not produce any adenoviral recombinants. Despite scrupulously monitoring each step, the generation of adenoviral

recombinants was not guaranteed. Nevertheless, VEGF adenoviral recombinants were, eventually, generated.

PCR analysis of isolated viral DNA, using the gene specific V1 and V2 primers, demonstrated the presence of VEGF (figure 5.10). Although, PCR analysis of isolated viral DNA demonstrated the absence of a complete E1 region in the adenoviral recombinant, feint bands corresponding to the size of this product could still be seen. This may represent wild-type adenovirus contamination, non-specific PCR or contamination of genomic DNA from the 293 cell line containing E1 sequences.

5.4.3 Ad $VEGF_{165}$ expression analysis

Recombinant protein expression from the $VEGF_{165}$ recombinant adenovirus was demonstrated using western analysis of Ad $VEGF_{165}$-infected NIH3T3 fibroblasts. It has been shown that a wide range of cell lines and tissues express VEGF (Berse et al., 1992; Levy et al., 1995; Namiki et al., 1995; Stavri et al., 1995). VEGF antibodies are unable to distinguish VEGF protein from different mammalian species. The presence of VEGF protein, derived from the adenoviral recombinant, could therefore only be eluded to by demonstrating a higher signal in the Ad $VEGF_{165}$-infected cell extracts than in un-infected or Ad-GFP infected cell extracts, the latter acting as a control for virally infected cells. Figure 5.11 demonstrates significantly higher levels of VEGF protein in Ad $VEGF_{165}$-infected cell extracts than Ad-GFP infected cell extracts and in particular, un-infected cells. The significantly higher level of VEGF signal in the Ad-GFP infected cells compared to the un-infected cells may be due to either the adenoviral vector itself or to the GFP protein expressed from it. Low levels of adenoviral proteins may be expressed if cellular transcription factors are able to activate transcription from the viral DNA. The presence of low levels of adenoviral proteins in Ad GFP infected cells may be able to stimulate production of VEGF protein.

5.4.4 Future Directions

The long term goal of this project is to investigate the possibility that adenoviral gene transfer of VEGF into the ischaemic myocardium can increase collateral perfusion to ischaemic regions of the myocardium, thereby improving myocardial performance. Gene transfer with adenoviral vectors have shown promise in delivering high levels of foreign gene expression in the myocardium (Guzman et al., 1993; Muhlhauser et al., 1996; Barr et al., 1994). VEGF is known to increase collateral circulation in the ischaemic heart (Banai et al., 1994a; Hariawala et al., 1996a; Pearlman et al., 1995a) and $VEGF_{165}$ expressed by a recombinant adenovirus induces angiogenesis *in vivo* when injected subcutaneously (Muhlhauser et al., 1995).

Gene transfer into the myocardium with replication-deficient recombinant adenovirus vectors either by intramuscular injection or intracoronary infusion has previously been demonstrated (Guzman et al., 1993; Muhlhauser et al., 1996; Barr et al., 1994). Intramuscular injection of adenoviral vectors was more efficient at infecting cells in a localised area but intracoronary infusion was less invasive (Muhlhauser et al., 1996). With the advances in angioplasty technology, intracoronary infusions also have direct clinical relevance. The secretable nature of VEGF (Ferrara et al., 1992; Leung et al., 1989) suggests that only a fraction of myocardial cells need to be infected to elicit a functional response. Indeed, despite the very low transfection rates of intramuscular injection of plasmid DNA, plasmid DNA constructs expressing VEGF injected into ischaemic skeletal muscle successfully augment collateral perfusion of the ischaemic muscle (Tsurumi et al., 1996). Intracoronary infusion of VEGF adenoviral vectors may therefore allow sufficient gene transfer to improve myocardial performance without the injury and invasive surgical procedures associated with intramuscular injections.

Unfortunately the functional analysis of Ad $VEGF_{165}$ was outwith the scope of this project. However other investigators in the group will be further characterising the Ad $VEGF_{165}$. In particular, the ability of media conditioned by cells infected with Ad

$VEGF_{165}$ to elicit a growth response of endothelial cells in culture will be an important functional characterisation. This induction of endothelial cell growth should be reduced and finally blocked by addition of increasing concentrations of VEGF antibody. This experiment would demonstrate that the Ad $VEGF_{165}$ is capable of expressing functionally active VEGF and would allow further *in vivo* assays of angiogenesis, such as the chick chorioallantoic membrane (Phillis and Kumar, 1979), to be investigated (possibly in collaboration with Prof. Struyker-Boudier, Maastricht). $VEGF_{165}$ expressed from a recombinant adenovirus has previously shown to elicit angiogenesis *in vivo* when resuspended into matrigel plugs and injected subcutaneously into mice (Muhlhauser et al., 1995). Furthermore, recombinant VEGF protein infused in a model of chronic ischaemia increases collateral flow to ischaemic areas of the myocardium (Pearlman et al., 1995). Having demonstrated the angiogenic potential of Ad $VEGF_{165}$, the ability of the Ad $VEGF_{165}$ to functionally improve the ischaemic myocardium would then be investigated.

Despite the potential of VEGF to improve collateral flow and tissue perfusion, the exact mechanism(s) by which this effect is accomplished remain unclear. VEGF has a number of diverse effects in relation to its angiogenic activity. In addition to acting as a specific mitogen for endothelial cells (Tischer et al., 1989; Leung et al., 1989), VEGF increases the permeability of vascular endothelium (Connolly et al., 1989), induces NO-dependent relaxations in coronary arteries (Ku et al., 1993), stimulates the migration of monocytes through endothelial monolayers (Clauss et al., 1996; Clauss et al., 1990) and promotes the migration of endothelial cells (Folkman, 1995; Waltenberger et al., 1994). The downstream intracellular signalling pathways responsible for such diverse actions are gradually being elucidated. VEGF's mitogenic, permeability and vasodilator actions are all mediated through the nitric oxide/guanylate cyclase signalling cascade (Van der Zee et al., 1997; Wu et al., 1996; Morbidelli et al., 1996; Ku et al., 1993). The mechanism by which VEGF stimulates the migration of monocytes through endothelial monolayers has yet to be identified. However, evidence implicates the involvement of $p125^{FAK}$, a member of the non-receptor protein tyrosine kinase family and its putative substrate,

paxillin, in the migration of endothelial cells in response to VEGF (Abedi and Zachary, 1997). Endothelial cell migration plays an essential role in angiogenesis and vascular remodelling (Folkman, 1995; Waltenberger et al., 1994). As details about the potential molecular pathways involved in the VEGF induced angiogenic response are identified, it may become possible to further enhance VEGF's actions.

There are a number of approaches that have been shown to potentiate the actions of VEGF. A number of growth factors including transforming growth factor β (TGFβ), epidermal growth factor (EGF), platelet derived growth factor-BB (PDGF-BB) and basic fibroblast growth factor (bFGF) induce the secretion of VEGF in various cell lines(Tsai et al., 1995; Pertovaara et al., 1994). Combined administration of recombinant VEGF and bFGF to the ischaemic hind limb of rabbits induces an angiogenic response in the ischaemic limb that is greater than either VEGF or bFGF alone (Asahara et al., 1995). Placenta growth factor (PlGF), a protein with 53% amino acid sequence identity to VEGF has also been shown to potentiate the bioactivity of VEGF *in vitro* and *in vivo* (Park et al., 1994). The mitogenic effect on endothelial cells of low, marginally efficacious concentrations of VEGF is significantly potentiated by co-administration of PlGF in a dose dependent manner (Park et al., 1994). *In vivo*, the ability of PlGF to potentiate the effect of low concentrations of VEGF to induce extravasation of evans blue dye in the Miles vascular permeability assay, which determines the permeability of blood vessels to evans blue dye, is more striking than the mitogenic effect on endothelial cells in culture(Park et al., 1994).

There are, potentially, a number of alternative approaches that may enhance the ability of VEGF to revascularise ischaemic myocardium. Overexpressing positive angiogenic factors such as VEGF may not be sufficient to induce angiogenesis. It may be necessary to decrease the levels of negative angiogenic regulators such as angiostatin or thrombospondin (Rastinejad et al., 1989; O'Reilly et al., 1994). Adenovirally-mediated gene transfer of VEGF and receptors for VEGF is another possible approach. Modulating the nitric oxide system, which has been shown to be involved in the mitogenic and

vascular permeability activities of VEGF (Van der Zee et al., 1997; Wu et al., 1996; Morbidelli et al., 1996; Ku et al., 1993), may also prove to be beneficial.

Chapter 6

CONCLUSIONS

6.1 Myocardial Ischaemia

Myocardial ischaemia is characterised by a pathological alteration of cardiac contractile, electrical or biochemical function caused by a reduction of coronary blood flow (Hearse, 1994). The myocardium initiates a variety of cellular pathways to adapt to the ischaemic stress (Das et al., 1995; Elliott et al., 1992; Allen and Orchard, 1987; Kloner et al., 1989). These adaptions include a number of structural and biochemical alterations to the myocardium often involving changes in the levels of gene expression. Levels of expression of a number of stress related genes are increased, including members of the heat shock protein family whose function is thought to involve the stabilisation and formation of intracellular protein complexes (Mestril and Dillmann, 1995). The antioxidant genes, Manganese-Superoxide dismutase (Mn-SOD) and perioxisomal catalase (Das et al., 1993), are also induced along with their corresponding enzymatic activity. An increase in the level and activity of antioxidants in the cell limits the effect of oxygen free radicals, the main source of oxidative damage (Steare and Yellon, 1995). Reactive oxygen species can react with fatty acids, leading to lipid peroxidation of membranes, and proteins, leading to the destruction and oxidation of amino acids, oxidation of sulfhydryl groups and polypeptide scission (Thompson and Hess, 1986).

A complex interaction of molecular signals result in the expression of such genes as part of the myocardial adaptive response to ischaemic stress. There is evidence that individual components of ischaemia such as hypoxia, hypoglycaemia and changes in the redox status of the cell may be involved in triggering the transcriptional activation of specific genes involved in the response of the myocardium to ischaemia (Goldberg and Schneider, 1994; Laderoute and Webster, 1997; Ausserer et al., 1994; Webster et al., 1994).

6.2 Transcriptional Activation of Genes by Ischaemia

Investigations into the inducibility of a hybrid promoter containing myosin heavy chain basal regulatory sequences plus four copies of the HIF-1 binding site presented in chapter 3 provide insight into possible mechanisms involved in the transcriptional activation of genes by ischaemia. The activity of a luciferase reporter gene construct was increased 4.1 and 4.7-fold after 15 minutes of ischaemia followed by 1 and 4 hours of reperfusion respectively (figure 3.2). The timing and magnitude of the response is determined by the rate of accumulation and activity of the hypoxia responsive element (HRE) binding factor HIF-1, during ischaemia and reperfusion (Huang et al., 1996; Wang et al., 1995). Although HIF-1 levels were not quantified in the ischaemic myocardium, previous studies in Hep3B and HeLa cells, indicate that HIF-1 binding activity is rapidly induced upon exposure to hypoxia (Huang et al., 1996; Wang et al., 1995). The results indicate that HIF-1 binding activity in the ischaemic myocardium is increased and that genes with promoter sequences containing HIF-1 binding sites are likely targets for HIF-1. Indeed, putative HIF-1 binding sites have been identified in the promoter regions of the gene encoding VEGF (Forsythe et al., 1996), the VEGF receptor flt-1 (Gerber et al., 1997), inducible nitric oxide synthase (Melillo et al., 1997), α_{1B}-adrenergic receptor (Eckhart et al., 1997) and several glycolytic enzymes (Semenza et al., 1996). Furthermore, the gene products of these genes have also been shown to be positively regulated by hypoxia. The work presented in this study therefore indicates that HIF-1 may play an important role in the induction of several genes as part of the myocardial adaptive response to ischaemic stress.

6.3 Transcriptional Regulation of the Skeletal α-actin Promoter

Studies into the transcriptional response of the human skeletal α-actin (SkAct) gene in experimental models of ischaemia with reperfusion demonstrated no significant inductions of either the full length or 5' deleted promoter at the time points assayed (chapter 4). Nevertheless, the study did successfully demonstrate the ability of plasmid DNA injected directly into the myocardium to express significant amounts of fully active recombinant protein.

The study also highlighted important differences between similar experiments performed in cell culture and those performed *in vivo*. The SkAct gene is positively regulated by c-fos and c-jun *in vitro* (Bishopric et al., 1992) yet the *in vivo* model employed demonstrated no such regulation (figure 4.4). Expression levels of a SkAct promoter construct with a mutation in the GATA-4 binding domain (SkAct-mt1) is more than 10-fold higher than the non-mutated SkAct promoter (SkAct) *in vivo* (figure 4.5). However, *in vitro*, the two promoter constructs have similar expression levels (N. Bishopric, personal communication). Such discordance between *in vivo* and *in vitro* data has been previously reported for the α-myosin heavy chain (α-MHC) gene which encodes for one of the major contractile proteins of the cardiac sarcomere (Buttrick et al., 1993). α-MHC constructs containing short upstream sequences are reversibly responsive to thyroid hormone in cell culture but remain active *in vivo* even in the absence of thyroid hormone.

Discordance between *in vitro* and *in vivo* observations may not only be due to the physical environment of the cells, but also to the developmental origin of the cells. The *in vitro* data on the transactivation of the SkAct promoter by c-fos and c-jun, the relative expression levels of the SkAct-mt1 promoter compared to the SkAct promoter and the comparison between full length and 5' deleted SkAct promoter constructs were all performed in neonatal rat cardiac myocytes. However, the *in vivo* study, presented here was performed in the adult rat myocardium. SkAct gene expression in the rat heart is

known to vary throughout development (Vandekerckhove et al., 1986; Mayer et al., 1984). During early development, SkAct and cardiac α-actin (CAct) mRNA levels accumulate in fetal and neonatal rat hearts. However, in the adult rat heart, CAct expression remains high whereas SkAct expression is very low or absent (Vandekerckhove et al., 1986; Mayer et al., 1984). It is therefore possible that not only is SkAct expression altered during development, but its transcriptional regulation may be altered also. The different developmental origin of the cardiac myocytes as well as the different physical environment may therefore account for the observed differences in SkAct transcriptional regulation. This work therefore warns against extrapolating *in vitro* data to the *in vivo* situation, particularly when the model systems employed involve cardiac myocytes at different developmental stages.

6.4 Gene Therapy in the Myocardium

With the advances in gene transfer technology, a novel therapeutic approach to the treatment of myocardial ischaemia may be available. Current clinical treatments involve drug therapy and in severe cases, surgery. Whereas drug therapy is aimed at treating the consequences of myocardial ischaemia, gene therapy may be aimed at treating not only the consequences but also the underlying pathogenesis of myocardial ischaemia.

Gene transfer strategies, directed at improving the contractile function of the ischaemic myocardium, must satisfy three important criteria. A safe and efficient gene transfer system must be used to deliver the therapeutic construct. Appropriate gene(s) must be identified as targets for manipulation and crucially, the therapeutic gene(s) must be accurately regulated and controlled. In the case of myocardial ischaemia candidate genes should be accurately and efficiently delivered to the myocardium and only express during periods of ischaemia.

The ability of adenoviral vectors to infect a wide range of cell types, including cardiac myocytes, and direct high levels of recombinant protein expression may make these vectors suitable candidates for gene transfer in the myocardium (Muhlhauser et al., 1996; Barr et al., 1994). Neither direct injection, nor intracoronary infusion of adenoviral recombinants into porcine myocardium was associated with clinically significant side effects (Muhlhauser et al., 1996). Evidence therefore suggests that adenoviral vectors are capable of delivering foreign genes to the myocardium with high efficiency and low toxicity.

Delivery of recombinant VEGF protein in a model of chronic myocardial ischaemia results in increased collateral flow to the ischaemic areas of the myocardium, improving cardiac function (Pearlman et al., 1995). Gene transfer of a DNA construct expressing VEGF in a model of peripheral limb ischaemia augments ischaemic muscle perfusion and collateral vessel development (Tsurumi et al., 1996). Adenoviral recombinants expressing recombinant VEGF, induce neovascularisation in the skin of mice immediately surrounding the site of injection (Muhlhauser et al., 1995). Expressing VEGF in the heart using adenovirus mediated gene transfer may therefore lead to increased collateral perfusion of the ischaemic myocardium and functionally improve cardiac performance. Indeed, adenovirus-mediated gene transfer of the angiogenic factor fibroblast growth factor 5 (FGF-5) in a porcine model of stress-induced myocardial ischaemia, significantly improved cardiac function and blood flow (Giordano et al., 1996). The stress-induced model involves placing an ameroid constrictor around a branch of the coronary artery. The ameroid constrictor is hygroscopic and slowly swells, leading gradually to complete closure of the artery with minimal infarction because of the development of collateral vessels. Myocardial function and blood flow are normal at rest but blood flow is insufficient to prevent ischaemia when myocardial oxygen demands increase (i.e. during pacing of the heart). Hearts receiving adenoviral-mediated gene transfer of FGF-5 show improved pacing-induced regional function and blood flow (Giordano et al., 1996).

Work presented here (chapter 5) demonstrates the construction of a functional, replication deficient recombinant adenovirus that expresses significant levels of VEGF protein. Evidence suggests that adenovirally-mediated gene transfer of VEGF to the ischaemic myocardium may improve cardiac function by increasing blood flow to ischaemic areas of the heart.

Accurate regulation of transferred genes is crucial if a true therapeutic benefit is to be achieved. The hybrid promoter, containing myosin heavy chain basal regulatory sequences plus four copies of the erythropoietin HIF-1 binding site (MHC-HRE), presented in chapter 3, conferred inducible expression of a luciferase reporter gene in response to myocardial ischaemia. This induction was rapid and reversible upon reperfusion of the myocardium. Combining the regulatory properties of the MHC-HRE with the adenoviral recombinant expressing VEGF may allow therapeutic levels of a potent angiogenic factor to be expressed only in ischaemic areas of the myocardium. Localised expression of VEGF in ischaemic areas of the heart may improve the therapeutic potential of VEGF, and reduce the possibility of serious side effects such as the growth and development of latent tumours (Plate et al., 1992; Kim et al., 1993). This regulated approach may represent an important step forward in gene transfer strategies designed to deliver highly selective therapeutic gene products to the ischaemic myocardium safely and efficiently.

Chapter 7

REFERENCES

Abate, C., Patel, L., Rauscher FJ, I.I.I., and Curran, T. (1990). Redox regulation of Fos and Jun DNA-binding activity *in vitro*. Science *249*, 1157-1161.

Abedi, H. and Zachary, I. (1997). Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. Journal of Biological Chemistry *272*, 15442-15451.

Aiello, L.P., Avery, R.L., Arrigg, P.G., Keyt, B.A., Jampel, H.D., Shah, S.T., Pasquale, L.R., Thieme, H., Iwamoto, M.A., Park, J.E., Nguyen, H.V., Aiello, L.M., Ferrara, N., and King, G.L. (1994). Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. New England Journal of Medicine *331*, 1480-1487.

Allen, D.G. and Orchard, C.H. (1987). Myocardial contractile function during ischemia and hypoxia. Circulation Research *60*, 153-168.

Alpert, N.R. and Mulieri, L.A. (1982). Increased myothermal economy of isometric force generation in compensated cardiac hypertrophy induced by pulmonary artery constriction in the rabbit. A characterization of heat liberation in normal and hypertrophied right ventricular papillary muscles. Circulation Research *50*, 491-500.

Ammendola, R., Mesuraca, M., Russo, T., and Cimino, F. (1994). The DNA-binding efficiency of Sp1 is affected by redox changes. European Journal of Biochemistry *225*, 483-489.

Ananthan, J., Goldberg, A.L., and Voellmy, R. (1986). Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. Science *232*, 522-524.

Angello, D.A., Headrick, J.P., Coddington, N.M., and Berne, R.M. (1991). Adenosine antagonism decreases metabolic but not functional recovery from ischemia. American Journal of Physiology *260*, H193-H200.

Aoki, M., Morishita, R., Muraishi, A., Moriguchi, A., Sugimoto, T., Maeda, K., Dzau, V.J., Kaneda, Y., Higaki, J., and Ogihara, T. (1997). Efficient *in vivo* gene transfer into the heart in the rat myocardial infarction model using the HVJ (Hemagglutinating Virus of Japan) - Liposome method. Journal of Molecular & Cellular Cardiology *29*, 949-959.

Aoyagi, T. and Izumo, S. (1993). Mapping of the pressure response element of the c-fos gene by direct DNA injection into beating hearts. Journal of Biological Chemistry *268*, 27176-27179.

Applegate, R.J., Walsh, R.A., and ORourke, R.A. (1990). Comparative effects of pacing-induced and flow-limited ischemia on left ventricular function. Circulation *81*, 1380-1392.

Arceci, R.J., King, A.A.J., Simon, M.C., Orkin, S.H., and Wilson, D.B. (1993). Mouse GATA-4: A retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Molecular & Cellular Biology *13*, 2235-2246.

Asahara, T., Bauters, C., Zheng, L.P., Takeshita, S., Bunting, S., Ferrara, N., Symes, J.F., and Isner, J.M. (1995). Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis *in vivo*. Circulation *92*, II365-II371.

Ausserer, W.A., BourratFloeck, B., Green, C.J., Laderoute, K.R., and Sutherland, R.M. (1994). Regulation of c-jun expression during hypoxic and low-glucose stress. Molecular and Cellular Biology *14*, 5032-5042.

Banai, S., Jaklitsch, M.T., Shou, M., Lazarous, D.F., Scheinowitz, M., Biro, S., Epstein, S.E., and Unger, E.F. (1994a). Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. Circulation *89*, 2183-2189.

Banai, S., Shweiki, D., Pinson, A., Chandra, M., and Lazarovici, G. (1994b). Upregulation of vascular endothelial growth factor expression inducedby myocardial ischaemia: Implications for coronary angiogenesis. Cardiovascular Research *28*, 1176-1179.

Barr, E., Carroll, J., Kalynych, A.M., Tripathy, S.K., Kozarsky, K., Wilson, J.M., and Leiden, J.M. (1994). Efficient catheter mediated gene transfer into the heart using replication-defective adenovirus. Gene Therapy *1*, 51-58.

Bauters, C., Asahara, T., Zheng, L.P., Takeshita, S., Bunting, S., Ferrara, N., Symes, J.F., Isner, J.M., and Callow, A.D. (1995). Site-specific therapeutic angiogenesis after systemic administration of vascular endothelial growth factor. Journal of Vascular Surgery *21*, 314-325.

Berk, B.C., Alexander, R.W., Brock, T.A., and et al. (1986). Vasoconstriction: A new activity for platelet-derived growth factor. Science *232*, 87-90.

Berse, B., Brown, L.F., Van de Water, L., Dvorak, H.F., and Senger, D.R. (1992). Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. Molecular Biology of the Cell *3*, 211-220.

Bett, A.J., Prevec, L., and Graham, F.L. (1993). Packaging capacity and stability of human adenovirus type 5 vectors. Journal of Virology *67*, 5911-5921.

Biben, C., Hadchouel, J., Tajbakhsh, S., and Buckingham, M. (1996). Developmental and tissue-specific regulation of the murine cardiac actin gene *in vivo* depends on distinct

skeletal and cardiac muscle-specific enhancer elements in addition to the proximal promoter. Developmental Biology *173*, 200-212.

Bishopric, N.H., Simpson, P.C., and Ordahl, C.P. (1987). Induction of the skeletal alpha-actin gene in alpha1-adrenoceptor- mediated hypertrophy of rat cardiac myocytes. Journal of Clinical Investigation *80*, 1194-1199.

Bishopric, N.H., Jayasena, V., and Webster, K.A. (1992). Positive regulation of the skeletal alpha-actin gene by Fos and Jun in cardiac myocytes. Journal of Biological Chemistry *267*, 25535-25540.

Bishopric, N.H. and Kedes, L. (1991). Adrenergic regulation of the skeletal alpha-actin gene promoter during myocardial cell hypertrophy. Proceedings of the National Academy of Sciences of the United States of America *88*, 2132-2136.

Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K., and Tjian, R. (1987). Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science *238*, 1386-1392.

Boveris, A. and Cadenas, E. (1975). Mitochondrial production of superoxide anion and its relation to the antimycin-sensitive respiration. FEBS Letters *54*, 311-314.

Brand, T., Sharma, H.S., Fleischmann, K.E., Duncker, D.J., McFalls, E.O., Verdouw, P.D., and Schaper, W. (1992). Proto-oncogene expression in porcine myocardium subjected to ischemia and reperfusion. Circulation Research *71*, 1351-1360.

Braunwald, E. (1987). The path to myocardial salvage by thrombolytic therapy. Circulation *76*, II-2-II-7.

Brogi, E., Schatteman, G., Wu, T., Kim, E.A., Varticovski, L., Keyt, B., and Isner, J.M. (1996). Hypoxia-induced paracrine regulation of vascular endothelial growth factor receptor expression. Journal of Clinical Investigation *97*, 469-476.

Buttrick, P.M., Kass, A., Kitsis, R.N., Kaplan, M.L., and Leinwand, L.A. (1992). Behavior of genes directly injected into the rat heart *in vivo*. Circulation Research *70*, 193-198.

Buttrick, P.M., Kaplan, M.L., Kitsis, R.N., and Leinwand, L.A. (1993). Distinct behavior of cardiac myosin heavy chain gene constructs *in vivo*: Discordance with *in vitro* results. Circulation Research *72*, 1211-1217.

Chen, E.P., Bittner, H.B., Davis, R.D., Folz, R.J., and Van Trigt, P. (1996). Extracellular superoxide dismutase transgene overexpression preserves postischemic myocardial function in isolated murine hearts. Circulation *94*, II412-II417.

Ching Yi Chen, Croissant, J., Majesky, M., Topouzis, S., McQuinn, T., Frankovsky, M.J., and Schwartz, R.J. (1996). Activation of the cardiac alpha-actin promoter depends upon serum response factor, Tinman homologue, Nkx-2.5, and Intact serum response elements. Developmental Genetics *19*, 119-130.

Chroboczek, J., Bieber, F., and Jacrot, B. (1992). The sequence of the genome of adenovirus type 5 and its comparison with the genome of adenovirus type 2. Virology *186*, 280-285.

Clark, J.M. (1988). Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. Nucleic Acids Research *16*, 9677-9686.

Clauss, M., Gerlach, M., Gerlach, H., Brett, J., Wang, F., Familletti, P.C., Pan, Y-CE, Olander, J.V., Connolly, D.T., and Stern, D. (1990). Vascular permeability factor: A tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. Journal of Experimental Medicine *172*, 1535-1545.

Clauss, M., Weich, H., Breier, G., Knies, U., Rockl, W., Waltenberger, J., and Risau, W. (1996). The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. Journal of Biological Chemistry *271*, 17629-17634.

Claycomb, W. (1975). Journal of Biological Chemistry *250*, 3229-3235.

Coetzee, I.H. and Lochner, A. (1993). Free radical effects on myocardial membrane microviscosity. Cardioscience *4*, 205-215.

Coffin, R.S., Howard, M.K., Cumming, D.V.E., Dollery, C.M., McEwan, J., Yellon, D.M., Marber, M.S., MacLean, A.R., Brown, S.M., and Latchman, D.S. (1996). Gene delivery to the heart *in vivo* and to cardiac myocytes and vascular smooth muscle cells *in vitro* using herpes virus vectors. Gene Therapy *3*, 560-566.

Coffin, R.S. and Latchman, D.S. (1996). Herpes simplex virus-based vectors. In Genetic manipulation of the nervous system. D.S. Latchmann, ed. (London: Academic Press), 99-111.

Cohen, M.V. (1995). Arterial and venous collaterals salvage ischemic myocardium. Journal of Myocardial Ischemia *7*, 152-161.

Conn, G., Bayne, M.L., Soderman, D.D., Kwok, P.W., Sullivan, K.A., Palisi, T.M., Hope, D.A., and Thomas, K.A. (1990). Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. Proceedings of the National Academy of Sciences of the United States of America *87*, 2628-2632.

Connolly, D.T., Olander, J.V., Heuvelman, D., Nelson, R., Monsell, R., Siegel, N., Haymore, B.L., Leimgruber, R., and Feder, J. (1989). Human vascular permeability factor. Isolation from U937 cells. Journal of Biological Chemistry *264*, 20017-20024.

Crews, S.T., Thomas, J.B., and Goodman, C.S. (1988). The Drosophila single-minded gene encodes a nuclear protein with sequence similarity to the per gene product. Cell *52*, 143-151.

Currie, R.W., Tanguay, R.M., and Kingma Jr, J.G. (1993). Heat-shock response and limitation of tissue necrosis during occlusion/reperfusion in rabbit hearts. Circulation *87*, 963-971.

Czyzyk-Krzeska, M.F., Furnari, B.A., Lawson, E.E., and Millhorn, D.E. (1994). Hypoxia increases rate of transcription and stability of tyrosine hydroxylase mRNA in pheochromocytoma (PC12) cells. Journal of Biological Chemistry *269*, 760-764.

Czyzyk-Krzeska, M.F. and Beresh, J.E. (1996). Characterization of the hypoxia-inducible protein binding site within the pyrimidine-rich tract in the 3'-untranslated region of the tyrosine hydroxylase mRNA. Journal of Biological Chemistry *271*, 3293-3299.

Das, D.K., Engelman, R.M., and Kimura, Y. (1993). Molecular adaptation of cellular defences following preconditioning of the heart by repeated ischaemia. Cardiovascular Research *27*, 578-584.

Das, D.K., Maulik, N., and Moraru, I.I. (1995). Gene expression in acute myocardial stress. Induction by hypoxia, ischemia, reperfusion, hyperthermia and oxidative stress. Journal of Molecular and Cellular Cardiology *27*, 181-193.

Defer, C., Belin, M., Caillet-Boudin, M., and Boulanger, P. (1990). Human adenovirus-host cell interactions: Comparative study with members of subgroups B and C. Journal of Virology *64*, 3661-3673.

Dolnick, B.J. and Pink, J.J. (1983). 5-Fluorouracil modulation of dihydrofolate reductase RNA levels in methotrexate-resistant KB cells. Journal of Biological Chemistry *258*, 13299-13306.

Donnelly, T.J., Sievers, R.E., Vissern, F.L.J., Welch, W.J., and Wolfe, C.L. (1992). Heat shock protein induction in rat hearts: A role for improved myocardial salvage after ischemia and reperfusion? Circulation *85*, 769-778.

During, M.J. (1997). Adeno-associated virus as a gene delivery system. Advanced Drug Delivery Reviews *27*, 83-94.

Elliott, A.C., Smith, G.L., Eisner, D.A., and Allen, D.G. (1992). Metabolic changes during ischaemia and their role in contractile failure in isolated ferret hearts. Journal of Physiology *454*, 467-490.

Ely, S.W. and Berne, R.M. (1992). Protective effects of adenosine in myocardial ischemia. Circulation *85*, 893-904.

Fedele, F.A., Gewirtz, H., Capone, R.J., Sharaf, B., and Most, A.S. (1988). Metabolic response to prolonged reduction of myocardial blood flow distal to a severe coronary artery stenosis. Circulation *78*, 729-735.

Ferrara, N., Houck, K.A., Jakeman, L.B., Winer, J., and Leung, D.W. (1991). The vascular endothelial growth factor family of polypeptides. Journal of Cellular Biochemistry *47*, 211-218.

Ferrara, N., Houck, K., Jakeman, L., and Leung, D.W. (1992). Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocrine Reviews *13*, 18-32.

Fett, J.W., Strydom, D.J., Lobb, R.R., and et al. (1985). Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. Biochemistry *24*, 5480-5486.

Fishbein, M.C., Maclean, D., and Maroko, P.R. (1978). Experimental myocardial infarction in the rat: Qualitative and quantitative changes during pathologic evolution. American Journal of Pathology *90*, 57-70.(Abstract)

Fishman, G.I., Kaplan, M.L., and Buttrick, P.M. (1994). Tetracycline-regulated cardiac gene expression *in vivo*. Journal of Clinical Investigation *93*, 1864-1868.

Fliss, H. and Gattinger, D. (1996). Apoptosis in ischemic and reperfused rat myocardium. Circulation Research *79*, 949-956.

Flotte, T.R. and Carter, B.J. (1995). Adeno-associated virus vectors for gene therapy. Gene Therapy *2*, 357-362.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Medicine *1*, 27-31.

Folkman, J. and Klagsbrun, M. (1987). Angiogenic factors. Science *235*, 442-447.

Gilgenkrantz, H., Duboc, D., Juillard, V., Couton, D., Pavirani, A., Guillet, J.G., Briand, P., and Kahn, A. (1995). Transient expression of genes transferred *in vivo* into heart using first-generation adenoviral vectors: role of the immune response. Human Gene Therapy *6*, 1265-1274.

Giordano, F.J., Ping, P., McKirnan, M.D., Nozaki, S., DeMaria, A.N., Dillmann, W.H., MathieuCostello, O., and Hammond, H.K. (1996). Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. Nature Medicine *2*, 534-539.

Goldberg, M.A., Dunning, S.P., and Bunn, H.F. (1988). Regulation of the erythropoietin gene: Evidence that the oxygen sensor is a heme protein. Science *242*, 1412-1415.

Goldberg, M.A. and Schneider, T.J. (1994). Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. Journal of Biological Chemistry *269*, 4355-4359.

Gordon, E.M. and Anderson, W.F. (1994). Gene therapy using retroviral vectors. Current Opinion in Biotechnology *5*, 611-616.

Gospodarowicz, D., Ferrara, N., Schweigerer, L., and Neufeld, G. (1987). Structural characterisation and biological functions of fibroblast growth factor. Endocrine Reviews *8*, 95-109

Gossen, M. and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. Proceedings of the National Academy of Sciences of the United States of America *89*, 5547-5551.

Gottlieb, R.A., Burleson, K.O., Kloner, R.A., Babior, B.M., and Engler, R.L. (1994). Reperfusion injury induces apoptosis in rabbit cardiomyocytes. Journal of Clinical Investigation *94*, 1621-1628.

Gou Young Koh, Kim, S., Klug, M.G., Park, K., Soonpaa, M.H., and Field, L.J. (1995). Targeted expression of transforming growth factor-beta1 in intracardiac grafts promotes vascular endothelial cell DNA synthesi. Journal of Clinical Investigation *95*, 114-121.

Gradin, K., McGuire, J., Wenger, R.H., Kvietikova, I., Whitelaw, M.L., Toftgard, R, Tora, L., Gassmann, M., and Poellinger, L. (1996). Functional interference between hypoxia and dioxin signal transduction pathways: Competition for recruitment of the Arnt transcription factor. Molecular & Cellular Biology *16*, 5221-5231.

Greber, U.F., Willetts, M., Webster, P., and Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. Cell *75*, 477-486.

Guzman, R.J., Lemarchand, P., Crystal, R.G., Epstein, S.E., and Finkel, T. (1993). Efficient gene transfer into myocardium by direct injection of adenovirus vectors. Circulation Research *73*, 1202-1207.

Hainaut, P. and Milner, J. (1993). Redox modulation of p53 conformation and sequence-specific DNA binding *in vitro*. Cancer Research *53*, 4469-4473.

Hariawala, M.D., Horowitz, J.R., Esakof, D., Sheriff, D.D., Walter, D.H., Keyt, B., Isner, J.M., and Symes, J.F. (1996). VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. Journal of Surgical Research *63*, 77-82.

Harris, H.A., Bisteni, A., Russell, R.A., Brigham, J.C., and Firestone, J.E. (1954). Excitatory factors in ventricular tachycardia resulting from myocardial ischaemia : Potassium a major excitant. Science *119*, 200-203.

Hashimoto, E., Ogita, T., Nakaoka, T., Matsuoka, R., Takao, A., and Kira, Y. (1994). Rapid induction of vascular endothelial growth factor expression by transient ischemia in rat heart. American Journal of Physiology - Heart and Circulatory Physiology *267*, H1948-H1954.

Hearse, D.J. (1994). Myocardial Ischaemia : can we agree on a definition for the 21st century ? Cardiovascular Research *28*, 1737-1744.

Henson, D. and Mufson, M.A. (1971). Myocarditis and pneumonitis with type 21 adenovirus infection: association with fatal myocarditis and pneumonitis. Am J Dis Child *121*, 334-336.

Hentze, M.W., Rouault, T.A., Harford, J.B., and Klausner, R.D. (1989). Oxidation-reduction and the molecular mechanism of a regulatory RNA- protein interaction. Science *244*, 357-359.

Herschman, H.R. (1991). Primary response genes induced by growth factors and tumor promoters. Annual Review of Biochemistry *60*, 281-319

Herskowitz, A., Choi, S., Ansari, A.A., and Wesselingh, S. (1995). Cytokine mRNA expression in postischemic/reperfused myocardium. American Journal of Pathology *146*, 419-428.

Hess, P. and Tsien, R.W. (1984). Mechanism of ion permeation through calcium channels. Nature *309*, 453-456.

Hochachka, P.W., Buck, L.T., Doll, C.J., and Land, S.C. (1996). Unifying theory of hypoxia tolerance: Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proceedings of the National Academy of Sciences of the United States of America *93*, 9493-9498.

Hoffman, E.C., Reyes, H., Chu, F., Sander, F., Conley, L.H., Brooks, B.A., and Hankinson, O. (1991). Cloning of a factor required for activity of the Ah (dioxin) receptor. Science *252*, 954-958.

Horwitz, M.S. (1990). Adenoviridiae. In Virology. B.N. Fields and D.M. Knipe, eds. (New York: Raven Press), 1679-1740.

Houck, K.A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D.W. (1991). The vascular endothelial growth factor family: Identification of a fourth molecular species and characterization of alternative splicing of RNA. Molecular Endocrinology *5*, 1806-1814.

Houck, K.A., Leung, D.W., Rowland, A.M., Winer, J., and Ferrara, N. (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. Journal of Biological Chemistry *267*, 26031-26037.

Huang, L.E., Arany, Z., Livingston, D.M., and Bunn, H.F. (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. Journal of Biological Chemistry *271*, 32253-32259.

Huang, R.P. and Adamson, E.D. (1993). Characterization of the DNA-binding properties of the early growth response-1 (Egr-1) transcription factor: Evidence for modulation by a redox mechanism. DNA and Cell Biology *12*, 265-273.

Huard, J., Goins, W.F., and Glorioso, J.C. (1995). Herpes simplex virus type 1 vector mediated gene transfer to muscle. Gene Therapy *2*, 385-392.

IhlVahl, R., Marquetant, R., Bremerich, J., and Strasser, R.H. (1995). Regulation of beta-adrenergic receptors in acute myocardial ischemia: Subtype selective increase of mRNA specific for beta1-adrenergic receptors. Journal of Molecular and Cellular Cardiology *27*, 437-452.

Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K, Usuki, K., Takaku, F., Risau, W., and Heldin, C. (1989). Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. Nature *338*, 557-562.

Isner, J.M., Pieczek, A., Schainfeld, R., Blair, R., Haley, L., Asahara, T., Rosenfield, K., Razvi, S., Walsh, K., and Symes, J.F. (1996). Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet *348*, 370-374.

Izumo, S., Nadal Ginard, B., and Mahdavi, V. (1988). Proceedings of the National Academy of Sciences of the United States of America *85*, 339-343.

Jakeman, L.B., Winer, J., Bennett, G.L., Altar, C.A., and Ferrara, N. (1992). Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. Journal of Clinical Investigation *89*, 244-253.

Jelkmann, W. (1992). Erythropoietin: Structure, control of production, and function. Physiological Reviews *72*, 449-489.

Jennings, R.B. and Reimer, K.A. (1983). Factors involved in salvaging ischemic myocardium: Effect of reperfusion of arterial blood. Circulation *68*, I25-I36.

Jennings, R.B. and Steenbergen, C., Jr. (1985). Nucleotide metabolism and cellular damage in myocardial ischemia. Annual Review of Physiology *47*, 727-749.

Kaipainen, A., Korhonen, J., Pajusola, K., Aprelikova, O., Persico, M.G., Terman, B.I., and Alitalo, K. (1993). The related FLT4, FLT1, and KDR receptor tyrosine kinases show distinct expression patterns in human fetal endothelial cells. Journal of Experimental Medicine *178*, 2077-2088.

Kallio, P.J., Pongratz, I., Gradin, K., McGuire, J., and Poellinger, L. (1997). Activation of hypoxia-inducible factor 1alpha: Posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proceedings of the National Academy of Sciences of the United States of America *94*, 5667-5672.

Kaplitt, M.G., Xiao, X., Samulski, R.J., Li, J., Ojamaa, K., Klein, I.L., Makimura, H., Kaplitt, M.J., Strumpf, R.K., and Diethrich, E.B. (1996). Long-term gene transfer in porcine myocardium after coronary infusion of an adeno-associated virus vector. Annals of Thoracic Surgery *62*, 1669-1676.

Karjalainen, J., Heikkila, J., Nieminen, M.S., and et al. (1983). Etiology of mild acute infectious myocarditis. Relation to clinical features. Acta Medica Scandinavica *213*, 65-73.

Karns, L.R., Kariya, K.I., and Simpson, P.C. (1995). M-CAT, CArG, and Sp1 elements are required for alpha1-adrenergic induction of the skeletal alpha-actin promoter during cardiac myocyte hypertrophy. Transcriptional enhancer factor-1 and protein kinase C as conserved transducers of the fetal program in cardiac growth. Journal of Biological Chemistry *270*, 410-417.

Kawana, M., Lee, M.E., Quertermous, E.E., and Quertermous, T. (1995). Cooperative interaction of GATA-2 and AP1 regulates transcription of the endothelin-1 gene. Molecular and Cellular Biology *15*, 4225-4231.

Keck, P.J., Hauser, S.D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D.T. (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science *246*, 1309-1312.

Kelly, T.J. and Lewis, A.M. (1973). Journal of Virology *12*, 643-648.

Kendall, R.L. and Thomas, K.A. (1993). Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proceedings of the National Academy of Sciences of the United States of America *90*, 10705-10709.

Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., and Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. Nature *362*, 841-844.

Kim, S.J., Glick, A., Sporn, M.B., and Roberts, A.B. (1989). Characterization of the promoter region of the human transforming growth factor-beta1 gene. Journal of Biological Chemistry *264*, 402-408.

Kitsis, R.N., Buttrick, P.M., McNally, E.M., Kaplan, M.L., and Leinwand, L.A. (1991). Hormonal modulation of a gene injected into rat heart *in vivo*. Proceedings of the National Academy of Sciences of the United States of America *88*, 4138-4142.

Klagsbrun, M. and D'Amore, P.A. (1991). Regulators of angiogenesis. Annual Review of Physiology *53*, 217-239

Kloner, R.A., Przyklenk, K., and Whittaker, P. (1989). Deleterious effects of oxygen radicals in ischemia/reperfusion. Resolved and unresolved issues. Circulation *80*, 1115-1127.

Kloner, R.A., Przyklenk, K., Whittaker, P., and Hale, S. (1995). Preconditioning stimuli and inadvertent preconditioning. Journal of Molecular and Cellular Cardiology *27*, 743-747.

Kloner, R.A. and Braunwald, E. (1980). Observations on experimental myocardial ischemia. Cardiovascular Research *14*, 371-395.

Komuro, I., Katoh, Y., Kaida, T., Shibazaki, Y., Kurabayashi, M., Hoh, E., Takaku, F., and Yazaki, Y. (1991). Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. Possible role of protein kinase C activation. Journal of Biological Chemistry *266*, 1265-1268.

Komuro, I. and Yazaki, Y. (1993). Control of cardiac gene expression by mechanical stress. Annual Review of Physiology *55*, 55-75.

Kontos, H.A., Wei, E.P., Povlishock, J.T., and Dietrich, W.D. (1980). Cerebral arteriolar damage by arachidonic acid and prostaglandin G2. Science *209*, 1242

Korecky, B. and Rakusan, K. (1978). Normal and hypertrophic growth of the rat heart. Changes in cell dimension and number. American Journal of Physiology - Heart and Circulatory Physiology *234*, H123-H128

Ku, D.D., Zaleski, J.K., Liu, S., and Brock, T.A. (1993). Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. American Journal of Physiology - Heart and Circulatory Physiology *265*, H586-H592.

Kurabayashi, M., Komuro, I., Tsuchimochi, H., Takaku, F., and Yazaki, Y. (1988). Molecular cloning and characterization of human atrial and ventricular myosin alkali light chain cDNA clones. Journal of Biological Chemistry *263*, 13930-13936.

Kusuoka, H., Jacobus, W.E., and Marban, E. (1988). Calcium oscillations in digitalis-induced ventricular fibrillation: Pathogenetic role and metabolic consequences in isolated ferret hearts. Circulation Research *62*, 609-619.

Laderoute, K.R. and Webster, K.A. (1997). Hypoxia/reoxygenation stimulates Jun kinase activity through redox signaling in cardiac myocytes. Circulation Research *80*, 336-344.

Lasley, R.D., Rhee, J.W., Van Wylen, D.G.L., and Mentzer, R.M., Jr. (1990). Adenosine A1 receptor mediated protection of the globally ischemic isolated rat heart. Journal of Molecular & Cellular Cardiology *22*, 39-47.

Lee, T.C., Chow, K.L., Fang, P., and Schwartz, R.J. (1991). Activation of skeletal alpha-actin gene transcription: The cooperative formation of serum response factor-binding complexes over positive cis-acting promoter serum response elements displaces a negative-acting nuclear factor enriched in replicating myoblasts and nonmyogenic cells. Molecular and Cellular Biology *11*, 5090-5100.

Lee, T.C., Shi, Y., and Schwartz, R.J. (1992). Displacement of BrdUrd-induced YY1 by serum response factor activates skeletal alpha-actin transcription in embryonic myoblasts. Proceedings of the National Academy of Sciences of the United States of America *89*, 9814-9818.

Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V., and Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. Science *246*, 1306-1309.

Levy, A.P., Levy, N.S., Loscalzo, J., Calderone, A., Takahashi, N., Yeo, K.T., Koren, G., Colucci, W.S., and Goldberg, M.A. (1995a). Regulation of vascular endothelial growth factor in cardiac myocytes. Circulation Research *76*, 758-766.

Levy, A.P., Levy, N.S., Wegner, S., and Goldberg, M.A. (1995b). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. Journal of Biological Chemistry *270*, 13333-13340.

Levy, A.P., Levy, N.S., and Goldberg, M.A. (1996). Post-transcriptional regulation of vascular endothelial growth factorby hypoxia. Journal of Biological Chemistry *271*, 2746-2753.

Li, S. and Huang, L. (1997). Protamine sulfate provides enhanced and reproducible intravenous gene transfer by cationic liposome/DNA complex. Journal of Liposome Research *7*, 207-219.

Lin, H., Parmacek, M.S., Morle, G., Bolling, S., and Leiden, J.M. (1990). Expression of recombinant genes in myocardium *in vivo* after direct injection of DNA. Circulation *82*, 2217-2221.

Lindquist, S. (1986). The heat-shock response. Annual Review of Biochemistry *55*, 1151-1191.

Liu, B., Earl, H.M., Baban, D., Shoaibi, M., Fabra, A., Kerr, D.J., and Seymour, L.W. (1995). Melanoma cell lines express VEGF receptor KDR and respond to exogenously added VEGF. Biochemical and Biophysical Research Communications *217*, 721-727.

MacLellan, W.R., Lee, T.C., Schwartz, R.J., and Schneider, M.D. (1994). Transforming growth factor-beta response elements of the skeletal alpha-actin gene. Combinatorial action of serum response factor, YY1, and the SV40 enhancer-binding protein, TEF-1. Journal of Biological Chemistry *269*, 16754-16760.

Maglione, D., Guerriero, V., Viglietto, G., DelliBovi, P., and Persico, M.G. (1991). Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proceedings of the National Academy of Sciences of the United States of America *88*, 9267-9271.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997). Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature *386*, 403-407.

Mangano, D.T. (1990). Perioperative cardiac morbidity. Anesthesiology *72*, 153-184.

Mann, D.L., Kent, R.L., and Cooper, G., IV (1989). Load regulation of the properties of adult feline cardiocytes: Growth induction by cellular deformation. Circulation Research *64*, 1079-1090.

Marban, E., Robinson, S.W., and Wier, W.G. (1986). Mechanisms of arrhythmogenic delayed and early afterdepolarizations in ferret ventricular muscle. Journal of Clinical Investigation *78*, 1185-1192.

Marber, M.S., Mestril, R., Chi, S.H., Sayen, M.R., Yellon, D.M., and Dillmann, W.H. (1995). Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. Journal of Clinical Investigation *95*, 1446-1456.

Marchuk, D., Drumm, M., Saulino, A., and Collins, F.S. (1991). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Research *19*, 1154

Matherne, G.P., Linden, J., Byford, A.M., Gauthier, N.S., and Headrick, J.P. (1997). Transgenic A1 adenosine receptor overexpression increases myocardial resistance to ischemia. Proceedings of the National Academy of Sciences of the United States of America *94*, 6541-6546.

Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (1993). Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: Evidence for a widespread oxygen-sensing mechanism. Proceedings of the National Academy of Sciences of the United States of America *90*, 2423-2427.

Mayer, Y., Czosnek, H., Zeelon, P.E., Yaffe, D., and Nudel, U. (1984). Expression of the genes coding for the skeletal muscle and cardiac actins in the heart. Nucleic Acids Research *12*, 1087-2000.

McCord, J.M. (1985). Oxygen-derived free radicals in postischemic tissue injury. New England Journal of Medicine *312*, 159-163.

McCord, J.M. and Day, E.D. (1978). Superoxide-dependent production of hydroxyl radical catalysed by iron-EDTA complex. FEBS Letters *86*, 139-142.

McHugh, K.M., Crawford, K., and Lessard, J.L. (1991). A comprehensive analysis of the developmental and tissue-specific expression of the isoactin multigene family in the rat. Developmental Biology *148*, 442-458.

McKay, R.G., Pfeffer, M.A., Pasternak, R.C., and et al. (1986). Left ventricular remodeling after myocardial infarction: A corollary to infarct expansion. Circulation *74*, 693-702.

Meggs, L.G., Tillotson, J., Huang, H., Sonnenblick, E.H., Capasso, J.M., and Anversa, P. (1990). Noncoordinate regulation of alpha-1 adrenoceptor coupling and reexpression of alpha skeletal actin in myocardial infarction-induced left ventricular failure in rats. Journal of Clinical Investigation *86*, 1451-1458.

Meggs, L.G., Huang, H.E., Tillotson, J., Capasso, J.M., and Anversa, P. (1992). Re-expression of alpha skeletal actin and regulation of alpha1 adrenoceptor signalling in DOCA-salt hypertension in rats. Cardiovascular Research *26*, 878-885.

Mestril, R. and Dillmann, W.H. (1995). Heat shock proteins and protection against myocardial ischemia. Journal of Molecular and Cellular Cardiology *27*, 45-52.

Miyazono, K., Okabe, T., Urabe, A., and et al (1987). Purification and properties of an endothelial cell growth factor from human platelets. Journal of Biological Chemistry *262*, 4098-4103.

Mizukami, Y., Yoshioka, K., Morimoto, S., and Yoshida, K.I. (1997). A novel mechanism of JNK1 activation. Nuclear translocation and activation of JNK1 during ischemia and reperfusion. Journal of Biological Chemistry *272*, 16657-16662.

Mizumura, T., Auchampach, J.A., Linden, J., Bruns, R.F., and Gross, G.J. (1996). PD 81,723, an allosteric enhancer of the A1 adenosine receptor, lowers the threshold for ischemic preconditioning in dogs. Circulation Research *79*, 415-423.

Molkentin, J.D., Kalvakolanu, D.V., and Markham, B.E. (1994). Transcription factor GATA-4 regulates cardiac muscle-specific expression of the alpha-myosin heavy-chain ge. Molecular & Cellular Biology *14*, 4947-4957.

Morbidelli, L., Chang, C., Douglas, J.G., Granger, H.J., Ledda, F., and Ziche, M. (1996). Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. American Journal of Physiology *270*, H411-H415.

Muhlhauser, J., Merrill, M.J., Pili, R., Maeda, H., Bacic, M., Bewig, B., Passaniti, A., Edwards, N.A., Crystal, R.G., and Capogrossi, M.C. (1995). VEGF165 expressed by a replication-deficient recombinant adenovirus vector induces angiogenesis *in vivo*. Circulation Research *77*, 1077-1086.

Muhlhauser, J., Jones, M., Yamada, I., Cirielli, C., Lemarchand, P., Gloe, T.R., Bewig, B., Signoretti, S., Crystal, R.G., and Capogrossi, M.C. (1996). Safety and efficacy of *in vivo* gene transfer into the porcine heart with replication-deficient, recombinant adenovirus vectors. Gene Therapy *3*, 145-153.

Nadal-Ginard, B. and Mahdavi, V. (1989). Molecular basis of cardiac performance. Plasticity of the myocardium generated through protein isoform switches. Journal of Clinical Investigation *84*, 1693-1700.

Nambu, J.R., Lewis, J.O., Wharton Jr, K.A., and Crews, S.T. (1991). The Drosophila single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. Cell *67*, 1157-1167.

Namiki, A., Brogi, E., Kearney, M., Kim, E.A., Wu, T., Couffinhal, T., Varticovski, L., and Isner, J.M. (1995). Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. Journal of Biological Chemistry *270*, 31189-31195.

Nishizawa, J., Nakai, A., Higashi, T., Tanabe, M., Nomoto, S., Matsuda, K., Ban, T., and Nagata, K. (1996). Reperfusion causes significant activation of heat shock transcription factor 1 in ischemic rat heart. Circulation *94*, 2185-2192.

O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H., and Folkman, J. (1994). Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell *79*, 315-328.

Pantely, G.A., Malone, S.A., Rhen, W.S., Anselone, C.G., Arai, A., Bristow, J., and Bristow, J.D. (1990). Regeneration of myocardial phosphocreatine in pigs despite continued moderate ischemia. Circulation Research *67*, 1481-1493.

Park, J.E., Chen, H.H., Winer, J., Houck, K.A., and Ferrara, N. (1994). Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, *in vitro* and *in vivo*, and high affinity binding to Flt-1 but not to Flk-1/KDR. Journal of Biological Chemistry *269*, 25646-25654.

Parker, T.G., Chow, K.L., Schwartz, R.J., and Schneider, M.D. (1990). Differential regulation of skeletal alpha-actin transcription in cardiac muscle by two fibroblast growth factors. Proceedings of the National Academy of Sciences of the United States of America *87*, 7066-7070.

Parker, T.G., Chow, K.L., Schwartz, R.J., and Schneider, M.D. (1992). Positive and negative control of the skeletal alpha-actin promoter in cardiac muscle. A proximal serum response element is sufficient for induction by basic fibroblast growth factor (FGF) but not for inhibition by acidic FGF. Journal of Biological Chemistry *267*, 3343-3350.

Pastan, I., Seth, P., Fitzgerald, D., and Willingham, M. (1986). Adenovirus entry into cells : some newobservations or an old problem. In Virus attachment and entry into cells. R.L. Crowell and K. Lonberg-Holm, eds. (Washington DC: American Society for Microbiology), 141-146.

Pearlman, J.D., Hibberd, M.G., Chuang, M.L., Harada, K., Lopez, J.J., Gladstone, S.R., Friedman, M., Sellke, F.W., and Simons, M. (1995). Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. Nature Medicine *1*, 1085-1089.

Pepper, M.S., Ferrara, N., Orci, L., and Montesano, R. (1991). Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochemical and Biophysical Research Communications *181*, 902-906.

Perlman, D. and Halvorson, H.O. (1983). A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. Journal of Molecular Biology *167*, 391-409.

Pernow, J. and Wang, Q. (1997). Endothelin in myocardial ischaemia and reperfusion. Cardiovascular Research *33*, 518-526.

Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O., and Alitalo, K. (1994). Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. Journal of Biological Chemistry *269*, 6271-6274.

Pfeffer, M.A. and Braunwald, E. (1990). Ventricular remodeling after myocardial infarction: Experimental observations and clinical implications. Circulation *81*, 1161-1172.

Phillis, R. and Kumar, S. (1979). Int J Cancer *23*, 82-89.

Pierce, G.N. and Czubryt, M.P. (1995). The contribution of ionic imbalance to ischemia/reperfusion-induced injury. Journal of Molecular and Cellular Cardiology *27*, 53-63.

Plate, K.H., Breier, G., Weich, H.A., and Risau, W. (1992). Vascular endothelial growth factor is a potential tumour angiogenssis factor in human gliomas *in vivo*. Nature *359*, 845-848.

Plumier, J.C.L., Ross, B.M., Currie, R.W., Angelidis, C.E., Kazlaris, H., Kollias, G., and Pagoulatos, G.N. (1995). Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. Journal of Clinical Investigation *95*, 1854-1860.

Plumier, J.C.L., Robertson, H.A., and Currie, R.W. (1996). Differential accumulation of mRNA for immediate early genes and heat shock genes in heart after ischaemic injury. Journal of Molecular and Cellular Cardiology *28*, 1251-1260.

Pognonec, P., Kato, H., and Roeder, R.G. (1992). The helix-loop-helix/leucine repeat transcription factor USF can be functionally regulated in a redox-dependent manner. Journal of Biological Chemistry *267*, 24563-24567.

Poller, W., Schneider-Rasp, S., Liebert, U., Merklein, F., Thalheimer, P., Haack, A., Schwaab, R., Schmitt, C., and Brackmann, H. (1996). Stabilization of transgene expression by incorporation of E3 region genes into an adenoviral factor IX vector and by transient anti-CD4 treatment of the host. Gene Therapy *3*, 521-530.

Poltorak, Z., Cohen, T., Sivan, R., Kandelis, Y., Spira, G., Vlodavsky, I., Keshet, E., and Neufeld, G. (1997). VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. Journal of Biological Chemistry *272*, 7151-7158.

Poole-Wilson, P.A. (1985). The nature of myocardial damage following reoxygenation. In Control and manipulation of calcium movement. J.R. Parrat, ed. (New York: Raven Press), 62-99.

Prentice, H., Kloner, R.A., Prigozy, T., Christensen, T., Newman, L., Li, Y., and Kedes, L. (1994). Tissue restricted gene expression assayed by direct DNA injection into cardiac and skeletal muscle. Journal of Molecular and Cellular Cardiology *26*, 1393-1401.

Prentice, H., Kloner, R.A., Li, Y., Newman, L., and Kedes, L. (1996). Ischemic/reperfused myocardium can express recombinant protein following direct DNA or retroviral injection. Journal of Molecular and Cellular Cardiology *28*, 133-140.

Prentice, H., Bishopric, N.H., Hicks, M.N., Discher, D.J., Wu, X., Wylie, A.A., Webster, K.A. (1997). Regulated expression of a foreign gene targeted to the ischaemic myocardium. Cardiovascular Research *35*, 567-574.

Quinn, T.P., Peters, K.G., De Vries, C., Ferrara, N., and Williams, L.T. (1993). Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proceedings of the National Academy of Sciences of the United States of America *90*, 7533-7537.

Quinones, M.J., Leor, J., Kloner, R.A., Ito, M., Patterson, M., Witke, W.F., and Kedes, L. (1996). Avoidance of immune response prolongs expression of genes delivered to the adult rat myocardium by replication-defective adenovirus. Circulation *94*, 1394-1401.

Rastinejad, F., Polverini, P.J., and Bouck, N.P. (1989). Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell *56*, 345-355.

Reed, J.C. (1997). Double identity for proteins of the Bcl-2 family. Nature *387*, 773-776).

Reimer, K.A., Lowe, J.E., Rasmussen, M.M., and Jennings, R.B. (1977). The wavefront phenomenon of ischaemic cell death, I: Myocardial infarct size vs. duration of coronary occlusion in dogs. Circulation *56*, 786-794.(Abstract)

Reimer, K.A., Jennings, R.B., and Tatum, A.H. (1983). Pathobiology of acute myocardial ischemia: Metabolic, functional and ultrastructural studies. American Journal of Cardiology *52*, 72A-81A.

Reimer, K.A., Murry, C.E., Yamasawa, I., and et al (1986). Four brief periods of myocardial ischemia cause no cumulative ATP loss or necrosis. American Journal of Physiology - Heart and Circulatory Physiology *251*, 20/6) H1306-H1315.

Reimer, K.A., Murry, C.E., and Jennings, R.B. (1990). Cardiac adaptation to ischemia. Ischemic preconditioning increases myocardial tolerance to subsequent ischemic episodes. Circulation *82*, 2266-2268.

Ring, C.J.A. (1997). Adenovirus Vectors. In Gene Therapy. D.N. Lemoine, ed. (Oxford: BIOS Scientific Publishers), pp. 61-76.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. Science *234*, 364-368.

Ross, R., Raines, E.W., and Bowen-Pope, D.F. (1986). The biology of platelet-derived growth factor. Cell *46*, 155-169.

Rudolphi, K.A., Keil, M., Fastbom, J., and Fredholm, B.B. (1989). Ischaemic damage in gerbil hippocampus is reduced following upregulation of adenosine (A1) receptors by caffeine treatment. Neuroscience Letters *103*, 275-280.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning : A laboratory manual. Cold Spring Harbour: Laboratory Press

Saraste, A., Pulkki, K., Kallajoki, M., Henriksen, K., Parvinen, M., Voipio-, and Pulkki, L. (1997). Apoptosis in human acute myocardial infarction. Circulation *95*, 320-323.

Schaper, W. (1984). Experimental Infarcts and the Microcirculation. In Therapeutic approaches to infarct size limitation. D.J. Hearse and D.M. Yellon, eds. (New York: Raven Press), 234-256.

Schiaffino, S., Samuel, J.L., Sassoon, D., Lompre, A.M., Garner, I., Marotte, F., Buckingham, M., Rappaport, L., and Schwartz, K. (1989). Nonsynchronous accumulation of alpha-skeletal actin and beta-myosin heavy chain mRNAs during early stages of pressure-overload-induced cardiac hypertrophy demonstrated by in situ hybridization. Circulation Research *64*, 937-948.

Schreiber, A.B., Winkler, M.E., and Derynck, R. (1986). Transforming growth factor-alpha: A more potent angiogenic mediator than epidermal growth factor. Science *232*, 1250-1253.

Schwartz, K., Lecarpentier, Y., Martin, J.L., and et al (1981). Myosin isoenzymic distribution correlates with speed of myocardial contraction. Journal of Molecular and Cellular Cardiology *13*, 1071-1075.

Schwartz, K., De la Bastie, D., Bouveret, P., and et al (1986). alpha-Skeletal muscle actin mRNA's accumulate in hypertrophied adult rat hearts. Circulation Research *59*, 551-555.

Schwartz, K., Carrier, L., Mercadier, J.J., Lompre, A.M., and Boheler, K.R. (1993). Molecular phenotype of the hypertrophied and failing myocardium. Circulation *87*, VII5-VII10.

Sellke, F.W., Wang, S.Y., Stamler, A., Lopez, J.J., Li, J., and Simons, M. (1996). Enhanced microvascular relaxations to VEGF and bFGF in chronically ischemic porcine myocardium. American Journal of Physiology - Heart and Circulatory Physiology *271*, H713-H720.

Semenza, G.L. (1994). Regulation of erythropoietin production: New insights into molecular mechanisms of oxygen homeostasis. Hematology/Oncology Clinics of North America *8*, 863-884.

Semenza, G.L., Roth, P.H., Fang, H.M., and Wang, G.L. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. Journal of Biological Chemistry *269*, 23757-23763.

Semenza, G.L. and Wang, G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Molecular and Cellular Biology *12*, 5447-5454.

Shlafer, M., Myers, C.L., and Adkins, S. (1987). Mitochondrial hydrogen peroxide generation and activities of glutathione peroxidase and superoxide dismutase following global ischemia. Journal of Molecular and Cellular Cardiology *19*, 1195-1206.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*, 843-845.

Singal, P.K., Beamish, R.E., and Dhalla, N.S. (1980). Potential oxidative pathways of catecholamines in the formation of lipid peroxides and genesis of heart disease. Adv Exp Med Biol *191*, 421-427.

Stavri, G.T., Zachary, I.C., Baskerville, P.A., Martin, J.F., and Erusalimsky, J.D. (1995). Basic fibroblast growth factor upregulates the expression of vascularendothelial growth factor in vascular smooth muscle cells: Synergistic interaction with hypoxia. Circulation *92*, 11-14.

Steenbergen, C., Hill, M.L., and Jennings, R.B. (1987). Cytoskeletal damage during myocardial ischemia: Changes in vinculin immunofluorescence staining during total *in vitro* ischemia in canine heart. Circulation Research *60*, 478-486.

Swynghedauw, B. (1986). Developmental and functional adaption of contractile proteins in cardiac and skeletal muscles. Physiological Reviews *66*, 710-749.

Swynghedauw, B. and Delcayre, C. (1982). Biology of Cardiac Overload. Pathobiol Ann *12*, 137-182.

Takala, T. (1981). Protein synthesis in the isolated perfused rat heart. Effects of mechanical work load, diastolic ventricular pressure and coronary pressure on amino acid incorporation and its transmural distribution into left ventricular protein. Basic Research in Cardiology *76*, 44-61.

Takeshita, S., Zheng, L.P., Brogi, E., Kearney, M., Pu, L.Q., Bunting, S., Ferrara, N., Symes, J.F., and Isner, J.M. (1994). Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. Journal of Clinical Investigation *93*, 662-670.

Takeshita, S., Rossow, S.T., Kearney, M., Zheng, L.P., Bauters, C., Bunting, S., Ferrara, N., Symes, J.F., and Isner, J.M. (1995). Time course of increased cellular proliferation collateral arteries after administration of vascular endothelial growth factor in a rabbit model of lower limb vascular insufficiency. American Journal of Pathology *147*, 1649-1660.

Terman, B.I., DougherVermazen, M., Carrion, M.E., Dimitrov, D., Armellino, D.C., Gospodarowicz, D., and Bohlen, P. (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochemical and Biophysical Research Communications *187*, 1579-1586.

Thomas, K.A., Rios-Candelore, M., Gimenez-Gallego, G., and et al (1985). Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin 1. Proceedings of the National Academy of Sciences of the United States of America *82*, 6409-6413.

Tischer, E., Gospodarowicz, D., Mitchell, R., Silva, M., Schilling, J., Lau, K., Crisp, T., Fiddes, J.C., and Abraham, J.A. (1989). Vascular endothelial growth factor: A new member of the platelet-derived growth factor gene family. Biochemical and Biophysical Research Communications *165*, 1198-1206.

Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J.C., and Abraham, J.A. (1991). The human gene for vascular endothelial growth factor: Multiple protein forms are encoded through alternative exon splicing. Journal of Biological Chemistry *266*, 11947-11954.

Tsai, J.C., Goldman, C.K., and Gillespie, G.Y. (1995). Vascular endothelial growth factor in human glioma cell lines: Induced secretion by EGF, PDGF-BB, and bFGF. Journal of Neurosurgery *82*, 864-873.

Tseng, Y.T., Waschek, J.A., and Padbury, J.F. (1995). Functional analysis of the 5' flanking sequence in the ovine beta1- adrenergic receptor gene. Biochemical and Biophysical Research Communications *215*, 606-612.

Tsika, R.W., Bahl, J.J., Leinwand, L.A., and Morkin, E. (1990). Thyroid hormone regulates expression of a transfected human alpha-myosin heavy-chain fusion gene in fetal rat heart cells. Proceedings of the National Academy of Sciences of the United States of America *87*, 379-383.

Tsurumi, Y., Takeshita, S., Chen, D., Kearney, M., Rossow, S.T., Passeri, J., Horowitz, J.R., Symes, J.F., and Isner, J.M. (1996). Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. Circulation *94*, 3281-3290.

Turrens, J.F. and boveris, A. (1980). Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochemical Journal *191*, 421-427.

Unemori, E.N., Ferrara, N., Bauer, E.A., and Amento, E.P. (1992). Vascular endothelial growth factor induced interstitial collagenase expression in human endothelial cells. Journal of Cellular Physiology *153*, 557-562.

Vaisman, N., Gospodarowicz, D., and Neufeld, G. (1990). Characterization of the receptors for vascular endothelial growth factor. Journal of Biological Chemistry *265*, 19461-19466.

Van der Zee, R., Murohara, T., Luo, Z., Zollmann, F., Passeri, J., Lekutat, C., and Isner, J.M. (1997). Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. Circulation *95*, 1030-1037.

Vandekerckhove, J., Bugaisky, G., and Buckingham, M. (1986). Simultaneous expression of skeletal muscle and heart actin proteins in various striated muscle tissues and cells. A quantitative determination of the two actin isoforms. Journal of Biological Chemistry *261*, 1838-1843.

Vandekerckhove, J. and Weber, K. (1978). Mammalian cytoplasmic actins are the products of at least two genes and differ in primary structure in at least 25 identified positions from skeletal muscle actins. Proceedings of the National Academy of Sciences of the United States of America *75*, 1106-1110.

Vandekerckhove, J. and Weber, K. (1979). The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle and rabbit slow skeletal muscle. Differentiation *14*, 123-133.

Vandekerckhove, J. and Weber, K. (1984). Chordate muscle actins differ distinctly from invertebrate muscle actins. The evolution of the different vertebrate muscle actins. Journal of Molecular Biology *179*, 391-413.

Von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucleic Acids Research *14*, 4683

Walder, C.E., Errett, C.J., Bunting, S., Lindquist, P., Ogez, J.R., Heinsohn, H.G., Ferrara, N., and Thomas, G.R. (1996). Vascular endothelial growth factor augments muscle blood flow and function in a rabbit model of chronic hindlimb ischemia. Journal of Cardiovascular Pharmacology *27*, 91-98.

Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C. (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. Journal of Biological Chemistry *269*, 26988-26995.

Walter, P. and Blobel, G. (1981). Translocation of proteins across the endoplasmic reticulum. III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. Journal of Cell Biology *91*, 557-561.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proceedings of the National Academy of Sciences of the United States of America *92*, 5510-5514.

Wang, G.L. and Semenza, G.L. (1993a). Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. Journal of Biological Chemistry *268*, 21513-21518.

Wang, G.L. and Semenza, G.L. (1993b). General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proceedings of the National Academy of Sciences of the United States of America *90*, 4304-4308.

Watanabe, M., Green, F.J., and Farmer, B.B. (1986). Preparation and use of cardiac myocytes in experimental cardiology. In The Heart and Cardiovascular System. H.A. Fozzard, E. Haber, R.B. Jennings, A.M. Katz, and H.E. Morgan, eds. (New York: Raven Press), pp. 241-252.

Webster, K.A., Discher, D.J., Sato, B., Bodi, I, and Bishopric, N.H. (1993). Induction of immediate-early genes and hypertrophic marker transcripts by hypoxia-reoxygenation cycles in rodent cardiac myocytes. Journal of Cellular Biochemistry *No.S17D SID*, 237

Webster, K.A., Discher, D.J., and Bishopric, N.H. (1994). Regulation of fos and jun immediate-early genes by redox or metabolic stress in cardiac myocytes. Circulation Research *74*, 679-686.

Webster, K.A. and Bishopric, N.H. (1992). Molecular regulation of cardiac myocyte adaptations to chronic hypoxia. Journal of Molecular & Cellular Cardiology *24*, 741-752.

Whittaker, P., Rakusan, K., and Kloner, R.A. (1996). Transmural channels can protect ischemic tissue: Assessment of long-term myocardial response to laser- and needle-made channels. Circulation *93*, 143-152.

Whittaker, P. and Kloner, R.A. (1991). Ventricular remodeling of the heart. Current Opinion in Cardiology *6*, 346-351.

Wolff, J.A., Malone, R.W., Williams, P., Wang Chong, Acsadi, G., Jani, A., and Felgner, P.L. (1990). Direct gene transfer into mouse muscle *in vivo*. Science *247*, 1465-1468.

Wu, H.M., Huang, Q., Yuan, Y., and Granger, H.J. (1996). VEGF induces NO-dependent hyperpermeability in coronary venules. American Journal of Physiology *271*, H2735-H2739.

Wu, X., Bishopric, N.H., Discher, D.J., Murphy, B.J., and Webster, K.A. (1996). Physical and functional sensitivity of zinc finger transcription factors to redox change. Molecular and Cellular Biology *16*, 1035-1046.

Yoshida, T., Watanabe, M., Engelman, D.T., Engelman, R.M., Schley, J.A., Maulik, N., Ho, Y.S., Oberley, T.D., and Das, D.K. (1996). Transgenic mice overexpressing glutathione peroxidase are resistant to myocardial ischemia reperfusion injury. Journal of Molecular and Cellular Cardiology *28*, 1759-1767.

Ytrehus, K. and Downey, J.M. (1993). Experimental models assessing the physiology of myocardial ischemia. Current Opinion in Cardiology *8*, 581-588.

Zhi Qing Zha, McGee, D.S., Nakanishi, K., Toombs, C.F., Johnston, W.E., Ashar, MS, and Vinten-Johansen, J. (1993). Receptor-mediated cardioprotective effects of endogenous adenosine are exerted primarily during reperfusion after coronary occlusion in the rabbit. Circulation *88*, 709-719.

Ziche, M., Jones, J., and Gullino, P.M. (1997). Role of prostaglandin E1 and copper in angiogenesis. J Nat Cancer Inst, Vol 69(2) 475-482.