Expression of Nuclear-Acting Early-Response Genes in the Rat Heart: implications for cardiac hypertrophy.

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PUBLICATIONS FROM THIS THESIS

1. Adrenergic agents, but not triiodo-L-thyronine induce c-*fos* and c-*myc* expression in the rat heart. **R.D. Hannan** and A.K. West. Basic Research in Cardiology, 86;154-164 (1991)

2. Localization of c-*fos* and related gene expression in the rat heart following norepinephrine expression. **R.D. Hannan,** F.A. Stennard and A.K. West. Journal of Molecular and Cellular Cardiology, 25; 1137-1148 (1993)

3. Localization of c-*myc* proto-oncogene expression in the rat heart *in vivo* and in the isolated, perfused heart following treatment with norepinephrine. **R.D. Hannan**, F.A. Stennard and A.K. West. *Biochimica et Biophysica Acta,* 1217;281-290 (1994)

ABBREVIATIONS

α-SkA: α-skeletal actin

ABC: avidin-biotin complex

aFGF: acidic fibroblast growth factor

ANG II: angiotensin II

ANP: atrial natriuretic peptide/factor

AP-1: activating protein 1

APAAP: alkaline phosphatase-antialkaline phosphatase

ATF: activating factor 1

bFGF: basic fibroblast growth factor

BSA: bovine serum albumin

cAMP: cyclic AMP

Cd: cadmium chloride

CRE: cAMP response element

CREB: cAMP response element binding protein

DAB: diaminobenzidine

DEX: dexamethasone

DMSO: dimethyl sulfoxide

EPI: epinephrine

ET-1: endothelin-1

FLI: Fos like immunostaining

HLH: helix-loop-helix

i.p: intraperitoneal

IP_3: inositol phosphate

LAB: labeled avidin-biotin

LR: leucine repeat

MHC: myosin heavy chain

MLC: myosin light chain

MOPS: morpholinopropane sulfonic acid

MT metallothionein

NE: norepinephrine

PAP: peroxidase-antiperoxidase

PBS: phosphate buffered saline

PCNA: proliferating cell nuclear antigen

PDC: 4a-phorbol 12b, 13a,-didecanoate

PDGF: platelet derived growth factor

PI: phosphatidyl inositol

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PMA: phorbol 12 myristate 13 acetate

SD: standard deviation

SDS: sodium dodecyl sulphate

SHR: spontaneously hypertensive rats

SR: sarcoplasmic reticulum

SRE: serum response element

SRF: serum response factors

T_3: triiodine-L-thyroxine

TBS: tris-buffered saline

TGF-β_1: transforming growth factor β_1

TNFα: tumor necrosis factor α

TPA: 12-*O*-tetradecanoylphorbol-13-acetate

TRE: thyroid response element

TrM: tropomyosin

Expression Of Nuclear-acting Early-Response Genes in the Rat Heart: Implications for Cardiac Hypertrophy

Post-natal growth of the mammalian heart is characterized by an increase in size of pre-existing cardiomyocytes (hypertrophy) rather than an increase in their number (hyperplasia). The primary stimuli for this growth process are not clearly understood but seem to involve both hemodynamic and hormonal factors. A major challenge to researchers has been to clearly define the signals that activate and regulate cardiac hypertrophy and to elucidate the intracellular transducing mechanisms which couple the hypertrophic stimuli to the long term changes in cardiac phenotype and function. Of the most likely candidate molecular signals the nuclear-acting early-response genes are of particular interest since their protein products are thought to play key roles in linking extracellular signals with terminal patterns of gene expression during growth and differentiation. The work in this thesis has examined the ability of various hypertrophic stimuli to modulate the expression of nuclear acting early response genes in the rat heart both *in vivo* and *in vitro*. A single injection of norepinephrine (2.5 μg/kg to 2.5 mg/kg) transiently increased mRNA levels of the nuclear acting early-response genes c-*myc*, c-*fos*, c-*jun*, *fra*-1 and *fra*-2 in the rat heart. Similar responses were also observed following chronic infusion of norepinephrine (100 μg/kg/h) but not in response to treatment with the hypertrophic hormone triiodo-L-thyroxine. Hybridization histochemistry and immunocytochemistry techniques were used to localize early response genes to particular cell types and regions of the heart. Following norepinephrine administration (2.5 mg/kg) Fos protein transiently accumulated in the cardiac myocytes and to a much lesser extent other cell types. In direct contrast, little Myc immunostaining was observed in the cardiac myocytes with greatest expression being localized to the cardiac non-myocyte population, presumably fibroblasts and cells of the vasculature system. The observed responses for both genes was not uniform but appeared greatest in the left atrium and left ventricle with lesser expression elsewhere. In order to differentiate the complex systemic interactions of norepinephrine from its direct actions upon the heart an isolated perfused heart system was employed. Both elevated perfusion pressure (60-120 mmHg) and the inclusion of norepinephrine (1 nM to 1 μM) in the perfusion buffer (60 mmHg) led to elevated mRNA levels of c-*myc*, c-*fos*, *fra*-1 and *fra*-2. These findings demonstrate the utitlity of the isolated perfused heart system as a model to study separately the effects of pressure load and NE on gene expression during the early stages of cardiac hypertrophy. Taken together with the *in vivo* results they lend further support to the notion that the products of early-response genes structurally or functionally related to c-*fos* may mediate the hypertrophic actions of norepinephrine and pressure overload. In contrast, c-*myc* expression may be associated with the proliferation of cardiac non-myocyte cells which occurs concomitant with cardiac hypertrophy.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 DEVELOPMENT OF THE HEART: A BRIEF OVERVIEW

1.1.1 Myocyte Formation

Cardiac myocytes develop by active proliferation of a population of undifferentiated myogenic cells (presumptive myoblasts) which are derived from the splanchnic mesoderm of the early embryo (Manasek, 1970; Zak 1974A, 1974B). Initially the myoblasts are indistinguishable from their parent presumptive myoblasts (Bugaisky and Zak, 1986), however synthesis of myofibrillar protein is slowly initiated and accumulates in the cells which become distinguishable as those of muscle (Zak 1974A, 1974B). This contrasts significantly with skeletal muscle development in which proliferation of skeletal myoblasts is mutually exclusive with the activation of muscle specific genes (Endo and Nadal-Ginard, 1986; Nguyen *et al.*, 1983; Schneider and Olson, 1988).

In the initial stages of cardiac morphogenesis the primitive heart consists only of myocytes, there being no fibroblasts, blood vessels or neuronal tissue (Manasek, 1970). These non-myocyte components of the myocardium have different origins, thought to be the epicardium region of the mesoderm (Manasek, 1968). They invade the myocardium later in its development but due to their rapid and continual proliferation they eventually outnumber the myocytes by 3 to 1 (Morkin and Ashford, 1968; Grove *et al.*, 1969; Bugaisky and Zak, 1986). Even so, myocytes account for more than 75% of the adult heart by volume as a result of their hypertrophic postnatal growth.

As growth of the heart continues and myofibrillar protein accumulates, myocytes change from the round or oval shape of the early fetal stage to become progressively more elongated and this event parallels development of intraluminal pressure (Bishop, 1990). However it is not until shortly after birth when cell division

ceases that cardiac myocytes rapidly assume all aspects (with the exception of size) of the adult cell. The external shape of the cell develops from the simple neonatal spindle cell to a complex structure containing fully developed laterally aligned sarcomeres, T-tubules, numerous intercalated disc regions and a cross sectional shape which is irregular in order to accommodate adjacent cells and blood vessels (Bishop, 1990). At this stage the previously immature vasculature cells, neuronal cells and connective tissue become fully developed and functional (Bishop, 1990).

1.1.2 Postnatal Development

Prenatal growth of heart occurs primarily as a result of an increase in cell number (hyperplasia) but shortly after birth myocyte cell division ceases and further myocardial enlargement stems largely from an increase in the size of pre-existing myocytes (hypertrophy) and to a lesser extent hyperplasia of nonmuscle cells (Clubb and Bishop, 1984; Zak 1974A, 1974B). The exact time of conversion from hyperplastic to hypertrophic growth varies from species to species. In the rat an early postnatal period of approximately 20 days (birth to time of weaning) (Mattfeldt and Mall, 1987) is characterized by both cell division and cell enlargement whilst in the late postnatal period (weaning onwards) further increase in cardiac myocyte mass occurs solely from hypertrophy of existing myocytes and hyperplasia of non-muscle cell types (Sasaki *et al.*, 1968; Claycomb, 1975; Korecky and Rakusan, 1978; Bing *et al.*, 1971; Spann *et al.*, 1971; Skosey *et al.*, 1972). This growth transition period is characterized by the appearance of multinucleate myocytes due to nuclear division which is not accompanied by cellular division (Clubb and Bishop, 1984). For example, in rats approximately 90% of adult ventricular myocytes have 2 or more nuclei (Bishop and Drummond, 1979; Clubb and Bishop, 1984; Muir, 1957; Challice and Edwards, 1961; Bugaisky and Zak, 1986) whilst in pigs adult myocytes contain as many as 4 to 16 nuclei (Grabner and Pfitzer, 1974). In contrast, during skeletal muscle development karyokinesis is not uncoupled from cytokinesis and formation of multinucleate myotubes occurs via cell fusion (Clubb and Bishop, 1984). Furthermore, postnatal growth of skeletal muscle differs in that it is mediated in part by recruitment of "satellite" precursor cells in addition to hypertrophy (Campion, 1984).

Postnatal growth of the heart is also associated with increasing circulatory demands of a rapidly growing animal and rather abrupt changes in the patterns of flow and circulatory resistance occur shortly after birth (Rudolph, 1979). There is a progressive increase in volume load on both sides of the heart whilst pressure load in the left ventricle is markedly altered, increasing from about 20-25 mmHg at birth to over 120 mmHg by several weeks of age (Bishop, 1990). This latter change correlates with faster hypertrophic growth of the left ventricular myocardium which is probably responsible for the relatively larger mass of this chamber in the adult heart (Rudolph, 1979; Bishop, 1990). Concomitant with cardiac myocyte development and the subsequent transition to hypertrophic growth is the coordinated shift in the expression of specific contractile genes and genes involved in cardiac metabolism and energetics (Katz, 1990; Nadal-Ginard and Mahdavi, 1989; Schneider and Parker, 1990). Generally these transitions involve selective transcription within multigene families or alternative mRNA splicing within the one gene (Breithart and Nadal-Ginard, 1987). Such "gene plasticity" results in a new cardiac phenotype whose functional properties differ from those of the fetal heart (Nadal-Ginard and Mahdavi, 1989). It is proposed that such changes allow the newly developed heart to better cope with the altered hemodynamic demand, circulating hormonal levels and higher oxygen dependence characteristic of the adult animal (Bishop, 1990).

1.2 CARDIAC HYPERTROPHIC STIMULI

The adult heart structure and composition are not, however, a fixed postnatal property, but can be modified even further in response to alterations in cardiovascular demand, altered circulating hormone levels and following ischemia. For instance, certain forms of exercise training or pulmonary artery stenosis are often associated with significant myocyte hypertrophy and heart growth beyond that normally expected. Altered levels of various circulatory hormones and neurotransmitters such as thyroxine, angiotensin II (ANG II) and norepinephrine (NE) are also associated with the hypertrophic growth process. However, since many of these agents affect cardiovascular hemodynamics in addition to interacting with their corresponding receptors within the myocardium, it has often been difficult to ascertain whether they

affect myocardial growth directly or whether their trophic actions are mediated by complex systemic interactions

1.2.1 Pressure and Volume Overload

Increased work placed upon the heart as a result of hemodynamic changes is one of the major factors associated with the initiation and maintenance of cardiac hypertrophy during both physiological growth and disease states.

Hemodynamic stimuli which may modulate cardiac growth are traditionally differentiated into two broad groups: those that result in *pressure overload* of the heart in which the ventricle must pump against a greater afterload (Batra and Rakusan, 1991) and those which give rise to *volume overload* of the heart in which the output is increased, often against a reduced peripheral resistance (Batra and Rakuson, 1991). Pressure overload following aortic (Schwartz *et al.*, 1978; Hess *et al.*, 1981; Caspari *et al.*, 1977; Oldershaw *et al.*, 1980; Krayenbuehl *et al.*, 1983) or pulmonary artery stenosis (Marino *et al.*, 1985; Cooper *et al.*, 1981) and renovascular or genetic hypertension is a major determinant of left ventricular hypertrophy in humans. This form of cardiac growth is generally associated with increased ventricular wall thickness and myocyte cross sectional area (Grossman *et al.*, 1975; Anversa *et al.*, 1986; Smith and Bishop, 1985) without chamber dilation and is termed "concentric" hypertrophy. Volume overload in humans is associated with certain forms of strenuous exercise training or a number of disease states including aortic insufficiency, arteriovenous fistula, tarsal septal defect or with hyperthyroidism (Grossman *et al.*, 1975; Ford, 1976; Hutchins *et al.*, 1973; Linzbach, 1960; Carabello *et al.*, 1989; Papadimitriou *et al.*, 1974; Thomas, *et al.*, 1984; Newman, *et al.*, 1982; Ross, 1974; Hultgren and Bove, 1981). In contrast to pressure overload, volume overload generally results in an enlargement of the ventricular circumference due to an increase in myocyte cell length (Grossman *et al., 1975;* Anversa *et al.*, 1986; Gerdes *et al.*, 1988) with variable changes in relative wall thickness (Linzbach, 1960; Grossman *et al.*, 1975) and is thus termed "eccentric" hypertrophy. Increased work load on the heart may also occur in response to a large variety of myocardial conditions such as ischemic cardiomyopathy or acute myocardial infarction in which muscle is lost in a diffuse or focal manner (Sonnenblick

et al., 1983). Events such as these may evoke significant hypertrophy of the remaining myocardium (reactive hypertrophy) which must assume the load of the tissue lost due to the cell death (Sonnenblick *et al.*, 1983). Myocyte growth in response to ischemia can be the result of either increased cell diameter and/or length (i.e. eccentric or concentric hypertrophy) and this appears to be largely dependent on the region and extent of cell damage, the precipitating cause and the status of the heart prior to the event (Anversa *et al.*, 1985; Rubin *et al.*, 1983).

1.2.1.1 Overload hypertrophy: an adaptive response

At least in the initial stages of cardiac hypertrophy, myocyte growth is viewed in terms of an adaptive response of the heart at the cellular and subcellular levels of organization which effectively minimizes the increase in pressure and volume overload (Rakusan, 1984). It is thought that the adaptive response may be attributed, at least in part, to changes in the relative amounts of cardiac specific isocontractile genes and enzymes of myocardial metabolism. For instance, rodent models of pressure overload are associated with an increase in cardiac mass and re-expression of genes associated with the neonatal heart such as β-myosin heavy chain (β-MHC) (Litten *et al.*, 1982; Lompre *et al.*, 1979; Martin *et al.*, 1983). The diminished actin-activated ATPase activity of β-MHC decreases the maximum velocity of shortening in unloaded muscle fibers (Schwartz *et al.*, 1981) and the lower energetic cost of developed work is proposed to be advantageous during high oxygen demand (Katz, 1990; Nadal-Ginard and Mahdavi, 1989). Other neonatal specific isocontractile genes which are also re-expressed include α-skeletal actin (α-SkA), β-tropomyosin (β-TrM) (Izumo *et al.*, 1988) and atrial myosin light chain-1 (MLC-1) (Katoh *et al.*, 1989). Similarly the neonatal isogenes encoding enzymes associated with myocardial metabolism such as the non-muscle subunits of creatine kinase (Ingwall *et al.*, 1985), lactate dehydrogenase (Hammond *et al.*, 1976) and the α_3-isoform of the sarcolemmal Na^+/K^+ ATPase (Zahler *et al.*, 1989) are up-regulated or reinduced during pressure overload hypertrophy and thus contribute to the increase in the glycolytic potential of the tissue (Bishop, 1990). Other genes whose expression is modulated positively or negatively during pressure overload hypertrophy include those encoding the sarcoplasmic reticulum (SR)

slow/cardiac Ca^{2+} ATPase (Komuro *et al.*, 1989) and atrial natriuretic peptide (ANP) (Izumo *et al.*, 1988) respectively. It is possible that down regulation of the SR Ca^{2+} ATPase may explain the alterations in function of the SR and the impairment of Ca^{2+} movements in the hypertrophic myocardium (Schwartz *et al.*, 1986).

However re-expression of fetal isogenes is not a characteristic of all forms of cardiac hypertrophy and does not appear to occur during cardiac growth associated with volume overload as elicited by hyperthyroidism or arteriovenous fistula (Swynghdauw, 1986). In fact in these forms of hypertrophy the relative amounts of isocontractile genes associated with the adult phenotype are increased (for review see Morkin *et al.*, 1983).

Isocontractile protein shifts also occur in the human heart, but they have been less well characterized and appear to differ in many cases from those in rodent hearts (Parker and Schneider, 1991). For instance β-MHC constitutes a major proportion of MHC in the adult human ventricle in contrast with rodents in which β-MHC is down-regulated. A comparison of some of the cardiac-specific genes in rodent and human ventricle and their alterations following hypertrophic stimulus are outlined in Table 1. Further application of molecular techniques such as polymerase chain reaction (PCR) will allow for better characterization of muscle-specific gene changes within the adult myocardium in the future. (Feldman *et al.*, 1991; Ito *et al.*, 1991 B).

Table 1.1 Expression of cardiac isocontractile genes in normal and pressure overloaded adult rat and human ventricles.

			Rat	Human
MHC	α	Normal	+++	+
		Pressure Overload	++	-
	β	Normal	+	+++
		Pressure Overload	+++	++++
α-Actin	Cardiac	Normal	+++	+++
		Pressure Overload	+++	?
	Skeletal	Normal	-/+	+
		Pressure Overload	+++	?

(-): not expressed; (+,++,+++): relative expression; (?): unknown or conflicting results. Adapted from Parker and Schneider (1991).

1.2.1.2 Experimental induction of overload hypertrophy

A number of experimental models have been developed in order to better characterize the effect of altered hemodynamic load on cardiac hypertrophy *in vivo*. Generally these models of hypertrophy are designed to mimic the changes in pressure and/or volume load observed clinically in animals during normal development and adaptation or in response to disease states.

Particular forms of physical activity involving dynamic exercise such as swimming or running endurance trials are common models used to study hypertrophic growth during volume overload. Typically, a moderate or strenuous exercise regimen imposed on rats results in 22% and 30% hypertrophy respectively of the right ventricle after 8 weeks (Anversa *et al.*, 1982; Loud *et al.*, 1984; Anversa *et al.*, 1984). It is worth noting however, that significantly increased adrenergic activity has been associated with swimming-exercised rats which may contribute to hypertrophy in its own right (Rupp, 1989) and may account for the observed increased proportion of α-MHC levels (Rupp and Wahl, 1990; Rupp, 1989; Rupp *et al.*, 1984; Schaible *et al.*, 1987; Pagani and Solaro, 1983). Other models of volume overload hypertrophy include chronic aortocaval fistula which can result in 20% to 100% increases in heart weight at 1-5 months compared to sham operated animals (Grossman *et al.*, 1975; Flaim *et al.*, 1987; Zimmer, 1983; Batra and Rakusan, 1991).

Cardiac hypertrophy following pressure overload can be rapidly induced in experimental animals by a wide variety of techniques, including abdominal aortic banding with right renal ischemia (Doering *et al.*, 1988; Jalil *et al.*, 1988; Jalil *et al.*, 1989), coarcation of 1 renal artery (Averill *et al.*, 1976; Marino *et al.*, 1985; Sen *et al.*, 1981; Thiedemann *et al.*, 1983; Sen and Bumpus, 1979), suprarenal abdominal aortic banding (Folkman and Klagsbrun, 1987; Slack *et al.*, 1987) and pulmonary artery vasoconstriction (Yoshida *et al.*, 1987). For instance, within 8 days following constriction of the abdominal aorta there is an approximate 50% increase in size of the left ventricle and cardiomyocyte transverse area (Anversa *et al.*, 1986). Spontaneously hypertensive rats (SHR) also develop cardiac hypertrophy and there is a gradual increase in cardiac mass as a function of an age-related rise in pressure. At 20 weeks of age both

hypertension and hypertrophy are established (Okamoto and Aoki, 1963; Okamoto *et al.*, 1966; Pfeffer *et al.*, 1979 A; Sen *et al.*, 1974).

Reactive cardiac hypertrophy may be induced experimentally by occlusion of a coronary artery. For instance complete ligation of the left anterior descending coronary artery in rats leads to transmural ischemia and a subsequent increase in myocardial volume of the surviving cardiac tissue (Zimmer *et al.*, 1990; Pfeffer *et al.*, 1979 B; Anversa *et al.*, 1984). In addition many models that induce acute cardiac overloading such as banding of the pulmonary artery of adult animals may result in multifocal areas of necrosis (Bishop and Melsen, 1976) and consequently reactive hypertrophy ensues.

1.2.1.3 Load: a primary and sufficent stimulus for cardiac hypertrophy

Changes in cardiac gene plasticity, metabolism and work parameters are readily characterized during experimentally-induced overload hypertrophy. In contrast it has proven very difficult to establish with any degree of certainty whether increased load itself is a direct and sufficient cause of cardiac hypertrophy in whole animals. Alterations in load are often accompanied by reflex changes in the levels of growth factors and neurotransmitters both locally and in circulation which may subsequently modulate heart growth.

i) models of load in vivo

One *in vivo* model which has proven particularly useful in dissecting the various parameters involved in overload hypertrophy is the heterotopical isotransplanted heart (Korecky *et al.*, 1987; Klein and Hong, 1986; Advani *et al.*, 1990; Klein *et al.*, 1990). In this model a heart is transplanted into the abdomen of a recipient animal of the same strain by attaching the stumps of aorta and pulmonary artery to the abdominal aorta and inferior vena cava respectively (Korecky and Masika, 1991). The transplant in effect functions as a denervated "non-working" Langendorff heart and is exposed to the same hormonal stimuli as the endogenous heart but does not support a hemodynamic load (Korecky and Masika, 1991). Several days following surgery an onset of atrophy is observed and this can not be reversed by the addition of growth-promoting hormones or neurotransmitters such as thyroxine or NE although expected shifts in the isomyosin spectrum due to these agents can be observed (Korecky and Masika, 1991; Klein and

Hong, 1986; Korecky *et al.*, 1987). In addition, moderate swimming exercise does not attenuate atrophy of isotransplanted hearts even though mild hypertrophy is observed in the endogenous heart (Advani *et al.*, 1990). In contrast, increased load placed upon the transplanted heart as a result of aortic insufficiency and/or stenosis of the aortic valve significantly attenuates the atrophy observed in the left ventricle (Korecky and Masika, 1991).

The papillary muscle, because of its easily dissectable, elongated form and highly oriented histological structure is another model often used for examining the direct effect of load on cardiac growth (Anversa *et al.*, 1986). In one study the tethering chordae tendineae of a single papillary muscle was cut to remove the load on this region of an otherwise normally loaded ventricle. This treatment very rapidly led to atrophy of the unloaded muscle (Cooper and Tomanek, 1982) even when the surrounding ventricular chamber was subjected to increased load secondary to aortic constriction and despite the influence of hormones supplied by the muscle's blood and nerve supply (Cooper *et al.*, 1985). The only means by which the mass could be returned to the atrophied papillary muscle was by surgical restoration of the original load (Thompson *et al.*, 1984; Kent *et al.*, 1985).

In vivo experiments such as these provide indirect evidence that increased work load is a major and sufficient factor contributing to the initiation and maintenance of cardiac hypertrophy following hemodynamic overload. Alternatively, these studies might simply indicate that load prevents atrophy, which might be an altogether different process to hypertrophy.

ii) models of load in vitro

In an attempt to better determine whether load itself is a primary and sufficient determinant for myocyte hypertrophy and cardiac growth a number of *in vitro* models have been developed including isolated superfused papillary muscle, Langendorff perfused hearts and cardiomyocyte cultures. These models have an advantage over their *in vivo* counterparts in that the complex systemic interactions of a given hypertrophic stimulus can be removed. Increased ventricular pressure has been shown to increase protein synthesis in isolated heart preparations (Hjalmarson and Isaksson, 1972; Morgan *et al.*, 1980; Schreiber *et al.*, 1966) and similarly, exposure of Langendorff perfused and working hearts to elevated aortic pressure increased the rate

of cardiac protein synthesis due to faster rates of both peptide chain initiation and elongation (Kira *et al.*, 1984). When ventricular pressure development was prevented by ventricular draining and hearts were arrested with tetrodotoxin, protein synthesis still increased as a function of perfusion pressure (Kira *et al.*, 1984). Further experiments demonstrated that oxygen consumption, glucose-6-phosphate levels and energy availability could be dissociated from the stimulatory effect of higher aortic pressure (Kira *et al.*, 1984). Taken together these results indicate that stretch of the ventricular wall in response to elevated aortic pressure is the parameter most closely related to increased protein synthesis *in vitro* (Kira *et al.*, 1984). In support of this other groups have demonstrated increased rates of protein synthesis in the left atrium of the perfused heart when the filling pressure of the chamber was elevated with respect to controls (Smith and Sugden, 1983). Similarly in isolated denervated papillary muscle preparations, an enhanced protein synthesis rate was found to be proportional to the load or tension applied to the muscle (Kent *et al.*, 1989).

The importance of load or stretch in the maintenance of cardiac muscle structure and function has become even more apparent from experiments with cardiomyocyte cell cultures. Unattached and thus externally unloaded feline cardiomyocytes maintained in cell suspension rapidly lose internal structure and organizational characteristics of differentiated striated muscle and come to resemble undifferentiated neonatal myocytes (Cooper *et al.*, 1989). In contrast, adult cardiomyocytes externally loaded by attachment to a laminin-coated substrate exhibited only a very gradual loss of their characteristic structural, biochemical and functional properties (Cooper *et al.*, 1986). Furthermore, linear deformation resulting in approximately 10% increase in cardiomyocyte cell length increased incorporation of [^{3}H]-uridine into nuclear RNA and [^{3}H]-phenylalanine into cytoplasmic protein (Mann *et al.*, 1989; Cooper *et al.*, 1989). Other studies have demonstrated that contractile arrest produced by either membrane depolarization or L-channel blockade inhibits not only growth of neonatal myocytes in culture but also expression of β-MHC (Samarel and Engelmann, 1991).

Thus *in vitro* experiments indicate that increased load itself is sufficient stimulus to accelerate RNA and protein synthesis and are supportive of the notion that

increased cardiac loading is one form of stimulus that is capable of initiating the biochemical events that form the basis of cardiac hypertrophy *in vivo*.

1.2.2 Hormones and Growth Factors

Although increased load or stretch of myocytes has been strongly implicated in the initiation and maintenance of cardiac hypertrophy, this does not obviate the possible importance to cardiac growth of other potential trophic factors such as norepinephrine (NE), thyroid hormones, angiotensin II (ANG II), adrenocorticoids and insulin. For many of these, it has been difficult to establish precisely what their contribution is to the hypertrophic growth process since they may modulate cardiac growth directly or as a secondary response via the alterations in hemodynamic parameters which they cause. In addition, recent studies have presented strong evidence that specific growth factors formed in the heart (Parker and Schneider, 1991; Schunkert *et al.*, 1990) or changes in the intracardiac activity of systems such as the renin-angiotensin system (Schunkert *et al.*, 1990; Baker *et al.*, 1990), may play an autocrine or paracrine role in the hypertrophic response of the myocardium.

It is possible that although the final result may be the same (i.e. increased cardiac muscle mass), different growth-promoting stimuli may accomplish this via alternative signaling pathways resulting in distinct changes in myocyte composition and cardiac design. By this means it is hypothesized that the heart may be better able to adapt to altered functional demand placed upon it during normal growth and disease states.

1.2.2.1 Catecholamines

The catecholamines, NE from the sympathetic nerves and epinephrine (EPI) from the adrenal medulla, have been identified as likely non-mechanical growth factors which may modulate the development of cardiac hypertrophy. Early evidence for this notion has stemmed from both clinical observations and from *in vivo* experiments but even stronger lines of evidence are now emerging from studies with isolated cardiac myocyte cultures.

i) in vivo studies

Numerous studies have been published documenting that chronic administration of NE *in vivo* rapidly leads to increases in myocardial mass and

hypertrophy of the cardiac myocytes (Laks *et al.*, 1973; Harri, 1978; King *et al.*, 1987). However the establishment of a direct causal relationship between adrenergic-receptor occupation and subsequent changes in myocardial metabolism *in vivo* have been difficult due to the complex cardiac and systemic effects that this hormone exerts. For instance, cardiac hypertrophy observed following chronic treatment with high doses of catecholamines is physiologically similar to that observed in pressure-overloaded hearts secondary to aortic stenosis since both models exhibit preferential hypertrophy of the left ventricle which is often accompanied by an increase in connective tissue (Marino *et al.*, 1985; Benjamin *et al.*, 1989). The similarity between these two forms of cardiac growth may be partly interpreted as the effect of high circulating levels of NE leading to increased vascular resistance (α_1-mediated vasoconstriction) and subsequent pressure-overload of the heart. Thus these findings argue for a secondary pressure component in the mediation of NE induced cardiac hypertrophy *in vivo*. Interpretation of results derived from animals treated with high doses of catecholamines *in vivo* may be further complicated due to their ability to cause myocardial damage (reviewed in Jiang and Downing, 1990). For instance it is well documented that acute high-dose administration of isoproterenol (a β-adrenergic agonist) can lead to significant cardiac cell necrosis (Stanton *et al.*, 1969) and thus a significant proportion of the cardiac enlargement observed under these conditions may be due to reactive hypertrophy and/or growth of connective tissue (Jiang and Downing, 1990) rather than a direct trophic effect of these drugs (Morgan and Baker, 1991).

However, direct evidence *in vivo* for a role of catecholamines in modulating cardiac hypertrophy independent of pressure comes from experiments in which cardiac hypertrophy is induced by levels of NE that do not alter hemodynamic parameters or in which changes in hemodynamics are reduced by peripherally-acting drugs. For instance chronic infusion or repeated injections of rats with subhypertensive doses of NE or isoproterenol results in significant cardiac hypertrophy (Laks *et al.*, 1973; Tse *et al.*, 1979; Chiba *et al.*, 1989; Marino *et al.*, 1991). Furthermore it has been shown that these changes in cardiac mass occur without concomitant cellular necrosis or fibrosis (Lin, 1973; Marino *et al.*, 1991). Similarly either infusion or repeated injections of hypertensive doses of either α- or β-adrenergic agonists rapidly and independently

induce cardiac hypertrophy and increase total RNA concentration even when hemodynamic parameters are normalized by co-infusion with the calcium channel blocker verapamil (Zierhut and Zimmer, 1989). Thus these experiments demonstrate that low doses of catecholamines can modulate cardiac hypertrophy independent of pressure load. In respect to this it is interesting to note that the morphological changes produced by low doses of NE appear to be different to those observed in response to hypertensive levels of this hormone. For instance subhypertensive doses of NE have been reported to lead to similar increases in both left and right ventricular mass which are directly paralleled by increases in left and right ventricular muscle cell cross-sectional area (Marino *et al.*, 1991). Furthermore, increased muscle mass is accompanied by proportional increases in interstitial volume and that of the vascular compartment of the heart but not with increased density of connective tissue (Marino *et al.*, 1991). In short it appears that low doses of NE produce morphological changes in the heart similar to those produced by volume-overload hypertrophy, while higher doses of NE can produce morphological growth and abnormalities similar to those produced by pressure-overload hypertrophy (Marino *et al.*, 1991).

In addition to the effect of NE on the heart described above, it appears that the adrenergic system may indirectly regulate other models of cardiac enlargement. For example a number of studies have reported that cardiac α_1- and β-adrenergic receptor numbers are increased during aortic stenosis and consequently it has been hypothesized that increased catecholamine sensitivity may play a mediator role in pressure-overload hypertrophy (Karliner *et al.*, 1980; Limas, 1979; Tamai *et al.*, 1989). For instance, pressure-overload of guinea pig hearts elicits an increase in myocardial α_1-adrenoceptor density and this occurs before the onset of cardiac hypertrophy. Furthermore α_1- but not β-blockade could prevent the observed development in hypertrophy (Tamai *et al.*, 1989). However, since α_1-blockade also decreased left ventricular pressure it was not possible to determine whether it affected cardiac metabolism directly or secondary to reducing after load. Other groups have demonstrated that long term treatment of spontaneously hypertensive rats with α_1-adrenergic blockers reduces blood pressure, leads to regression of cardiac hypertrophy and shifts the myosin isoenzyme pattern towards α-MHC (Takeda *et al.*, 1991). In contrast a number of other studies, a

reduction in sympathetic activity was only moderately successful or unsuccessful in reducing cardiac mass (Cooper *et al*, 1985; Prasad *et al.*, 1984; Zimmer and Gerlach, 1982; Cutilletta *et al.*, 1977; Oparil and Cutilletta, 1979; Tomanek *et al.*, 1982)

In a number of physiological and pathological conditions leading to hypertrophy, sympathetic nervous activity is enhanced and the subsequent augmentation of catecholamine release has been implicated in the cardiac growth process. For instance patients with congestive heart failure invariably exhibit elevated plasma levels of catecholamines (Cohn *et al.*, 1984), whilst certain forms of exercise-induced hypertrophy in rats can be ameliorated by chemical sympathectomy and β-adrenergic blockade (Rupp and Wahl, 1990).

In summary, experiments with subhypertensive doses of NE are highly supportive of a direct metabolic role for catecholamines in the initiation and maintenance of cardiac hypertrophy *in vivo*. However interpretation of results derived from studies conducted with higher doses of catecholamines are less clear due to the accompanying hemodynamic and cardiotoxic effects that these hormones cause. Whether altered catecholamine and receptor levels modify the development of hypertrophy in response to mechanical overload is a controversial subject which is yet to be resolved. It is likely that the model of overload and the animal species under study are important factors in these instances (Booth and Thomason, 1991).

ii) in vitro studies

In an attempt to circumvent the complicating systemic effects of catecholamines, investigators have turned to the isolated perfused heart and other *in vitro* myocardial preparations. Early studies demonstrated that pretreatment with catecholamines *in vivo* enhanced protein synthesis in ventricular slices subsequently incubated *in vitro*, but there was no acute effect of EPI on protein synthesis in slices *in vitro* (Mallov, 1973). A number of groups have demonstrated that catecholamines can modulate protein synthesis in isolated perfused heart preparations. For instance NE, EPI and isoproterenol (selective β-agonist) added *in vitro* to perfused hearts caused a dose-dependent increase in the incorporation of [^{14}C]-phenylalanine into heart protein during the first 60 min of perfusion but only EPI was effective in increasing protein synthesis after 90 min of perfusion (Kallfelt *et al.*, 1976). In fact other groups have

detected inhibition of protein synthesis by β-adrenergic agonists and other agents which raise intracellular cyclic AMP (cAMP) concentrations in the perfused heart during sustained perfusion (Fuller and Sugden, 1988). This apparent lack of effect or decrease in protein synthesis during extended perfusion periods with catecholamines has been postulated to be the result of decreased cardiac ATP levels. It has been demonstrated that catecholamines perfused *in vitro* decrease cardiac ATP levels and total adenosine nucleotide contents (Fuller and Sugden, 1988; Chua *et al.*, 1978), probably due to insufficient O_2 delivery by the simple physiological saline solutions used in perfusion buffers, in the face of positive chronotropy and inotropy. Furthermore, since protein synthesis is an endogonic process, decreased nucleoside triphosphate contents may explain the apparent decrease in protein synthesis elicited by β-adrenergic agents during the second hour of perfusion (Fuller and Sugden, 1988). Other groups have avoided problems of β-receptor-mediated reduction in cardiac ATP by using hearts arrested with tetrodotoxin and in these preparations drugs that increased cAMP content such as glucagon and forskolin did not reduce cardiac ATP levels but were able to increase protein synthesis after 90 min of perfusion (Xenophontos *et al.*, 1989).

In addition to β-agonists, increased protein synthesis has also been demonstrated in isolated perfused hearts in response to selective α_1-adrenergic agents and this has been shown to be due to faster rates of translation of pre-existing mRNA (Fuller *et al.*, 1990).

In contrast to perfused heart preparations, *in vitro* models using cardiac cell culture permit the direct analysis of a single variable at the myocyte level. The most inclusive studies examing the effects of catecholamines on cardiomyocyte hypertrophy *in vitro* have been undertaken by Paul Simpson's group (Simpson, 1983; Simpson, 1985; Simpson, 1989; Henrich and Simpson, 1988; Bishopric *et al.*, 1987; Waspe *et al.*, 1990) using isolated neonatal cardiocyte cultures and have subsequently been confirmed by a number of other groups (Meidell *et al.*, 1986; Inuzuka, 1986). With these preparations it has been demonstrated that quiescent neonatal myocytes maintained in serum-free medium respond equipotentially to NE and EPI with an increase in protein content, cell surface area or cell volume, with no associated cell division. Furthermore, cardiac growth was not restricted to non-specific increases in

proteins since mRNAs encoding for the muscle-specific isocontractile genes α-skeletal actin (α-SkA), β-MHC (Waspe *et al.*, 1990; Bishopric *et al.*, 1987) and MLC-2 (Lee *et al.*, 1988) were also up-regulated. Thus the growth of these cells, both in terms of phenotypic changes and cellular enlargement, in response to NE appears to closely model cardiac hypertrophy observed *in vivo* in response to pressure overload. The growth-promoting effect of NE in these cell cultures was demonstrated to be mainly mediated via the α_1-adrenergic receptors since both β- and α_2-specific blockers failed to inhibit hypertrophic growth whilst β-adrenergic agonists did not result in increased cell size (Simpson, 1983). In addition it was demonstrated that the growth response could be regulated independently of beating since growth inhibition with cyclohexamide did not prevent induction of beating by NE and conversely inhibition of beating with β-antagonists did not prevent stimulation of hypertrophy by NE or EPI (Simpson, 1985). These experiments provided the first direct evidence that catecholamines could modulate cardiac growth independent of changes in cardiac mechanics.

The notion that the NE growth response of neonatal cells is specific to α_1-adrenergic agents may have to be revised since Simpson's group have subsequently demonstrated that the β-adrenergic receptor is linked to myocyte hypertrophy although it has a 1000-fold lower EC_{50}, is dependent on certain culture conditions, may require contractility and may not activate transcription (Simpson *et al.*, 1991).

The trophic actions of catecholamines have also been investigated in myocyte cultures isolated from adult animals and they appear to differ markedly from those observed in neonatal myocyte preparations. For example β- but not α-adrenergic stimulation increased α-MHC expression in contracting adult myocytes (Rupp *et al.*, 1991) and this response was shown not to be linked to the high mechanical activity induced by β-adrenergic agents since no differences in expression were observed between contracting and arrested myocytes. The finding that α-MHC expression is increased in these experiments rather than β-MHC, as was demonstrated in neonatal myocytes treated with NE, is intriguing but not unexpected since β-adrenergic administration in adult rats has been shown to lead to an increased proportion of α-MHC (Sreter *et al.*, 1982; Rupp *et al.*, 1983; Buttrick *et al.*, 1988; Rupp *et al.*, 1991).

However, since β-adrenergic stimulation increases NE release from presynaptic nerve terminals (Mueller and Axelrod, 1968) it is difficult to determine whether β-agonists act primarily via β- or α-adrenergic mechanisms *in vivo*. In direct contrast, others have shown that β-adrenergic agonists do not stimulate contractile protein synthesis in quiescent adult myocytes but instead give rise to a general augmentation of non-contractile protein levels (Dubus *et al.*, 1990). Interestingly in another study of adult rat myocyte preparations, elevated rates of protein synthesis (20-30%) were induced by acute administration of α_1-agonists and this was shown to be due to faster rates of translation of pre-existing mRNA (Fuller *et al.*, 1990). However, Cooper *et al* (1986 and 1987) showed no trophic response was observed in adult feline cardiomyocytes treated with NE (Cooper *et al.*, 1986) and thus the growth promoting effects of NE may be species-specific or the significant differences in the experimental culture conditions may be critical (Rupp *et al.*, 1991).

The above results clearly demonstrate that adrenergic agents can directly mediate hypertrophy and isocontractile gene expression in neonatal cardiac myocytes. However, despite recent advances in the preparation and culture of adult cardiac myocytes (Bugaisky and Zak, 1989), studies concerning the effect of catecholamines on myocyte hypertrophy with these cells are less conclusive and appear to differ from those observed in neonatal myocytes. It has been suggested that this discrepancy could result from differences in the animal developmental stage from which the myocytes were obtained. In the heart of adult mammals the sensitivity of α_1-agents is decreased with respect to neonatal hearts (Graham and Lannier, 1986; Schaffer and Williams, 1986) whilst cardiac responsiveness to β-adrenergic agents is increased (Longabaugh *et al.*, 1986). Alternatively it is possible that growth stimulation in adult myocytes is more dependent on the inotropic (Watanabe and Lindemann, 1984; Winegrad, 1984) and chronotropic effects (Watanabe and Lindemann, 1984) of adrenergic stimulation than in neonatal cells. Importantly, recent studies indicate that growth factor(s) released from cardiac non-myocytes that act in a paracrine fashion may be important for hypertrophy of adult hearts (Parker and Schneider, 1991; Long *et al.*, 1991) and thus future *in vitro* experiments will need to investigate the effects of adrenergic agents on adult myocytes in the presence of other myocardial cell types.

In conclusion, *in vivo* studies indicate that adrenergic agents can stimulate cardiac hypertrophy independent of hemodynamic changes and these findings are supported by experiments with isolated neonatal myocytes. However when adult myocytes are removed from their complex three dimensional surrounds and cultured *in vitro* the effect of adrenergic agents on their growth is less conclusive. It is likely that cardiac non-myocytes may play an important role in mediating the effects of NE on hypertrophic growth of adult myocytes *in situ* and that this may be mediated by the release of specific paracrine growth factor(s).

1.2.2.2 Angiotensin II

The vasoactive octapeptide hormone ANG II is the major circulatory component of the renin-angiotensin system, an important hormone system that regulates volume and fluid homeostasis in humans and other animals. In addition to these well established actions, recent studies indicate that ANG II may directly increase protein synthesis and cardiac hypertrophy by coupling to its cardiac membrane receptor. Furthermore, evidence has mounted recently suggesting that a locally active, intracardiac renin-angiotensin system may have an autocrine or paracrine influence on myocyte growth and cardiac hypertrophy during pressure overload.

i) in vivo studies

ANG II may indirectly affect cardiac growth by causing increased blood pressure and total vascular resistance due to coupling to its receptors in the vasculature system. However, direct *in vivo* evidence for a role of ANG II in modulating myocardial growth has come from studies in which animals administered subhypertensive doses (Morgan and Baker, 1991) or hypertensive doses of ANG II in which the pressor activity of the drug was inhibited, (Khairallah and Kanabus, 1983), still developed marked cardiac hypertrophy. From these studies it has been suggested that ANG II stimulates protein synthesis and cell growth in cardiac tissue by direct coupling to its cardiac reeceptors including those on the cardiomyocytes (Baker *et al.*, 1984; Baker and Khosla, 1986; Wright *et al.*, 1983). However the mechanism by which occupation of its receptor is coupled to protein synthesis is not known.

In addition to a role for circulating ANG II in cardiac growth, evidence supporting the presence of local renin-angiotensin systems in the heart (Jin *et al.*, 1988;

Dzau, 1988) has recently accrued. Studies indicate that both angiotensinogen (the substrate for angiotensin converting enzyme) and renin mRNA are found in the myocardium and are developmentally regulated in this tissue (Chernin *et al.*, 1990; Jin *et al.*, 1988; Dzau *et al.*, 1987; Kanapuli and Kumar, 1987; Ohkubu, *et al.*, 1986). For instance, both renin and angiotensinogen mRNA are present in all chambers of the neonatal heart but soon after birth, expression is localized to the left and right atria (Chernin *et al.*, 1990). Furthermore, the angiotensin converting pathway appears to be amplified during pressure-overload hypertrophy since in hypertensive rats angiotensinogen levels are several-fold higher in the left ventricle than in normotensive, non-hypertrophic rats (Li *et al.*, 1989). Similarly, aortic constriction results in up-regulation of angiotensinogen and angiotensin converting enzyme mRNA levels in the hypertrophying left ventricle (Baker *et al.*, 1990; Schunkert *et al.*, 1990). It has been suggested that this may represent a regression of the ventricular myocyte toward the neonatal cell type exemplified by cardiac specific isocontractile proteins shifts (Chernin *et al.*, 1990). This developmental regulation and re-expression during pressure overload is similar to that observed for ANP mRNA (Chernin *et al.*, 1990; Wei *et al.*, 1987).

The exact physiological roles of the local ANG II generating pathways have not been defined but within the heart it has been suggested that in addition to effects on cardiac contractility, coronary vasomotor tone and arrhythmogenesis, they may play a permissive role in modulating cardiac growth and development (Dzau, 1988; Lindpaintner *et al.*, 1988). For instance, treatment with angiotensin converting enzyme inhibitors resulted in prevention or reversal, of left ventricular hypertrophy brought about by aortic stenosis in rats (Schunkert *et al.*, 1990; Kromer and Riegger, 1988) and in response to chronic pressure overload in humans (Wakashima *et al.*, 1984; Devereaux *et al.*, 1987). Furthermore, this occurred even when the levels of angiotensin converting enzyme inhibitors did not decrease peripheral resistance (Schunkert *et al.*, 1990).

These data indicate ANG II may directly affect myocardial growth by coupling to its cardiac receptors and that this may occur independently of the well established endocrine functions such as increased peripheral vascular resistance and arterial pressure, that this hormone can produce. In addition it appears that locally

generated ANG II may have an important role in mediating cardiac cell growth and hypertrophy *in vivo* during pressure-overload hypertrophy.

ii) in vitro studies

A number of studies have demonstrated that ANG II can stimulate protein synthesis and hypertrophy in cultured vascular smooth muscle cells (Berk *et al.*, 1989; Geisterfer *et al.*, 1988). More recently a direct trophic role for angiotensin has been demonstrated in cardiac tissue. Cultured embryonic chick myocytes treated with ANG II for 3 h exhibited increased total RNA levels, total cellular protein (32%) and enhanced cellular hypertrophic growth within 5 to 6 days compared to untreated cells (Baker and Aceto, 1990). The growth-related effects of ANG II could be prevented by ANG II antagonists but not adrenergic antagonists and were accompanied by increased cytosolic Ca^{2+} influx. In addition the hypertrophic effects of ANG II were not dependent on the chronotropic state of the cells since increases in cellular protein were not inhibited by K^+ depolarization (Baker and Aceto, 1990). In contrast studies with adult myocyte cultures found that ANG II did not increase protein synthesis except under high Ca^{2+} conditions (Fuller *et al.*, 1990). It is possible that the discrepancy in results between these culture systems stems from quantitative and/or qualitative differences in the ANG II receptors. Further studies are needed to fully characterize the role of components of the renin angiotensin system in both adult and neonatal cell cultures.

In summary, both *in vivo* and *in vitro* data indicate that ANG II may modulate cardiac hypertrophy by direct activation of its cardiomyocyte receptors. In addition ANG II may indirectly affect cardiac growth during hypertension by either paracrine and/or autocrine functions related to a locally-acting intracardiac renin-angiotensin system.

1.2.2.3 Endothelin-1

Endothelin-1 (ET-1) is a 21-residue vasoconstrictive peptide derived from the endothelium that induces a potent and sustained vasoconstrictive effect on a number of blood vessel types (Yanagisawa *et al.*, 1988; Moravec *et al.*, 1989). It is considered to play an important role in the regulation of blood pressure and local blood flow (Ito *et al.*, 1991 A) and possesses powerful inotropic and chronotropic actions on isolated atria in a number of species (Moravec *et al*, 1989; Ishikawa *et al.*, 1988; Hu *et al.*, 1988).

ET-1 receptors in cardiac membrane (Gu *et al.*, 1989) and in cultured cardiomyocytes have been recently characterized and it now appears that ET-1, in addition to its vasopressor activity, may act as a myocyte growth factor in a similar fashion to α_1-adrenergic agonists.

Direct support linking ET-1 activity to cardiac growth has come from *in vitro* experiments with neonatal myocytes cultured in serum-free medium. In this system ET-1 has been shown to augment protein synthesis and hypertrophy in cardiac neonatal myocytes (Suzuki *et al.*, 1990; Ito *et al.*, 1991 A) with concomitant increases in mRNA transcripts (2-5 fold) of the muscle specific genes MLC-2, α-SkA and troponin-1 within 6 h (Ito *et al.*, 1991 A). Run-on transcription assays indicated that contractile isogene expression was regulated by increases at the level of transcription. Furthermore, similar responses were observed following treatment with TPA or ionomycin (Ito *et al.*, 1991 A) and were blocked by protein kinase C (PKC) inhibitors (Suzuki *et al.*, 1990) indicating that activation of PKC and calcium ion influx may mediate ET-1 induced myocyte hypertrophy. Recent studies indicate that the addition of ET-1 to cultured adult myocytes more than doubles the rate of protein synthesis (Neyses *et al.*, 1991).

These results indicate that ET-1 is a potential modulator of hypertrophy in neonatal myocytes and protein synthesis in adult myocytes, however its relevance to pathophysiological hypertrophy *in vivo* has not been determined. It is possible that ET-1 may act in a paracrine fashion to mediate pressure overload hypertrophy in certain models of hypertension as is hypothesized for the renin angiotensin system. In support of this, plasma ET-1 levels have been reported to be increased in patients with hypertension (Shichiri *et al.*, 1990) and acute myocardial infarction (Miyauchi *et al.*, 1989).

1.2.2.4 Thyroid hormones

The heart is a major target organ of thyroid hormone action and clinical symptoms associated with thyroid dysfunction indicate that this hormone exerts multiple effects on cardiac structure and function. In particular, the ability of thyroid hormone to modulate cardiac hypertrophic growth during disease states and in

experimental studies *in vivo* and *in vitro* has received considerable attention from both clinicians and researchers alike.

i) in vivo studies

It is well documented that the administration of thyroid hormones (T_4 and T_3) to rodents produces rapid and reversible cardiac hypertrophy (Bonnin *et al.*, 1983; Carter *et al.*, 1982; Crie *et al.*, 1983; Sandford *et al.*, 1978; Siehl *et al.*, 1985; Zahringer *et al.*, 1984; Yazaki and Raben, 1975). For instance, repeated injections or chronic infusion of T_3 results in a significant increase in RNA concentrations (approximately 20% within 1 day) (Morgan *et al.*, 1978; Kao *et al.*, 1977), protein synthesis (46% within 3 days) and myocardial mass (45% within 7 days) (Clarke and Ward, 1983). This has been shown to be largely due to faster rates of protein synthesis rather than decreased rates of protein degradation (Sandford *et al.*, 1978; Cohen *et al.*, 1966; Wildenthal *et al.*, 1978).

In addition to non-specific changes in protein synthesis, increased thyroid hormone levels are associated with specific alterations in expression of genes coding for isocontractile proteins. Hypothyroid states in the rat results in a complete switch in the normal α/β-MHC distribution (Hoh *et al.*, 1978; Lompre *et al.*, 1984) by inducing the progressive disappearance of α-MHC mRNA and the appearance of β-MHC mRNA (Talafih *et al.*, 1984). Conversely, thyroxine replacement has the opposite effect, suggesting that the α- and β-MHC genes are regulated by thyroid hormone in an antithetic fashion (Mahdavi *et al.*, 1984) and directly opposed to the isoform shifts observed during pressure-mediated cardiac hypertrophy. As with pressure-overload hypertrophy, it is thought that alterations in myosin composition during hypo- and hyper-thyroidism are physiologically significant, since the relative proportions of the isoforms seem to be directly related to the intrinsic speed of contraction (Barany, 1967; Schwartz *et al.*, 1981).

The mode by which thyroid hormone affects cardiac metabolism is a contentious issue. Convincing evidence exists that a significant proportion of the physiological action of thyroid hormone depends on its interaction with nuclear receptors with which it has high affinity (Oppenheimer *et al.*, 1972; Lazar and Chin, 1990). Following occupation of its receptor, it is internalized where the complex binds

to sites on sensitive DNA known as thyroid-response elements, and mediates or modulates both positively and negatively transcriptional processes. Thus thyroid hormone responsiveness of cardiac α-MHC has been implicated by the identification of a thyroid response elements within the 5' flanking region of this gene (for reviews see Mahdavi *et al.,* 1989; Nadal-Ginard and Mahdavi, 1989). By analogy it is possible that other changes in gene expression characteristic of thyroid hormone-induced cardiac hypertrophy may mediated by direct binding of the thyroid receptor to the appropriate sites on DNA.

Alternatively, the trophic actions of thyroid hormones may be the result of secondary alterations in hemodynamic and metabolic parameters since increased cardiac rate, left ventricular systemic pressure, CO_2 output and decreased peripheral resistance are all associated with hyperthyroidism (Buccino *et al.*, 1967; Morkin *et al.*, 1983). The observation that many of these changes are mimicked by the stimulated sympathetic nervous system has led to the hypothesis that alterations in adrenergic neurotransmitter sensitivity may have an importance in thyroid hormone-induced hypertrophy. In support of this, changes in cardiac NE levels (Levin *et al.*, 1982) and in NE turnover (Coulombe *et al.*, 1977) have been found in hypo- and hyperthyroidism. In addition, direct receptor binding studies have demonstrated that thyroid hormone administration leads to up-regulation of β-receptors (Williams *et al.*, 1977) by directly controlling the rate of transcription of the β_1-adrenergic receptor gene (Bahouth, 1991). Conversely α-receptor numbers are down regulated in cardiac tissue under these conditions (Ishac *et al.*, 1983; Williams and Lefkowitz, 1979). Clinically, β-adrenoceptor blockade has been used in combination with anti thyroid drugs for the treatment of thyrotoxicosis, and β-adrenoceptor antagonists have been shown to be partially effective in reducing T_4-mediated hypertrophy in animal experiments (Eliades and Harvey, 1989). However not all studies support this mode of therapy. For instance, reserpine treatment does not reduce cardiac hypertrophy during thyrotoxicosis (Malcolm, 1972). Furthermore, co-infusion of either α- and/or β-adrenergic blockers during thyroid hormone administration was found not to reduce cardiac hypertrophy even when left ventricular mechanical parameters were reduced (Zeihut and Zimmer, 1989). These studies

indicate that thyroid hormones can modulate cardiac hypertrophy independently of α- and β-adrenergic stimuli or changes in mechanical parameters of the heart.

In contrast, *in vivo* studies with heterotopically transplanted hearts (Korecky *et al.*, 1987; Klein *et al.*, 1990) or papillary muscle (Cooper and Tomanek, 1982; Cooper *et al.*, 1985) indicate that changes in myocardial mechanics are the more likely mediators of thyroid hormone-induced cardiac hypertrophy rather than direct metabolic effects (for a more detailed discussion see section 1.2.1.3)

ii) in vitro studies

Studies with *in vitro* preparations are generally supportive of a direct effect of thyroid hormones on protein synthesis. In hearts from T_4 injected rats that were perfused as working preparations with blood simulants, an increase in the number of ribosomes was associated with faster rates of protein synthesis (Siehl *et al.*, 1985). Similar results were obtained from investigations using fetal mouse hearts in organ culture and in addition, increased protein synthesis was demonstrated in absence of the usual systemic metabolic and hemodynamic effects associated with thyroid hormone administration *in vivo* (Crie *et al.*, 1983). In chicken cardiomyocyte cultures, both augmented protein synthesis and myocyte growth were observed following T_3 treatment (Carter *et al.*, 1985). Similarly, the addition of T_3 to K^+-depolarized noncontracting myocytes increased rates of protein synthesis by approximately 35% (unpublished from Morgan and Baker, 1991). In feline and rodent fetal cardiomyocyte cultures, thyroid hormone treatment increased α-MHC gene expression and down regulated β-MHC expression (Gustafson *et al.*, 1987; Nag and Cheng, 1984). Thus *in vitro* studies support a direct action of thyroid hormones on cardiac growth and isocontractile gene expression which does not seem to be dependent on secondary changes in contractile properties of these cells.

In summary it seems likely that both peripheral and direct effects of thyroid hormones contribute to the cardiac hypertrophy observed *in vivo*. However the relative contribution to these two trophic components of thyroid action has proven difficult to determine and further investigation is required.

1.2.2.5 Other myocardial hypertrophic factors

In addition to the afore mentioned trophic factors, other circulatory substances such as adrenocorticoids, insulin and growth hormone have been implicated to some degree in the initiation or maintenance of cardiac hypertrophy. It is likely however that in the majority of cases these substances play a permissive role as opposed to an initiatory role in this trophic process (Morgan and Baker, 1991). Even so, evidence is accumulating that cardiac myocytes may be the targets for the action of a number of peptide growth factors which may act in an autocrine or paracrine manner to mediate hypertrophic growth during cardiac overload. For instance when pressure load was applied to the right ventricle of an *in vitro* perfused rabbit heart, increased incorporation of [^{3}H]-lysine into cardiac contractile protein was observed not only in the right but also the left ventricle where pressure load was not applied (Kira *et al.*, 1982). Furthermore, perfusate washout from a heart with a pressure-loaded right ventricle stimulated increased [^{3}H]-lysine incorporation into both left and right ventricular chambers of a co-perfused unloaded heart (Kira *et al.*, 1982). More recently it was demonstrated that extracts from hypertrophied left ventricle of dogs with experimentally induced renal hypertension increased [^{3}H]-uridine uptake in cultured rat cardiac myocytes. These experiments suggest that growth factor(s) are released from pressure loaded ventricle which can initiate hypertrophy in unloaded ventricles and isolated myocytes (Honda *et al.*, 1988). Such trophic factor(s) may not be species-specific since water soluble extracts from pressure-loaded hypertrophying dog hearts can cause increased mRNA synthesis and initiate hypertrophy in unloaded rat hearts (Hammond *et al.*, 1982). The nature of these putative growth factor(s) is unknown but recently a number of known growth factors have been localized to cardiac tissue including acidic fibroblast growth factor (aFGF), basic FGF (bFGF), platelet-derived growth factor (PDGF), tumor necrosis factor α (TNFα) and transforming growth factor β_1 (TGF β_1) (for a review see Parker and Schneider, 1991). Furthermore, the administration of these growth factors to cardiac myocyte cultures stimulated reexpression of neonatal specific contractile genes similar to those observed in pressure overloaded hearts *in vivo* (see Table 1.2). In addition to these well characterized growth factors a novel growth factor has been isolated from cardiac non-myocyte cells which stimulates hypertrophy but not hyperplasia of neonatal cardiac myocytes maintained in serum free medium (Long *et al.*, 1991).

Thus considerable evidence is accumulating to suggest that cardiac non-myocytes may modulate myocyte growth during normal development and in response to hypertrophic stimuli by the production of an array of growth factors which act in a paracrine fashion. Such findings further emphasize the complexity of the hypertrophic growth response *in vivo* and underline the need to consider all cell types of the heart when investigating this growth phenomena.

Table 1.2 Changes in the expression of cardiomyocyte genes in response to peptide growth factors.

Gene/isoform	**Growth Factor**
MHC	
α	aFGF, bFGF, TGFβ$_1$
β	bFGF, TGFβ$_1$
α-actin	
Cardiac	bFGF, TGFβ$_1$
Skeletal	bFGF, TGFβ$_1$
Smooth	aFGF, bFGF, TGFβ$_1$
ANF	aFGF, bFGF, TGFβ$_1$
Slow/cardiac Ca^{2+} ATPase	aFGF, bFGF, TGFβ$_1$

aFGF: acidic fibroblastic growth factor; bFGF: basic fibroblastic growth factor; TGFb$_1$:transforming growth factor β$_1$. Adapted from Parker and Schneider (1991).

1.3 HYPERTROPHIC SIGNAL TRANSDUCTION PATHWAYS AND GENE TRANSCRIPTION

1.3.1 Gene Transcription

The data presented clearly illustrates that cardiac hypertrophy is a heterogeneous process involving both quantitative changes in general protein synthesis and also qualitative alterations in the levels of specific isocontractile and metabolic proteins. In the majority of studies the observed changes in myocyte size and phenotype were attributed to increased protein synthesis rather than decreased protein degradation (Kira *et al.*, 1984; Xenophontos *et al.*, 1989; Fuller *et al.*, 1990; Simpson, 1983). Theoretically a number of potential sites for the regulation of protein synthesis exist

including transcription and translation. However, during cardiac hypertrophy, changes in myocyte levels of contractile and non-contractile proteins were generally preceded by corresponding alterations in the level of their appropriate mRNA (Waspe *et al.*, 1990; Bishopric *et al.*, 1987; Lee *et al.*, 1988). Thus gene plasticity during cardiac hypertrophy appears to be the result of changes above the level of translation. Furthermore, using nuclear run-on assays to quantify the initiation of transcription it was shown that an increased rate of DNA transcription rather than increased RNA stability was responsible for selective increases in isocontractile mRNA (Lee *et al.*, 1988; Ito *et al.*, 1991 B; Simpson, 1990).

Considered together these studies demonstrate that extracellular hypertrophic stimuli are able to effect changes in gene expression within the nuclei of cardiac myocytes and establish that gene transcription is a critical regulatory event during cardiac hypertrophy.

1.3.2 Signal Transduction Pathways

The intracellular transducing mechanisms which couple hypertrophic stimuli to long term changes in cardiac gene transcription have yet to be clearly elucidated. In other systems it has been shown that growth promoting stimuli regulate gene expression via intracellular pathways (second messenger systems) which culminate in the increased activity of a number of protein transcription factors (third messenger systems) which subsequently interact with the promoter elements of target genes (for review see Lenardo and Baltimore, 1989; Mitchell and Tjian, 1989; Yamamoto, 1985). It is likely then that a similar regulatory strategy occurs during hypertrophic growth following mechanical load or exposure to hormones such as NE. In accordance with this hypothesis there are a number of general but distinct second messenger systems which have been tentatively implicated in the transduction of these hypertrophic stimuli from the myocyte cell membrane to the nucleus including (1) cAMP formation, (2) PKC activation and (3) increases in ion fluxes. Thyroid hormone is able to affect gene transcription directly by binding to thyroid response element target sites in DNA and this has been discussed previously, however evidence also exists that this hormone may mediate hypertrophy indirectly through some of the above pathways.

1.3.2.1 cAMP

Intracellular content of cAMP may play a significant role in mediating cardiac hypertrophy in response to a variety of trophic stimuli. A number of studies indicate that cellular stretch or deformation of cardiac muscle increases cAMP as well as protein synthesis and ribosome formation (Morgan *et al.*, 1989; Watson, 1989). Elevated levels of adenyl cyclase have been reported in guinea pig hearts 10 min following hemodynamic overload (Schreiber *et al.*, 1971). Furthermore, elevated aortic pressure increased protein synthesis, cAMP concentration and cAMP dependent protein kinase activity in isolated perfused rat hearts (Xenophontos *et al.*, 1989, Haneda *et al.*, 1990; Watson *et al.*, 1989). Occupation of β-adrenergic receptors by catecholamines has been reported to increase adenyl cyclase activity, lead to cardiac hypertrophy *in vivo,* and increased protein synthesis and cell growth in adult myocyte cell cultures. The thyroid hormone T_3 has been shown to increase cAMP levels in the heart within 12 h of administration (Zimmer and Peffer, 1986) and accordingly it has been suggested that this may be an intracellular transducing signal for cardiac hypertrophy induced in response to this hormone (Zimmer and Peffer, 1986).

1.3.2.2 PKC

PKC activity has been strongly implicated in myocyte hypertrophy in response to catecholamine, ANG II or ET-1 administration or following stretch. For instance, PKC activity has been associated with increased general protein synthesis, isocontractile gene transcription and myocyte hypertrophy in isolated neonatal myocyte cultures following α_1-adrenergic or ET-1 stimulation (Henrich and Simpson, 1988; Suzuki *et al.*, 1990). Secondly, phorbol myristate (PMA) activates PKC and also gives rise to trophic responses similar to those seen in response to α_1-adrenergic and ET-1 administration (Henrich and Simpson, 1988; Suzuki *et al.*, 1990). Whether PKC activity is coupled to cardiac hypertrophy mediated by these hormones, in adult myocytes, is still uncertain since it has proven difficult to establish whether growth in isolated adult cardiomyocytes responds to α_1-adrenergic stimulation (Rupp *et al.*, 1991; Dubus *et al.*, 1990).

Although not formally demonstrated in cardiac myocyte cultures, ANG II has been shown to elevate PKC translocation and induce phosphorylation of nuclear laminae in cultured vascular smooth muscle (Tsuda and Alexander, 1990). It is possible therefore that increased translocation of PKC may be a means by which increased intracardiac renin-angiotensin system activity may directly modulate cardiac growth independent of hemodynamic changes. PKC has also been implicated in stretch-induced hypertrophy since atrial distention causes increased phosphatidylinositol (PI) turn-over and this has been shown not to be the result of NE or acetylcholine release (Von Harsdorf *et al.*, 1989).

1.3.2.3 Ion fluxes

Changes in ion fluxes, particularly altered calcium ion current (Keung, 1989; Kleiman and Houser, 1988), Na^+-Ca^{2+} exchange and calcium release from SR (Lecarpentier *et al.*, 1987; Lorell *et al.*, 1986) have been reported in hypertrophic myocardium. Increased aortic pressure has been shown to significantly increase $[Ca^{2+}]i$ in isolated perfused hearts (Schreiber *et al.*, 1977) although elevation of Ca^{2+} in the perfusate did not alter rates of protein synthesis (Haneda *et al.*, 1989). The mechanism by which $[Ca^{2+}]i$ increases in response to perfusion pressure is not known but it is likely to be modulated through stretch-activated ion channels which have been identified in the lumen of adult and neonatal cardiac myocytes (Bustamante *et al.*, 1991). Furthermore, stretch of myocytes in culture increases protein synthesis, alters isocontractile protein content and elevates Ca^{2+} influx (Komuro *et al.*, 1989 A). It has also been demonstrated that activated β-adrenergic receptors can increase Ca^{2+} flux through cardiac calcium channels via direct G-protein interaction or by phosphorylation of cAMP-dependent protein kinases (Haung *et al.*, 1990; Yatawi and Brown, 1989). Finally, α_1-adrenergic agonists, ANG II, ET-1 and stretch stimulate calcium ion release from the endoplasmic reticulum via increased phosphoinositide-phospholipase C (PI PLC) turnover and inositol phosphate (IP_3) formation (Brown *et al.*, 1985; Suzuki *et al.*, 1990; Baker and Aceto, 1989). Thus increased $[Ca^{2+}]i$ due to mobilization from intracellular stores in addition to influx through membrane channels, may be a means by which hypertrophic stimuli may modulate cardiac growth.

Sodium ion flux has been associated with growth processes in cardiac tissue. Deformation of cardiocytes increases Na^+ flux through a stretch-activated ion channel in the SR (Bustamante *et al.*, 1991). The normal influx of Na^+ induced by spontaneous contraction in neonatal rat myocytes is associated with increased general protein and isocontractile protein synthesis, compared to non-contracting cells (McDermott and Morgan, 1989). Partial block of this mechanotransducer by the polycationic antibiotic streptomycin (Ohmori, 1985) reduces both Na^+ uptake in stretched myocardium and isolated cardiocytes by 15% and reduces contractile protein synthesis (Kent *et al.*, 1991). In differentiated avian skeletal myotubles both increased sodium influx and protein synthesis have been observed in response to mechanical stretch (Guharay and Sachs, 1984).

1.3.2.4 Other mechanisms of transduction

In addition to the cellular messengers described above it is possible that rapidly activated, unknown molecular signals, secondary to trophic stimuli could bind directly to DNA and regulate cellular growth and phenotype (Komuro *et al.*, 1990). It has also been postulated that stretch-dependent plasmalemma alterations could be translated directly into changes in nuclear structure via transmission of mechanical forces across cytoskeletal structures that physically link the plasma membrane to the nuclear envelope (Georgatos and Blobel, 1987). The importance of the cytoskeleton and the interaction of the cytoskeleton with intracellular matrix in regulation of cell growth has been the subject of a number of reviews (Lazarides, 1980; Lazarides, 1985 A; Craig, 1985; Lazarides, 1985 B; Bissel *et al.*, 1982). Furthermore, it was recently demonstrated that during cardiac hypertrophy a number of cytoskeletal networks are modified (Rappaport and Samuel, 1988; Samuel *et al.*, 1990).

1.3.2.5 Molecular mechanisms by which PKC, cAMP and ion flux may modulate gene transcription.

Mechanisms by which increased PKC activity, cAMP levels, ion flux or mechanical transducers may regulate hypertrophic growth processes are not known but it is well established that protein phosphorylation by kinases is one key regulatory mechanism for signal transduction pathways that link cell surface events to alterations in

gene expression (see Schonthal *et al.*, 1991). For instance increased cardiac cAMP levels or increased membrane phospholipid turnover following hypertrophic stimuli can activate cAMP dependent protein kinase A (PKA) or PKC respectively. In turn, activation of these kinases may result in the modulation of nuclear transcription factors which could then bind to regulatory regions of the target genes (e.g. an isocontractile gene) or to another protein bound to a regulatory region, and thereby activate RNA polymerase II (Simpson *et al.*, 1989). Activation of transcription factors by PKC in other systems has been previously demonstrated (Elsholtz *et al.*, 1986; Sen and Baltimore, 1986; Angel *et al.*, 1987; Bohmann *et al.*, 1987) and often involves 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TREs) (Angel *et al.*, 1987). Genes that are regulated by cAMP have also been identified (Roesler *et al.*, 1988) and usually contain a cAMP response element (CRE) in their 5' control regions (Franza *et al.*, 1987). For a more detailed discussion of the above see section 13.3. Increased [Ca^{2+}] may also regulate transcription during hypertrophy via kinase activity since increased ion flux or release from intracellular stores following trophic stimuli, may cause phosphorylation of a variety of cellular proteins including transcription factors (Morgan and Curran, 1986), by Ca^{2+} activated, calmodulin-independent PKC (Colbran *et al.*, 1989) or by PKC (Blinks *et al.*, 1982; Rasmussen and Barrett, 1984). Alternatively Ca^{2+} may bind and activate a transcription factor directly in a similar manner to which it activates calmodulin. At present no second messenger system is known to be dependent on Na^+ uptake but it is possible that increased [Na^+]i may be acting by secondary alterations in [Ca^{2+}]i via Na^+-Ca^{2+} exchange (Reeves, 1985). Mechanisms by which mechanotransducers may activate transcription in nucleus are not yet known although protein dephosphorylation may be important in these instances.

1.3.3 Cardiac Gene Transcription Factors: A Possible Role for the Nuclear-Acting Early-Response Genes

Transcriptional regulation of genes mediated through second messenger systems appears to be a pivotal control point for the development and regression of many forms of cardiac hypertrophy. Accordingly, significant effort has been channeled into elucidating the nature of the implied transcription factors and the possible mechanisms by which they interact with trophic signals to regulate cardiac gene

plasticity. It has been suggested that hypertrophic-specific changes in gene expression are exerted by the products of a limited number of critical regulatory genes (Simpson, 1988 A). If this is true, then the products of such genes would likely to be located within the nucleus where they could bind to specific DNA sequence elements embedded in promoters, enhancers and silencers and where they could interact with other proteins to initiate or block RNA transcription. Furthermore they would be likely to exist in low levels compared to other gene products, but be rapidly inducible for instance following a phosphorylation cascade by specific kinases so that they could couple short term signals elicited at the cell surface to long term alterations in cellular phenotype and function. At present the best candidates for such regulatory factors would appear to be the products of a number of nuclear acting early-response genes including c-*myc*, c-*fos* and c-*jun* (reviewed in Herich and Ponta, 1989).

c-*myc*, c-*fos* and c-*jun* belong to families of nuclear acting, DNA binding genes whose expression, whilst normally low in quiescent cells, is induced rapidly and transiently in response to a great variety of extracellular signals including mitogenic and differentiation-inducing factors and agents that cause depolarization of neuronal cells. Classically these genes are characterized by their ability to respond rapidly with increased transcription rates following stimulation with phorbol esters in a sequential manner: c-*fos* mRNA is detected within the first few minutes followed by increased c-*myc* mRNA (Greenberg and Ziff 1984; Muller 1984). Although originally termed proto-oncogenes due to their involvement in neoplasia, many of these genes also appear to play vital roles during normal mitosis and cell growth and perhaps memory formation and the term immediate-early or nuclear-acting early-response genes has become more appropriate.

1.3.3.1 c-*fos* and c-*jun* families

The c-*fos* gene was originally found as the cellular homologue of oncogenes carried by two murine retroviruses, FBR and FBJ that were involved in the generation of radiation-induced osteosarcoma (for review see Curran, 1988). The c-*jun* gene was discovered independently from c-*fos* as the cellular homologue of v-*jun*, the transforming gene of avian sarcoma virus 17 (Maki *et al.*, 1987). The protein products of c-*fos* (Fos) and c-*jun* (Jun) interact together via a region of periodically repeated

leucine zippers to form a heterodimeric complex named activator protein 1 (AP-1) (for review see Curran and Franza, 1988). Jun, unlike Fos, also forms homodimers which appear to be less stable than Fos/Jun heterodimers (Nakabeppu and Nathans, 1991; Rauscher *et al.*, 1988). In response to a variety of extracellular signals AP-1 binds to, and activates transcription from nanonucleotide sequences within promoter regions of target genes. These sequences are often components of complex regulatory elements containing binding sites for multiple transcription factors that are responsive to extracellular stimuli (Lee *et al.*, 1987 B; Distel *et al.*, 1988; Sonnenberg *et al.*, 1989). These include the TPA response element located in the promoter of a number of genes responsive to elevated levels of PKC such as collagenase and also both c-*fos* and c-*jun* themselves (Angel *et al.*, 1987; Lee *et al.*, 1987 A; Lee *et al.*, 1987 B). Another such site is the cAMP response element (CRE) that binds transcription factor CRE binding protein (CREB) and confers the response to adenyl cyclase and PKA pathways (Franza *et al.*, 1987; Sassone-Corsi *et al.*, 1990). Furthermore, c-*fos* and c-*jun* products form heterodimeric complexes with certain members of the CRE/Activating factor 1 (ATF) families that interact preferentially with CRE sites (Benbrook and Jones, 1990). The CRE site is also a target for transcription factors activated by second messengers other than cAMP, for example calcium (Sheng *et al.*, 1990). AP-1 proteins have also been shown to be involved in the regulation of the cis-acting motif, serum response element (SRE) of c-*fos* and other serum growth factor-inducible genes that binds a different class of transcription factor, the serum response factors (SRF) (for reviews see Treisman, 1990; Rivera and Greenberg, 1990).

Several genes related to c-*fos* and c-*jun* have also been isolated including *fra*-1, *fra*-2 and *fos*-b (Cohen and Curran, 1988; Nishina *et al.*, 1990; Zerial *et al.*, 1989) (related to c-*fos*), and *jun*B and *jun*D (Ryder *et al.*, 1988; Ryder *et al.*, 1989) (related to c-*jun*). The protein products in these families contribute to an array of mono- and heterodimeric complexes that bind AP-1 and CRE sites (Cohen *et al.*, 1989; Nakabeppu *et al.*, 1988; Hai and Curran, 1991; Curran, 1991) but have different binding affinities and trans-activation properties (Chiu, 1989; Hirai *et al.*, 1989; Schutte *et al.*, 1989; Lucibello *et al.*, 1990; Ryseck and Bravo, 1991). In addition *jun*B is involved in the negative regulation of c-*jun* expression (Chiu *et al.*, 1989). It is possible then that

differential expression of these proteins may be one mechanism to ensure diversity and specificity of cellular responses to extracellular stimuli and allow for target gene selectivity (Angel and Karin, 1991). Recently, interactions of Fos and Jun with several members of the steroid receptor family have been described and these observations and those described above indicate that Fos and Jun can regulate transcription cooperatively with other transcription factor families (Schule *et al.*, 1990; Yang-Yen *et al.*, 1990). The products of these genes have been implicated as nuclear "third messenger" molecules in signal transduction processes and thus may contribute to the coupling of short term signals elicited by cell-surface stimulation to alterations in cellular phenotype by regulating expression of target genes (Curran, 1991).

1.3.3.2 c-*myc*

c-*myc* was first identified as the mammalian homologue of the transforming gene of an avian retrovirus, myelocytomatosis (Roussel *et al.*, 1979) and its expression appears to be critical for determining the proliferative, differentiative and oncogenic potential of a wide variety of cell types (Reviewed by Cole, 1986; Luscher and Eisenman, 1990). Regulation of the c-*myc* gene is complex and occurs at the transcriptional and post-transcriptional level and appears to be tissue and stimulus specific (Dean 1986; Greenburg and Ziff, 1984; Kelly and Siebenlist, 1988; Siebenlist, 1988). Like c-*fos* and c-*jun*, c-*myc* is rapidly induced in response to growth factors and it is possible that they may share common regulatory elements since all are induced in the absence of protein synthesis (Kelly *et al.*, 1983; Lau and Nathans 1987; Muller, 1984).

c-*myc* is one of a family of nuclear phosphoproteins including N-*myc* (Kohl *et al.*, 1986; De Pinto *et al.*, 1986), L-*myc* (Nau *et al.*, 1985), R-*myc*, P-*myc* and B-*myc* (Ingvarsson *et al.*, 1988) which have similar exon structures and appear to be differentially regulated during development (for a review see Marcu *et al.*, 1992). The protein products of the c-*myc* family have long been thought to be involved in the regulation of gene expression because of their nuclear localization and ability to bind DNA via either basic/helix-loop-helix (HLH) (Mure *et al.*, 1989) or basic/leucine repeat (LR) structures common to several binding proteins (Landschulz *et al.*, 1988). However

a convincing demonstration of this has remained elusive until the recent cloning of a heterodimeric partner of Myc, called Max, that facilitates sequence-specific DNA-binding activity (Blackwood and Eisenman, 1991). Max like Myc contains adjacent HLH and LR domains and forms heterodimers with c-, N- and L-*myc* but not with other HLH proteins (Beckmann *et al.*, 1990; Gregor *et al.*, 1990; Hu *et al.*, 1990).

c-*myc* expression is generally higher in proliferating cells than quiescent cells and is activated in quiescent cells by mitogenic stimuli (Dean *et al.*, 1986; Campisi *et al.*, 1984; Kelly *et al.*, 1983) including PDGF in Balb/c-3T3 fibroblasts and this increased expression is associated with entry into, and progression through the cell cycle (Armelin *et al.*, 1984, Kaczmareck *et al.*, 1985, Kelly *et al.*, 1983). During terminal differentiation of cells, when proliferation ceases, there is concurrent reduction in expression of the c-*myc* gene (Endo and Nadal-Ginard, 1986; Schneider *et al.*, 1987). Furthermore, constitutive c-*myc* expression has been shown to block differentiation of mouse cell lines (Coppola and Cole 1986). Current opinion is that the differential or combinatorial expression of the *myc* gene family has a role in regulating multiple differentiation pathways (Luscher and Eisenman, 1990).

1.4 NUCLEAR-ACTING EARLY-RESPONSE GENE EXPRESSION IN HEART AND CARDIAC MYOCYTES

Although the expression of nuclear-acting early-response genes has been traditionally linked with hyperplasia in a number of settings, they have more recently become the focus of considerable attention in the context of cardiac growth and hypertrophy. For instance, the loss of proliferative capacity and altered isogene expression of myocytes during myocardial development is paralleled by decreased expression in the cardiac levels of c-*myc* (Schneider *et al.*, 1986). Similarly, other members of the myc family including N-*myc* and B-*myc* are also down regulated in the maturing heart (Schneider and Parker, 1990; Parker and Schneider, 1991; Claycomb and Lanson, 1987) and this is perhaps not surprising given the role these genes are hypothesized to play in cellular differentiation processes. Down regulation of c-*myc* is an early event during skeletal muscle differentiation (Endo and Nadal-Ginard, 1986;

Schneider *et al.*, 1987) which fails to occur in muscle cell lines that cannot differentiate (Payne *et al.*, 1987; Sejersen *et al.*, 1985). Whilst decreased expression of c-*myc* is not obligatory for muscle development, autonomous expression of c-*myc* can delay or partially inhibit the myogenic phenotype (Caffrey *et al.*, 1987; Schneider *et al.*, 1987). Furthermore, v-*myc* can inhibit differentiation in skeletal muscle cells (Falcone *et al.*, 1985), impair cardiac differentiation and produce cardiac rhabdosarcomas in avian embryos (Saule *et al.*, 1987).

In contrast to c-*myc*, constitutive expression of c-*fos* appears not to be detectable (Barka *et al.*, 1987) or is extremely low in neonatal or adult heart tissue *in vivo* (Claycomb and Lanson, 1987; Izumo *et al.*, 1988; Schneider *et al.*, 1986) although it has been localized to specific regions of the myocardium during embryogenesis (T. Curran, personal communication) and thus may play a role in the early development of the heart. However, c-*fos* expression seems to be sensitive to cell isolation procedures since it is abundantly expressed in freshly isolated neonatal and adult cardiocytes (Claycomb and Lanson, 1987). The potential for c-*fos* to play a role in cardiomyocyte differentiation is highlighted by the increased expression of this gene associated with the decreased expression of several muscle specific genes and the subsequent loss of several differentiated traits in skeletal muscle (Lassar *et al.*, 1989 B).

However, c-*myc* and c-*fos* expression, like the expression of neonatal-specific isocontractile genes, do not appear to be irreversibly repressed in terminally differentiated cardiac myocytes since their expression can be elicited by a number of interventions including some that provoke hypertrophic growth both *in vivo* and *in vitro*. For instance, in 28 day old rats, constriction of the abdominal aorta results in approximately 10% increase in cardiac mass and increased c-*myc* expression in both atria and the left ventricle 48 h following imposition of the overload (Mulvagh *et al.*, 1987). However aortic constriction in 80 day old rats results in increased c-*myc* mRNA in the atria alone indicating that up regulation of c-*myc* during pressure overload is both age- and tissue-dependent (Mulvagh *et al.*, 1987). In another study acute aortic constriction resulted in increased cardiac c-*fos* and c-*myc* mRNA levels within 30 min and 2 h respectively (Komuro *et al.*, 1988). Maximal levels were observed after 8 h and had returned to baseline by 48 h following surgery. Direct mechanical load also appears

to modulate early response gene expression in neonatal cardiocytes since stretching of these cells *in vitro* increases total cell RNA content, protein content and mRNA levels of c-*fos* (15 fold after 30 min) and the fetal specific isocontractile gene α-SkA (Komuro *et al.*, 1991). Thus cardiocyte stretching *in vitro* may mimic hemodynamic load *in vivo* in terms of hypertrophy and gene expression.

Similarly adrenergic treatment has been shown to modulate early-response gene expression in both the adult heart and isolated myocyte cultures. Thus administration of β-adrenergic agonists *in vivo* led to a rapid and transient increase in the levels of c-*fos* in the hearts of mice, rats and hamsters (Barka *et al.*, 1987). Increased c-*fos* levels were first observable within 30 min of administration of the drug and peaked after 60 min but had returned to near basal levels by 2 h. An increase in calcium through voltage-dependent channels appeared not to be required for this activation since calcium channel blockers, verapamil, nifedipine and diltiazem had no effect. A similar increase in c-*fos* expression was also obtained following administration an α-adrenergic agonist, histamine and prostaglandin (Barka *et al.*, 1987). In quiescent non-dividing neonatal cardiac myocytes cultures both increased cell size and selective up-regulation of several neonatal specific isocontractile genes was preceded by a transient increase in c-*myc* mRNA levels following treatment with α_1-adrenergic agents (Starksen *et al.*, 1986). Increased c-*myc* mRNA levels were detectable at 30 min, peaked at 2 h (10 fold above control), and returned to basal by 6 h after stimulation. Serum and the phorbol ester promoter PMA (phorbol 12 myristate 15 acetate) which activates PKC, also enhanced c-*myc* expression and hypertrophy in these cell cultures (Starksen *et al.*, 1986).

Taken together these studies provide indirect evidence that mechanical load and adrenergic administration may be linked to modulation of cardiac mass and myocyte size via transduction pathways which act through transcription factors such as c-*myc* and c-*fos*. In addition, up-regulation of c-*myc* and c-*fos* in response to hypertrophic stimuli appears to precede a general regression of the heart to the developmental phenotype as indicated by the subsequent re-expression of neonatal-specific isocontractile genes. Thus the response of adult cardiomyocytes to hypertrophic stimulus has similarities to the mitogenic response of other differentiated

cell types, which often involves the suppression of the adult phenotype and re-expression of the fetal program (Ruoslahi *et al.*, 1984).

1.5 THESIS AIM

The central aim of this thesis is to establish whether the expression profile of nuclear-acting early-response genes in cardiomyocytes is consistent with their mediating aspects of adrenergic initiated cardiac hypertrophy. More specifically, the work in this thesis will seek to;

i) examine in detail the temporal changes in expression of c-*myc* and c-*fos* and related genes in the heart following both acute and chronic administration of catecholamines *in vivo* and to determine the relative contribution of each type of adrenergic receptor to these responses.

ii) localize the expression of c-*myc* and c-*fos* mRNA and protein following adrenergic administration, to particular regions and cell types of the heart by gross dissection techniques and by *in situ* hybridization histochemistry and immunocytochemistry techniques.

iii) establish whether the isolated perfused heart system is a suitable model in which to study both the early and terminal of stages of gene expression associated with cardiac hypertrophy *in vivo.* and if so to differentiate the possible direct and indirect affects of adrenergic hormones on c-*myc* and c-*fos* expression using this system.

CHAPTER 2

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMAL TECHNIQUES

2.1.1 Drugs and Animals

2.1.1.1 Drugs

DL-Propranolol.HCl	Sigma
Heparin	CSL
Methoxamine.HCl	Sigma
Nembutal	Bumac Labs.
Nifedipine	Sigma
(-)Norepinephrine.HCl	Sigma
4a-Phorbol 12b 13a-didecanoate (PDC)	Sigma
Phenoxybenzamine.HCl	Sigma
Phentolamine.HCl	Ciba-Geigy
Phorbol 12-myristate 13-acetate (PMA)	Sigma
Pyruvate	Boehringer
Forskolin	Sigma

All other drugs and chemicals were of analytical grade.

2.1.1.2 Preparation of drugs

For experiments *in vivo*, adrenergic agents were prepared freshly each day in 0.9% saline and 0.1% ascorbic acid. For *in vitro* perfusion experiments adrenergic agents were prepared in perfusion buffer (see section 5.2.1) containing 0.1% ascorbic acid. Triiodo-L-thyroxine (T_3) was prepared daily in phosphate buffered saline (pH 11.0). Phorbol esters were prepared as stock 10 mg/ml solution in dimethyl sulfoxide (DMSO) and kept at -20°C when not in use.

2.1.1.3 Animals

Male hooded Wistar rats (180-200 g) reared in the animal house, University of Tasmania were used for all experiments unless otherwise stated. Rats were maintained *ad libitum* on a standard laboratory chow diet.

2.2 NORTHERN ANALYSIS

Specific mRNA transcripts were detected amongst the total RNA extracted from the heart by standard northern blotting techniques (Maniatis *et al.*, 1989). Briefly, purified RNA extracts were separated according to size by electrophoresis through denaturing agarose gels and then transferred by capillary action to solid support nylon membranes. RNA attached to the membrane (which had retained its relative position during transfer) was then hybridized to radiolabeled cDNA or oligonucleotide probes and the position of any complimentary bands identified by autoradiography. For slot blotting, RNA samples were applied directly to the membrane support by suction through holding wells in a specially designed apparatus. Membranes were then hybridized directly to radiolabeled probes to determine the relative levels of target RNA transcripts (Maniatis *et al.*, 1989).

2.2.1 Materials

2.2.1.1 Reagents

[α-^{32}P]dCTP	Bresatec
[γ-^{32}P]dATP	Bresatec
8-hydroxyquinoline	BDH
Agarose gel (HGT, LGT)	FMC Bioproducts
Dextran Sulphate	Pharmacia
DNA (type III from Salmon testis)	Sigma
Ethidium bromide	BDH
Guanidinium thiocyanate	Serva or Fluka
Morpholinopropane sulfonic acid	Sigma
Oligo(dt)-cellulose	P-L Biochemicals

Phenol	BDH
Proteinase K	Boehringer-Mannheim
Sodium dodecyl sulphate (SDS)	Serva
Zeta Probe transfer membrane	BioRad

Reagents for bacterial propagation were obtained from Difco, restriction enzymes were sourced from Pharmacia, all other reagents were of molecular biology grade.

2.2.1.2 Solutions

Normal procedures were taken to ensure that solutions were free of ribonuclease contamination. All glassware was heated at 180°C overnight whilst heat sensitive equipment was soaked in 0.5 M NaOH and then rinsed in Milli Q water. All solutions unless otherwise stated were made up in distilled/deionized (Milli Q) water and autoclaved. The ribonuclease inhibitor diethyl pyrocarbonate was used to treat water in certain instances.

2.2.2 Extraction of Total RNA and Poly(A)$^+$ RNA Selection

2.2.2.1 Total RNA extraction

Total RNA was extracted according to the method of Chomcyznski and Sacchi (1987) with slight modification. 1 g of frozen tissue was finely ground under liquid nitrogen and transferred to a sterile plastic tube containing 10 ml of homogenizing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and given two bursts at high speed with an ultra-Turrax homogenizer. To the homogenate was added sequentially 1 ml of 2 M sodium acetate pH 4.0, 10 ml phenol (saturated with 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.1% 8-hydroxyquinoline) and 2 ml of chloroform: isoamyl alcohol mixture (49:1), with thorough mixing after each addition. The final mixture was shaken vigorously for 4 min then centrifuged in glass tubes at 10 000 g for 20 min at 4°C. The upper aqueous phase was mixed with 10 ml of isopropyl alcohol in a fresh tube and cooled at -20°C for 1 h. Total RNA was pelleted by centrifugation at 15 000 g for 10 min at 4°C, drained and redissolved in 1.5 ml 1 mM EDTA pH 7.4. The RNA was

reprecipitated by addition of 2 ml 4.5 M potassium acetate pH 6.0 and incubated at -20°C for 1 h. Following centrifugation the RNA pellet was washed consecutively in 70% and 95% ethanol, dried under vacuum for 1 h and dissolved at 65°C in 200 µl of 0.5% SDS. For smaller amounts of tissue volumes of extraction buffers were adjusted accordingly.

2.2.2.2 Selection of poly(A)$^+$

mRNA purification was facilitated by virtue of the polyadenylate tail carried at the 3' ends of most mRNA species allowing selective retention on oligo(dt) cellulose. The method described below is essentially the same as that of Edmonds *et al.*, (1971) and Aviv and Leder, (1972).

A small piece of sterile glass wool was placed in the outlet of a 1 ml syringe and 0.1 g of oligo(dt) cellulose saturated with binding buffer (0.01 M Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.5% SDS) was added. The column was packed down by gentle tapping to give a final volume of 0.2-0.3 ml. RNA (1 mg) dissolved in 500 µl of binding buffer was heated to 65°C then applied to the column followed by a further 3 ml of binding buffer and 1.5 ml of wash buffer (0.01 M Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA). Bound poly(A)$^+$ RNA was eluted with 1.5 ml of elution buffer (0.01 M Tris-HCl pH 7.5, 1 mM EDTA) and adjusted to a final concentration of 0.5 M NaCl, and the binding, washing and elution repeated as before. Poly(A)$^+$ RNA in the final eluate was recovered by precipitation with 2 volumes of 95% ethanol at -20°C overnight. After centrifugation at 15 000 g for 10 min poly(A)+ RNA pellets were drained, dried under vacuum for 1 h and dissolved at 65°C in 0.5% SDS.

2.2.2.3 Analysis of extracted RNA

Integrity of total RNA and poly(A)$^+$ RNA was determined visually by ethidium bromide fluorescence after separation on 0.75% agarose slab gels. RNA was quantified by spectrophotometric analysis at 260 nm against reagent blanks. An optical density (O.D) reading of 1 corresponds to 40 µg of RNA (Maniatis *et al.*, 1989).

2.2.3 Northern and Slot Blotting

2.2.3.1 Northern blotting

i) denaturing gels

Denaturing formaldehyde gels (1.2%) were prepared by dissolving the appropriate amount of HGT agarose in distilled water, 5 x MOPS running buffer (0.1 M morpholinopropane sulfonic acid pH 7, 40 mM sodium acetate and 5 mM EDTA) and formaldehyde (12.3 M) to give 1 x and 2.2 M final concentrations, respectively. Gels were poured in a large gel apparatuses (20 x 20 cm) as described by Maniatis *et al.*, (1989) and once set were submerged in 1 x MOPS running buffer ready for electrophoresis. RNA samples (10-50 μg in 10 l) were mixed with 5 μl of 10 x MOPS, 8 μl of formaldehyde, and 22.5 μl of formamide and then incubated at 65°C for 15 min, cooled on ice and mixed with 1/5 volume of loading dye (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.5 mg/ml ethidium bromide). After samples were loaded into the appropriate wells the gel was electrophoresed at 15-30 V overnight before being photographed on a U.V light box and transferred to a solid support membrane as described below. In some instances standard RNA molecular weight ladders (BRL Cat. 5620SA) were also included in the same gel as the experimental RNA in order to determine the relative size of hybridizing transcripts.

ii) northern transfer

RNA was quantitatively transferred from denaturing gels to nylon membranes (Zeta Probe) by capillary action under mildly alkaline conditions by standard methods. Mild alkaline conditions were chosen since they facilitate elution of RNA from agarose by promoting partial hydrolysis of large RNA species and also induce binding of RNA to the nylon membrane (Vrati *et al.*, 1987). After transfer, usually between 5-6 h, membranes were briefly rinsed twice in 2 x SSC (0.3 M NaCl, 0.03 M trisodium citrate pH 7.0), blotted dry and then baked at 80°C for 2 h to ensure covalent binding of the RNA to the membrane. Membranes prepared in this manner were directly hybridized to radiolabeled probes (see section 2.5.6), or alternatively, stored between dry filter paper in sealed plastic bags at room temperature.

2.2.3.2 Slot blotting

Slot blotting of extracted RNA was performed using a Milliblot-S system (Millipore) and Zeta Probe nylon membranes (Maniatis *et al.*, 1989). After assembling

the apparatus according to the manufacturers instructions, RNA samples (50 ng-50 µg) were diluted to 500 µl with 30 mM NaOH and heated to 65°C for 15 min before being cooled on ice. Samples were then applied to the appropriate wells of the apparatus and transferred to the nylon membrane by means of suction from a vacuum pump. When completed the apparatus was disassembled and the nylon membrane rinsed briefly in 2 x SSC to neutralize the NaOH and then blotted dry and baked at 80°C for 2 h. Membranes prepared in this manner were directly hybridized to radiolabeled probes.

2.2.4 cDNA Clones

cDNA clones used as probes for northern analysis and slot blotting included a 1.06 kb *Pst*1 fragment from v-*fos*, a 1.5 kb (*Hind*III) fragment from β-rat-tubulin, and a 2 kb *Hin*dIII fragment from rat c-*myc* supplied by Dr. R. Crawford (Howard Florey Institute), a 1.5 kb *Eco*R1 fragment from mouse *fra*-1, a 1.2 kb *Eco*RI fragment from mouse *fra*-2, supplied by Dr. D Cohen (John Curtin School of Medical Research) and a 1.4 kb *Hin*dIII/*Eco*RI fragment from human c-*jun* supplied by Professor M. Karin (U.C.S.D).

2.2.4.1 Amplification of cDNA clones

Plasmids containing cDNA clones were transformed into competent *E.coli* (strain MC1061) cells by standard techniques and propagated in normal L-broth medium containing appropriate antibiotics as described by Maniatis *et al.*, (1989). Plasmid DNA was subsequently isolated by the alkaline lysis method (Birnboim and Doly, 1979) and following restriction digests with the appropriate enzymes, cDNAs were purified from plasmid DNA by electrophoresis through 1.5% LMP agarose gels (Maniatis *et al.*, 1989) and purified by silica bead adhesion using a commercially available kit ("Gene Clean", Bio 101 Inc). cDNA inserts prepared in this manner were used directly in the preparation of radiolabeled probes (section 2.2.5).

2.2.5 Synthetic Oligonucleotides

Atrial natriuretic peptide- and calcitonin gene-specific oligonucleotide probes were a kind gift from Mrs Jenny Penschow and Dr. J. Haralambidis (Howard

Florey Institute). The metallothionein (Mt) probe was obtained from Dr. A. K. West (University of Tasmania). The 40 mer exon 1 specific c-*fos* probe (rat-c-*fos* PR-1) and the 40 mer exon 2 specific c-*myc* probe (rat-c-*myc* PR-1) were obtained from Oncogene Science, USA. The remaining oligonucleotide probes described below, were synthesized by the author on a Pharmacia Gene Assembler Plus synthesizer by the solid phase method using phosphoramidite chemistry. Probes were synthesized on 0.2 μmole scale and the base protecting groups removed by treatment with ammonia at 60°C for 16 h. Following removal of solvent by vacuum the residue was redissolved in 1 ml of 0.2 mM EDTA and purified by PAGE according to the method of (Current Protocols). Purified oligonucleotides were precipitated and adjusted to 50 ng/μl in 0.2 mM EDTA before radiolabeling.

FOS2130:

30 mer exon 2 specific c-*fos* probe (rat)

5' GGG CTG CAC CAG CCA CTG CAG GTC TGG GTC 3'

FOS2230:

30 mer exon 2 specific c-*fos* probe (rat)

5' TCT GGT CTG CGA TGG GGC CAC GGA GGA GAC 3'

FOS4130:

30 mer exon 4 specific c-*fos* probe (rat sense strand)

5' GGC AGG GTG AAG GCC TCC TCA CAC CTC GGG 3'

MYC2130:

30 mer exon 2 specific c-*myc* probe (rat)

5' CCT GTT GGT GAA GCT AAC GTT GAG GGG CAT 3'

MYC3130:

30 mer exon 3 specific c-*myc* probe (rat, sense strand).

5' CGC ACA AGA GTT CCG TAG CTG TTC AAG TTT 3'

α-SkA20:

20 mer α-skeletal actin specific probe (rat) (Gustafsen *et al.*, 1986).

5' GCA ACC ATA GCA CGA TGG TC 3'

α-MHC20:

20 mer α-myosin heavy chain specific probe (rat) (Gustafsen *et al.*, 1985).

5' TTG TGG GAT AGC AAC AGC GA 3'

2.2.6 Labeling cDNA and Oligonucleotide Probes

2.2.6.1 cDNA restriction fragments were labeled to a high specific activity (~1 x 10^{9} cpm/μg) by the random-primed method of Feinberg and Vogelstein (1989) using a commercially available kit (OLK, Bresatec) as described below.

50-100 ng (5-10 μl) of DNA was heated for 5 min at 95°C, cooled on ice and briefly centrifuged to bring down condensation. To this was added 12.5 μl of nucleotide/random primer buffer (20 μM dATP, dGTP, dTTP, 50 mM Tris HCl pH 7.6, 50 mM NaCl, 20 mM $MgCl_2$, 100 μg/ml gelatin, 12.5 μg hexanucleotide primers) and 4 μl of [α-^{32}P]dCTP (4000 Ci/mM, Bresatec) and the solution mixed well. The reaction was initiated by the addition of 5-10 units of DNA polymerase I (Klenow fragment) and after incubation at 37°C for 20 min, unincorporated nucleotides were separated from the labeled strand by exclusion chromatography using a Sephadex G-50 column as described by Maniatis *et al.*, (1989).

2.2.6.2 Labeling oligonucleotide probes

Oligonucleotide probes were 5' end labeled to a high specific activity (0.5-1 x 10^{9} cpm/μg) using a commercially available kit (TKK-1, Bresatec) as described below.

Oligonucleotide DNA, generally (50-200 ng) was heated for 5 min at 70°C then cooled on ice. After briefly centrifuging 2 μl of buffer (60 mM Tris-HCl pH 7.6, 90 mM $MgCl_2$), 2 μl dithiothreitol (100 mM), 2 molar equivalents of [γ-^{32}P]dATP, i.e., 10 μl of a 10 mCi/ml solution of [γ-^{32}P]dATP (4000 Ci/mmol) for 100 ng of a 30 mer

oligonucleotide (average MW=9207), 5 U of polynucleotide kinase and distilled water to 20 µl were added. After mixing well the tube was incubated at 37°C for 15 min after which the unincorporated nucleotides were separated from the labeled oligonucleotide probe using exclusion chromatography through a Sephadex G-25 "spun column" (Penschow *et al.*, 1989).

2.2.7 Hybridization with cDNA and Oligonucleotide Probes

Nylon membranes containing immobilized RNA were sandwiched between two sheets of hardened filter paper (Whatman no. 54) and soaked in 25 ml of cDNA hybridization buffer (Vrati *et al.*, 1987), (50% deionized formamide, 0.5% milk powder, 1% SDS, 50% dextran sulphate, 50 mM phosphate buffer pH 6.5, 4 x SSC, 0.5 mg/ml salmon sperm DNA sheared and denatured) or oligonucleotide hybridization buffer, (20% deionized formamide, 5 x SSC, 50 mM phosphate buffer pH 6.8, 1 mM pyrophosphate, 0.1% BSA, 0.1% polyvinylpyrollidone, 0.1% ficoll, 2% SDS and 100 µg/ml salmon sperm DNA sheared and denatured) and then placed in a pre-made polyethylene plastic bag. The remaining hybridization solution (25 ml) containing the radiolabeled probe (denatured immediately before adding to the hybridization mixture by heating to 95°C for 5 min) was poured into the bag which was then heat sealed being careful to exclude any air bubbles. The bag was then placed between two glass plates and incubated at 37°C to 42°C (depending on the homology of the probe and the stringency required) overnight. After hybridization membranes were removed from the bags and non-specific "background" hybridization removed by consecutive washings in the following buffers:

(1) 2 x SSC at room temperature for 5 min

(2) 2 x SSC, 0.1% SDS at room temperature for 15 min

(3) 0.2 x SSC, 1% SDS at 65°C (cDNA), 42°C (oligo) for 15 min

(4) 0.2 x SSC at room temperature for 5 min

Membranes were subsequently blotted dry on filter paper, covered in plastic cling wrap and placed immediately next to a sheet of Kodak X-OMAT x-ray film between two $CaWO_4$ intensifying screens (Cronex Lightning Plus, Dupont) in an x-ray cassette. Following exposure at -80°C between 8 h and 1 week, films were developed

for 5 min in standard Kodak developer and fixed for 5 min in Kodak fixer and then rinsed and dried.

Membranes could be rescreened several times with additional probes after removal of the previously hybridized probe. This was achieved by incubating membranes at 98°C in 0.1 x SSC and 0.5% SDS twice for 15 min and then briefly in 0.2 x SSC after which membranes were re-baked at 80°C for 2 h (Vrati *et al.*, 1987).

2.2.8 Treatment of Results

For graphical representation of results, autoradiograms were quantified using a scanning laser densitometer and corrected for loading by comparison to readings for the control probe β-tubulin. Results in each case were expressed relative to control values and expressed +/- standard error mean (SEM) when number of experiments was greater than 3. For n=2 results were expressed as standard deviation (SD).

2.3 *In situ* HYBRIDIZATION HISTOCHEMISTRY

In this study *in situ* hybridization was used in an attempt to localize early-response gene mRNA to discrete cell populations and regions of the heart following hypertrophic stimulus. The principal nucleic acid probes used in this study were synthetic oligonucleotides since they were readily synthesized by the author using phosphoramidite chemistry (see chapter 3) and could be designed to exploit regions of maximum difference between closely homologous mRNAs whilst "sense" probes could be prepared and used as highly specific negative controls. Furthermore, due to their relatively short length, problems associated with tissue penetration and self hybridization, encountered with other probe systems, could be largely avoided (Penschow *et al.*, 1989).

In addition to oligonucleotide probes, the use of cDNA probes complimentary to early-response genes was also attempted since they were readily available and had been well characterized in terms of gene specificity by northern analysis (see section 2.2.6). The choice of labeling for oligonucleotide and cDNA probes was via ^{32}P since it has a high specific activity and permits rapid visualization on x-ray film and with liquid emulsion with resolution to single cells in some cases

(Penschow *et al.*, 1989). Although [^{3}H] labeling can potentially give much higher resolution than ^{32}P the low energy of this radioisotope makes x-ray film exposure less practical and limits sensitivity of the signal. Similarly ^{35}S gives higher resolution than ^{32}P but requires longer exposure times and has greater potential for background even under reducing conditions (Penschow *et al.*, 1989). Numerous nonradioisotopic labels such as photobiotin/avidin were available at the time of study however it was considered that their sensitivity and ease of use was not yet equal to that achievable with ^{32}P labeling and were consequently not used.

Effective tissue fixation is one of the most important steps towards obtaining satisfactory *in situ* hybridization results (Moench, 1987; Hofler, 1987; Angerer *et al.*, 1987; Cumming and Fallon, 1988). In these studies crosslinking fixatives such as formaldehyde and gluteraldehyde were used due to their superior ability to prevent loss of target mRNA and to preserve tissue morphology in comparison to precipitative fixers such as acetone or methanol (McAllister & Rock, 1985). Crosslinking fixatives are limited in that they tend to hinder tissue penetration of long probes (Penschow *et al.*, 1989) such as labeled cDNA. In such circumstances it is necessary to employ secondary permeabilization steps such as mild enzymatic digestion with proteinases to ensure probe access to target mRNA sites. Fixation and permeabilization tend to work in opposition and thus achievement of optimal conditions can only be measured in terms of final hybridization signal (Penschow *et al.*, 1989).

Of the considerable variety of *in situ* hybridization protocols attempted during the course of these studies, only those used on a routine basis with some degree of success are outlined below.

2.3.1 Reagents

3-aminopropyltriethoxy-silane	Aldrich
Amersham LM-1 Liquid Emulsion	Amersham
D.P.X mountant	BDH
Eosin Y	Sigma
Ethylene glycol	Sigma
Formaldehyde	Merc
Gelatin	BDH

Glutaric dialdehyde	Merc
Hematoxylin	Sigma
Hexane	Ajax
Ilford K-5 Liquid Emulsion	Ilford
O.C.T	Miles Inc.
Pronase E	Sigma
Xylene	BDH

Other reagents were of molecular biological grade.

2.3.2 *In situ* Hybridization Protocol

2.3.2.1 *In situ* hybridization with oligonucleotide probes

The oligonucleotide hybridization procedure described below is essentially the same as that described by Penschow *et al.*, (1989) with minor modification.

i) tissue preparation, fixation and prehybridization

Tissue was removed from experimentally treated or control animals and immediately embedded with tissue-Tek in a dry ice/hexane bath and stored at -80°C until processed. Control tissue was similarly treated and in some cases was included on the same block as experimental tissue. When processed, embedded tissue was allowed to warm to -15°C in a cryostat and then sectioned to 5 μm. Sections were picked up onto slides (pre-coated with gelatin or poly-lysine (Penschow *et al.*, 1989)) and immediately laid on dry ice for 30 min to freeze the section rapidly (this reduces the risk of mRNA degradation and improves cell morphology, (J. Penschow, personal communication). Slides were subsequently transferred to fixative (4% gluteraldehyde, 0.1 M phosphate buffer pH 7.4, 20% ethylene glycol) and fixed at 5°C for 4 min and then rinsed twice in 2 X SSC and prehybridized by immersion in oligonucleotide *in situ* hybridization buffer (600 mM NaCl, 50 mM phosphate buffer pH 7.0, 5 mM EDTA, 0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrollidone, 0.01% sheared/denatured DNA, 40% deionized formamide) at 40°C for 1 h. After pre-hybridization sections were dehydrated through 50%, 70% ,95%, 100%, 100% ethanol containing 300 mM ammonium acetate, at which stage they were either stored at -20°C for several weeks in moisture proof containers or hybridized immediately.

ii) probe preparation and hybridization

Prior to hybridization radiolabeled probes were precipitated by the addition of 0.01 volume of tRNA (10 mg/ml in TE buffer pH 7.4), 0.1 vol 3 M sodium acetate pH 7.4 and 2 volumes of 100% ethanol. After incubation overnight at -20°C, probe DNA was collected by centrifugation at 10 000 g, washed consecutively in 70% and 95% ethanol and vacuum dried for 30 min before being dissolved at 65°C in the appropriate volume of hybridization buffer. Probe concentrations for hybridization were determined empirically but were usually in the range of 0.5 to 10 ng/µl. Immediately prior to hybridization probes were denatured by boiling at 95°C for 5 min and then chilled on ice. Approximately 25 to 30 µl of probe was then spotted onto clean glass cover slips (25 x 24 mm) and picked up onto experimental slides which were subsequently placed on a plastic grid and overlaid with plastic wrap. After incubation overnight in a humidified chamber at 37°C cover slips were removed from slides by gentle agitation in 4 x SSC after which non-specific probe binding was removed by incubating the slides in 2 x SSC at 40°C for 1 h with occasional agitation and then in 1 X SSC at 40-50°C for a further hour. Finally slides were dehydrated through 50%, 70% ,95% and 100% ethanol and processed for autoradiography (see section 2.3.3.3).

2.3.3.2 *In situ* hybridization with cDNA probes

The cDNA hybridization described below is a modification of that described by Closs *et al.*, (1990).

i) tissue fixation and prehybridization

Tissue was sectioned as described above and thawed onto slides pre-coated with 2% 3-aminopropyltriethoxy-silane (Penschow *et al.*, 1989) and immediately placed on a heated plate at 50°C for 5 min (to remove endogenous ribonuclease activity) before being fixed at room temperature for 30 min in 0.1 M phosphate buffered saline (PBS) containing 4% formaldehyde. Slides were then thoroughly rinsed in PBS and incubated in 0.2 M HCl for 20 min at room temperature and then rinsed again in 2 X SSC. Slides were then subjected to mild protease treatment by incubation in 1 X SSC containing 125 µg/ml pronase E (this buffer had been autodigested for 2 h to remove potential ribonucleases) after which they were rinsed in PBS and fixed again in formaldehyde as before. After rinsing again in PBS slides were prehybridized by immersion in cDNA *in situ* hybridization buffer (600 mM NaCl, 10 mM Tris.HCl pH 7.4, 1 mM EDTA, 0.02%

Ficoll, 0.02% BSA, 0.02% polyvinylpyrrollidone, 0.01% sheared denatured DNA, 0.01% tRNA, 0.1 mg/ml polyadenylate, 50% deionized formamide) at 40°C for 1 h. After pre-hybridization sections were dehydrated through 50%, 70% ,95%, 100%, 100% ethanol containing 300 mM ammonium acetate, at which stage they were either stored at -20°C for several weeks in moisture proof containers or hybridized immediately.

ii) probe preparation and hybridization

The hybridization for cDNA probes was essentially the same as that described for oligonucleotide probes. Hybridization temperatures varied between 37°C and 42°C depending on percentage homology of the cDNA probes. Following hybridization non-specific probe binding was removed by incubating the slides in formamide buffer (50% formamide, 0.6 M NaCl, 10 mM Tris HCl pH7.4, 1 mM EDTA) at room temperature for 1 h followed by washing in 2 x SSC at 40°C for 1 h with occasional agitation and then in 0.1 X SSC at 60-65°C for a further 2 h. After dehydration through 50%, 70%, 95%, 100%, 100% ethanol, slides were processed for autoradiography as described below.

2.3.2.3 Probe detection by autoradiography

i*) regional analysis using high resolution film*

Slides prepared in section 2.3.3.2 were taped to a piece of 3MM filter paper and exposed in an x-ray cassette to Amersham high resolution β-max film. After the appropriate exposure time films were processed as usual (section 2.2.6).

ii) microscopic analysis using liquid emulsion.

Under dark room conditions Ilford K-5 or Amersham LM-1 nuclear emulsion gel was diluted 1 in 2 with distilled water and warmed to 40°C for 2 h. Slides were briefly dipped into the prewarmed emulsion and excess liquid allowed to drain away by standing slides vertically. When the emulsion had dried slides were loaded into racks and placed in light proof boxes over silica gel and exposed for 1 to 4 weeks. Exposure time was approximately 10 times that required to give a medium gray image following exposure next to fast (low resolution) x-ray films such as Amersham MP or Kodak K5. Following exposure slides were developed and fixed in filtered Kodak fixer and developer, rinsed in 3% acetic acid, hardened in formalin for 1 min and counter-stained with Haematoxylin/eosin (Penschow *et al.*, 1989) and then dehydrated

through graded ethanol, cleared into xylene and mounted with D.P.X mountant. Slides were photographed between 100 and 400 time magnification under blue light using a standard photomicroscope system.

2.3.2.4 Probe specificity and controls.

in situ hybridization results may be misleading due to unexpected homologies or to short regions within a probe hybridizing to unknown target sequences. Similarly interactions between proteins and probes may occur whilst a variety of autoradiographic artifacts can provide convincing hybridization signals or alternatively, reduce genuine hybridization signals. In an attempt to counter any potential spurious results an array of experimental controls were used in the present *in situ* hybridization experiments and these are outlined in table 2.

Table 2. *In situ* hybridization controls

Factor of interest	*Procedure*
Non-specific probe binding	Sense DNA probe
	Nonhomologous probe
	Digestion of target RNA
	Non-expressing tissue
Probe specificity	Northern blot
Autoradiography	No probe
	Tissue only
	Blank slide
Target distribution	Immunocytochemistry

2.4 IMMUNOCYTOCHEMISTRY

c-*myc* and c-*fos* protein products were localized to specific cardiac regions and cell types by immunocytochemistry using both monoclonal and affinity purified polyclonal antibodies. A variety of immunodetection systems were experimented with including peroxidase-antiperoxidase (PAP) (Sternberger *et al.*, 1970), alkaline phosphatase-antialkaline phosphatase (APAAP) (Cordell *et al.*, 1984), avidin-biotin complex (ABC) (Hsu and Soban, 1982), and labeled avidin-biotin (LAB) technique (Guesdon *et al.*, 1979) in an attempt to obtain the highest sensitivity and lowest background possible. In these studies the technique which consistently gave the best results was the LAB method. In this immunostaining procedure, target tissue sections, either frozen or formalin-fixed and paraffin-embedded, were first incubated with a monoclonal antibody or affinity-purified polyclonal antibody (primary antibody) to the antigen of interest. Specifically bound antibody was then visualized by incubation with a biotinylated second-step antibody (link antibody) against immunoglobulins of the relevant species (i.e. biotinylated goat anti-mouse IgG for a primary antibody raised in mice), followed by incubation with a streptavidin-enzyme conjugate and chromagen-substrate. Two different enzyme/chromagen systems, the calf intestinal alkaline phosphatase fast red TR and the horseradish peroxidase/ diaminobenzidine (DAB) systems were used in the course of these studies. The former of these systems gives a brilliant red end product which is easily detectable to the human eye even in low concentrations (Rainbow, 1988) but is soluble in alcohol which means it must be mounted with aqueous mountants. The latter system gives a brown end product which can be mounted in both aqueous and organic mountants and its sensitivity can be enhanced with salts of heavy metals such as nickel, silver or osmium (Hsu and Soban, 1982; Johannson and Beckman, 1983; Rodriguez *et al.*, 1984).

A number of fixation methods were experimented with in an endeavor to obtain the best antigen preservation whilst retaining morphological detail of the specimen. In this author's hands fixation of fresh frozen tissue with cross linking fixatives (i.e. formaldehyde) or paraffin embedded tissue gave no or very poor immunostaining for Myc and Fos protein, possibly because these conditions either altered or destroyed the Myc and Fos epitopes. In contrast, although the tissue morphology was of lower quality than that using cross linking fixatives, fresh frozen

sections fixed with precipitative fixers (i.e. acetone, methanol, ethanol) resulted in relatively strong immunostaining for both the anti-Myc and -Fos antibodies. Due to the lower resultant background, methanol fixation in conjunction with peroxidase /DAB was routinely used for the majority of studies with anti-Myc and -Fos early-response gene antibodies. For detection of tissue marker proteins such as α-smooth muscle actin formaldehyde fixation in conjunction with alkaline phosphatase/ fast red TR was routinely used with satisfactory results.

2.4.1 Materials

2.4.1.1 Reagents and chemicals

Aqueous Mounting medium	BioGenex
Crystal/Mount	Biomed Corp.
D.P.X Mountant	BDH
Diaminobenzidine	Sigma
Gelatin	Davis Gelatin
Imidazole	Sigma
Levamisole	Sigma
N,N Dimethyl Formamide	Sigma
Napthol AS MX Phosphate	Sigma
Osmium tetroxide	Sigma
Potassium Dichromate	BDH
Protease type XXIV	Sigma
Thymol	BDH

2.4.1.2 Immunological-link and -labels

Immunological-link and -labels were obtained from BioGenex (USA) and are listed below.

Alkaline phosphatase-conjugated streptavidin	(HK350-5K)
Biotinylated anti-mouse immunoglobulins	(HK335-5M)
Biotinylated anti-rabbit immunoglobulins	(HK336-5R)
Normal Goat Serum	(HK112-5K)

Peroxidase-conjugated streptavidin (HK330-5K)

2.4.1.3 Primary antibodies

Primary antibodies were obtained from commercial sources and are outlined below.

1) Fos

For detection of Fos and related proteins a rabbit, affinity purified polyclonal antibody raised against the peptide (S G F N A D Y E A S S S R C) corresponding to residues 4 to 17 of human *fos* (Oncogene Science, USA, Cat # HCS17) was used at a concentration of 2.5 μg/ml. This antibody potentially cross-reacts with all members of the *fos* gene familly. In addition, polyclonal antibodies specific for Fos, Fra-1 and Fra-2 were obtained as a kind gift fom Dr. D. Cohen (A.N.U) and were used in some preliminary studies. These antibodies were raised to non-conserved regions within each protein and therefore are unlikely to cross-react with other members of the *fos* gene family (Dr. D. Cohen, pers. comm.)

2) Myc

Myc protein was detected using a mouse monoclonal antibody from ascites fluid raised against the peptide (A P S E D I W K K F E L C) corresponding to residues 44-55 of rat c-*myc* (Cambridge Research Biochemicals, UK, Cat. # OM-11-904) and was used at a concentration of 25 μg/ml.

3) Smooth muscle Actin

Vascular smooth muscle was detected using a mouse monoclonal antibody from ascites fluid corresponding to a conserved decapeptide region of human and rat α-smooth muscle actin (Skalli *et al.*, 1986) (ICN ImmunoBiologicals, Israel, Cat. # 63-793) and was used at a dilution of 1:400.

2.4.2 Immunodetection Procedure

2.4.2.1 Immunodetection of early-response gene proteins by the LAB-immunoperoxidase method

This method is a modification of the original method of Guesdon *et al.*, (1979).

Experimental hearts embedded in OCT compound as described in section 2.1.4 were warmed slowly to -22°C, mounted on a chuck in a freezing microtome and sectioned to 5 μm. Sections were then immediately thawed onto gelatin-dichromate coated slides (Penschow *et al.*, 1989) and fixed at once in 95% methanol for 2 min and then air dried for 2 h at room temperature. Sections prepared in this manner were stored in moisture proof containers at -20°C for several weeks or at -80°C for up to several months without any apparent decrease in immunosensitivity. For immunodetection sections were allowed to thaw to room temperature and then incubated in methanolic H_2O_2 (0.5%) for 30 min to remove endogenous peroxidase activity. After rinsing in PBS they were then incubated successively in 0.1% avidin in PBS pH 7.4 and then 0.01% biotin in PBS for 20 min to remove endogenous avidin binding activity (Wood and Warnke, 1981). After rinsing in PBS sections were incubated in 0.1% Triton X-100 in order to permeabilize the tissue and then non-specifically blocked by incubating in normal goat serum (1%) for 30 min. Sections were subsequently incubated with the primary antibody overnight at 4°C at the appropriate dilution in diluent (PBS pH 7.4, 0.1% BSA). After rinsing in PBS, sections were incubated for 1 h at room temperature with biotinylated anti-rabbit immunoglobulin or biotinylated anti-mouse immunoglobulin for polyclonal and monoclonal primary antibodies respectively. After rinsing, sections were incubated for 1 h at room temperature with peroxidase-conjugated streptavidin. After rinsing again with PBS, peroxidase activity was visualized by incubating slides in DAB/imidazole (0.02% DAB, 0.135% imidazole in PBS) for 2 min and then in DAB/imidazole containing 0.005% H_2O_2 for 3 min. Sections were then rinsed with distilled water and incubated in freshly prepared Osmium tetroxide (0.002% in PBS) for 3 min and rinsed in distilled water, dehydrated through ethanol, cleared into xylene and mounted with D.P.X. mountant. Slides were photographed as described in section 2.3.3.3.

2.4.2.2 Immunodetection of cell marker proteins by the LAB-alkaline phosphatase method

This method is a modification of the original method of Guesdon *et al.*, (1979).

Fresh frozen tissue was sectioned as described previously and immediately fixed by incubating in tri-fixative (2% formaldehyde, 49% methanol, 49% acetone) for 90 s at 5°C and then rinsed in tris buffered saline (TBS) for 5 min. Sections were then incubated successively in 0.1% avidin in TBS (0.1 M Tris.HCl pH7.6, 0.9 g/l NaCl) and then 0.01% biotin in Tris-saline for 20 min to remove endogenous avidin binding activity. After rinsing in TBS sections were incubated in 0.1% Triton X-100 in order to permeabilize the tissue and then non-specifically blocked by incubating in normal goat serum (1%) for 30 min. Sections were subsequently incubated with the primary antibody overnight at 4°C at the appropriate dilution in diluent (TBS pH 7.6, 0.1% BSA). After rinsing in TBS, sections were incubated for 1 h at room temperature with biotinylated anti-rabbit immunoglobulin or biotinylated anti-mouse immunoglobulin for polyclonal and monoclonal primary antibodies respectively. After rinsing, sections were incubated for 1 h at room temperature with alkaline phosphatase-conjugated streptavidin. following rinsing with TBS once again, phosphatase activity was visualized by preincubating sections in 0.1 M Tris.HCl pH 8.2 for 2 min then in developing solution (Napthol AS MX 0.2 mg/ml, Fast red TR 0.75 mg/ml, levamisole 0.2 mg/ml (an inhibitor of endogenous phosphatases) in 0.1 M Tris HCl pH 8.2) for a further 20 min. After rinsing with water sections were protected with crystal mount, dried at 45°C and then mounted with D.P.X. Slides were photographed as described in section 2.3.3.3.

CHAPTER 3

EFFECTS OF HYPERTROPHIC AGENTS ON EARLY-RESPONSE GENE EXPRESSION *IN VIVO*

3.1 INTRODUCTION

Studies by Simpson's group and others have demonstrated that NE acting via the α_1-adrenergic receptors is capable of directly and independently inducing hypertrophy of neonatal cardiac myocytes *in vitro* and that this process is qualitatively similar to that observed in adult hearts following pressure overload (reviewed in Simpson, 1990). It has been speculated that the intracellular pathways linking occupancy of the adrenergic receptors to qualitative changes in protein synthesis observed during hypertrophy might be transduced by one or more members of a group of nuclear-acting early response genes (Simpson, 1988 A). This notion is consistent with the observations that elevated expression of some of these genes is an early event which occurs during NE-mediated neonatal myocyte hypertrophy (Starksen *et al.*, 1986; Iwaki *et al.*, 1990). However, cardiac myocytes isolated from neonatal hearts have demonstrable physiological, pharmacological and metabolic differences compared to adult cells (for an inclusive list see table 3.1) and results from studies with these immature, artificially cultured cells may not be directly applicable to the adult heart *in vivo* (Bugaisky and Zak, 1989).

In addition it is possible that other cells may modify the response of adjacent cardiac myocytes by the release of growth factors. In support of this it has been shown that acidic and basic fibroblast growth factors can modify isocontractile gene expression and increase expression of c-*fos* and c-*jun* in isolated neonatal myocytes (unpublished data from Parker and Schneider, 1991) and it is possible that their release from fibroblasts may be enhanced by adrenergic agents. Accordingly it is of considerable interest to determine if NE might also modulate a similar program of early response gene expression in myocytes of the adult heart *in situ*. As a first step in this direction the work in this chapter has examined the expression of common and distinct members of the early-response gene program including c-*myc*, c-*fos*, c-*jun*, *fra*-1

and *fra*-2 in whole adult rat hearts following acute and chronic administration of adrenergic agonists *in vivo*. For comparison the effects of the hypertrophic hormone T_3 on cardiac early-response gene expression was also analyzed since this hormone results in a qualitatively different form of hypertrophy to that observed in response to α_1-adrenergic agents .

Table 3.1 Comparison of cultured embryonic/neonatal and adult cardiac myocytes.

	Embryonic/neonatal	Adult
Initial striated structure	Immediately lost	Lost with time
Striated structure regained	Yes	Yes
Morphology	Unique	Unique
T-tubule system	Rudimentary	Well-developed
Beating rate	70-100	175-220
Mass beating	Synchronous	Asynchronous initially; becomes synchronous in time
Isozymes	V_1: V_3 1:1	V_1 Only (during 1st week)
PAS positive	Yes	Yes
Cell division	Yes	No
DNA synthesis	Yes	Yes
Epinephrine responsive	Yes	Yes
Resting potential	-38.2 mV	-76.3 mV

(Adapted from Bugaisky and Zak, 1989)

3.2 EXPERIMENTAL PROTOCOLS

3.2.1 Acute Administration of Hypertrophic Agents *in vivo*

Rats were administered a single 0.5 ml intraperitoneal (i.p.) injection of the appropriate drug dissolved in vehicle (see section 2.1.2). Each animal received an equivalent dose per kilogram of body weight and control animals were injected with an equal volume of vehicle alone. Where used, adrenergic antagonists were injected twice,

1 h and again 10 min before NE administration. At the appropriate time after administration of drugs, rats were anaesthetized with an i.p. injection of 0.4 ml sodium pentobarbitone, 0.2 ml heparin and 0.4 ml of 0.9% saline and the hearts quickly excised and rinsed in ice-cold saline. Hearts for northern analysis were left whole or dissected into the various chambers and snap frozen in liquid nitrogen to await RNA extraction (section 2.2.2).

3.2.2 Chronic Administration of Hypertrophic Agents *in vivo*

3.2.2.1 Norepinephrine administration

Alzet mini-osmotic pumps purchased from Alzet Corporation (U.S.A. Model 2002) were filled according to the accompanying instructions with NE in vehicle or vehicle alone (see section 2.1.2). Pumps were then equilibrated for 24 h in 0.9% saline before use and implanted in animals as follows. Rats were anaesthetized in an ether box and unconsciousness maintained by use of a nose cone. The intrascapular region was shaved, swabbed with antiseptic and a 1 cm incision made by blunt dissection to accommodate the pump. Following insertion of the pump, the wound was closed with surgery clips and the animals were allowed to recover from surgery and thereafter maintained as usual. At the appropriate time after surgery animals were anaesthetized, hearts removed and weighed and prepared for analysis as described in section 2.2.

3.2.2.2 T_3 administration

Rats were injected subcutaneously every 24 h with T_3 and weighed on a daily basis. Control animals received an equivalent volume injection of vehicle alone (see section 2.1.2). After the appropriate number of days rats were anaesthetized and hearts removed, blotted dry and weighed. Hearts were then immediately prepared for analysis as described in section 2.2.2.

3.3 RESULTS

3.3.1 Acute Effect of Adrenergic and Thyroid Hormones on Early-Response Gene Expression *in vivo*

3.3.1.1 Adrenergic agents

Total RNA was extracted from hearts and analyzed for early-response gene expression by northern blotting as described in the methods. Basal levels of c-*myc* but not c-*fos* mRNA could be detected in the hearts of both untreated and saline-injected animals, although this required over-exposure of the appropriate autoradiographs (Fig 3.1-3.4). A single injection of NE (2.5 mg/kg) however, greatly elevated both c-*fos* and c-*myc* mRNA levels, with c-*fos* transcripts increasing 15 min after injection to peak at 1-2 h and returning to basal by 6 h (Fig 3.1, Lanes 3-10). Elevated c-*myc* mRNA was observed at 30 min after injection of NE, peaked at 2-3 h and returned to basal levels after 6 h (Fig 3.1, Lanes 3-10). Thus, the increase in mRNA levels of these two genes in response to NE was both transient and sequential. Similar but lesser increases of c-*fos* and c-*myc* were observed following single doses of NE at levels as low as 2.5 μg/kg, indicating that this response is not restricted to extremely high doses of the hormone (Fig 3.2).

In order to determine which component of NE action was responsible for the change in c-*myc* and c-*fos* mRNA levels, rats were treated with the α-adrenergic agonist phenylephrine (2.5 mg/kg) or the β-adrenergic agonist, isoproterenol (2.5 mg/kg). Both agents caused an increase in c-*fos* and c-*myc* mRNA which was similar in magnitude to that due to NE (Fig 3.3 & 3.4). Minor crossover affinity of phenylephrine for β-adrenergic receptors was not responsible for the observed gene inductions in response to this agent since animals were co-treated with the selective β-adrenergic antagonist propranolol (50 mg/kg). Interestingly, the time required for c-*myc* mRNA to reach maximal levels appeared to be dependent on which agent was administered, peaking at 1 h, 2 h, 3 h following treatment of rats with NE (αβ), phenylephrine (α) and isoproterenol (β) respectively (Fig 3.5).

Recent studies indicate that c-*fos* is one of a related family of rapidly inducible genes including *fra*-1 (Cohen and Curran, 1988) and *fra*-2 (Yoshida *et al.*, 1993) whose products form heterodimers with products of the c-*jun* gene family and

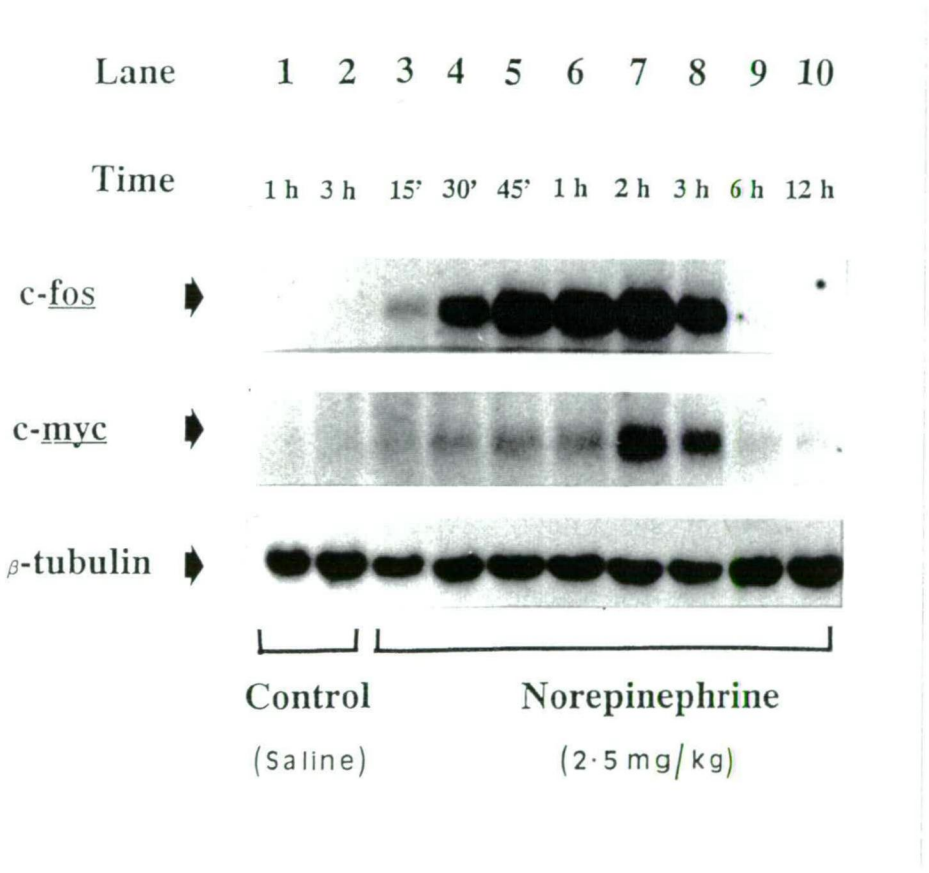

Fig 3.1. Cardiac expression of c-*fos* and c-*myc* in response to a single injection of norepinephrine.
Total RNA was extracted from rat hearts removed at the various times indicated following an i.p. injection of 0.9% saline (Lanes 1 & 2) or norepinephrine (2.5 mg/kg, Lanes 3-10). After electrophoresis and northern blotting, the RNA (50 μg) was hybridized to c-*fos* (upper tracks), c-*myc* (middle tracks) and β-tubulin (lower tracts).

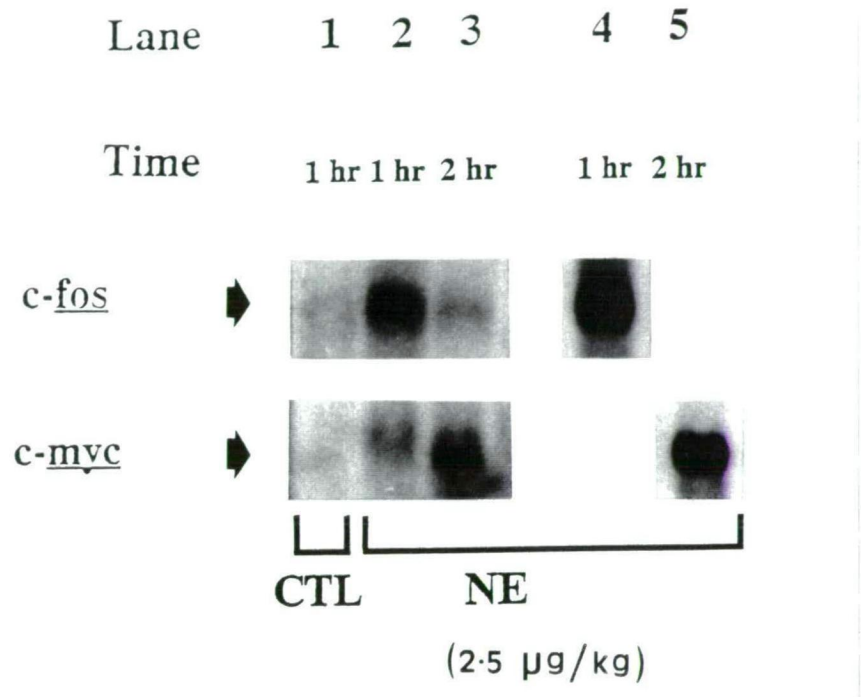

Fig 3.2. Cardiac expression of c-*fos* and c-*myc* in response to a single low dose injection of norepinephrine.

Total RNA was extracted from rat hearts removed at the various times indicated following an i.p. injection of 0.9% saline (Lane 1) or norepinephrine (2.5 mg/kg, Lanes 2 & 3) and 50 µg was hybridized to c-*fos* or c-*myc*. In addition, 7 µg of poly(A)$^+$ RNA was isolated from the 1 h and 2 h total RNA samples for norepinephrine-treated rats and hybridized to c-*fos* (Lane 4) and c-*myc* (Lane 5) probes respectively.

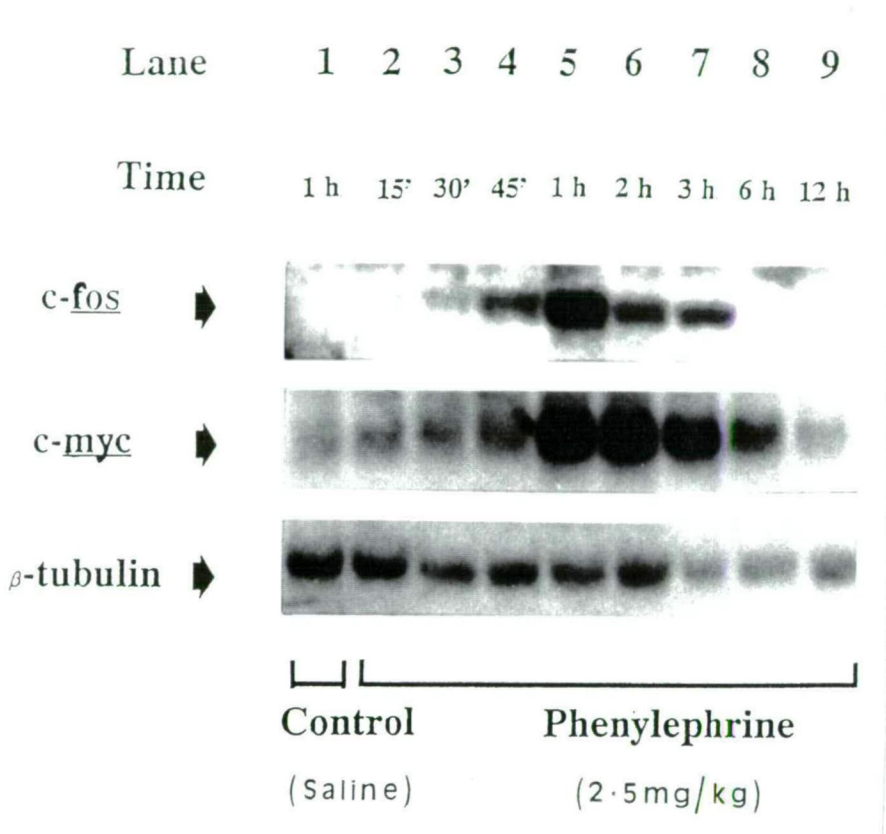

Fig 3.3. Cardiac expression of c-*fos* and c-*myc* in response to a single injection of phenylephrine. Total RNA was extracted from rat hearts removed at the various times indicated following an i.p. injection of 0.9% saline (Lane 1) or phenylephrine (2.5 mg/kg, Lanes 2-9). c-*fos*, c-*myc* and β-tubulin transcripts were analyzed as described previously.

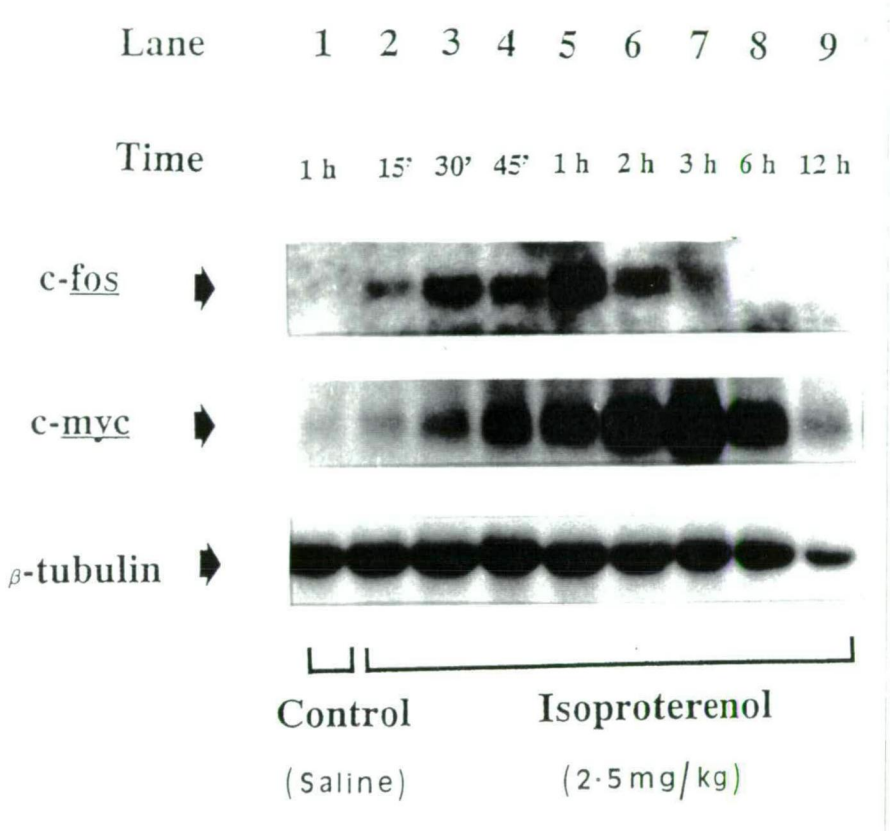

Fig 3.4. Cardiac expression of c-*fos* and c-*myc* in response to a single injection of isoproterenol.
Total RNA was extracted from rat hearts removed at the various times indicated following an i.p. injection of 0.9% saline (Lane 1) or isoproterenol (2.5 mg/kg, Lanes 2-9), c-*fos* , c-*myc* and β-tubulin transcripts were analyzed as described previously.

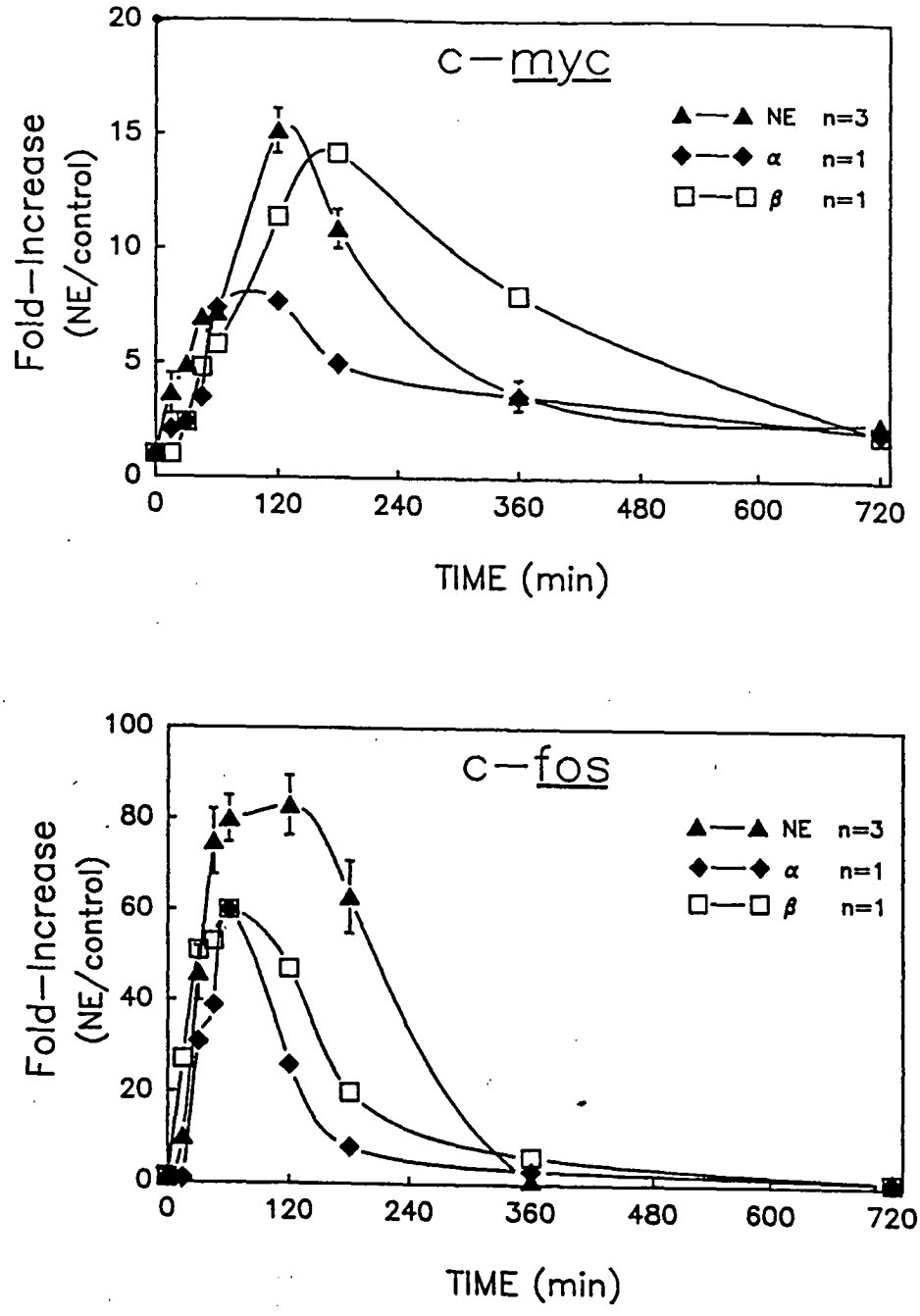

Fig 3.5. Quantification of c-*myc* and c-*fos* in response to norepinephrine or selective α– and β-adrenergic agonists.

The hybridization signals obtained by northern blotting in figures 3.1, 3.3 and 3.4 were quantitated via laser densitometry and, after standardization to β-tubulin levels in each track, were expressed as the fold increase over control (basal) signals observed in hearts from untreated rats. α–agonist, phenelepherine. β–agonist, isoproterenol. Vertical bars indicate standard error mean (S.E.M.).

bind to AP-1 like regions of target genes to modulate their transcription (Reviewed in Angel and Karin, 1991). Accordingly it was of interest to determine whether these genes were also expressed in the heart following NE administration. c-*jun* and *fra*-2 but not *fra*-1 mRNA was endogenously expressed at low levels in the hearts of both adult untreated and saline-injected animals (Fig 3.6, Lanes 1-3). Injection of NE (2.5 mg/kg) however, greatly elevated the mRNA for all three genes: increased *fra*-1 mRNA appeared maximal after 1 h but remained elevated above control levels up to 6 h following administration of NE. Increased *fra*-2 and c-*jun* mRNA reached maximal levels after 2 h and had returned to near basal levels by 12 h and 6 h respectively following NE treatment. Interestingly, a second transcript of lower molecular weight (4 kb) appeared for *fra*-2 following NE stimulation which is not detectable in basal tissue (Fig 3.6, Lanes 4-8). These results indicate that an array of early-response genes are induced in a sequential fashion in the rat heart following acute administration of NE.

In order to determine the percentage contribution of the α- and β-receptors to NE mediated early response gene expression, rats were exposed to selective α- or β-adrenergic blockade during NE administration (Fig 3.7 & 3.8). Co-treatment with propranolol (50 mg/kg), a synthetic β_1, β_2-antagonist, did not attenuate the induction of any early-response genes investigated. In fact in most cases (c-*fos*, c-*jun*, *fra*-1 and *fra*-2) the observed induction was increased by 5-30% with respect to animals treated with NE alone. In contrast co-treatment with phentolamine (25 mg/kg) a synthetic α_1, α_2-adrenergic antagonist, significantly attenuated, but did not abolish the induction of c-*fos* and c-*jun* whilst the mRNA levels of c-*myc*, *fra*-1 and *fra*-2 where not significantly reduced. It was noted again that the time of maximal c-*myc* expression and also *fra*-1 expression was altered in response to a specific component of NE action; thus β-blockade resulted in greatest c-*myc* and *fra*-1 levels at 1 h compared to 3 h with α-blockade. Blockade with both α- and β-adrenergic antagonists significantly reduced NE induction of all early-response genes to near basal levels indicating that the hormone indeed acts via the adrenergic receptors (Fig 3.7).

3.3.1.2 T_3 administration

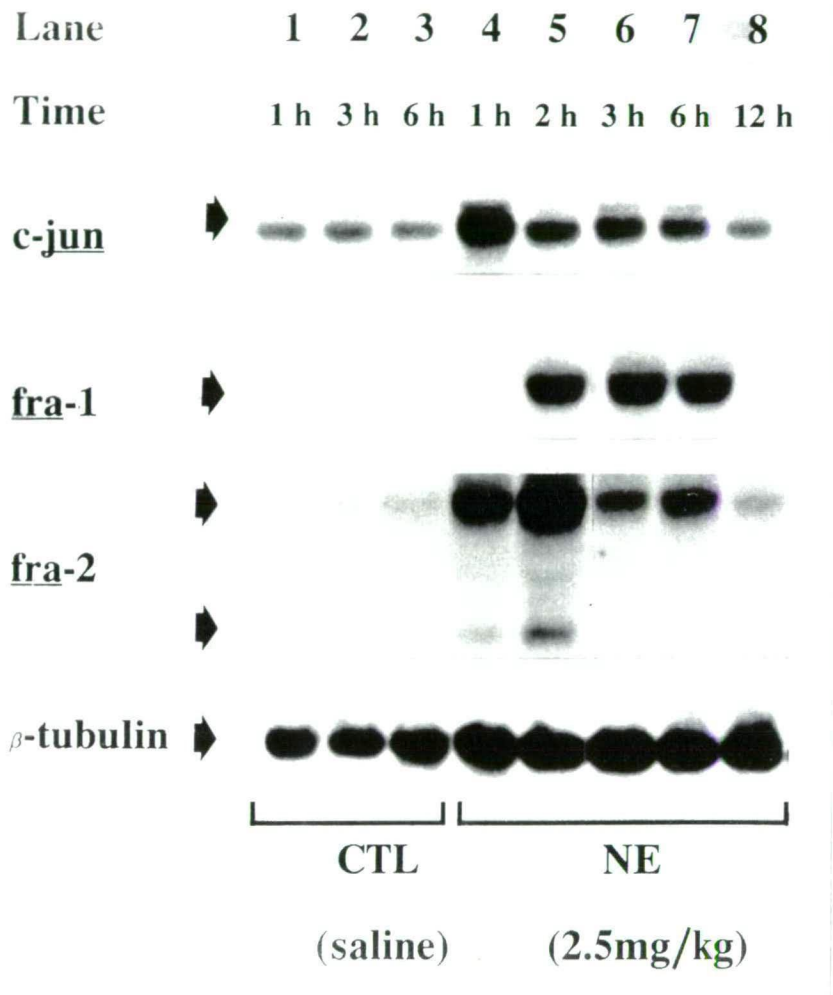

Fig 3.6. Cardiac expression of c-*jun*, *fra-1* and *fra-2* in response to a single injection of norepinephrine. Total RNA was extracted from rat hearts removed at the various times indicated following an i.p. injection of 0.9% saline (Lanes 1-3) or norepinephrine (2.5 mg/kg, Lanes 4-8). After electrophoresis and northern blotting, the RNA (50 μg) was hybridized to c-*jun*, *fra*-1, *fra*-2 and β-tubulin .

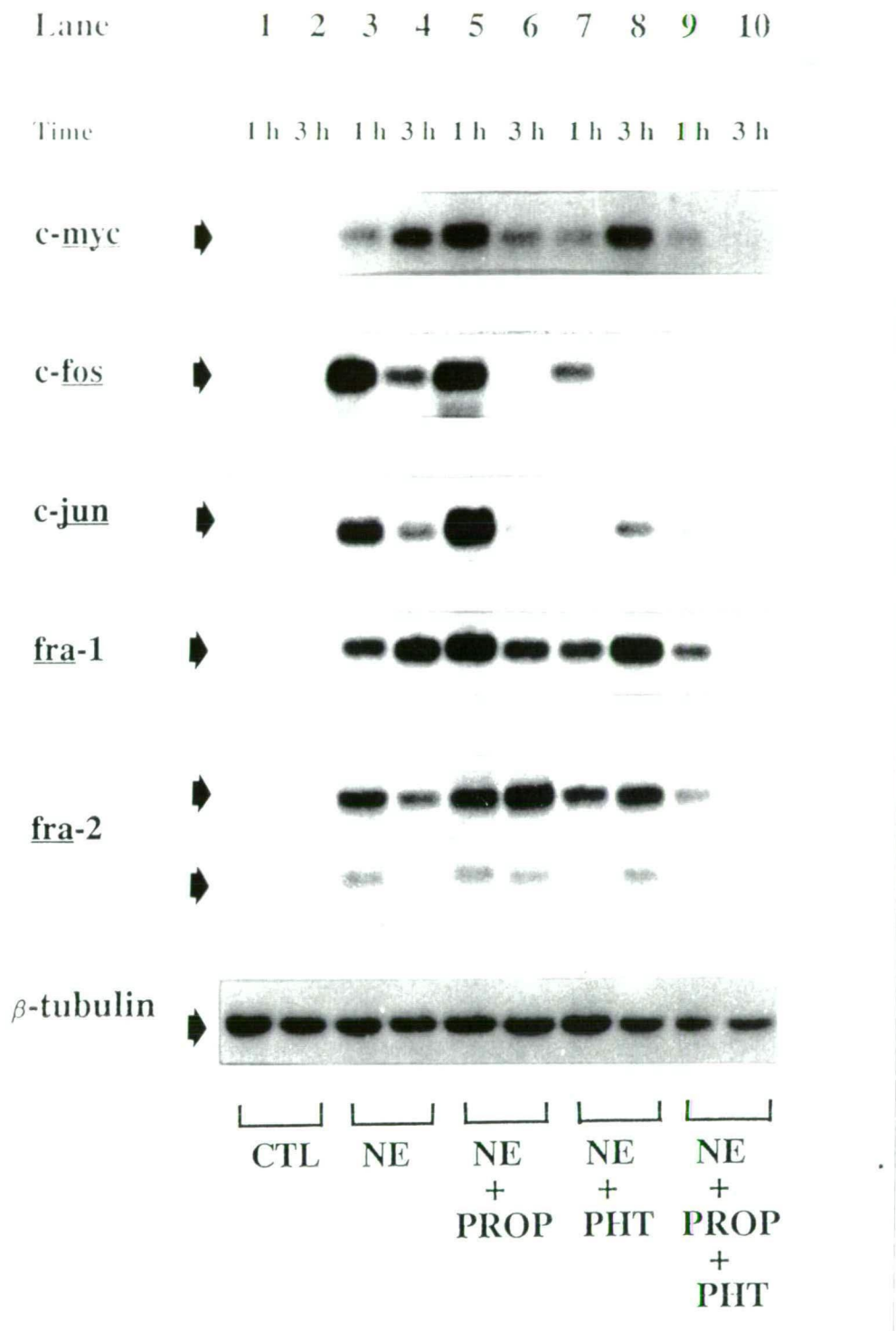

Fig 3.7. Cardiac expression of c-*fos* and c-*myc*, c-*jun*, *fra*-1 and *fra*-2 in response to α- or β-adrenergic blockade prior to NE administration.

Total RNA was extracted from rat hearts removed 1 h and 3 h following an i.p. injection of 0.9% saline (CTL, Lanes 1 and 2) or norepinephrine (2.5 mg/kg, Lanes 3 & 4). The β-antagonist propranolol (PROP, 50 mg/kg, Lanes 5 & 6) or α-antagonist phentolamine (PHT, 25 mg/kg, Lane 7 & 8) or a combination of both (lanes 9 & 10) were given twice, 1 h and 10 min prior to norepinephrine administration. c-*myc*, c-*fos*, c-*jun*, *fra*-1, *fra*-2 and β-tubulin transcripts were analyzed as described previously.

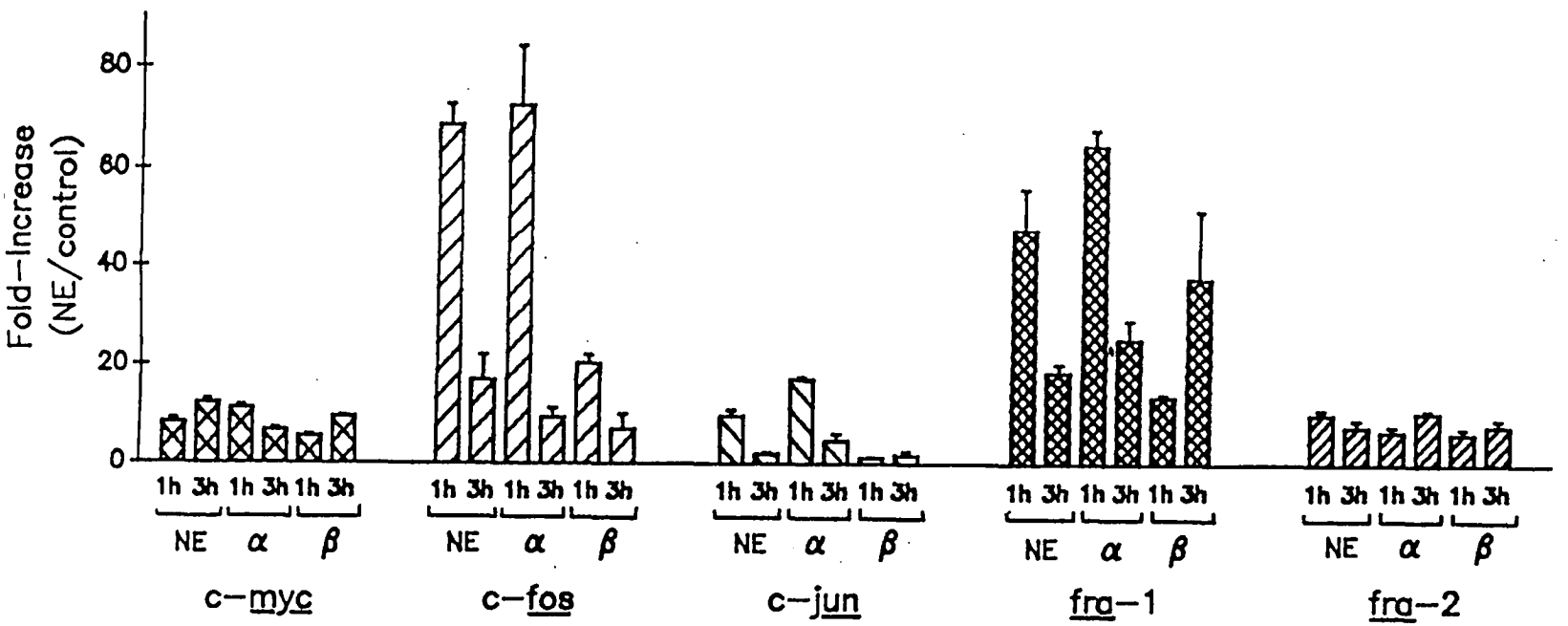

Fig 3.8.Quantification of c-*fos*, c-*myc*, c-*jun*, *fra*-1 and *fra*-2 expression following α- or β-adrenergic blockade of NE-stimulated rats.
The hybridization signals obtained by northern blotting in figure 3.7 were quantitated via laser densitometry and, after standardization to β-tubulin levels in each track, were expressed as the fold increase over control (basal) signals observed in hearts from rats treated with vehicle. Basal signals for c-*fos* and c-*jun* were arbitrarily assigned the value of one. α=NE (2.5 mg/kg) + propranolol (50 mg/kg). β=NE (2.5 mg/kg) + phentolamine (25 mg/kg). Vertical bars indicate standard error mean (S.E.M.), n=3.

Rats were administered a single injection of the thyroid hormone T_3 (0.25 mg/kg) and hearts were removed for analysis as described in the methods. No significant increase in c-*myc*, c-*fos*, or *fra*-1 expression compared to vehicle injected animals was observed up to 12 h following hormone administration (Fig 3.9). In contrast *fra*-2 mRNA levels (6.0 kb transcript) appeared to be slowly upregulated by T_3 administration. Elevated *fra*-2 mRNA was first observable 3 h following treatment and by 12 h mRNA levels were similar to that observed following NE treatment. Cardiac c-*jun* transcripts in response to T_3 administration were not assessed.

3.3.2 Chronic Administration of Hypertrophic Agents *in vivo*

3.3.2.1 Chronic norepinephrine administration

Continued infusion of NE (100 μg/kg/h) by means of mini-osmotic pumps implanted in the intrascapular region of rats led to observable cardiac hypertrophy (assessed by wet weight of the whole heart) within 3 days (Fig 3.10). These results are comparable with those of others (Johnson *et al.*, 1983). No evidence of fibrous scar formation was immediately obvious in any region of the heart following this treatment (Jiang and Downing, 1990). Increased heart to body weight ratios were also accompanied by increased expression of the neonatal-specific isocontractile gene α-SkA, with increased transcript levels appearing after 2 h of infusion, rising to a maximum after 24 h and declining, but still above basal, after 72 h of NE infusion (Fig 3.11 & 3.12). Similarly, mRNA levels for the early-response genes c-*myc*, c-*fos*, c-*jun*, *fra*-1 and *fra*-2 were also elevated in response to NE infusion (Fig 3.11, Lanes 1-12 & Fig 3.12), although their expression profiles differed with respect to each other and were of lesser intensity than the corresponding levels following a single injection of NE (2.5 mg/kg) *in vivo* (Fig.3.11, Lanes 12-13). Elevated c-*fos* transcripts appeared within 1 h of NE infusion, peaking at 3 h and had returned to basal by 6 h of infusion. In contrast c-*myc*, c-*jun*, *fra*-1 and *fra*-2 exhibited more complex expression profiles. Elevated c-*myc* mRNA levels demonstrated an initial minor peak between 1 and 4 h of infusion after which transcript levels declined to basal values by 6 h before increasing again by 12 h of infusion to reach a second, more sustained peak of greater intensity between 24

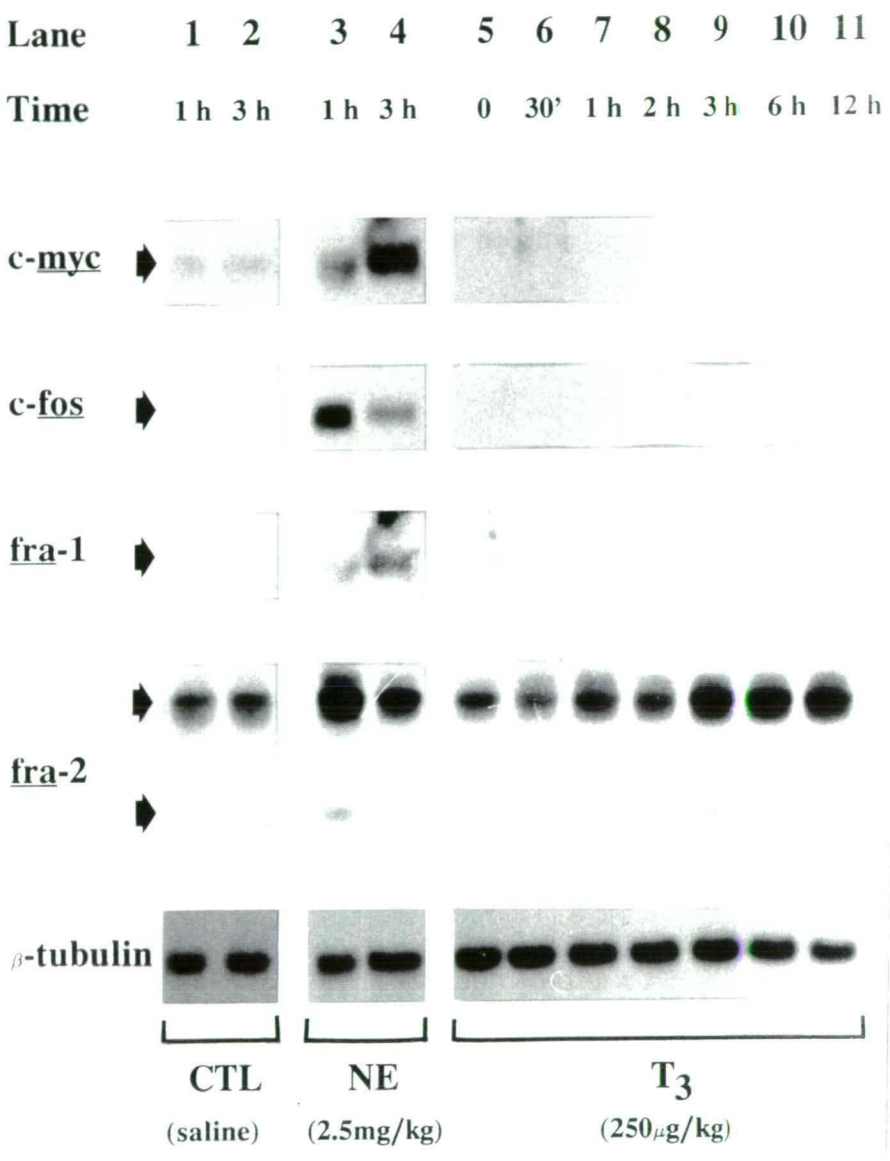

Fig 3.9. Cardiac expression of c-*myc*, c-*fos*, *fra-1* and *fra-2* in response to a single injection of triiodo-L-thyronine.

Total RNA was extracted from rat hearts removed at various times following an i.p. injection of vehicle (Lanes 1 & 2) or triiodo-L-thyronine (0.25 mg/kg, T_3, Lanes 5-12). c-*myc*, c-*fos*, *fra*-1, *fra*-2 and β-tubulin transcripts were analyzed as described previously. As a positive control, cardiac RNA obtained from rats treated i.p. 1 h or 3 h previously with norepinephrine (2.5 mg/kg, NE, Lanes 3 & 4) have been included.

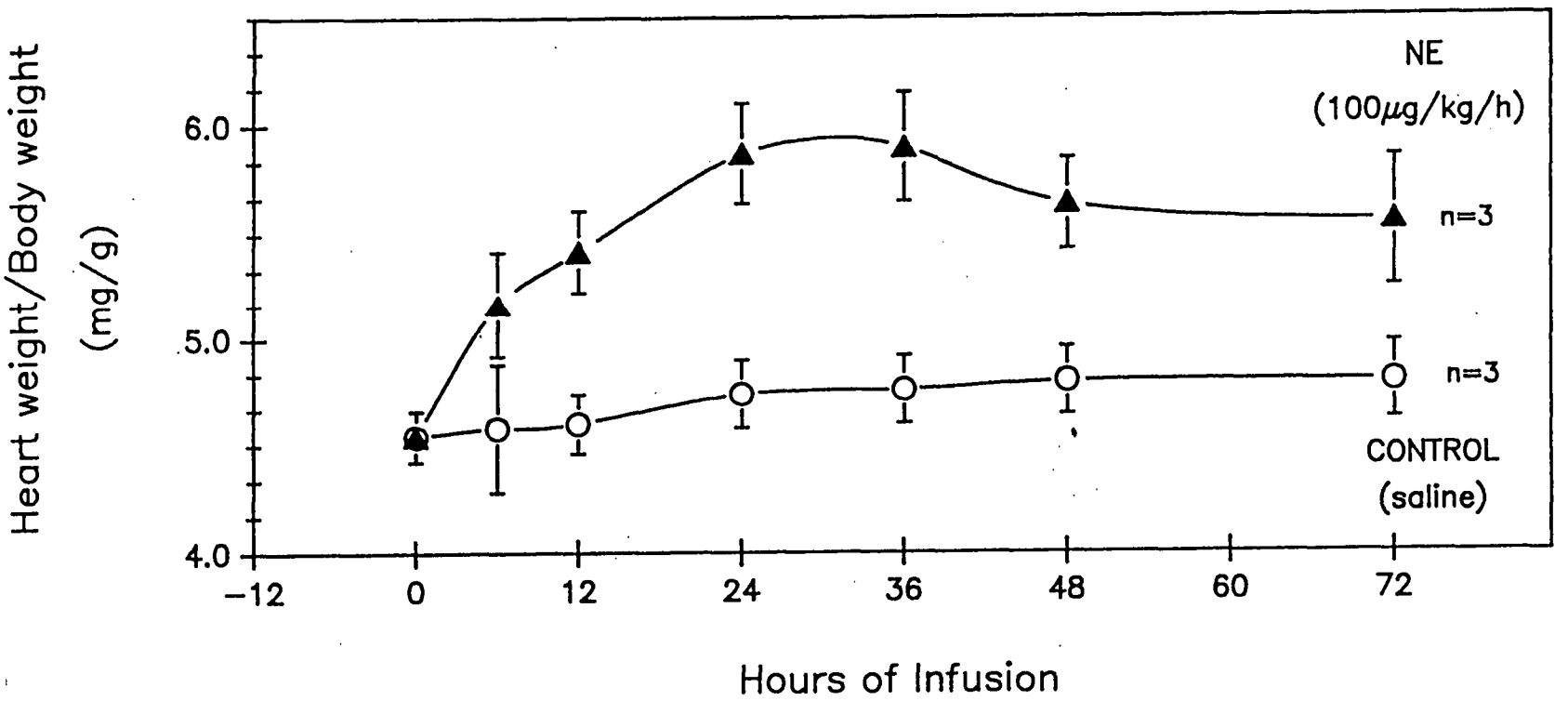

Fig 3.10. Induction of cardiac hypertrophy during chronic norepinephrine infusion.
Rats were infused continuously with norepinephrine (100 μg/kg/h) via implanted mini-osmotic pumps and after the times indicated the animals were sacrificed and heart and body weights measured. Vertical bars indicate standard error mean (S.E.M.).

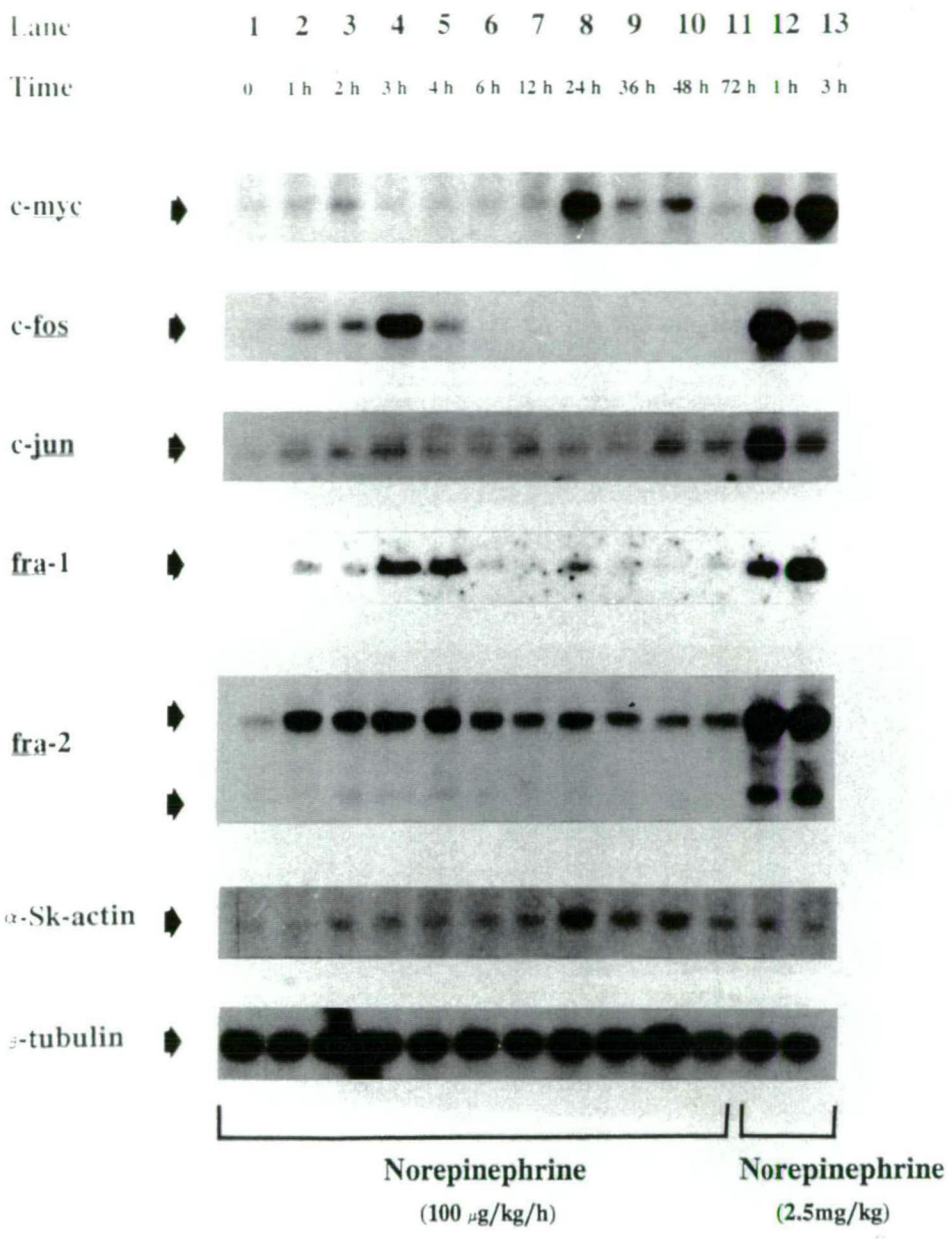

Fig 3.11 Cardiac expression of c-*myc*, c-*fos*, c-*jun*, *fra*-1, *fra*-2 and α-skeletal actin during continuous infusion of norepinephrine.

Rats were infused chronically with saline or norepinephrine (100 μg/kg/h, Lanes 1-10) for the times indicated, following which their hearts were removed and RNA isolated. c-*myc*, c-*fos*, c-*jun*, *fra*-1, *fra*-2 , α-skeletal actin and β -tubulin mRNA levels were analyzed as described previously. As a positive control, cardiac RNA obtained from rats treated i.p. 1 h or 3 h previously with norepinephrine (2.5 mg/kg, NE, Lanes 12 & 13) have been included.

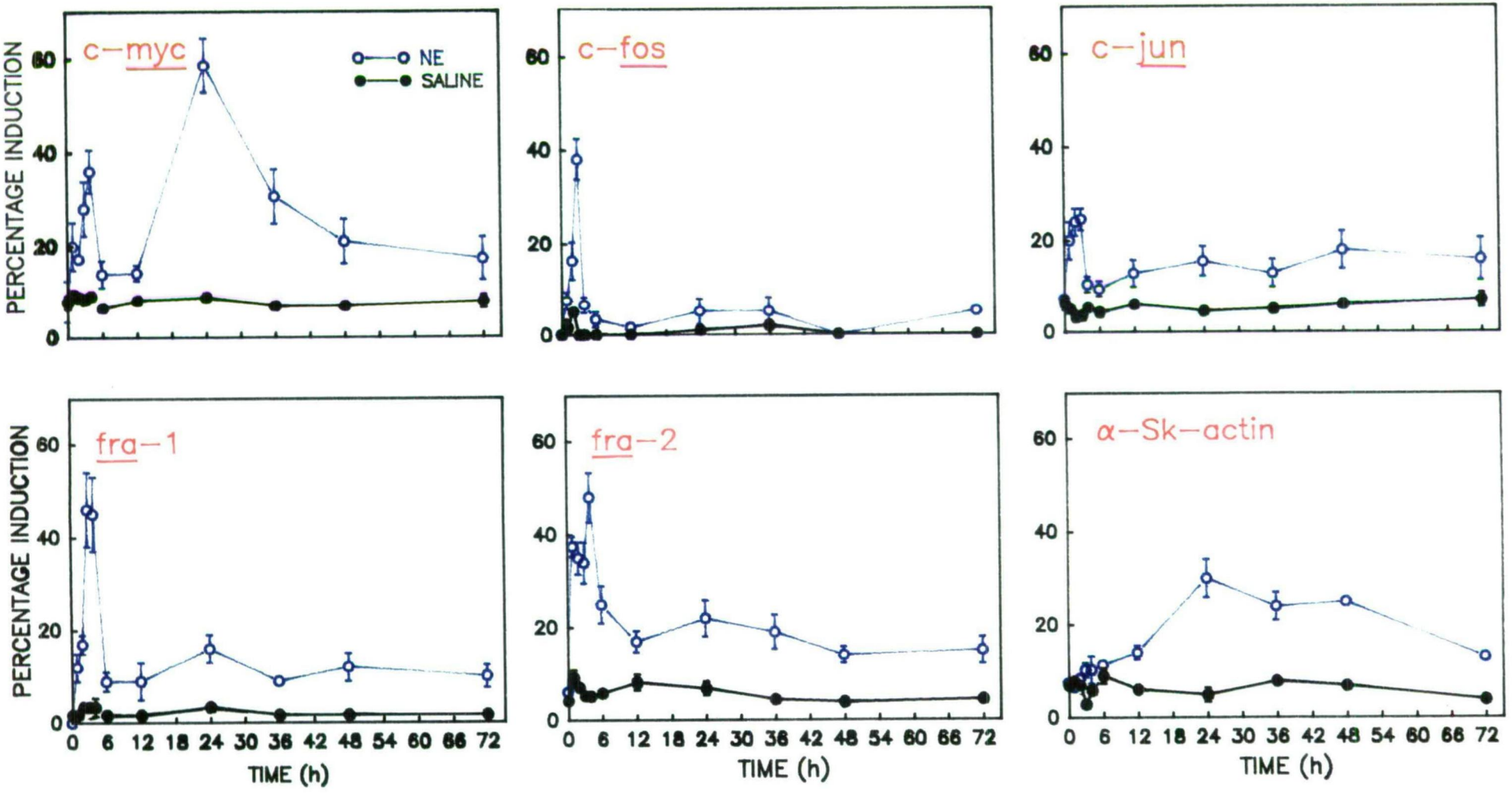

Fig 3.12 Quantification of cardiac c-*myc*, c-*fos*, c-*jun*, *fra*-1, *fra*-2 and α-skeletal actin induction during continuous infusion of norepinephrine.
The hybridization signals obtained by northern blotting in figure 3.11 were quantitated via densitometry and, after standardization to β-tubulin levels in each track, were expressed as percentage of the equivalent signal observed 1 h (c-*fos*, c-*jun*) or 3 h (*fra*-1, *fra*-2, c-*myc*) after acute norepinephrine administration (2.5 mg/kg). Vertical bars indicate standard error mean (S.E.M.), n=3.

h and 48 h. By 72 h of NE infusion c-*myc* transcript levels had returned to near basal values. *fra*-1 and *fra*-2 and c-*jun* also exhibited initial transitory peaks between 1 and 6 h of infusion of NE after which the level of these genes declined but remained above basal even after 72 h of infusion. Animals infused with saline did not exhibit increased heart to body weight ratios, nor were the transcript levels for α-SkA or the early-response genes elevated significantly above levels observed in untreated animals.

3.3.2.2 Chronic T_3 administration

Rats administered a daily injection of T_3 (0.2 mg/kg) exhibited significant increases in heart weight and heart to body weight ratios within 2-3 days (Fig 3.13) and these results are similar to those obtained by others (Sanford *et al.*, 1978; Clarke and Ward, 1983). Increased cardiac mass was accompanied by increased expression of the isocontractile gene α-MHC (Fig 3.14), an event which can be used as a marker for the hyperthyroid state and is characteristic of thyroid hormone-induced cardiac hypertrophy in the rat (Everett *et al.*, 1984; Dillmann *et al.*, 1989). Similar increases in α-MHC transcripts were also observed following daily administration of T_3 at doses as low as 2-20 μg/kg/day although changes in cardiac mass were not analyzed at these doses of T_3. However, no change in the level of c-*fos* and c-*myc* mRNA was observed compared to control animals at any of the time points studied during T_3 administration (Fig 3.14). c-*jun* *fra*-1 and *fra*-2 mRNA levels were not assessed following chronic treatment with T_3.

3.4 DISCUSSION

The nuclear-acting, early-response genes encode known or putative transcription factors, the induction of which are thought to modulate gene transcription during normal growth and differentiation (reviewed in Angel and Karin, 1991). Recently it has been proposed that these genes may also play an active role in the postnatal growth of the heart (Simpson, 1988 B), a process in which myocytes increase in size without concomitant cell division (Clubb and Bishop, 1984; Zak, 1974 A and B). This study has examined whether hormonal agents such as NE and T_3 which promote

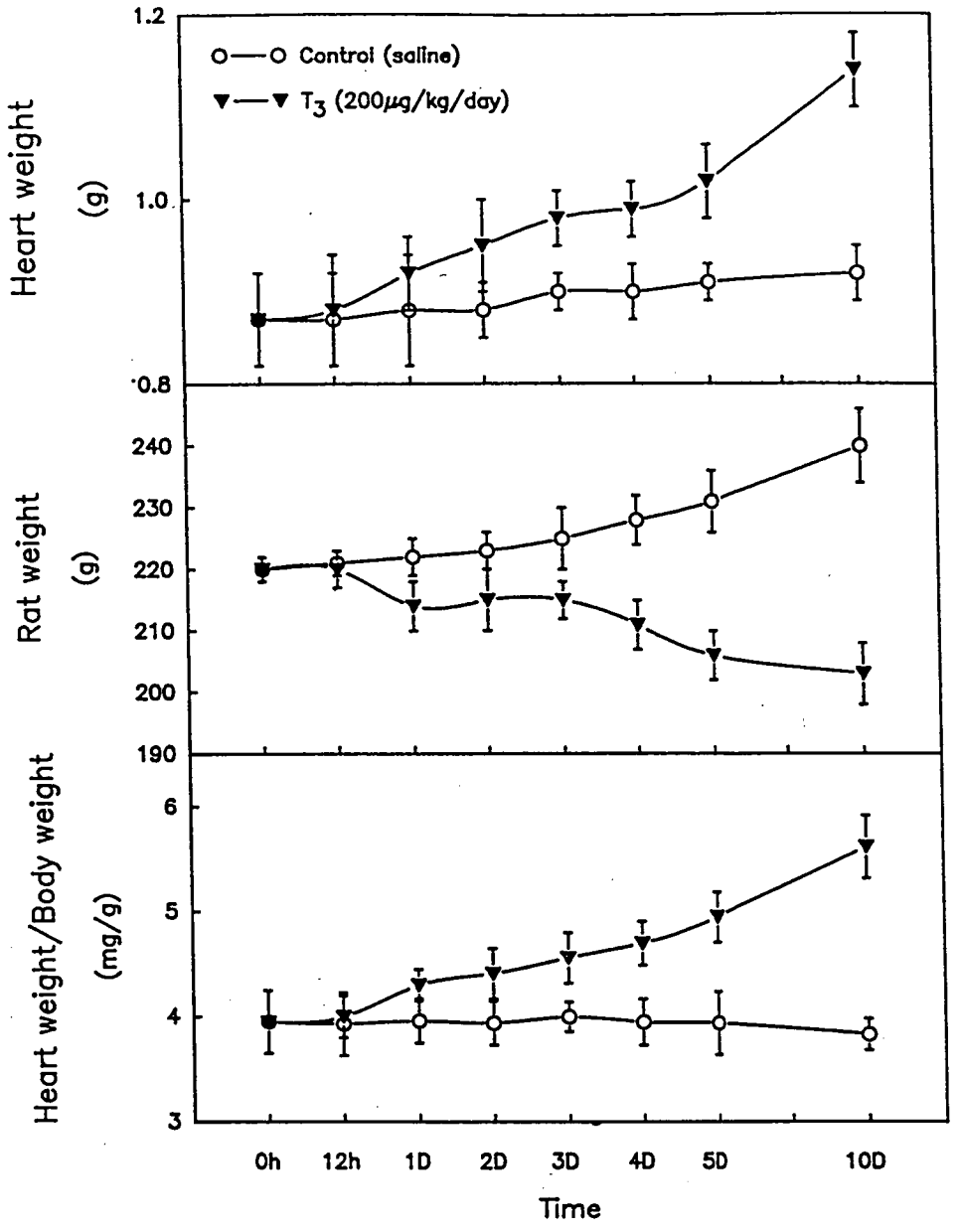

Fig 3.13. Induction of cardiac hypertrophy in response to daily injections of triiodo-L-thyronine.
Rats were administered a daily injection of T_3 (0.2 mg/kg) and at various times indicated body and heart weights were measured and expressed as a ratio of heart weight to body weight. Vertical bars indicate standard error mean (S.E.M.), n=3.

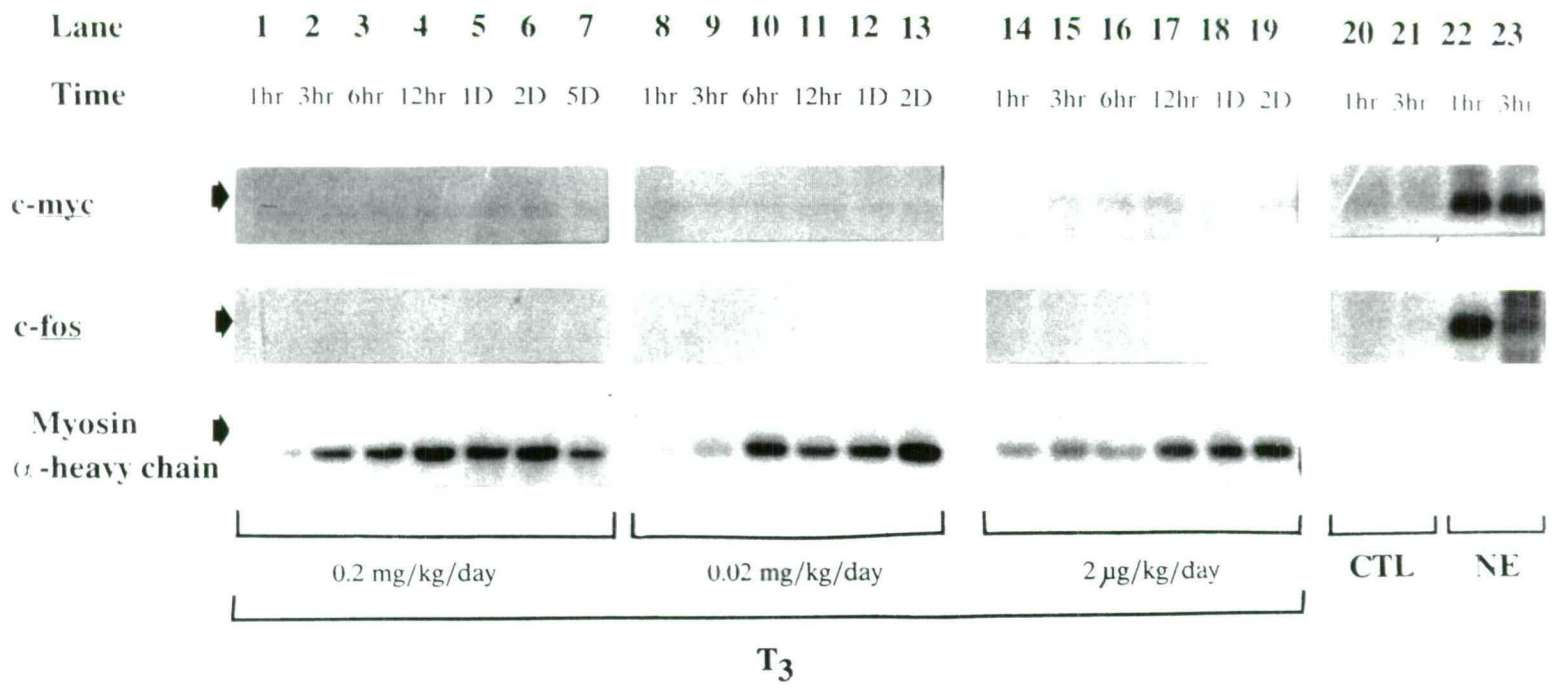

Fig 3.14. Cardiac expression of c-*myc*, c-*fos*, and myosin α-heavy chain in response to various doses of triiodo-L-thyronine.
Total RNA was extracted from rat hearts removed at the various times indicated following daily injections of triiodo-L-thyronine of 0.2 mg/kg/day (Lanes 1-7), 0.02 mg/kg/day (Lanes 8-13) and 2 μg/kg/day (Lanes 14-19). c-*myc*, c-*fos*, and myosin heavy chain transcripts were analyzed as previously described. As positive controls cardiac RNA extracted from rats treated with an i.p. injection of vehicle (CTL, Lanes 20 & 21) or norepinephrine (NE, 2.5 mg/kg, Lanes 22 & 23) was included.

cardiac hypertrophy will influence the expression of early response genes in the adult heart *in vivo*.

i) induction of the early-response gene program in response to NE administration.

In this study c-*myc*, c-*jun* and *fra*-2 mRNAs were constitutively expressed at very low levels in the hearts of untreated adult rats. It is possible that this basal expression is localized to cells that are actively and continuously dividing such as fibroblasts or endothelial cells since c-*myc*, and the *fos*-related gene family have been associated with cellular proliferation (Angel and Karin, 1991). Interestingly expression of c-*fos* and *fra*-1 mRNAs were not observed in untreated adult hearts and taken with the above results is clear evidence that differential expression of the early-response gene program contributes to the function of adult hearts during normal growth.

However the administration of a single injection of the hypertrophic agent NE (2.5 mg/kg) led to a rapid and transient increase in the cardiac mRNA levels for all of these genes with respect to control animals. The observed responses were not due to stress of the injection since little or no elevation in early-response gene expression was observed following an intraperitoneal (i.p.) injection of isotonic saline. Similarly increased gene expression cannot be explained simply as a result of the relatively large NE administration since the expression of some of these genes was characterized following doses of NE as low as 2.5 μg/kg. The majority of experiments however, were conducted with the higher dose of adrenergic agents since the responses were qualitatively similar to that of the lower dose, and also because others have shown that significant cardiac hypertrophy can result following repeated daily injections of adrenergic agents in the range 2.5-5 mg/kg (Clarke and Ward, 1983). These results are consistent with the hypothesis that early-response gene expression might play an important role in the adaptive response of the heart to NE stimulation.

The sequential pattern of early-response gene expression following NE administration (e.g. c-*fos* preceding c-*myc* induction) was generally similar to that observed in other cell systems in response to mitogen stimulation (reviewed in Curran *et al.*, 1989). However the duration of these responses appeared to be slightly longer than has been observed previously in response to acute stimulus (Angel and Karin, 1991).

For instance, the transient c-*fos* mRNA response peaked around 1-2 h rather than 30-60 min as is commonly observed in other systems. Similarly, *fra*-1 and *fra*-2 mRNA levels were still significantly elevated above basal 6 h following NE treatment. The reason for this relatively sustained response is not clear since it can be expected that NE injected i.p. would be rapidly turned over and cleared from the animals system. These findings may indicate that multiple cell types are contributing to this response and demonstrates that even brief exposure of the heart to the trophic hormone NE, can have a lasting effect on this tissue in terms of gene expression. This finding is in accordance with the notion that these nuclear-acting genes couple short term signals elicited by cell surface stimuli to long term alterations in cellular phenotype (Curran, 1991) and is supportive of the proposed role for these genes in NE-mediated cardiac hypertrophy (Starksen *et al.*, 1986; Lee *et al.*, 1988).

There are no previous reports demonstrating expression of *fra*-1 and *fra*-2 expression in the heart or cardiac myocyte preparations and the observations here are of particular significance since different members of the *fos* and *jun* families have distinct transcriptional properties (Angel and Karin, 1991), thus changes in the composition of their protein heterodimer complexes (AP-1) in the heart may be one means by which the sequential and diverse expression of target genes involved in the hypertrophic response are coordinated. In this regard it is of interest to note that at least two different *fra*-2 mRNA transcripts are observed during northern analysis and this has been observed in other tissues previously (Yoshida, *et al.*, 1993). The higher molecular weight *fra*-2 transcript but not the lower molecular weight transcript is detectable at low levels in basal heart tissue but both transcripts are readily induced following acute NE administration. The size difference between the two transcripts is attributable to the heterogeneity of the 3'-end, probably reflecting utilization of different polyadenylation sites (Yosida, *et al.*, 1993). It will be interesting to to determine their physiological relevance in terms of cardiac function.

In the present studies a single administration of NE caused transient increases in the cardiac levels of early-response genes. However a continuous exposure of NE is required for up to 24 to 48 h before overt hypertrophy can be observed *in vivo*. It is likely then that if early-response genes play a trans-activating role in this process, that they should also be expressed either during, or immediately prior, to this

hypertrophic growth period. Accordingly early-response gene expression was examined in rat hearts actively hypertrophying as a result of chronic infusion of NE. Rats were infused at a rate of 100 μg/kg/h since this treatment is a commonly used model producing cardiac hypertrophy within 24-48 h (Johnson *et al.*, 1983), a finding which was confirmed in the present experiments. Cardiac hypertrophy was preceded by transient increases in all early-response genes examined and this response was qualitatively similar to that observed in response to a single administration of NE further supporting the conclusion that early-response gene expression was not limited to a bolus, high dose injection of NE. In addition, a second significant and sustained increase in c-*myc* mRNA levels was observed between 24 h and 72 h of NE infusion and this was of particular interest since it coincided with observable increases in cardiac mass and increased expression of the isocontractile gene α-SkA. In neonatal cardiac myocytes elevated c-*myc* expression has been associated with increased expression of the fetal gene program including reexpression of α-SkA and β-MHC (Simpson, 1990) and taken together with the present results, suggests that induction of the early-response gene and fetal isocontractile gene programs are a conserved response of both the neonatal myocytes and the adult heart to NE stimulation.

However induction of the early-response gene program is not associated with all forms of cardiac hypertrophy (Izumo *et al.*, 1987; Izumo *et al.*, 1988). For example, treatment of rats with a single injection of T_3 did not increase cardiac mRNA levels for c-*myc*, c-*fos*, or *fra*-1 although continued daily injections of this dose of T_3 caused significant cardiac hypertrophy and up regulation of α-MHC. It is important to note in this respect that T_3 is removed from the body at a much slower rate than NE and thus repeated daily injections can be considered as a single chronic treatment rather than a series of acute treatments These results are in agreement with those of a later study which also found no increase in myocardial c-*myc* levels following T_3 administration (Green *et al.*, 1991). In direct contrast, acute T_3 administration resulted in a gradual upregulation of the constitutively expressed *fra*-2 transcript. The physiological significance of this is not known but it is interesting to speculate that this gene may play a transducing role in thyroid hormone-induced hypertrophy. Further studies are needed to confirm this preliminary and intriguing result.

ii) early-response gene expression is primarily an α -adrenergic response

In addition to temporal differences in expression, these genes also exhibited differential responses to the α- and β-components of NE action. For instance, elevation of c-*fos* and c-*jun* following α-adrenergic stimulation was significantly greater than that induced by β-adrenergic agents as shown by blockade of the α-and β-components of NE respectively. c-*fos* has previously been reported to be expressed in cardiac tissue *in vivo* following α- and β-adrenergic stimulation (Moalic *et al.*, 1989; Barka *et al.*, 1987), however these experiments did not establish the relative contribution of the α- and β-receptors to this response or characterize the temporal pattern of expression of this gene.

In contrast to the above, c-*myc*, *fra*-1 and *fra*-2 also have a significant β-adrenergic component to their response. Elevation of c-*myc* in response to α-adrenergic stimulation in the adult, *in vivo* model is not unexpected since both hypertrophy and c-*myc* expression can be induced in cultured neonatal myocytes by an α_1-adrenergic mechanism (Starksen *et al.*, 1986). However, c-*myc* levels in cultured neonatal myocytes are not increased by β-adrenergic agents (Starksen *et al.*, 1986) therefore the increased c-*myc* expression observed in these experiments following β-stimulation may indicate that the gene induction is occurring via a secondary mechanism or in a non myocyte cell type. Alternatively the discrepancy may be due to the well documented differences in α- and β-receptor numbers between adult and neonatal cardiac tissue (Graham and Lannier, 1986; Schaffer and Williams, 1986; Longbaugh *et al.*, 1986).

It is interesting to note that the time course of c-*myc* and *fra*-1 expression is dependent on the particular component of adrenergic action: maximal c-*myc* and *fra*-1 mRNA levels were observed at 1 h, 2 h and 3 h in response to α-, αβ- and β-adrenergic treatment. This differential response might be due to the distinct intracellular signaling mechanisms activated by the α- and β-adrenergic receptors (Brown and Jones, 1986; Homcy and Graham, 1985; Tomlinson *et al.*, 1985) or alternatively might be a further indication that the β-adrenergic response of c-*myc* and *fra*-1 involves a secondary pathway perhaps involving several cell types and growth factors. Another possibility is

that c-*myc* and *fra*-1 transcription is elevated by α-adrenergic stimulation, an event that would produce a rapid rise in c-*myc* and *fra*-1 mRNAs, whilst β-adrenergic agents increase c-*myc* and *fra*-1 mRNA accumulation by reducing their degradation. Regulation of c-*myc* expression in other systems at least, is known to include both transcriptional and post-transcriptional mechanisms (Alitalo *et al.*, 1987) and resolution of these possibilities will require a detailed examination of c-*myc* and *fra*-1 transcript synthesis and turnover. Finally, it is conceivable that a significant proportion of the early response gene expression following β-agonist treatment is actually mediated by increased release of NE from sympathetic nerve endings induced by stimulation of the presynaptic β_2-receptors (Simpson, 1985), and consequently would depend on postsynaptic α-adrenoceptor stimulation. However in these studies early-response gene expression was observed during β-adrenergic stimulation even when the α-adrenergic receptors were blocked with the α-antagonist phentolamine. It is possible but unlikely that this treatment did not adequately block the α-adrenergic receptors during NE administration since all animals were pretreated twice, 1 h and 10 min prior to NE administration with a 10 fold excess of α-adrenergic blocker.

iii) NE and pressure-overload hypertrophy: common and distinct effects on early-response gene expression

Recent studies with cell cultures indicate that c-*myc*, c-*fos* and c-*jun* are inducible in neonatal myocytes following NE treatment (Starksen *et al.*, 1986; Iwaki *et al.*, 1990). Thus it is possible that at least part of the responses observed in the present *in vivo* experiments with adult rats are a result of direct NE activation of cardiac myocyte adrenergic receptors (Bruckner *et al.*, 1985; Buxton and Brunton, 1986; Kauman and Lemoine, 1987; Brodde, 1987). However, in addition to cardiac myocytes, α_1- and β-receptors are located on a number of other cell types including smooth muscle of the coronary and peripheral vascular systems where they mediate vasoconstriction and vasodilation respectively (Hyman, 1986). It is well established that high doses of NE or moderate doses of α_1-agonists lead to increased vascular resistance and pressure load on the heart (Zierhut and Zimmer, 1989) and pressure overload itself can cause increased cardiac expression of c-*myc*, c-*fos*, c-*jun* during aortic stenosis (Black *et al.*,

1991; Rockman *et al.*, 1991; Izumo *et al.*, 1988). It is not possible in the present study to determine whether the α-adrenergic stimulated early-response gene expression is mediated in part or wholly by a secondary pressure response and further experiments in which changes in hemodynamic parameters are controlled need to be conducted. Nevertheless the fact that continuous subcutaneous infusion of NE at the dose used in these chronic infusion experiments, leads to a rapid and sustained elevation of systolic blood pressure (Johnson *et al.*, 1983) is supportive of a pressure component in the early-response gene program.

However, it is important to note that although the activation of the early-response gene program following NE administration appears to be similar to that documented in response to pressure-overload, significant temporal and qualitative differences in this induction process are observable. For instance, c-*fos* induction following chronic NE infusion is similar to that observed following a single administration of NE with mRNA levels returning to basal by 3-6 h. In contrast, c-*fos* levels in pressure overloaded hearts remain elevated for up to 24 h following aortic stenosis (Yazaki *et al.*, 1989). The results presented here thus indicate that continued expression of c-*fos* is not necessary for NE mediated cardiac hypertrophy and re-expression of the neonatal gene program to occur. Furthermore, pressure overloaded rodent hearts do not express *fra*-1 (Rockman *et al.*, 1991) yet this gene is upregulated in animals treated with both acutely and chronically with NE. Thus the adrenergic induction of this gene observed here is probably a direct response to cardiac α- and β-receptor stimulation independent of load. c-*myc* expression also differs between pressure overloaded hearts and NE-treated hearts since expression of this gene is bi-phasic during chronic NE infusion whereas only one sustained peak is observed following aortic stenosis (Yazaki *et al.*, 1989). Again this stimulus-specific behavior may reflect different physiological outcomes of each treatment. Clearly, to resolve these differences, further *in vivo* studies are needed in which early-response gene expression and subsequent phenotypical changes (e.g. isocontractile genes) are examined in response to subhypertensive doses of adrenergic agents and compared to those changes which occur in response to high doses of these hormones or pressure overload.

In summary the results presented in this chapter demonstrate that an array of early-response genes are rapidly and sequentially induced in the adult rat heart following adrenergic stimulation and that this response occurs via both the α- and β-adrenergic receptors. Furthermore, the expression of some of these genes is not transient but is sustained at high levels during the period when overt cardiac hypertrophy occurs and at the same time that the neonatal gene program is reactivated. These results are supportive of the hypothesis that induction of the early-response gene program is associated with, and may be necessary for, the modulation of the program of late response gene expression (e.g. isocontractile genes) that is characteristic of adrenergic-, but not thyroid hormone-mediated cardiac hypertrophy. Since some of the early response genes such as c-*myc* and c-*fos* are modulated by both NE and pressure-overload but others such as *fra*-1 are only sensitive to NE treatment, these studies also provide the first evidence that adrenergic agents and pressure-overload can activate both common and distinct subsets of these trans-activating genes.

CHAPTER 4

REGIONAL AND CELLULAR LOCALIZATION OF EARLY-RESPONSE GENE EXPRESSION *IN VIVO*

4.1 INTRODUCTION

Results presented in the previous chapter clearly demonstrated that administration of NE *in vivo* leads, either directly or indirectly, to a rapid activation of the early-response gene program in the rat heart. It is likely that at least part of this response can be attributed to the cardiac myocytes since others have shown that early-response genes are expressed in neonatal myocyte cultures following adrenergic treatment (Lee *et al.*, 1988; Starksen *et al.*, 1986). However, at least 70% of the cells in the heart are not myocytes, but those associated with neuronal, vascular and fibroblastic tissue of which any or indeed all may contribute to the cardiac early-response gene expression observed *in vivo*. For instance, it has been shown that c-*myc* and c-*fos* are induced in vascular smooth muscle cells during culture and whole aorta by vasoactive agents including NE and ANG II indicating that an analogous response may occur in the intact heart (Naftilan *et al*, 1989; Moalic *et al,* 1989). One necessary step then, in the establishment of a direct association between early-response gene expression in the heart and NE mediated hypertrophy of adult myocytes is to localize the mRNAs for these genes to specific cell types following adrenergic treatment and to demonstrate that they are translated to their cognate protein products. Further evidence might stem from the ability to correlate regional localization of this expression with the differential growth of the cardiac chambers during NE mediated cardiac hypertrophy. Already preliminary evidence for such a correlation has been obtained from pressure-overload models of cardiac hypertrophy since experimental aortic stenosis leads to not only a greater cardiac muscle growth of the left ventricle but also increased early-response gene expression in this chamber (Mulvagh *et al.*, 1987; Komuro *et al.*, 1988).

In an attempt to address some of these points, the work in this chapter has assessed regional and cellular expression of the early-response gene families in the heart

following NE stimulation, utilizing a combination of hybridization histochemistry (*in situ* hybridization) and immunocytochemical techniques. In addition, regional expression of early-response gene mRNA was also detected on a gross scale by dissecting hearts obtained from experimental animals into the various component chambers and analyzing their mRNA levels separately by northern analysis.

4.2 EXPERIMENTAL PROTOCOLS

4.2.1 Hybridization Histochemistry

For detection of metallothionein gene (Mt) expression rats were administered a single injection of either cadmium chloride (Cd, 10 μg/kg) or dexamethasone (DEX, 1 μg/kg) dissolved in 0.9% saline. Control animals were similarly injected with saline alone. After 4 or 6 h animals were sacrificed and livers were removed and prepared for northern analysis or hybridization histochemistry as described in section 2.3. ANP and calcitonin mRNA was detected in hearts removed from untreated adult rats as described in section 2.3. For detection of c-*myc* and c-*fos* mRNA, animals first received a single injection of NE (2.5 mg/kg) or saline and were sacrificed 1 to 3 hours later. Hearts were subsequently removed and processed for northern analysis and hybridization histochemistry as before.

4.2.2 Regional Localization of Early-Response Gene Expression by Northern Analysis.

Rats were treated with a single injection of NE (2.5 mg/kg) or saline as described previously and sacrificed after the appropriate time. Hearts were immediately removed and quickly dissected into the various chambers, rinsed in saline and snap-frozen in liquid nitrogen to await RNA extraction and northern analysis (section 2.2). Due to their small mass, left and right atria from up to five animals were combined for each experimental time point. Results presented are representative of at least 3 separate experiments.

4.2.3 Cellular Localization of Myc and Fos Protein by Immunocytochemistry.

Rats were administered a single injection of NE (2.5 mg/kg) as described in section 3.2.2.2. After the appropriate time animals were sacrificed, hearts removed and processed for immunocytochemistry using anti-early-response gene antibodies as described in section 2.4. Results are representative of at least 3 separate experiments.

4.3 RESULTS

4.3.1 Hybridization Histochemistry

Since hybridization histochemistry had not been performed previously in the present laboratory, a suitable protocol was devised using a number of trial oligonucleotide and cDNA probes which had been successfully used for *in situ* detection of mRNA by other laboratories Detection of Mt was chosen as a suitable experimental trial since; 1) it is easily induced to high levels in the kidney and liver of rats in response to heavy metal or glucocorticoid treatment; 2) its mRNA remains induced for a relatively long period of time giving plenty of opportunity for its detection and finally; 3) specific Mt-specific oligonucleotide and cDNA probes were readily available. The second trial experiment was designed specifically to optimize conditions for the detection of mRNA in cardiac tissue *in situ*. Atrial naturetic peptide (ANP) was an obvious choice for this study since its expression in the atria of the heart has been well characterized and its relative absence from adult ventricles means that these chambers may act as internal negative control tissue. For a negative control probe, the calcitonin gene was chosen since its expression is not detectable in the rat heart (J. Penschow, personal communication.).

4.3.1.1 Detection of Metallothionein mRNA in rat liver

Mt oligonucleotide and cDNA probe specificity was confirmed by northern analysis. Mt mRNA was not detectable in basal liver tissue but was rapidly induced 4 to 6 h following a single injection of Cd (10 μg/kg) or DEX (1 μg/kg) (Fig. 4.1). Both oligonucleotide and cDNA radiolabeled probes were specific for Mt mRNA since they hybridized to the expected 300 nucleotide transcript. However the cDNA probe exhibited the more intense hybridization signal even though both probes were

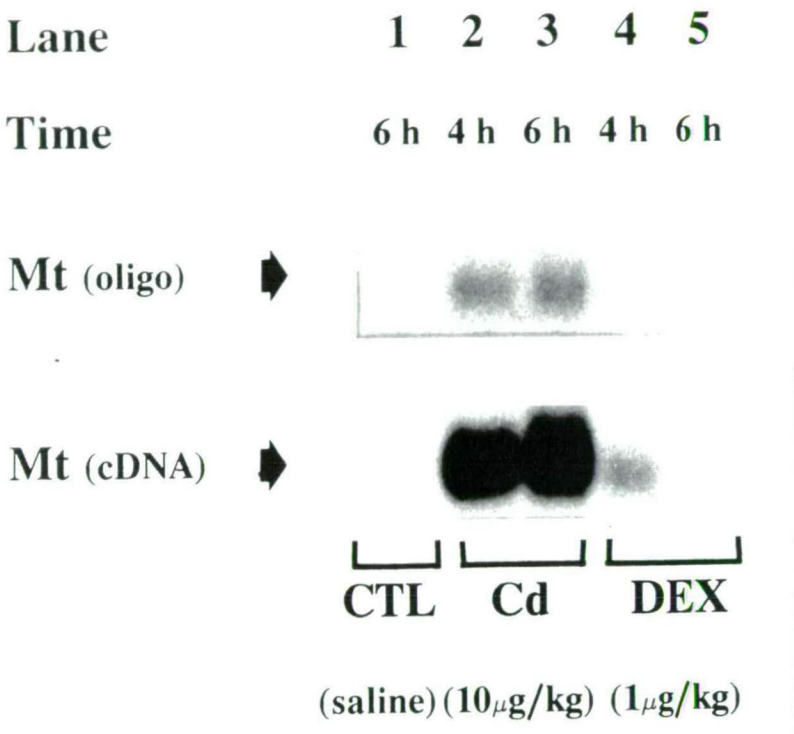

Fig 4.1 Hybridization of metallothionein-specific cDNA and oligonucleotide probes to rat liver following a single injection of cadmium chloride or dexamethasone.
Total RNA was extracted from rat liver at the various times indicated following an i.p. injection of 0.9% saline (Lane 1), cadmium chloride (10 μg/kg, Lanes 2 & 3) or dexamethasone (1 μg/kg, Lanes 4 & 5). After electrophoresis and northern blotting, the RNA (50 μg) was hybridized with a metallothionein specific oligonucleotide probe (upper track) or a cDNA probe (lower track).

radiolabeled to approximately the same specific activity (1 x 10^9 cpm/μg, Fig. 4.1). It is possible that these differences in intensity of signal could be explained by sub-optimal hybridization conditions for the oligonucleotide-probe. Nevertheless, these results established that both Mt cDNA and oligonucleotide probes were potentially suitable for *in situ* detection of Mt mRNA. Cd administration was used for subsequent *in situ* studies since this treatment increased Mt expression in the liver to a greater extent than did DEX.

Mt-specific probes were subsequently hybridized *in situ* to tissue sections of liver removed from animals previously treated with Cd as described in the methods. Typical results from one such an experiment are presented in Fig. 4.2 which shows expression of Mt mRNA in liver sections detected by exposure to high resolution autoradiographic film. Hybridization is significantly greater in tissue from animals treated with Cd (lower section) compared to saline (upper section) for both oligonucleotide (Fig. 4.2, Lanes 1 & 2) and cDNA (Fig. 4.2, Lanes 3 & 4) probes. The hybridization signal is due to specific binding to mRNA rather than DNA or protein since signal accumulation is prevented when tissue is pre-incubated with a broad spectrum RNase enzyme (Fig. 4.2, Lanes 5 & 6). These experiments establish that the present experimental protocols are suitable for the *in situ* detection of specific mRNA in rat tissue using both cDNA and oligonucleotide probes.

4.3.1.2 Detection of ANP mRNA in rat heart

Hybridization histochemistry protocols were further optimized for cardiac tissue using oligonucleotide probes specific for rodent ANP and by comparison to a negative control probe specific for the calcitonin gene. Two ANP oligonucleotide probes were used for this study and their specificity for ANP was tested by northern analysis (Fig. 4.3). Both rat-specific and rat/mouse-specific probes hybridized strongly to atrial RNA extracts and this regional distribution is in accordance with the results of others (J. Penschow pers. comm.). Subsequent hybridization of the negative control probe, calcitonin, to the same RNA gave no hybridization signal and this was expected since transcripts of this gene are not detectable in heart tissue (Fig. 4.3; J Penchow pers. comm.). These results establish that the ANP and calcitonin probes are suitable positive and negative probes respectively for *in situ* analysis of cardiac mRNA.

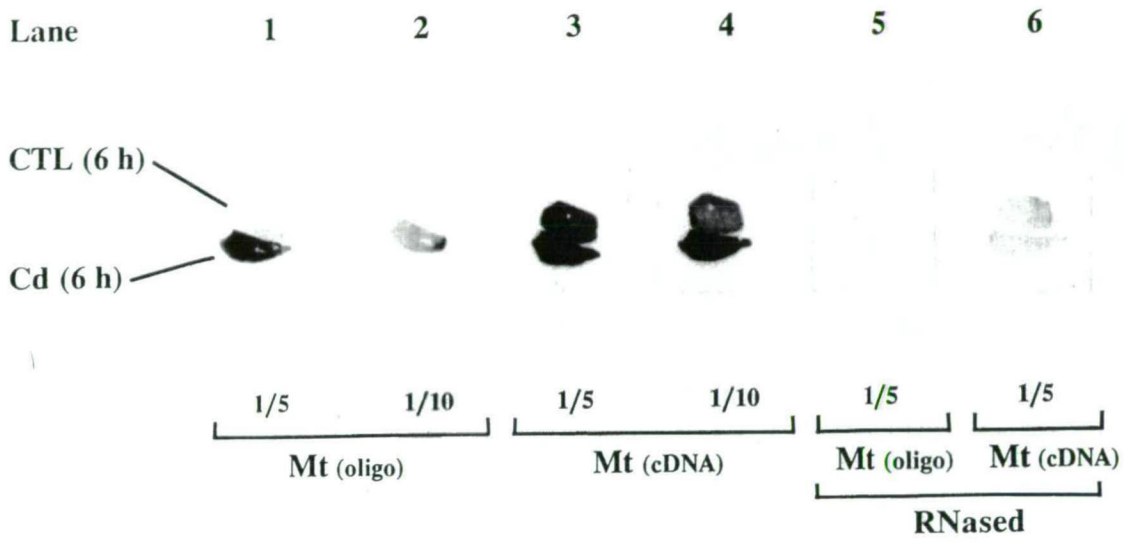

Fig 4.2 Effect of cadmium on expression of metallothionein in rat liver.
Liver was removed 6 h following treatment with either 0.9% saline (upper section) or cadmium chloride (10 µg/kg, lower section) and prepared for *in situ* hybridization (section 2.3). The sections were hybridized *in situ* with metallothionein specific oligonucleotide (Lanes 1 & 2) or cDNA (Lanes 3 & 4) probes at a 1/5 or 1/10 dilution. As a control the same probes were incubated with liver tissue pre-incubated with RNase A (Lanes 5 & 6).

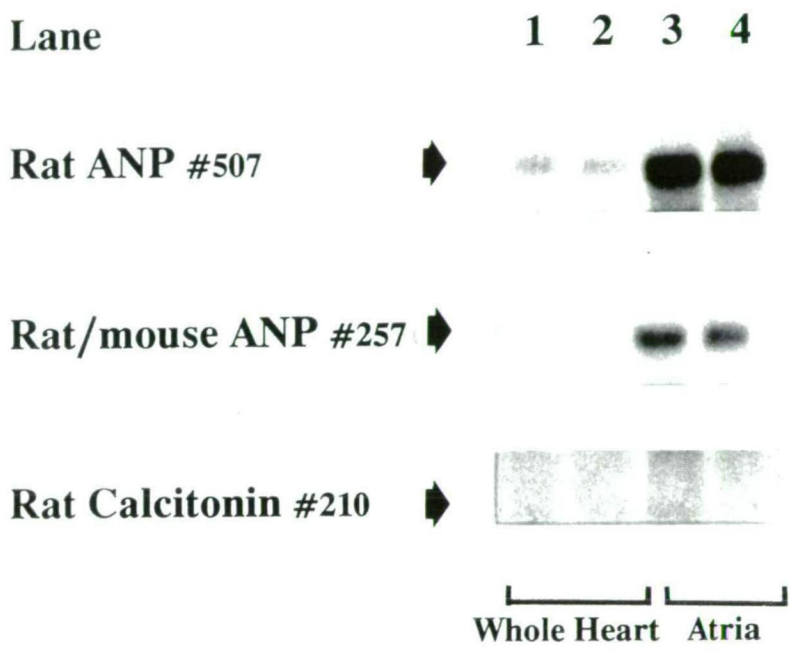

Fig 4.3 Analysis of ANP and calcitonin oligonucleotide probes in rat heart.
Total RNA was extracted from rat ventricle (Lanes 1 & 2) or rat atria (Lanes 3 & 4) and 50 µg of RNA was hybridized to two oligonucleotide probes (Rat ANP #507 and Rat/Mouse ANP #257). As a negative control the same RNA samples were hybridized to Rat calcitonin # 210, a gene not expressed in the heart.

ANP-specific probes were subsequently hybridized *in situ* to sections of heart tissue. Both rat-specific and rat/mouse-specific ANP probes hybridized strongly to atrial tissue (Fig. 4.4 & Fig. 4.5) but not to ventricular, skeletal or liver tissue (Fig. 4.4). The results cannot be explained as non-specific hybridization of probes to atrial tissue since no hybridization was observed for the negative control probe calcitonin, in this tissue (Fig. 4.4). These results established that the current *in situ* hybridization protocols were potentially suitable for the detection and localization of specific mRNA species within the rat heart and accordingly were used for the detection of early-response gene expression in this tissue.

4.3.1.3 Detection of c-*myc* and c-*fos* mRNA in rat heart

Anti-sense and sense probes to the expressed regions of c-*myc* and c-*fos* were designed and synthesized by the author as described in the methods. In addition, oligonucleotide probes specific for c-*myc* and c-*fos* were purchased from commercial sources and were used for comparison. Specificity of the probes was determined by hybridization to cardiac RNA extracted from animals treated with NE. Both c-*myc* anti-sense probes designed by the author hybridized to a 2.3 kb mRNA transcript indicating that they were indeed specific for this gene (Fig. 4.6, upper 2 tracks). The intensity of the observed hybridization signal was relatively weaker than similar hybridization with c-*myc* cDNA probes (results not shown). This difference in hybridization may once again be due to sub-optimal hybridization conditions for the oligonucleotide probes. In contrast a commercially obtained c-*myc* oligonucleotide probe did not hybridize to c-*myc* mRNA transcripts (Fig. 4.6, bottom track). This may have been due to the difficulty experienced with labeling this oligonucleotide to a high specific activity and seemed to be a problem intrinsic with this particular probe since no similar difficulty was experienced when labeling other oligonucleotide probes. As expected no hybridization was observed for the sense c-*myc* probe.

Specificity of c-*fos* oligonucleotide probes was similarly determined by northern analysis. All c-*fos* probes hybridized to a 2.2 kb mRNA transcript indicating that they were specific for this gene (Fig. 4.7, upper two tracks). In addition, the temporal pattern of the observed expression was similar to that observed following

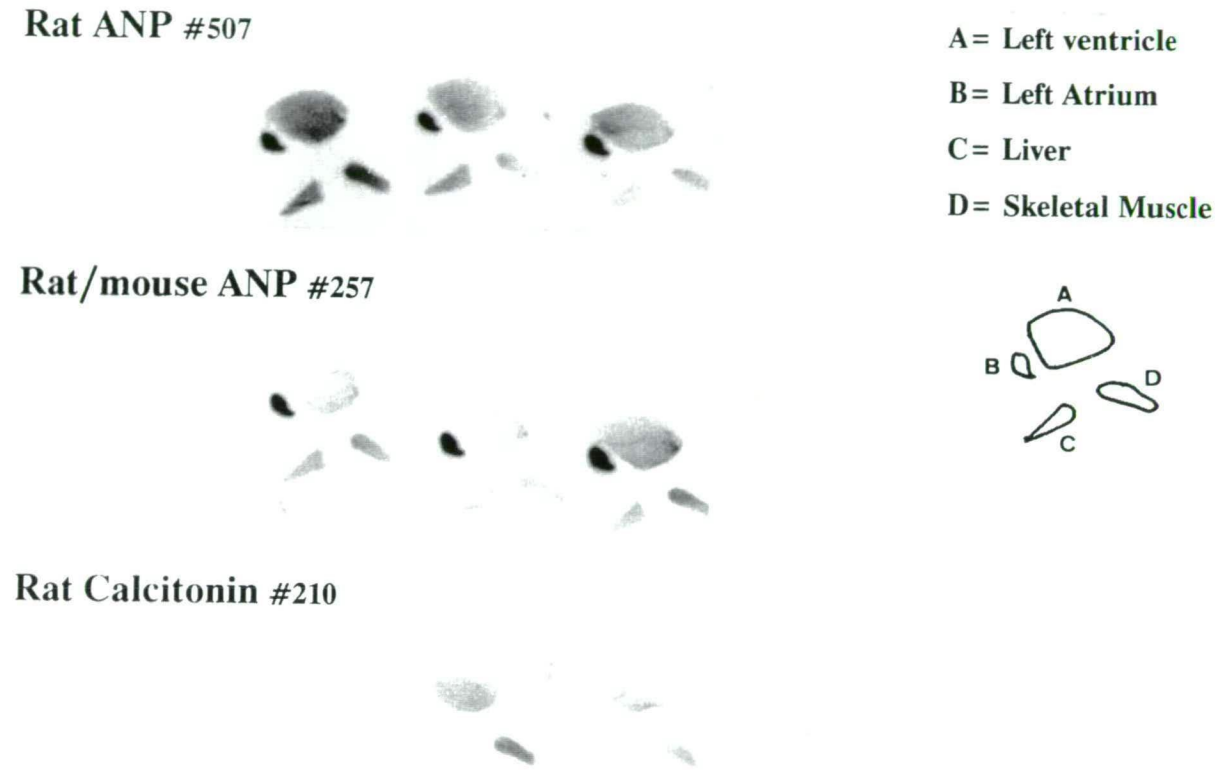

Fig 4.4 *in situ* hybridization of ANP in rat heart, liver and skeletal muscle.
Tissue sections of the left ventricle (A), left atrium (B), liver (C) and skeletal muscle (D) were prepared as described previously and hybridized *in situ* to the following oligonucleotide probes rat ANP #507 (upper panel), rat/mouse ANP #257 (middle panel) or rat calcitonin #210.

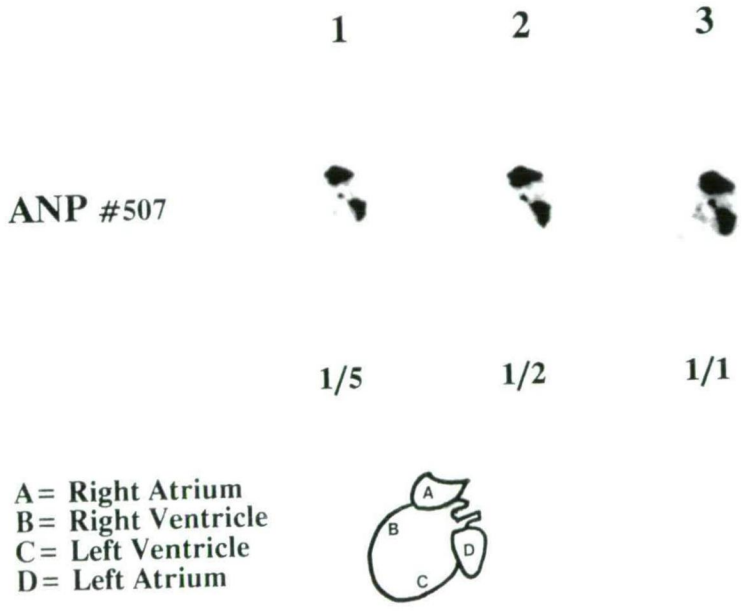

Fig 4.5 Regional localization of ANP expression in the rat heart.
Whole tissue sections of the rat heart were prepared as described previously and hybridized *in situ* to rat ANP #507 at various dilutions (1/5, 1/2, 1/1).

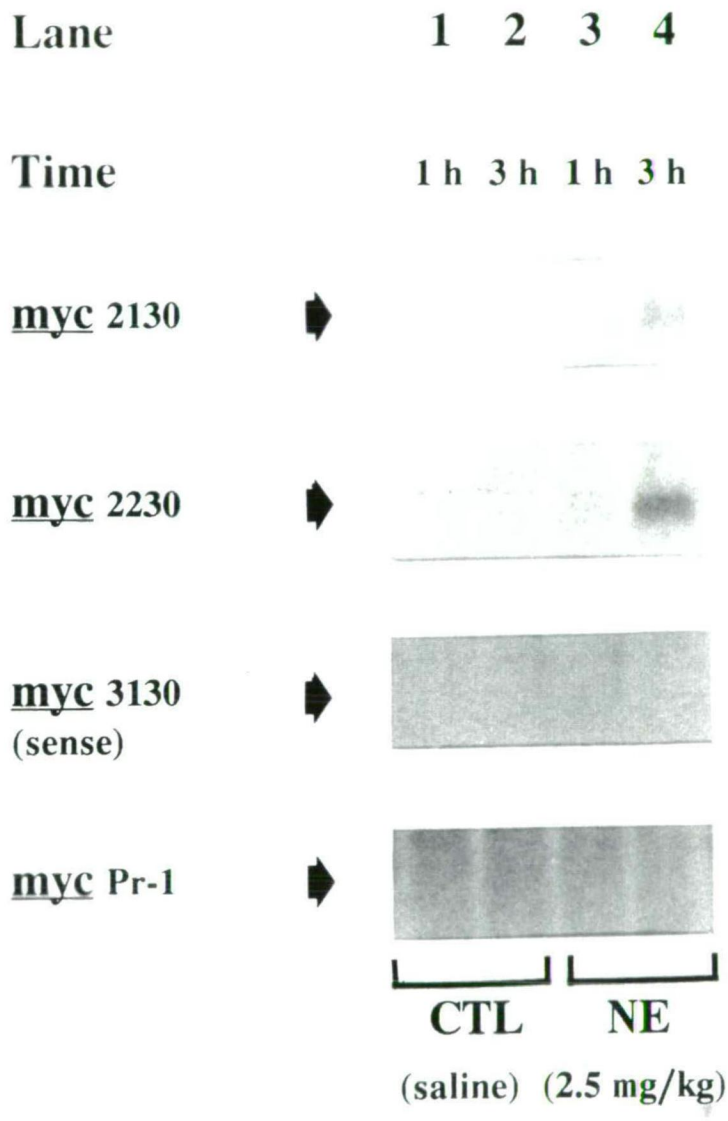

Fig 4.6 Analysis of c-*myc* oligonucleotide probes.
Total RNA was extracted from rat hearts at the various times indicated following an i.p. injection of 0.9% saline (Lane 1 & 2) or norepinephrine (2.5 mg/kg, Lanes 3 & 4). c-*myc* transcripts were analyzed using two anti-sense probes (*myc* 2130 and *myc* 2230), a sense probe (*myc* 3130) and a commercially available anti-sense probe (*myc* Pr-1).

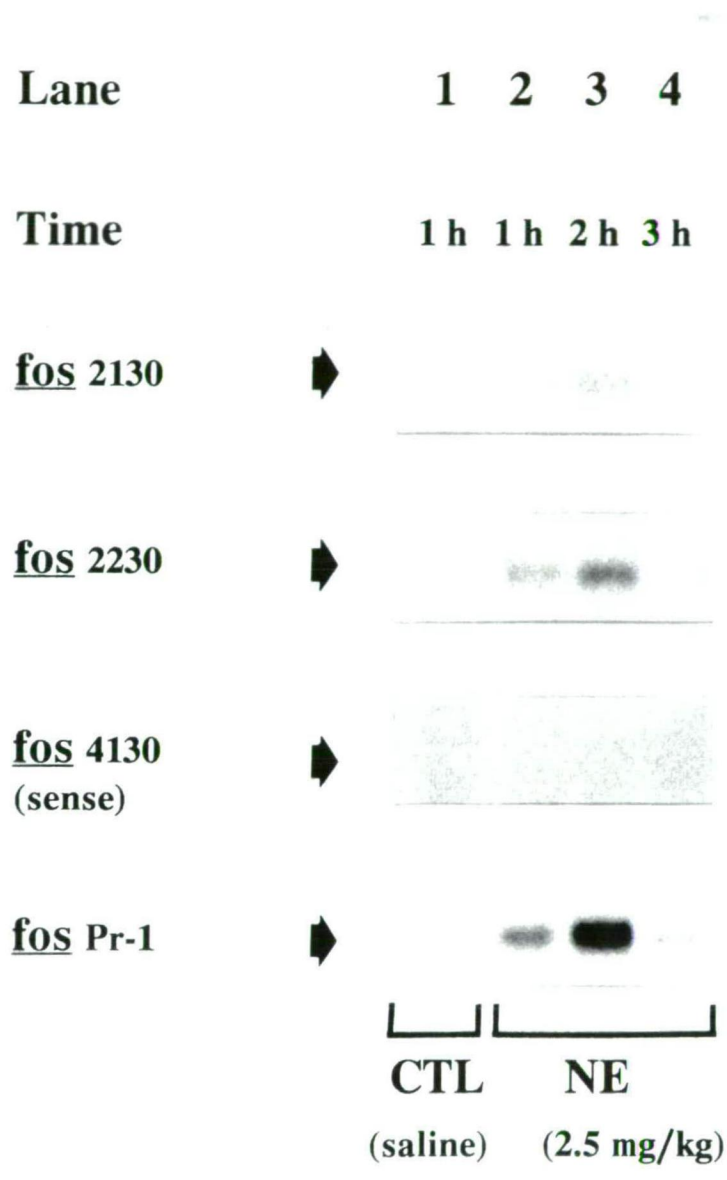

Fig 4.7 Analysis of c-*fos* oligonucleotide probes.
Total RNA was extracted from rat hearts at the various times indicated following an i.p. injection of 0.9% saline (Lane 1) or norepinephrine (2.5 mg/kg, Lanes 2-4). c-*fos* transcripts were analyzed using two anti-sense probes (*fos* 2130 and *fos* 2230), a sense probe (*fos* 4130) and a commercially available anti-sense probe (*fos* Pr-1).

hybridization with a c-*fos* cDNA probe. Hybridization signals varied considerably between probes (all probes in Fig. 4.7 were exposed for the equivalent period) with the commercially obtained *fos* Pr-1 probe consistently giving the highest sensitivity (Fig. 4.7, bottom track). As expected no hybridization was observed with the sense c-*fos* probe.

Specific c-*myc* and c-*fos* oligonucleotide probes were subsequently hybridized to sections of heart tissue obtained from animals treated previously for 1-3 h with NE. Results from a typical experiment are presented in Fig. 4.8 which shows tissue sections of experimental and control tissue which have been hybridized to *myc* and *fos* oligonucleotides and exposed to autoradiographic film. No increased hybridization was observed for NE-treated tissue (lower tissue section) compared to control tissue (upper tissue section) for any of the anti-sense c-*myc* or c-*fos* oligonucleotides synthesized by the author and this was despite considerable manipulation of the experiment protocol. As expected, no hybridization was observed for the sense probes. Since c-*myc* and c-*fos* mRNA have relatively short half-lives it was possible that the fixation technique used here allowed mRNA degradation during tissue processing. To test this possibility, previously fixed NE-treated tissue was scrapped off slides, processed for RNA extraction and northern analysis and subsequently hybridized to radiolabeled c-*myc* and c-*fos* cDNA probes. Results in Fig. 4.9 indicate that intact and hybridizable transcripts for both c-*myc* and c-*fos* were obtained from the tissue sections, thus the inability of the synthesized oligonucleotides to hybridize to c-*myc* and c-*fos* *in situ* is not due to degradation of their respective mRNA during tissue processing. Alternatively it is possible that in fact hybridization of oligonucleotide probes to target mRNA *in situ* had occurred but the hybridization signal was so weak that it was masked by the background non-specific binding. This possibility is especially likely given the weak hybridization signals observed for these probes during northern analysis. Finally it is possible that the target sequences in the transcript RNA's may not have been available for hybridization *in situ* due to secondary structure of the mRNA or due to associated proteins. Such problems would not be observable during northern analysis since RNA is stripped of any associated protein and efficiently linearized by the RNA extraction and northern blotting procedures.

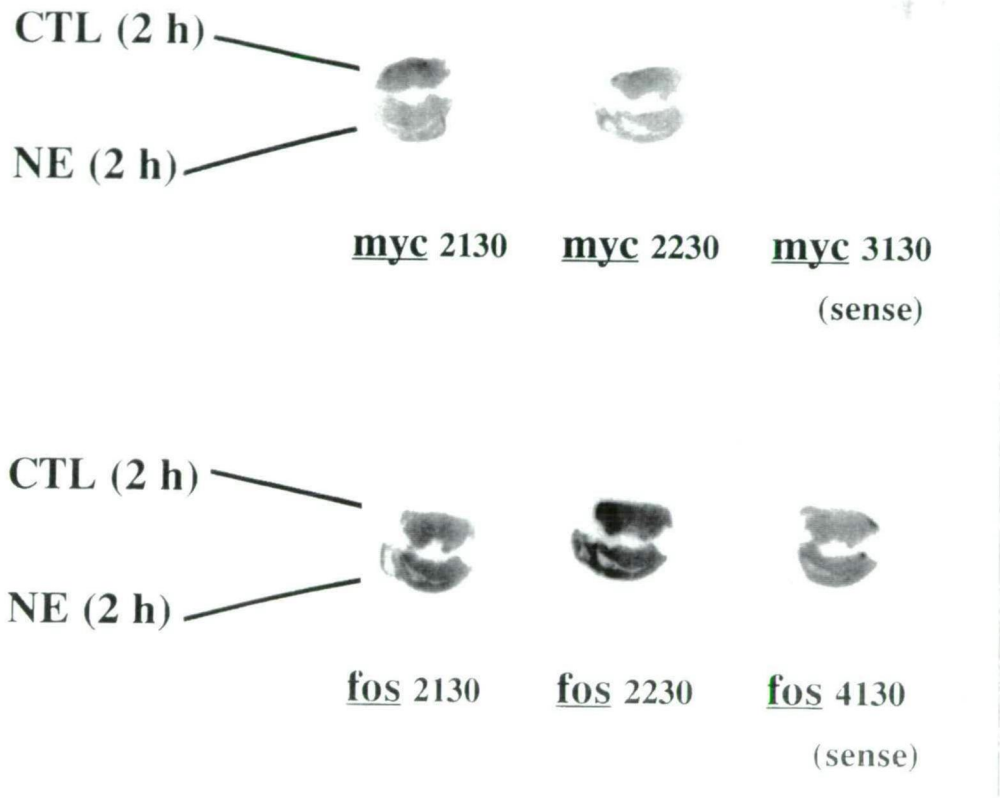

Fig 4.8 Cardiac *in situ* hybridization of c-*myc* and c-*fos* following a single injection of norepinephrine. Two hours following a single i.p. injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) hearts were removed, sectioned and then hybridized *in situ* with the c-*myc* probes (upper panel) utilized in Fig 4.6 or with c-*fos* probes (lower panel) utilized in Fig 4.7.

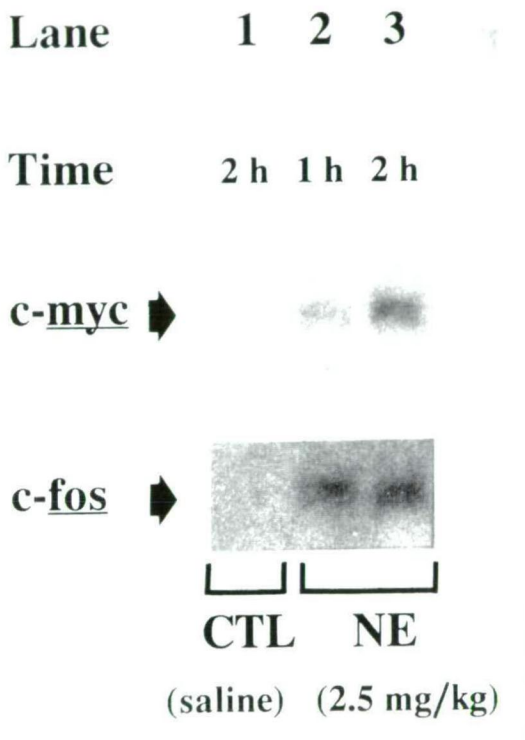

Fig 4.9 Northern analysis of c-*myc* and c-*fos* expression in cardiac tissue processed for *in situ* hybridization.

Tissue samples used in figure 4.8 were scrapped from the slide and total RNA extracted. After electrophoresis and northern blotting, the RNA was hybridized with c-*myc* (upper panel) or c-*fos* (lower panel) cDNA probes.

Due to the poor results obtained with these oligonucleotide probes, two commercially available oligonucleotide probes *myc* Pr-1 and *fos* Pr-1 specific for c-*myc* and c-*fos* respectively, were purchased and used for *in situ* analysis (see Figs. 4.6 & 4.7, bottom tracks) for northern analysis. NE-treated sections of heart tissue were hybridized as before to the commercially obtained *myc* and *fos* oligonucleotide probes (Fig. 4.10). Once again no difference between NE-treated and control hearts was observed for the *myc* Pr-1 probe (results not shown) but this was not unexpected in light of the difficulties experienced with this probe during northern analysis (see Fig. 4.6, bottom track). In contrast, after considerable manipulation of the experimental protocol, specific hybridization of the *fos* Pr-1 probe to *fos* mRNA *in situ* compared to control tissue was achieved (Fig. 4.10) The temporal pattern of this expression was similar to that observed for c-*fos* induction during northern analysis with maximal expression after 2 h. Hybridization was specific for mRNA since signal accumulation was prevented by preincubation of tissue with RNase A. The regional expression of c-*fos* was not uniform throughout the heart but greatest in the left ventricle and septum. Hybridization in atrial sections was variable and this was probably due to the problems encountered with this tissue partially coming off slides during extended post-hybridization washes. The inherent background problems encountered severely restricted the interpretation of these *in situ* results and for this reason further *in situ* hybridization was conducted using labeled cDNA probes.

The results from one such *in situ* experiment using cDNA probes for c-*myc* and c-*fos* are shown in Fig. 4.11. Increased hybridization is observed for both c-*fos* and c-*myc* in NE-treated tissue compared to control tissue. The temporal pattern of this expression is similar to that observed during northern analysis and is specific for mRNA since signal hybridization is prevented by preincubation with RNase. Regional distribution of c-*myc* and c-*fos* mRNA was less clearly discernible following hybridization to cDNA probes and this may have been due to the high background. Indeed this high background made localization of the responses to specific cells using liquid emulsion film impossible despite a number of attempts.

Because of these above described experimental problems encountered during *in situ* analysis and also due to time constraints, this work was not pursued

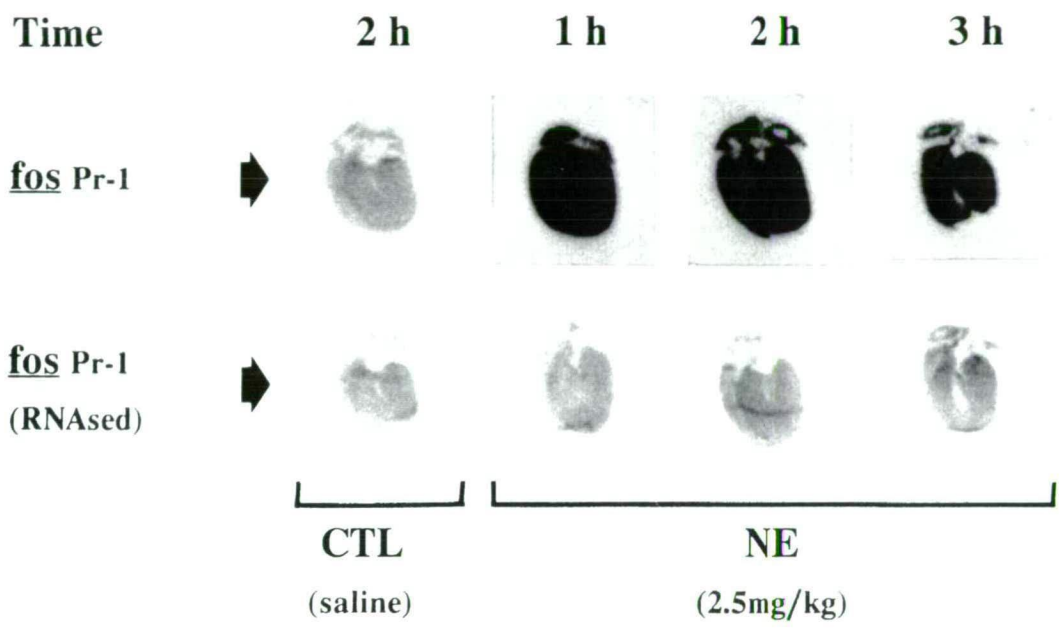

Fig 4.10 *in situ* hybridization of c-*fos* in the rat heart after a single injection of norepinephrine.
At various times after an injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) rat hearts were removed, sectioned and hybridized *in situ* with *fos* Pr-1. As a control some heart sections were also treated with RNase A before hybridization with the *fos* probe.

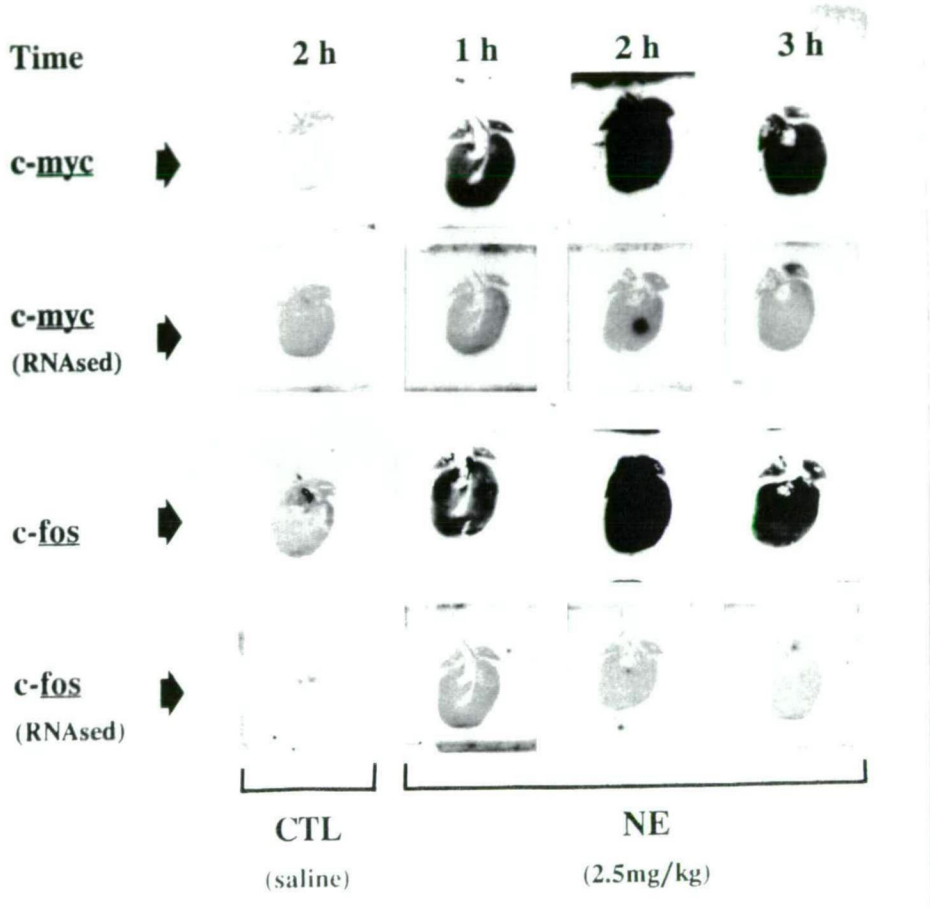

Fig 4.11 *in situ* hybridization of c-*myc* and c-*fos* in the rat heart after a single injection of norepinephrine. At various times after an injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) rat hearts were removed, sectioned and hybridized *in situ* with cDNA probes for c-*myc* and c-*fos*. As a control some heart sections were also treated with RNase A before hybridization with the probes.

further and subsequent regional and cellular analysis of c-*myc* and c-*fos* and other early-response genes was determined using northern analysis of mRNA obtained from separate heart chambers and by immunocytochemistry on whole cardiac sections.

4.3.2 Regional Northern Analysis

Hearts were removed from animals treated with NE or saline and dissected into the various chambers and early-response gene expression assessed by northern analysis (Fig. 4.12). All chambers of the heart contributed to the basal expression of c-*myc*, c-*jun* and *fra*-2 although this required considerable over-exposure of the appropriate autoradiographs. Administration of NE significantly increased mRNA levels of c-*myc*, c-*jun*, *fra*-1 and *fra*-2 above that of control tissue. Increased early-response gene mRNA levels were transient with maximal expression occurring for all genes at around 2 h and had returned to basal by 3 h with the exception of *fra*-1 and *fra*-2 which remained above basal for up to 6 h (results not shown but see chapter 3). The regional expression of early-response genes also differed following NE administration. For instance little or no c-*fos* transcripts were observed in the combined atria sample for any of the time points whilst *fra*-1, a structurally related gene exhibited maximal expression in this chamber. In contrast *fra*-2 expression was relatively uniform throughout the various chambers whilst c-*myc*, c-*fos* and c-*jun* expression was greatest in the left ventricle with progressively lesser expression in the right ventricle and septum. This variation in early-response gene expression is not due to different loading levelsor RNA as demonstrated by hybridization to the control probe β-tubulin. Thus early-response gene expression in the rat heart following NE treatment exhibits both regional and temporal differences.

4.3.3 Immunocytochemistry

Fos and Myc protein products were detected in control and experimental hearts by immunocytochemistry using a number of polyclonal anti-Fos antibodies and a monoclonal anti-Myc antibody respectively.

c-fos

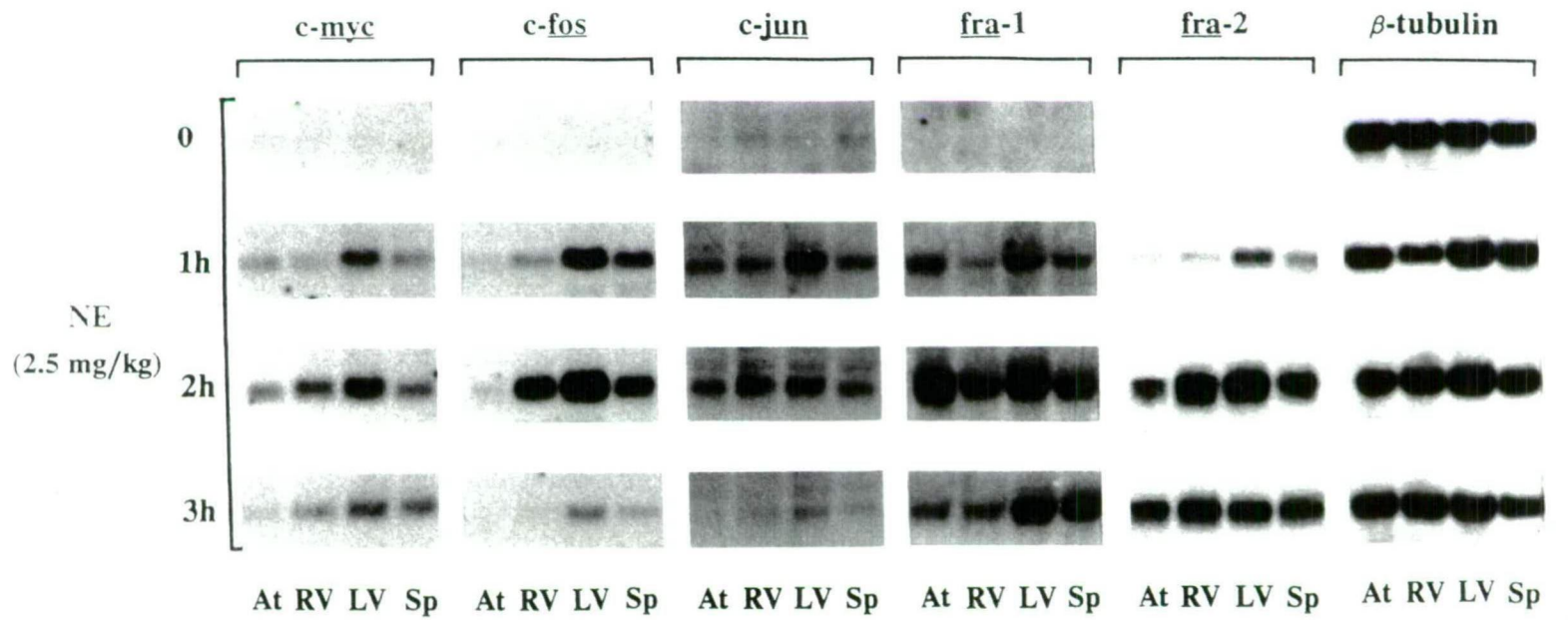

Fig 4.12 Regional localization of c-*myc*, c-*fos*, c-*jun*, *fra*-1 and *fra*-2 in the rat heart following norepinephrine administration.
Total RNA was extracted from rat heart chambers (At: atria, RV: right ventricle, LV: left ventricle, Sp: septum) at various times indicated following an i.p injection of saline (0) or norepinephrine (2.5 mg/kg, 1-3 h). After electrophoresis and northern blotting, the RNA (25 μg) was sequentially hybridized to c-*myc*, c-*fos*, c-*jun*, *fra*-1 and *fra*-2 (6.0 kb transcript only). Hybridization to β-tubulin was used to confirm that equal amounts of RNA were loaded in each track.

c-*fos* protein was localized using a polyclonal antibody raised to a synthetic peptide (Oncogene science). This antibody potentially cross-reacts with other members of the *fos* gene family (*fra*-1, *fra*-2 & *fos*-B) due to the relatively high conservation of the antigenic sequence. Accordingly immunostaining with this antisera was termed Fos-like immunostaining (FLI).

Little if any FLI was observed in any chambers of the hearts from untreated animals, although occasionally weak immunostaining was localized to the non- myocyte fraction, possibly fibroblasts, in hearts from saline treated animals (Fig. 4.13, Panel A and C). However, following administration of a single injection of NE (2.5 mg/kg) FLI increased dramatically in most chambers of the heart with greatest expression localized to the nuclei of the cardiac myocytes (Fig. 4.13, Panel B). In addition weaker expression was also observed in nuclei of smaller non-myocyte cells, presumably fibroblast although these cells were not positively identified (Fig. 4.13, Panel D). FLI was also observed in the cardiac vasculature, mainly in the vicinity of the smooth muscle cells and to a much lesser extent in the adjacent endothelial cells (Fig. 4.14, Panels A and C) In most cases immunostaining was restricted to the nuclei and this is in accordance with the proposed nuclear function of *fos* and *fos*-related proteins. On occasion weaker cytoplasmic staining was also evident in some myocyte and non-myocyte cells (Fig. 4.13, Panel D) but the frequency and intensity of this staining seemed to depend largely on the fixation method (see methods for a more detailed discussion). Maximal FLI was observed 1-3 h following NE administration although immunostaining was observable within 15 min following NE treatment and was still detectable in some hearts for up to 5-6 h post-treatment (Table 4.1). Although FLI was distributed relatively homogeneously throughout each chamber, the intensity of staining and number of nuclei stained per field of view was greatest in the left ventricle (Fig. 4.15 & Table 4.1) and this is in good agreement with the regional northern analysis data presented above. Interestingly, FLI in the left atrium was very high despite the low c-*fos* mRNA levels observed in this chamber during regional northern analysis (compare Fig. 4.15, Panel E with Fig. 4.12). This finding strengthens the idea that the anti-Fos antibody might cross-react with other proteins structurally related to Fos. For example both *fra*-1 and *fra*-2 mRNA are expressed at high levels in the atria following NE administration and cross-reactivity of the Fos antibody with these proteins may

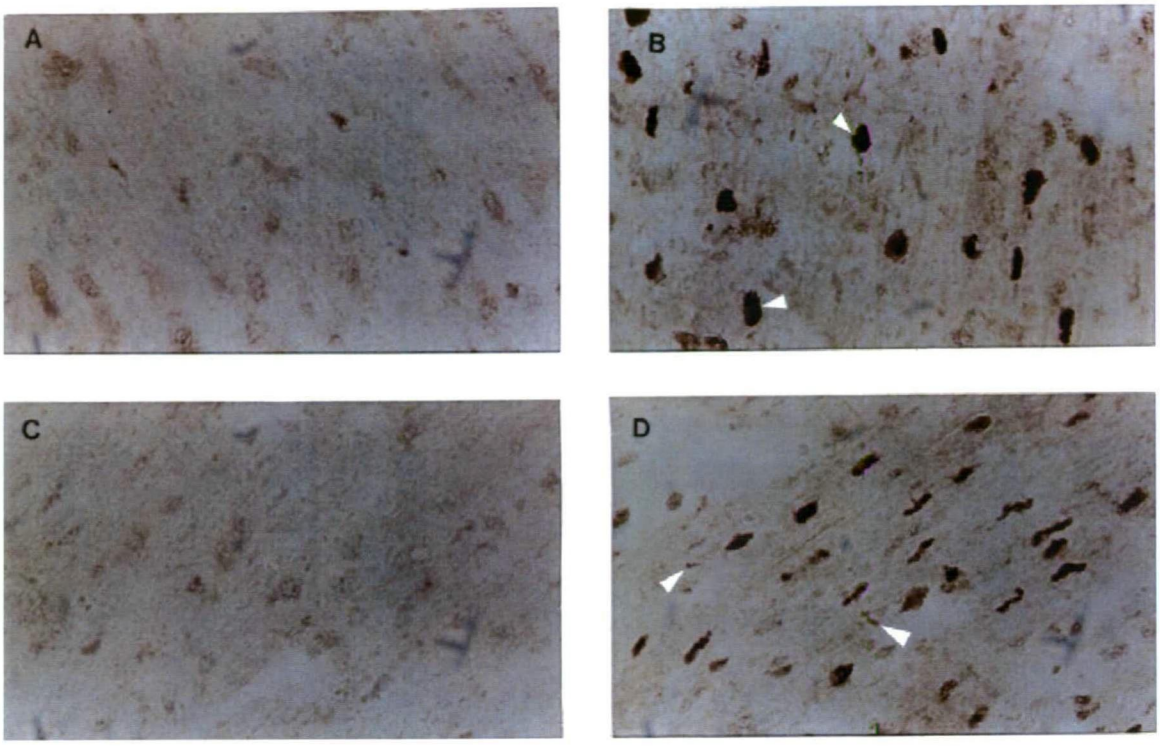

Fig 4.13 Cellular localization of Fos-like immunoreactivity in the left ventricle of rat hearts following norepinephrine administration.
After a single injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) the left ventricle of rat hearts were removed and immunostained for Fos-like immunoreactivity as described in "materials and methods". Results are representative of 3 independent experiments. *Panels A & C*, CTL 2 h left ventricle; *Panels B & D*, NE 2 h left ventricle. Arrows in *Panels B & D* show myocyte nuclear and non-myocyte nuclear staining respectively. 400 X magnification.

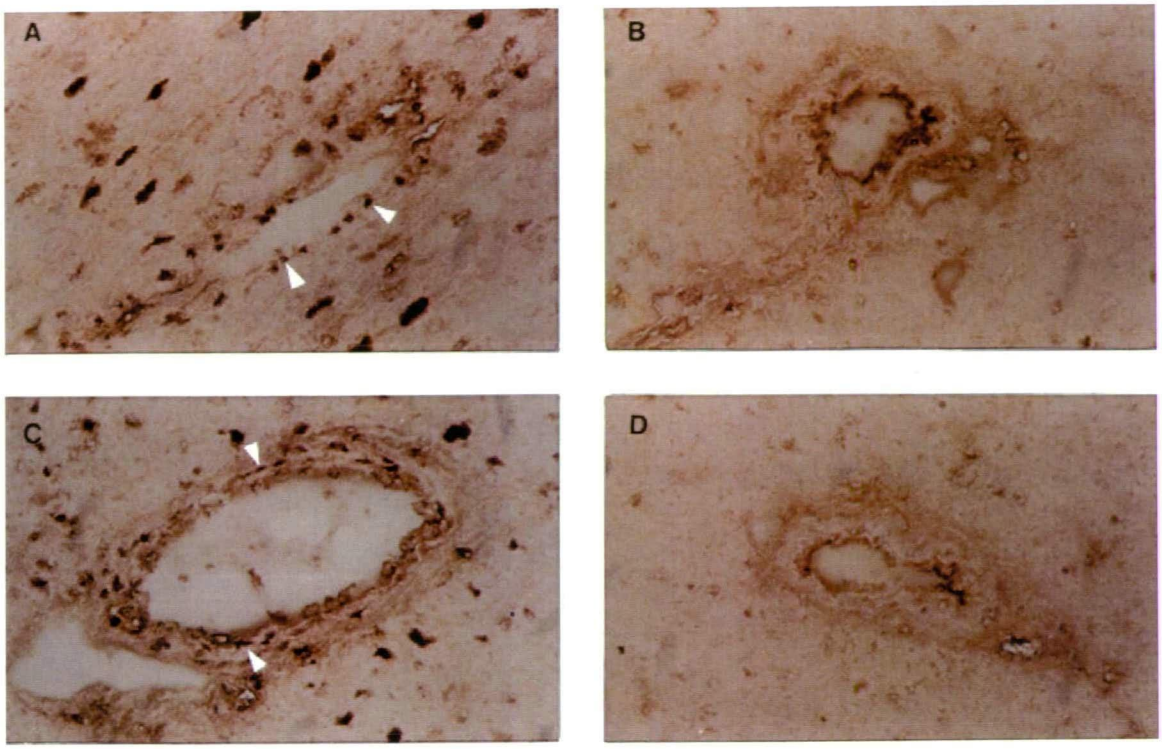

Fig.4.14 Cellular localization of Fos-like immunoreactivity in cardiac vascular tissue following norepinephrine administration.
After a single injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) the left ventricle of rat hearts were removed and immunostained for Fos-like immunoreactivity as described in "materials and methods". Results are representitive of 3 independent experiments. *Panels B & D*, CTL 2 h left ventricular vascular tissue; Panels *A & C*, NE 2 h left ventricular vascular tissue. Arrows in *Panels A & C* show staining of smooth muscle and endothelial nuclei. 400 X magnification

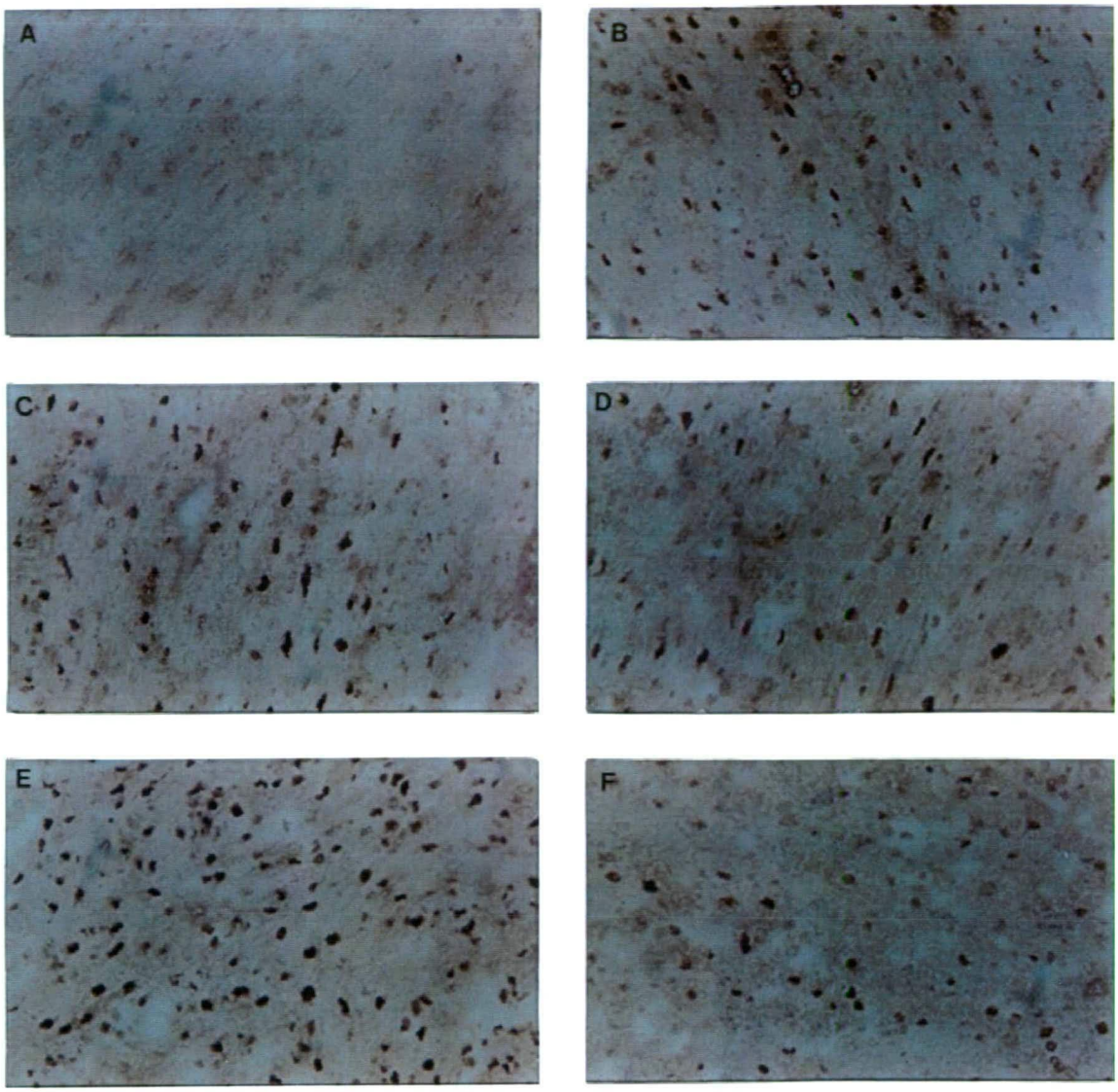

Fig 4.15 Regional localization of Fos-like immunoreactivity in rat heart following norepinephrine administration.

After a single i.p. injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and immunostained for Fos-like immunoreactivity as described in "materials and methods". Results are representative of 3 independent experiments. *Panel A*, CTL 2 h left ventricle; *Panel B*, NE 2 h septum; *Panel C*, NE 2 h left ventricle; *Panel D*, NE 2 h right ventricle; *Panel E*, NE 2 h left atrium; *Panel F*, NE 2 h right atrium. 200 X magnification.

Table 4.1 Cardiac Fos-like immunostaining following norepinephrine administration.

	CTL	-	-	-	-	-
	1 h	++	-	++	+	+
NE	2 h	++++	+	++++	++	+++
2.5 mg/kg	3 h	+	-	+++	+	++
	6 h	-	-	+	-	+
		LA	RA	LV	RV	Sp

Following a single i.p. injection of norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and stained for Fos-like immunoreactivity as described in "materials and methods". Relative immunostaining in three representative fields was scored as non-detectable (-) up to maximal (++++) as described elsewhere (Snoeckx *et al.*, 1991). LA: left atrium, RA: right atrium, LV: left ventricle, RV: right ventricle, Sp: septum. Type of labelled cell (muscle/non-muscle) was not considered.

Table 4.2 Cardiac Fos-specific immunostaining following norepinephrine administration.

	CTL	-	-	-	-	-
	1 h	-	-	++	+	+
NE	2 h	+/-	-	++++	++	+++
2.5 mg/kg	3 h	-	-	++	+	+
	6 h	-	-	+/-	-	-
		LA	RA	LV	RV	Sp

Following a single i.p. injection of norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and stained for Fos-specifc immunoreactivity as described in "materials and methods". Relative immunostaining in three representative fields was scored as non-detectable (-) up to maximal (++++) as described elsewhere (Snoeckx *et al.*, 1991). LA: left atrium, RA: right atrium, LV: left ventricle, RV: right ventricle, Sp: septum. Type of labelled cell (muscle/non-muscle) was not considered.

account for the high immunoreactivity observed in these chambers. In order to further explore this possibility preliminary immunostudies were performed using antibodies raised to peptide regions specific to c-*fos*, *fra*-1 and *fra*-2 (gift from Dr. D. Cohen).

Fig. 4.16 demonstrates immunostaining with an anti-Fos-specific antibody in sections of rat heart 2 h following treatment with saline or NE. Sections have been counter-stained with haematoxylin (blue nuclei) in order to demonstrate the relative proportion of cells expressing c-*fos* protein. Little immunostaining was observable in saline treated cells (Fig. 4.16, Panel D) however, Fos rapidly accumulated in the myocyte nuclei and cytoplasm following NE treatment (Panels B and C). Lesser expression was also observable in fibroblast-like nuclei and those of the vasculature (not shown). Greatest immunostaining was found in the left ventricle 2-3 h following treatment (Table 4.2) and in marked contrast to the expression pattern obtained with FLI, little expression is observed in the atria (Fig. 4.16, Panel A) at any time point. These results are in good agreement with those obtained by northern analysis (Fig. 4.12).

As with Fos, little Fra-1 immunoreactivity was observed in saline treated hearts when immunolabelled with the Fra-1 specific antibody (Fig. 4.17, Panel A). However following treatment with NE, *fra*-1 protein accumulated in myocyte nuclei and to a lesser extent non-muscle cells (Fig. 4.17, Panels B and C). Both nuclear and perinuclear expression was observed. Weaker Fra-1 immunostaining of cells of the vasculature system was also observed following NE administration (results not shown). In contrast to Fos-specific antibody, significant Fra-1 protein accumulation was observed in the left atria (Fig. 4.17, Panel C & Table 4.3), and this is in close agreement with distribution of *fra*-1 mRNA observed during regional northern analysis (Fig. 4.12). Fra-1 protein remained elevated for up to 6-12 h (results not shown) and this is also in accordance with the northern analysis results.

In contrast to Fos and Fra-1, immunostaining with an anti-Fra-2 specific antibody exhibited significant immunostaining in cardiac tissue from control and saline treated animals (Fig. 4.18, Panel A). Expression was restricted mainly to the smaller non-myocyte nuclei, perhaps fibroblasts and to a much lesser extent in the myocytes. These results were in accordance with the observation of *fra*-2 mRNA in hearts of control and saline treated animals (Fig. 3.6). Following treatment with NE, Fra-2

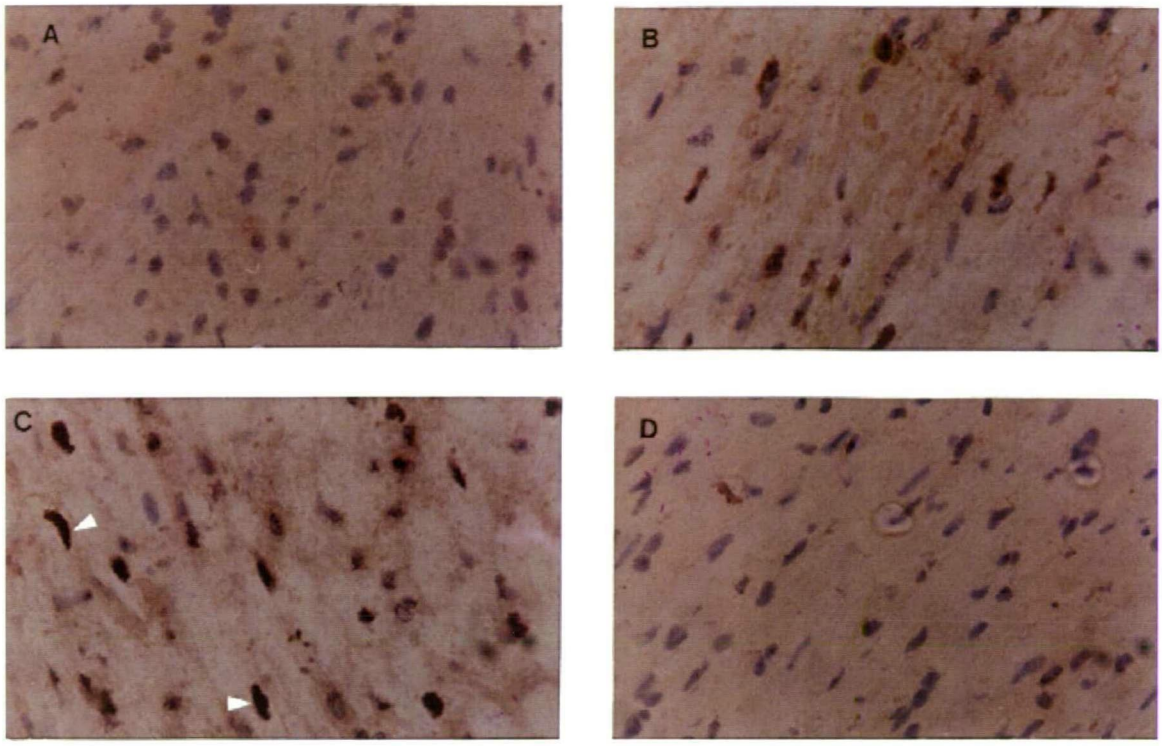

Fig 4.16 Regional and cellular localization of Fos-specific immunoreactivity in rat heart following norepinephrine administration.

After a single injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and immunostained with an anti-Fos specific antibody and then counter stained with hematoxylin (blue nuclei) as described in "materials and methods". Results are representitve of 3 independent experiments. *Panel A*, NE 2 h left atrium; *Panel B*, NE 2 h right ventricle; *Panel C*, NE 2 h left ventricle; *Panel D*, CTL 2 h left ventricle. Arrows in *Panel C* indicate myocyte nuclear staining. 200 X magnification.

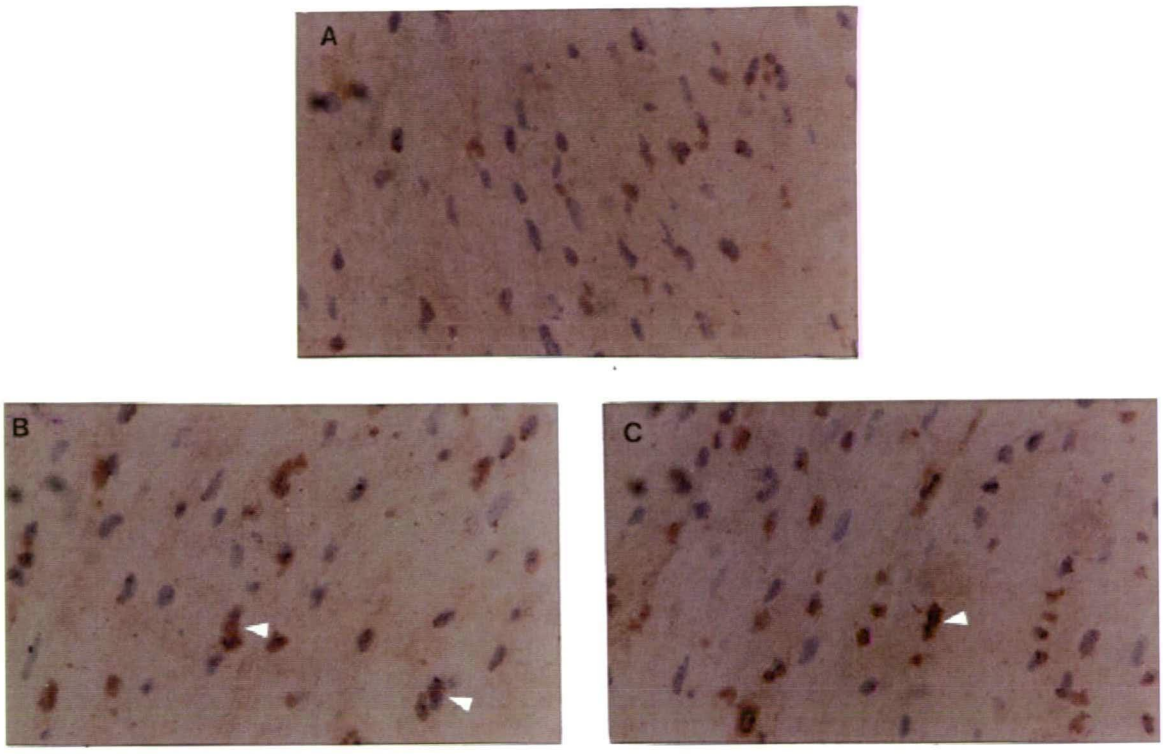

Fig 4.17 Regional localization of Fra-1 immunoreactivity in rat heart following norepinephrine administration.

After a single i.p injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and immunostained for Fra-1 immunoreactivity and then counter stained with hematoxylin (blue nuclei) as described in "materials and methods". Results are representative of 3 independent experiments. *Panel A*, CTL 2 h left ventricle; *Panel B*, NE 2 h left ventricle; *Panel C*, NE 2 h left atrium. Arrows in *Panels B & C* indicate nuclear staining of myocytes. 400 X magnification.

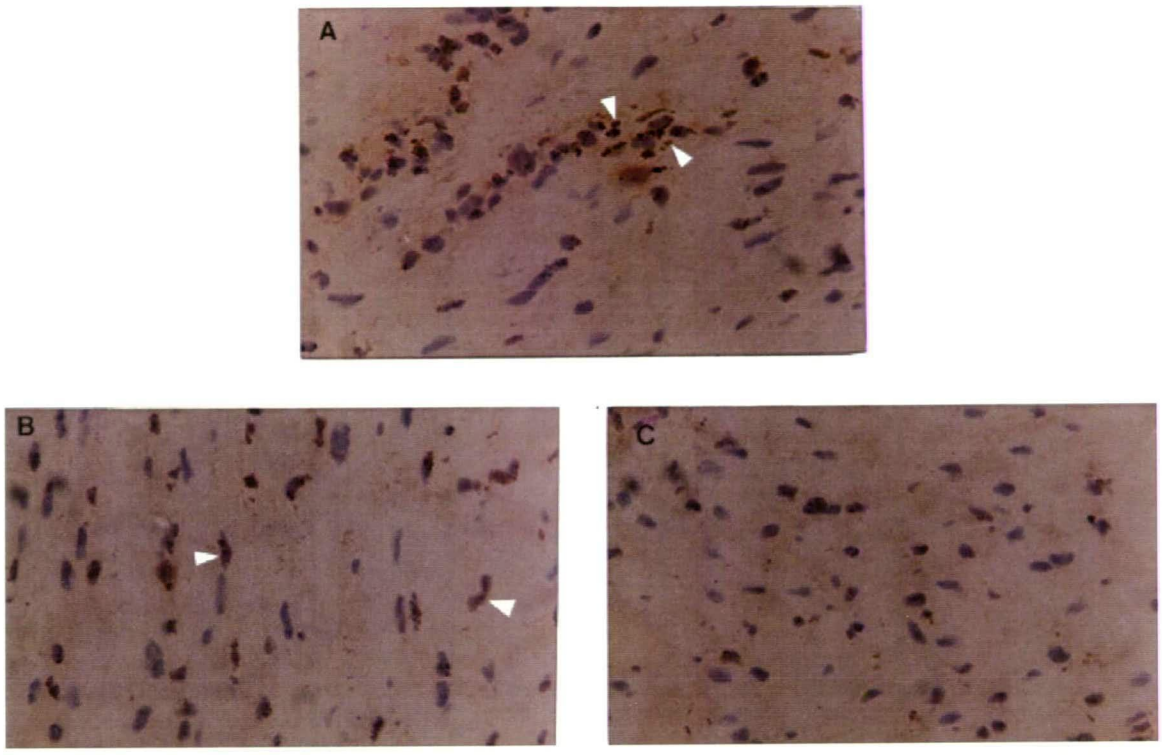

Fig 4.18 Regional localization of Fra-2 immunoreactivity in rat heart following norepinephrine administration.

After a single i.p injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and immunostained for Fra-2 immunoreactivity and counter stained with hematoxylin (blue nuclei) as described in "materials and methods". Results are representitve of 3 independent experiments. *Panel A*, CTL 2 h left ventricle; *Panel B*, NE 2 h left ventricle; *Panel C*, NE 2 h left atrium. Arrows in *Panel A* indicate basal non-myocyte nuclear staining. Arrows in *Panel B* indicate myocyte nuclear staining. 400 X magnification.

Table 4.3 Cardiac Fra-1 immunostaining following norepinephrine administration.

	CTL	-	-	-/+	-	-
	1 h	++	+	++	+	+
NE	2 h	+++	+	++++	++	++
2.5 mg/kg	3 h	++	+	++++	+++	++
	6 h	+/-	-	+	++	+/-
	12 h	+/-	-	+	++	+/-
		LA	RA	LV	RV	Sp

Following a single i.p. injection of norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and stained for Fra-1 immunoreactivity as described in "materials and methods". Relative immunostaining in three representative fields was scored as non-detectable (-) up to maximal (++++) as described elsewhere (Snoeckx *et al.*, 1991). LA: left atrium, RA: right atrium, LV: left ventricle, RV: right ventricle, Sp: septum. Type of labelled cell (muscle/non-muscle) was not considered.

Table 4.4 Cardiac Fra-2 immunostaining following norepinephrine administration.

	CTL	+	+	+	+	+
	1 h	+	+	+	+	+
NE	2 h	++	+	++	+	+
2.5 mg/kg	3 h	+++	+	++++	++	+++
	6 h	+/-	+	+++	+	++
	12 h	+	+	++	+	+
		LA	RA	LV	RV	Sp

Following a single i.p. injection of norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and stained for Fra-2 immunoreactivity as described in "materials and methods". Relative immunostaining in three representative fields was scored as non-detectable (-) up to maximal (++++) as described elsewhere (Snoeckx *et al.*, 1991). LA: left atrium, RA: right atrium, LV: left ventricle, RV: right ventricle, Sp: septum. Type of labelled cell (muscle/non-muscle) was not considered.

protein accumulated to a much greater extent in myocyte nuclei (Fig. 4.18, Panel B) and to a lesser extent in the smaller fibroblast-like nuclei and those of the vasculature (not shown). As with Fos and Fra-1 immunostaining, maximum expression occurred in the left ventricle 2-4 hr following NE treatment with lesser staining elsewhere including the atria (Fig. 4.18 C and Table 4.4). Stained nuclei were still observable 6-12 h following treatment (Table 4.4 and results not shown).

c-*myc*

c-*myc* protein was immunostained with a monoclonal antibody as described in the materials and methods. Weak Myc immunostaining was observed in hearts from untreated and saline injected animals in the non-myocyte cell population, probably fibroblasts (Fig. 4.19, Panel A) but little in the vascular cells or myocytes (Fig. 4.19, Panel C). This basal expression was homogeneous throughout all chambers of the heart although the intensity varied from animal to animal. The cells in Figure 4.19 have been counter-stained with haematoxylin (blue nuclei) in order to demonstrate the relative proportion and size of cells expressing c-*myc* protein. Administration of a single injection of NE resulted in rapid accumulation c-*myc* protein throughout all chambers of the heart. Importantly, and in significant contrast to FLI staining, Myc immunostaining was greatest in non-muscle cells, probably fibroblasts, with much lesser staining apparent in the larger myocyte nuclei (Fig. 4.19, Panel B and Fig. 4.20, Panels B-F). Significant increase in Myc expression was observed in the coronary vasculature cells, presumably the endothelium and to a lesser extent smooth muscle cells (Fig. 4.19, Panel D). Smooth muscle staining (red staining) was confirmed by staining of adjacent slides with an anti-smooth muscle actin antibody (compare Fig. 4.19, Panel D with Panel E). Myc protein was first observable within 30 min following treatment, rising to a peak after 1-3 h and had returned to basal levels by 6-12 h (see Fig. 4.20, Panels A-F, Table 4.5 and results not presented). These result indicates that c-*myc* may be associated with the hyperplastic response of cardiac non-myocyte cells rather than the hypertrophic response of cardiomyocytes following NE stimulation.

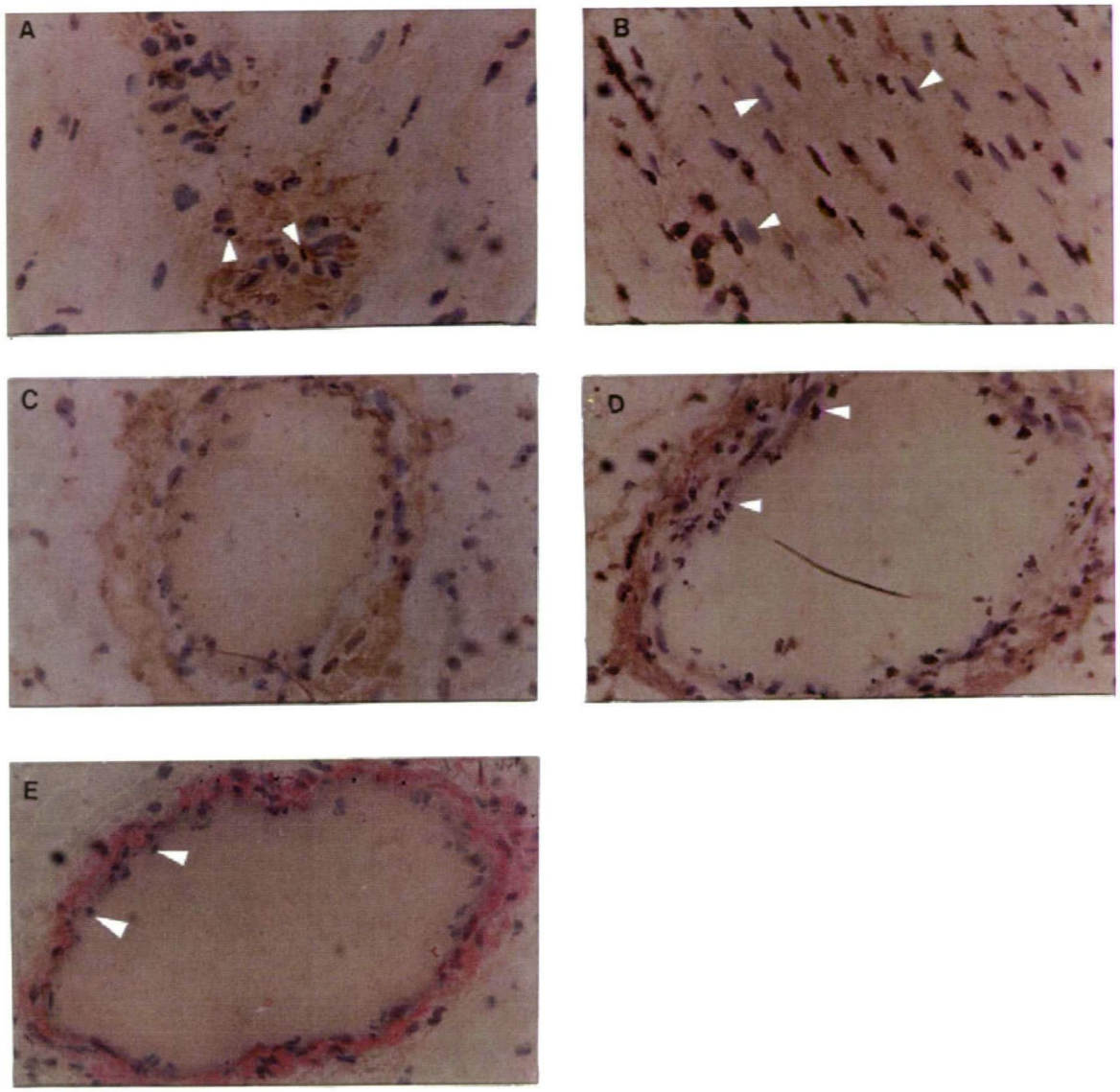

Fig 4.19 Cellular localization of Myc immunoreactivity in the left ventricle of rat hearts following norepinephrine administration.

After a single injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) the left ventricle of rat hearts were removed and immunostained for Myc or smooth muscle actin (red cytoplasmic staining) immunoreactivity and counterstained with hematoxylin (blue nuclei) as described in "materials and methods". Results are representative of 3 independent experiments. *Panel A*, CTL 2 h left ventricle; *Panel B*, NE 2 h left ventricle; *Panel C*, CTL 2 h left ventricle vascular tissue; *Panel D*, NE 2 h left ventricular vascular tissue; *Panel E*, serial section from *Panel D* stained with anti-smooth muscle actin. Arrows in Panel A show basal non-myocyte nuclear staining. Arrows in Panel B indicate the majority of myocyte nuclei are not stained with the anti-Myc antibody. Nuclei that stain positively for Myc are probably due to inter-cardic fibroblasts . Arrows in *Panel D* show staining of nuclei associated with vascular tissue. Arrows in *Panel E* confirm that Myc immunostained nuclei in *Panel D* probably belong to vascular endothelial and smooth muscle cells. 400 X magnification.

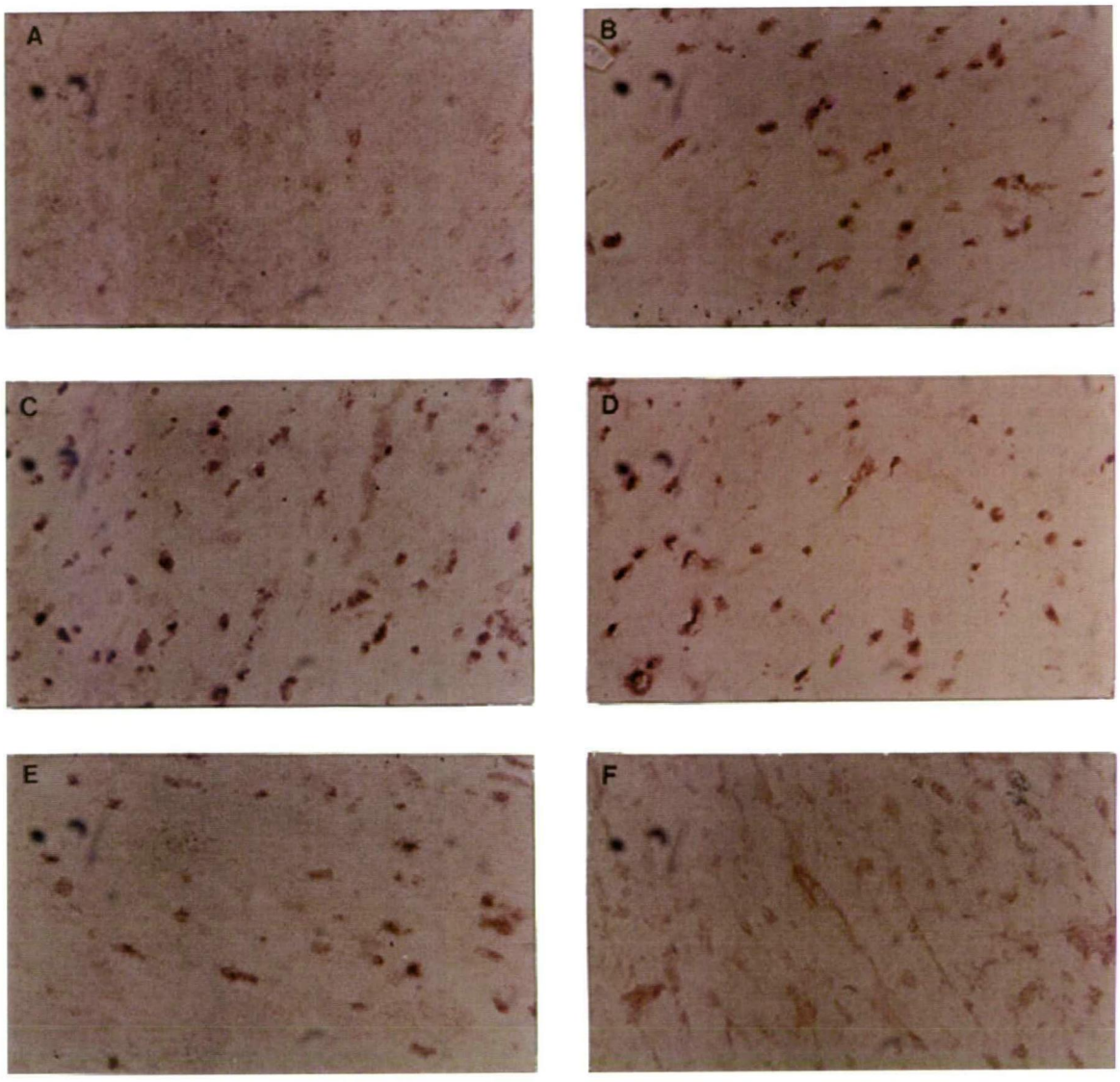

Fig 4.20 Regional localization of Myc immunoreactivity in rat heart following norepinephrine administration.

After a single i.p injection of saline (CTL) or norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and immunostained for Myc immunoreactivity as described in "materials and methods". Results are representative of 3 independent experiments. *Panel A*, CTL 2 h left ventricle; *Panel B*, NE 2 h septum; *Panel C*, NE 2 h left ventricle; *Panel D*, NE 2 h right ventricle; *Panel E*, NE 2 h left atriim; *Panel F*, NE 2 h right atrium, 200 X magnification.

Table 4.5 Cardiac Myc immunostaining following norepinephrine administration.

	CTL	+/-	+/-	+	+/-	+
	1 h	+	+	+	+	+
NE	2 h	++	++	+++	++	++
2.5 mg/kg	3 h	++	+	++++	++	+++
	6 h	+	+/-	+	+/-	+
	12 h	+/-	+/-	+/-	+/-	+/-
		LA	RA	LV	RV	Sp

Following a single i.p. injection of norepinephrine (NE, 2.5 mg/kg) rat hearts were removed and stained for Myc immunoreactivity as described in "materials and methods". Relative immunostaining in three representative fields was scored as non-detectable (-) up to maximal (++++) as described elsewhere (Snoeckx *et al.*, 1991). LA: left atrium, RA: right atrium, LV: left ventricle, RV: right ventricle, Sp: septum. Type of labelled cell (muscle/non-muscle) was not considered.

4.4 DISCUSSION

Studies from chapter 3 established that induction of the early-response genes occurred rapidly in the heart following NE administration *in vivo*. These experiments however, did not shed light on the specific cell types or areas within the heart that were responsible for this expression or reveal whether their corresponding protein products were produced. The work in this chapter has sought answers to these questions using a combination of *in situ* hybridization, northern and immunohistochemical approaches.

i) detection of c-myc and c-fos mRNA by hybridization histochemistry

Hybridization histochemistry (*in situ* hybridization) is a molecular technique which has been used widely for the detection and localization of gene expression in tissue samples. In this study the technique was employed in an attempt to characterize the regional, cellular and temporal expression of two early-response genes, c-*myc* and c-*fos* in the rat heart following acute NE administration.

While it was possible to demonstrate competency in the detection and localization of mRNA for some control genes (ANP and Mt) in both cardiac and liver tissue sections respectively, results obtained for the c-*myc* and c-*fos* analysis in the heart were not satisfactory. Complete resolution of these problems was compounded by the inherently long period which elapsed between the initial animal treatment and final interpretation of data and this was largely due to the extended exposure times needed to obtain adequate autoradiographic results. However, after considerable manipulation of the established experimental protocol, results were attained using a commercially obtained *fos* oligonucleotide (*fos* Pr-1) and c-*myc* and c-*fos* specific cDNA probes. These results demonstrated that c-*myc* and c-*fos* mRNA accumulated mainly in the left ventricle with lesser expression elsewhere and that the temporal pattern of this expression correlates with that observed during northern analysis. Thus c-*fos* induction preceded c-*myc* with maximal expression at 1-2 and 2-3 h respectively. Hybridization within the atria was variable and this was probably due to experimental difficulties involved in fixing this chamber to the slide. Analysis of c-*fos* and c-*myc* expression at a cellular level was inconclusive due to persistent background problems which made localization of mRNA to specific cell types impossible.

It is likely that the reasons for the poor results achieved with this method were due to the particular c-*myc* and c-*fos* probes and further studies using *in situ* hybridization would require the preparation and testing of more c-*myc* and c-*fos* specific oligonucleotide probes. Alternatively, riboprobes might be employed due to their greater hybridization stability. Further regional and cellular localization of early-response genes was undertaken by northern analysis or RNA extracted from individual heart chambers or by immunocytochemistry.

ii) regional localization of early-response gene expression by northern analysis.

Early-response gene expression was further examined in the various chambers of the heart by northern analysis following a single injection of NE (2.5 mg/kg). Constitutive expression of c-*myc*, c-*jun* and *fra*-2 mRNA observed previously in whole myocardial extracts, was localized to all chambers of the heart with only slight variation in regional intensity. Thus elements which control the constitutive expression of these genes do not appear to be chamber specific.

Following acute administration of NE, mRNA of all early-response genes investigated, was rapidly and transiently induced above basal levels, however each gene displayed a distinct regional and temporal pattern of distribution. For instance c-*fos* expression was barely detectable in the combined atrial sample, at any time point, in contrast to the structurally and functionally related gene *fra*-1 which exhibited near maximal expression in this chamber at 2 h. In a further example c-*fos* and c-*jun* mRNA levels were only transiently increased with mRNA levels back to basal levels by 3-4 h whilst *fra*-2 mRNA remained elevated for up to 6 h. These results, taken together with those of the previous chapter, indicate that the cardiac induction of early-response gene expression following NE administration is not only temporally and stimulus-specific, but also a tissue-specific phenomenom and further supports the idea that differential expression of these genes is a mechanism by which diversity and specificity of the hypertrophic response to trophic stimuli might be achieved.

The differences in regional distribution of these genes following acute NE treatment is suggestive that these genes have a different threshold for the same signal and/or are triggered by different signals. For instance the effect of NE on the heart *in vivo* is complex involving both direct receptor-mediated and secondary hemodynamic

events both of which are likely to contribute to the differential expression of early-response genes observed here. Thus the maximal expression of c-*myc* and c-*fos* observed in the left ventricle might be a function of the increased pressure that this chamber is exposed to as a result of peripheral vasoconstriction during NE treatment (see chapter 1). Indeed preliminary data from this laboratory (Eldridge, Hannan and West unpublished data) demonstrate that c-*myc* accumulates primarily in the left ventricle following chronic infusion of rats with hypertensive levels of α_1-adrenergic agents. This pattern of expression is similar to that observed in pressure-overloaded rat hearts following aortic stenosis and may support the hypothesis that c-*myc* and c-*fos* products mediate qualitative changes in protein synthesis which occur in the left ventricle during this growth process. For example ANP, TGF-β and α-SkA are all expressed at high levels in the left ventricle in pressure-overloaded rat hearts and contain potential AP-1 binding sites in their promoter regions (Parker *et al.*, 1991). Thus these observations in combination, are suggestive that increased pressure in the left ventricle is a major stimulus directing c-*myc* and c-*fos* expression in the heart following a single high dose injection of NE.

In contrast however, *fra*-2 mRNA levels are similar in the left and right ventricle and only slightly less in the septum and combined atrial sample despite the different pressures to which these regions might be transiently exposed following high dose NE treatment. Thus at least part of this response is probably due to direct cardiac NE receptor-mediated events independent of changes in pressure loading. These findings are interesting, in light of a recent study reporting that cats chronically administered doses of NE similar to those used in these experiments, exhibited cardiac hypertrophic growth which was of similar magnitude in both the left and right ventricles and which was physiologically similar to that observed following volume overload (Mariano *et al.*, 1991). It is intriguing to speculate that *fra*-2 may be involved in the modulation of quantitative or qualitative processes which occur in both the left and right sides of the heart during this form of hypertrophy. In support of this, it was observed earlier (chapter 3) that T_3 administration, a treatment which leads to volume overload hypertrophy, also produced an upregulation of *fra*-2 mRNA

fra-1 mRNA exhibits greatest expression in the left ventricle and combined atrial sample following NE treatment and like c-*fos* and c-*myc* described above, this may be due to the pressor effect of the treatment. However, *fra*-1 expression has been reported to be unresponsive to pressure in rodents (Rockman *et al*, 1991) and if true, this would seem to indicate that although all chambers of the heart are presumably exposed to the same circulating amounts of NE, their sensitivity to this stimuli differs. Clearly further studies are needed to determine which of these two hypotheses is correct.

Further explanation of the regional distribution of early-response gene mRNA in the heart is complicated by a number of factors. Firstly, both α- and β-components of NE appear to modulate early-response gene expression independently, thus the regional distribution of these genes following stimulation by these two components of NE action needs to be examined in detail. To this end, preliminary data from this laboratory (Hannan and West, unpublished data) indicates that infusion of α-adrenergic agents leads to expression of c-*myc* predominantly in the left side of the heart whilst infusion with β-adrenergic agents results in distribution of mRNA evenly in both the left and right ventricle. Secondly, it is possible that the observed early-response gene induction may occur in different cell types which themselves may also exhibit differential sensitivity to the direct or indirect effects of NE, or alternatively, they may release factors which may differentially modify the expression of early response genes in adjacent myocytes. For instance b-FGF is present in heart tissue and has been demonstrated to induce c-*fos*, c-*jun* and *jun*-B in cardiac muscle cells (Parker *et al.*, 1991), but while cell-type contribution to early-response gene expression can be assessed by immunohistochemistry (see below), elucidation of the possible role of autocrine and paracrine growth factors in this process will probably require the use of adult cardiac cell co-cultures.

iii) cellular localization of early-response genes by immunocytochemistry

Cellular localization of NE-induced early response gene expression was achieved by immunocytochemistry using commercially available monoclonal anti-Myc antibody and a polyclonal anti-Fos antibody. The anti-Fos antibody had been raised to a peptide sequence in the amino terminus which is conserved within the *fos* gene family.

Thus this antibody potentially cross-reacts with other members of the *fos* gene family and consequently positive immunostaining was termed Fos-like immunoreactivity (FLI).

Fos-like immunoreactivity (FLI) was not observed in any cell type in hearts removed from untreated animals but weak immunostaining was localized to the occasional myocyte of saline treated animals. Thus it is unlikely that the Fos antibody used in this study cross reacts to any significant degree with Fra-2 since mRNA for this gene was clearly observable in control cardiac tissue. These results are in agreement with the recent study of (Schunkert *et al.*, 1991) who also did not observe Fos immunostaining in untreated rat hearts (Schunkert *et al.*, 1991), but once again, differed to those of Rappaport's group who demonstrated significant basal Fos expression in the smooth muscle cells of the cardiac vasculature system. However Rappaport's study did not discuss the specificity, or lack there of, of their anti-Fos antibody with respect to other members of the Fos protein family. Thus it is possible that in fact their anti-Fos antibody strongly cross reacts with other Fos like proteins which are observed in basal cardiac tissue or alternatively, it may cross-react nonspecifically with some unrelated protein in smooth muscle. In addition the results of Rappaport's group differed further from the those presented here since they observed significant induction of Fos following anesthesia which they attributed to stress of the injection and/or alterations in cardiac pressure that this treatment might cause (Snoeckx *et al.*, 1991). However this response appeared to be age dependent since less FLI was observed in 12 week old rats than in 3 week old rats. The animals used in the present study were between 8 to 12 weeks old which may explain why little or no FLI was observed in anesthetized animals. More over, it appears that a decreased sensitivity to certain types of stress is a common feature of the maturing heart in terms of early-response gene expression since it has been demonstrated that aortic constriction leads to increased c-*myc* mRNA in both the left ventricle and in both atria of 28 day old rats but only the atria in similarly treated 80 day old rats (Mulvagh *et al.*, 1987). It will be interesting to see if NE-mediated early-response gene expression is similarly down regulated in the hearts of older animals and to determine whether this affects the ability of older animals to adapt to physiological effects of this hormone.

Low level endogenous expression of c-*myc* characterized previously in this study at the RNA level, was observed in all chambers of the heart, in non-muscle cells nuclei, possibly fibroblasts but to a much lesser extent in vascular smooth muscle or cardiac myocytes. In a recent study by Rappaport's group (Snoeckx *et al.*, 1991), Myc immunostaining was similarly observed in the non-muscle cells of the left ventricle of control animals and in addition, in the endothelium of large coronary arteries (Snoeckx *et al.*, 1991). The reason for the failure to observe similar Myc immunostaining in the endothelium by the present study is puzzling but may indicate that the immunofluorescence detection system employed by Rappaport's group is more sensitive than the immunodetection system used here.

Acute administration of NE led to the rapid and transient accumulation of c-*fos* like proteins and c-*myc* protein in the nuclei of heart cells. FLI was primarily restricted to the nuclei to the striated muscle cells and this is in accordance with results obtained from cultured myocyte cells following treatment with NE. In further support of these results, Schunkert's group (Schunkert *et al.*, 1991) demonstrated that c-*fos* proteins accumulate in the myocyte nuclei of pressure-overloaded hearts and taken together they give further support for the idea that c-*fos* and structurally related genes may play an active role in NE and pressure mediated hypertrophy of adult myocytes

Interestingly c-*fos* expression following NE administration was not restricted to the cardiac myocytes alone since increased FLI staining was also observed in the vascular smooth muscle nuclei which is in good agreement with previous reports of expression of c-*fos* mRNA in these cells and in whole aorta in response to NE treatment (Naftilan *et al.*, 1989; Moalic *et al.*, 1989). In addition much weaker accumulation of FLI relative to control was observed throughout the heart in smaller, presumably non-myocyte nuclei, a response that was also observed in the pressure overload studies of Rappaport's group (Snoeckx *et al.*, 1991). This weaker expression was possibly due to activity of these genes in a subset of cardiac fibroblasts or other cells, but a much more stringent identification of this cell type is required to confidently explore this possibility. It is worthy to note, however, that expression of early-response genes in other cell types which are probably actively dividing does not negate a specific role for these genes in the hypertrophic growth of differentiated myocytes. For instance, it is possible that in response to the same trophic stimuli (i.e. NE), increased

early-response gene expression in different cell types may coordinate alternative growth responses depending on their post-transcriptional modification, the availability of other transcription factors or nuclear chromatin structure. An alternative explanation is that increased cardiac myocyte early-response gene expression simply indicates that these cells are attempting to re-enter the cell cycle. In corroboration with this is the observation that NE administration results in increased rat cardiocyte proliferating cell nuclear antigen (PCNA), an auxiliary protein of DNA polymerase gamma which may indicate that the cells are capable of DNA synthesis (Marino *et al.*, 1990).

The temporal and spatial pattern of Fos immunostaining closely correlated with that observed during regional northern analysis. For example, increased Fos staining was observed in the left ventricle 30 min following NE treatment and preceded increases in Myc levels. Interestingly however, although c-*fos* mRNA was hardly detectable in the atria of NE treated animals at any time point, this chamber exhibited significant FLI 1-2 h following treatment. These results were interpreted as the result of cross reactivity of the anti-Fos antibody with other Fos-like proteins, for example Fra-1, since mRNA levels for this gene accumulate to high levels in the atria following acute NE stimulation. As a first preliminary step in determining which proteins structurally related to Fos that might contribute to the observed FLI, studies were undertaken using polyclonal antibodies raised to non-conserved regions between Fos, Fra-1 and Fra-2. These experiments demonstrated that c-*fos*, *fra*-1 and *fra*-2 protein products do indeed accumulate in the myocytes and to a lesser extent other cell types of adult hearts following NE treatment. In addition, the chamber-specific distribution correlated well with that observed during northern regional analysis. For example, protein products for all three genes accumulated in the left ventricle following NE treatment but only Fra-1 and Fra-2 immunostaining was observable in the atria. Thus it would appear that the observed FLI in the atria following NE stimulation is the result of cross reactivity of the anti-Fos antibody with other members of the Fos gene family (*fra*-1, *fra*-2, *fos*-B) or other as yet undiscovered *fos*-related genes, although western analysis is required for their confident identification. Unfortunately due to time constraints and limitations in the availability of the various anti-early-response gene antibodies, Western analysis was not able to be performed.

In direct contrast to FLI, Myc immunoreactivity following NE administration was greatest in non-cardiomyocyte cell types. A definite identification of these expressing cells was not made but their localization strongly suggests they are cardiac fibroblasts, the cell type that constitutes 90-95 % of the non-cardiomyocyte fraction (Eghbali *et al.*, 1991). In addition significant Myc immunostaining was observed in the cardiac vasculature. Staining of serial sections with an anti-snooth muscle actin antibody indicated that these nuclei were probably of endothelial or smooth muscle origin but once again further studies are required to definitively identify these cells. Importantly, these results would seem to indicate that prior expression of c-*myc* in cardiac myocytes is not required for NE mediated cardiac hypertrophy in adult rat hearts. In direct contrast to these findings Simpson and co-workers demonstrated accumulation of c-*myc* mRNA in neonatal myocyte cultures following NE administration and implicated this gene in mediating the growth response of these cells. One possible resolution of these differences is that c-*myc* expression observed in the developing heart of neonatal animals represents at least a partial contribution from proliferating and or hypertrophying cardiomyocytes. This is not unreasonable since evidence exists implicating a role for c-*myc* in the maturation of neonatal cardiomyocytes to terminally differentiated, non-dividing cells (see chapter 1 for a more detailed discussion). It is worthy to note however, that the present immunostudies were performed in cardiac tissue obtained 1-6 h following acute administration of NE. It will be interesting to see if the non-cardiac cell population is also the major site of c-*myc* expression during chronic infusion of NE since, in addition to this transient rise in Myc between 1-6 h, this gene also exhibits a second sustained rise in expression after 12 h of NE infusion (see chapter 3). It is possible for example that the initial rise in Myc observed here represents the proliferation of non-cardiomycytes which is concomitant with cardiomyocyte hypertrophy and this hypothesis is supported by the observation that c-*myc* mRNA levels are elevated in proliferating fibroblasts and endothelial cells. By analogy the second sustained rise in cardiac c-*myc* mRNA observed during NE infusion might be restricted to hypertrophying cardiomyocytes. Further studies are required in order to determine which of these possibilities is correct.

Interestingly, in the studies by Rappaport group (Snoeckx *et al.*, 1991) aortic stenosis did not lead to a further increase in Myc in the rat heart in any cell type, despite the well documented changes in c-*myc* mRNA levels during this treatment (Izumo *et al.*, 1988; Mulvagh *et al.*, 1987). Such findings are further evidence that pressure-load and NE administration lead to both similar and dissimilar responses at the cellular level during cardiac hypertrophy and this may correlate with the different pathologies which may ultimately arise from each treatment.

In summary this study provides the first evidence that administration of the hypertrophic hormone NE leads to accumulation of an array of similar and distinct early-response gene products in the adult heart *in vivo*. Fos and related gene products accumulated primarily in the cardiomyocyte nuclei and this in accordance with proposals that this gene family may play a transducing role during the initial stages of NE-mediated cardiac hypertrophy. In contrast Myc expression is mainly restricted to non-cardiomyocyte cell types such as fibroblasts and endothelial cells and consequently may be associated with the proliferation of these cells which is concomitant with cardiomyocyte hypertrophy. The regional and temporal pattern of distribution was confirmed at both the mRNA and protein levels and was found to be specific for each gene. Maximal expression of c-*myc*, c-*fos* and c-*jun* was observed in the left ventricle which might correlate with the increased pressure that this chamber is likely to experience during high dose NE administration. However the regional expression of *fra*-1 and *fra*-2 did not seem to correlate with changes in hemodynamics and indicates that these genes may be more responsive to the direct effect of NE. Taken together, these results provide indirect evidence that certain subsets of the early-response genes may play a trans-activating role in the chamber-specific responses observed in the heart following high doses of NE or aortic stenosis whilst a distinctly different subset might be important in coordinating global responses to hypertrophic growth stimuli.

CHAPTER 5

EXPRESSION OF EARLY-RESPONSE GENES IN THE ISOLATED PERFUSED HEART

5.1 INTRODUCTION

It is virtually impossible to dissociate the effects of stimuli that act directly on the cardiac myocyte from those that are secondary to systemic alterations. This is particularly so for the adrenergic hormones since they are not only capable of altering cardiac inotropy, chronotropy and metabolism, but they also increase work-load on this organ secondary to an increase in peripheral resistance (see chapter 1 for more in depth discussion). As a consequence of this some investigators have turned to cultured cardiac myocytes in order to examine the direct effect of NE and other trophic factors on myocyte growth independent of systemic influences. As discussed previously, the work of Simpson's group and subsequently others established that NE can directly mediate cardiac hypertrophy and modulate gene expression in isolated neonatal cardiomyocytes. However, it has been questioned whether it is appropriate to extrapolate results obtained from immature cells in an *in vitro* system to describe the responses of the fully mature myocyte *in vivo* (Bugaisky and Zak, 1989). For this reason other investigators have attempted to isolate and culture myocytes from the mature heart, but only recently has the preparation and culture of these cells been sufficiently advanced to allow for the long term study of adequate numbers of cells for biochemical and molecular analysis (Bugaisky and Zak, 1989). Even so, the effect of NE on heart cell growth *in vitro* appears to vary, with some groups reporting that adrenergic agents have a general anabolic effect on adult cardiac myocytes but do not modulate shifts in isocontractile protein synthesis (Dubbus *et al.*, 1990) whilst others have demonstrated specific phenotypic alterations following adrenergic treatment (Rupp *et al.*, 1991). Furthermore, there have been few reports of the effect of NE on early-response gene expression in isolated adult myocytes, possibly because stress of the cell isolation procedure itself has been shown to induce substantial early-response gene mRNA levels (Claycomb, 1987; Hannan and West unpublished data).

However an alternative model for investigating the direct effects of NE or other humoral factors on adult cardiac myocyte growth during controlled haemodynamic conditions, is the isolated (and therefore denervated) perfused heart system. In this model hearts are removed from the animal and perfused *in vitro* with a defined media either in a working or non-working capacity and in such a manner that the direct and indirect actions of NE can be studied separately whilst potentially critical interactions between different cardiac populations of cells are preserved. For instance, simulation of factors thought to be important in the development of cardiac hypertrophy, for example inclusion of NE in the perfusate, or elevation of perfusion pressure, have been shown to increase RNA, protein synthesis and ribosome formation (for review see Morgan *et al.*, 1992). If early-response gene expression is involved in any of these processes, it should be possible to observe their expression, and to mediate this experimentally in isolated perfused hearts. Thus, while overt hypertrophy cannot be observed in this system due to the time required for this to occur, it is possible to model the biochemical initiation of this event.

Accordingly the work in this chapter has sought to use the non-working perfused heart system in order to explore separately the direct and indirect effects of NE on cardiac early-response gene expression and to determine the specific chamber contribution to this response.

5.2 METHODS

5.2.1 *in vitro* Coronary Perfused Hearts

Rats were anaesthetized as described previously (section 3.2.1) and hearts removed and placed in ice-cold isotonic saline until contraction had stopped. The aorta was stripped of connective tissue and tied directly onto the cannula of the perfusion apparatus and the hearts were then perfused at a constant pressure of 60, 90 or 120 mmHg in the Langendorff (non-recirculating) manner as described by Williamson (Williamson, 1964). The perfusion cabinet was maintained at 37°C and a perspex surround was placed around the hearts to prevent evaporative cooling. With the apparatus available up to four hearts could be perfused simultaneously thus allowing a direct comparison to be made between untreated and experimental hearts. All hearts

were allowed a 15 min stabilization period and only hearts which had a stable contractile activity at the end of this period were accepted for further study. Following this period, designated "zero time" the drugs under investigation were introduced continuously into the perfusion medium of some hearts by means of a peristaltic pump or included directly in the perfusion medium reservoir (see section 5.3). Following perfusion, hearts were removed from the apparatus, rinsed in ice-cold isotonic saline and prepared for analysis as described in section 2.2. The perfusion medium was modified Krebs-Henseleit bicarbonate buffer (Williamson, 1964) containing in addition 1.27 mM $CaCl_2$, 0.05 mM EDTA, 5 mM glucose, and 2 mM pyruvate which was filtered through a 45 μm Millipore filter and continuously equilibrated against a gas mixture of 95% O_2, 5% CO_2 and maintained at 37°C by passage through a heated water jacket. See Fig. 5.1 for a diagram of the perfusion setup.

5.3 EXPERIMENTAL PROTOCOLS

5.3.1 Expression of Early-Response Genes in Hearts Perfused at 60 mmHg.

To assess the basal cardiac expression of early-response genes during perfusion, hearts were removed from anaesthetized animals and perfused at a constant pressure of 60 mmHg with Krebs-Henseleit buffer. At the end of the appropriate time (15 min-5 h), hearts were frozen in liquid nitrogen and processed for northern analysis as described in section 2.2. In order to confidently determine the baseline induction of early-response genes, 8 or more hearts were perfused at each time point and the results quantified by laser densitometry and analyzed statistically as described below (section 5.3.5)

5.3.2 Effect of Adrenergic Agents and Second Messengers.

To study the effect of NE on early-response gene expression independent of pressure load, hearts were perfused at a constant pressure of 60 mmHg with buffer containing NE at final concentrations between 1 nM and 1 μM. When used, adrenergic antagonists methoxamine (10 μM) and dl-propranolol (20 μM) were introduced into the perfusate 15 min prior to NE and thereafter maintained in the perfusate for the duration

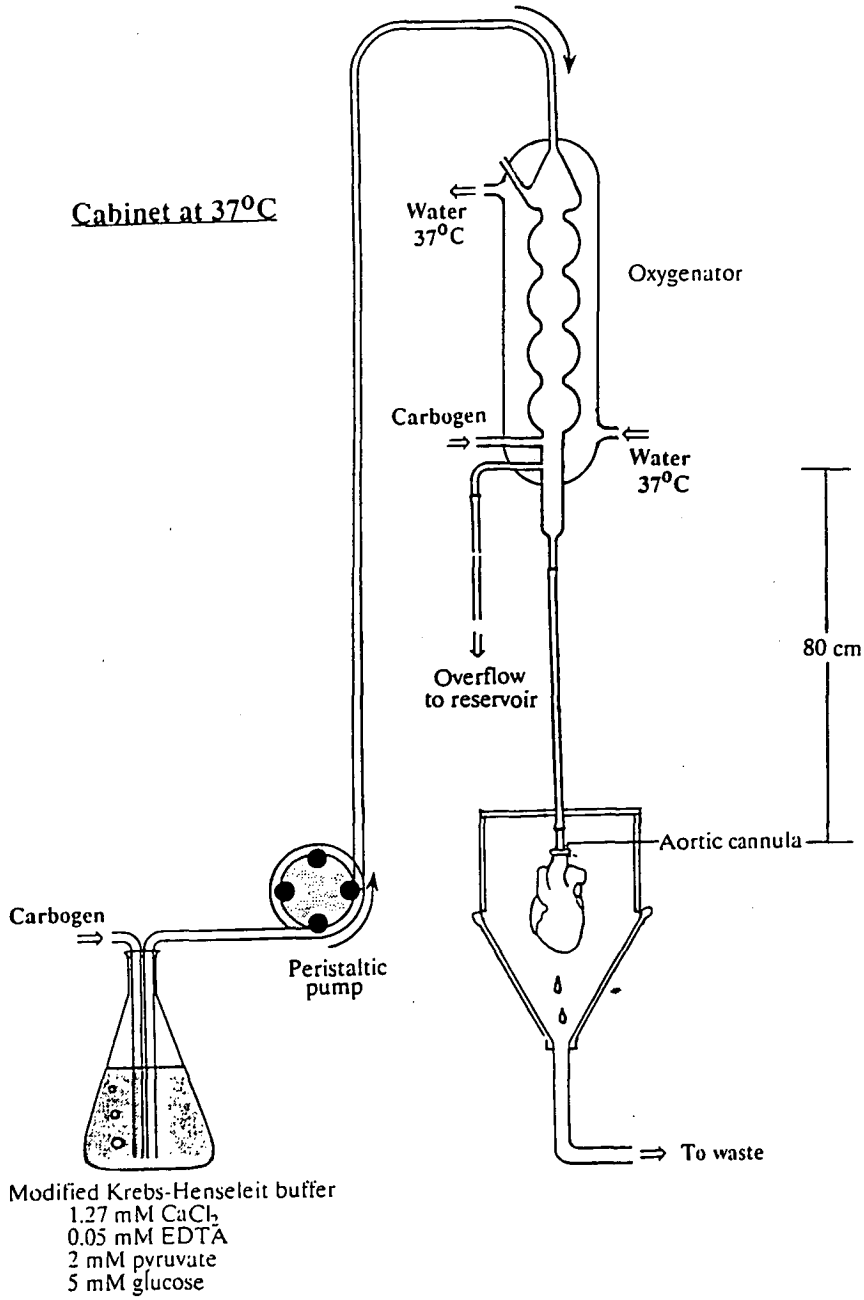

Fig 5.1 Perfusion apparatus.
A schematic representation of the non-working isolated perfused heart apparatus. For control perfusions (60 mmHg) the reservoir height was 80 cm above the aorta. For perfusions at elevated pressures (90 or 120 mmHg) the perfusion reservoir was raised to 120 and 160 cm respectively.

of the perfusion. In order to establish whether early-response gene expression was mediated by increased PKC activity or elevated cAMP levels hearts were similarly perfused at 60 mmHg in the presence of phorbol myristate (PMA) or forskolin at final concentrations of 20 nM and 2 μM respectively. At the end of the perfusion period, hearts were processed for northern analysis and analyzed as before.

5.3.3 Effect of Elevated Perfusion Pressure

In order to establish whether increased perfusion pressure and thus stretch of the ventricular wall, could modulate early-response gene expression independent of NE, hearts were perfused at 60, 90 or 120 mmHg for 1 to 3 h in the presence of buffer alone and early-response gene mRNA levels were analyzed as before.

5.3.4 Regional Localization of Early-Response Genes in the Perfused Heart

In order to assess the regional expression of early-response genes, hearts were perfused at 60 mmHg with NE or at 120 mmHg with buffer alone and then dissected into the various chambers and analyzed separately for early-response gene expression by northern analysis.

5.3.5 Treatment of Results

Following northern analysis autoradiograms were quantified by laser densitometry. Each sample was corrected for loading by comparison to the hybridization with the control probe β-tubulin and results expressed as a multiple of basal expression (fold basal). When the experimental number was n=3 or better, error bars represent standard error mean (S.E.M) and for n=2 error bars represent standard deviation (S.D.)

5.4 RESULTS

All hearts were pre-perfused for 15 min following removal from anaesthetized animals to allow them to equilibrate with experimental conditions. Low levels of c-*myc* but not c-*fos* mRNA could be detected in hearts directly after removal, although this required over exposure of the appropriate autoradiogram (Fig. 5.2, Lane

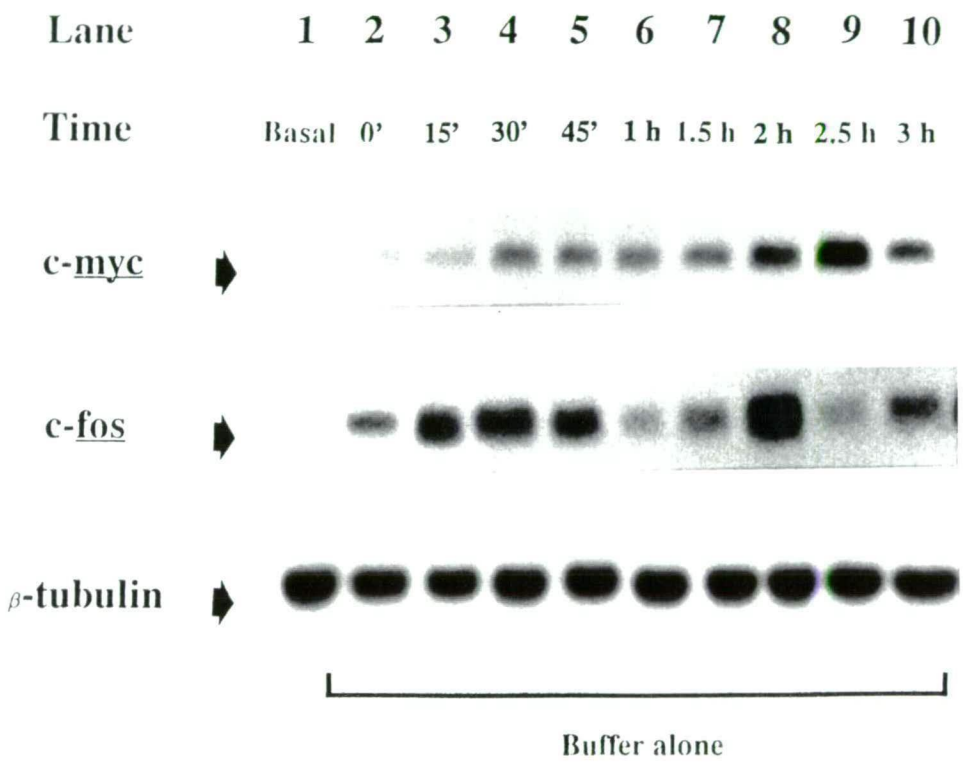

Fig 5.2 Cardiac expression of c-*myc* and c-*fos* in rat hearts perfused at a constant coronary pressure (60 mmHg).

Total RNA was extracted from rat hearts directly after removal from the animal (Basal, Lane 1) or at the various times indicated following perfusion at constant pressure of 60 mmHg (Lanes 2-10) with modified Krebs-Henseleit buffer. After electrophoresis and northern blotting, the RNA (50 μg) was hybridized to c-*myc* (upper tracks), c-*fos* (middle tracks) and β-tubulin (lower tracts).

1). However mRNA levels for both genes rose significantly as a consequence of perfusion with Krebs-Henseleit buffer at a constant pressure of 60 mmHg (Fig. 5.2, Lanes 2-10). Increased levels of c-*myc* mRNA could be observed after 15 min of perfusion and reached a peak between 2-3 h of perfusion. c-*fos* mRNA levels demonstrated a more complex profile since they reached a peak at 15-45 min and a second peak at approximately 2 h. These characteristic patterns for c-*myc* and c-*fos* expression were determined at each time point in 8 independent experiments with slight variation in the time course and intensity of the profile (see Fig. 5.10). Similar perfusion with 10 % bovine serum included in the perfusate did not alter the c-*myc* and c-*fos* expression profiles observed with buffer alone (results not presented). Similarly, prior treatment of hearts with α- and β–adrenergic antagonists before removal from the animals, also did not remove basal c-*myc* and c-*fos* expression (results not shown). All filters were rehybridized to the control probe β–tubulin to confirm that equal levels of mRNA were loaded in each track.

Inclusion of NE (0.1 μM, 1 μM) in the perfusion buffer significantly elevated the levels of c-*myc* and c-*fos* mRNA above that observed during perfusion with buffer alone at 60 mmHg, although c-*fos* was more responsive of the two genes (Fig. 5.3). The observed increase in mRNA levels was dose-dependent with greater expression observed with 1 μM NE than 0.1 μM NE although significant elevation of c-*myc* and c-*fos* above basal could be observed following perfusion with NE at levels as low as 1 nM (results not shown). Expression of some other genes structurally or functionally related to c-*fos* were also examined in hearts following perfusion with buffer alone or buffer containing NE (1 μM, Fig. 5.4). As with c-*myc* and c-*fos*, perfusion with buffer alone elevated mRNA levels of c-*jun*, *fra*-1 and *fra*-2. Inclusion of NE in the perfusate greatly elevated *fra*-1 and *fra*-2 mRNA levels however the increase in c-*jun* mRNA in response to NE was less intense with respect to hearts perfused with buffer alone (Fig. 5.4).

In order to determine the relative contribution of the α– and β-components of NE action on early-response gene expression, hearts were co-perfused with NE and the β-adrenergic blocker propranolol, or with NE and the α–adrenergic blocker phentolamine (Fig. 5.5 & Fig. 5.6). c-*myc*, c-*fos* and *fra*-1 were only responsive to

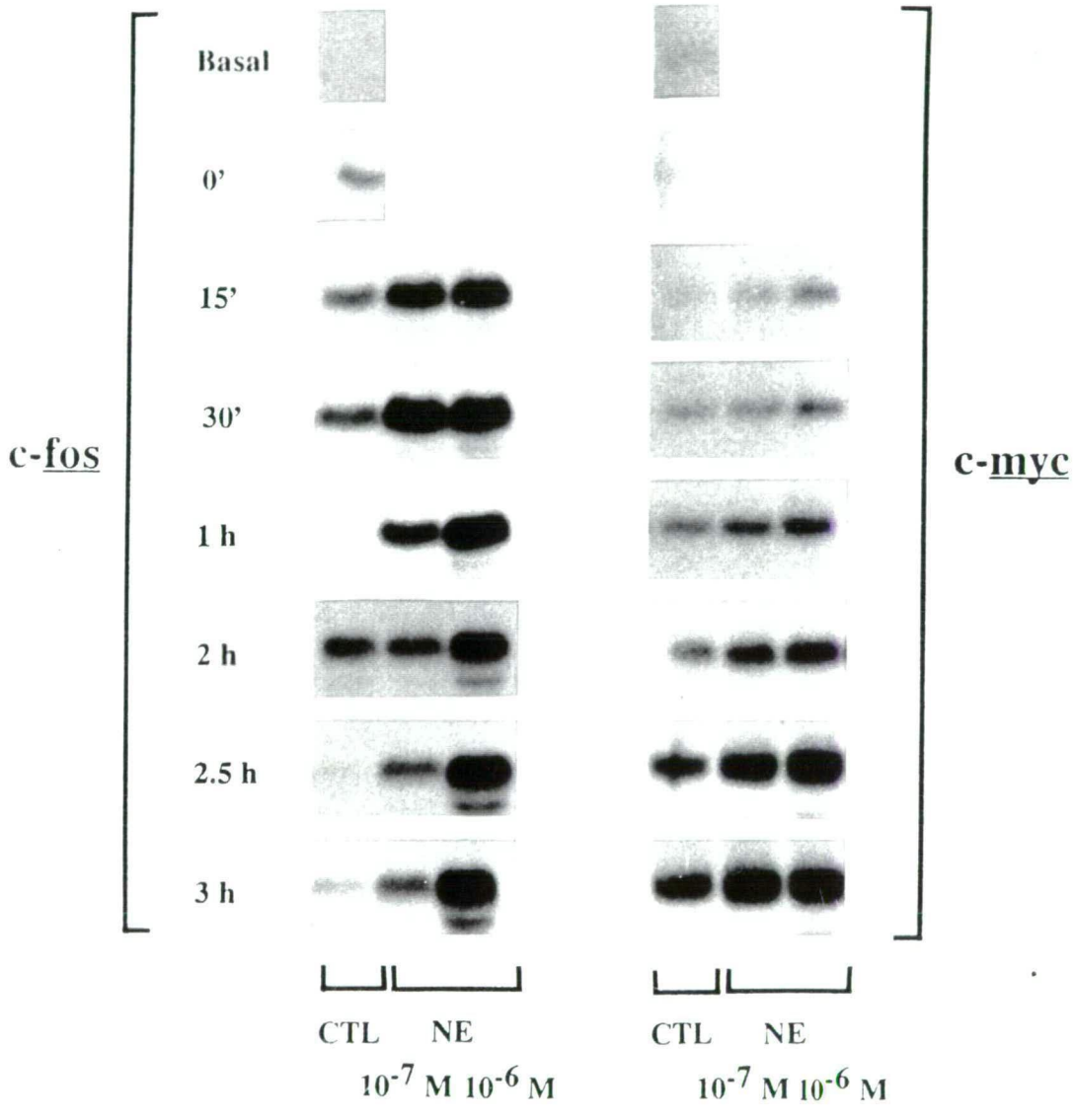

Fig 5.3 Induction of c-*myc* and c-*fos* in the perfused rat heart in response to norepinephrine.
Total RNA was extracted from rat hearts directly after removal from the animal (Basal) or at the various times indicated following constant perfusion at 60 mmHg with Krebs-Henseleit buffer alone (CTL) or with Kreb-Henseleit buffer containing norepinephrine (NE, 10^{-7}, 10^{-6} M). c-*myc* and c-*fos* transcripts were detected as previously described.

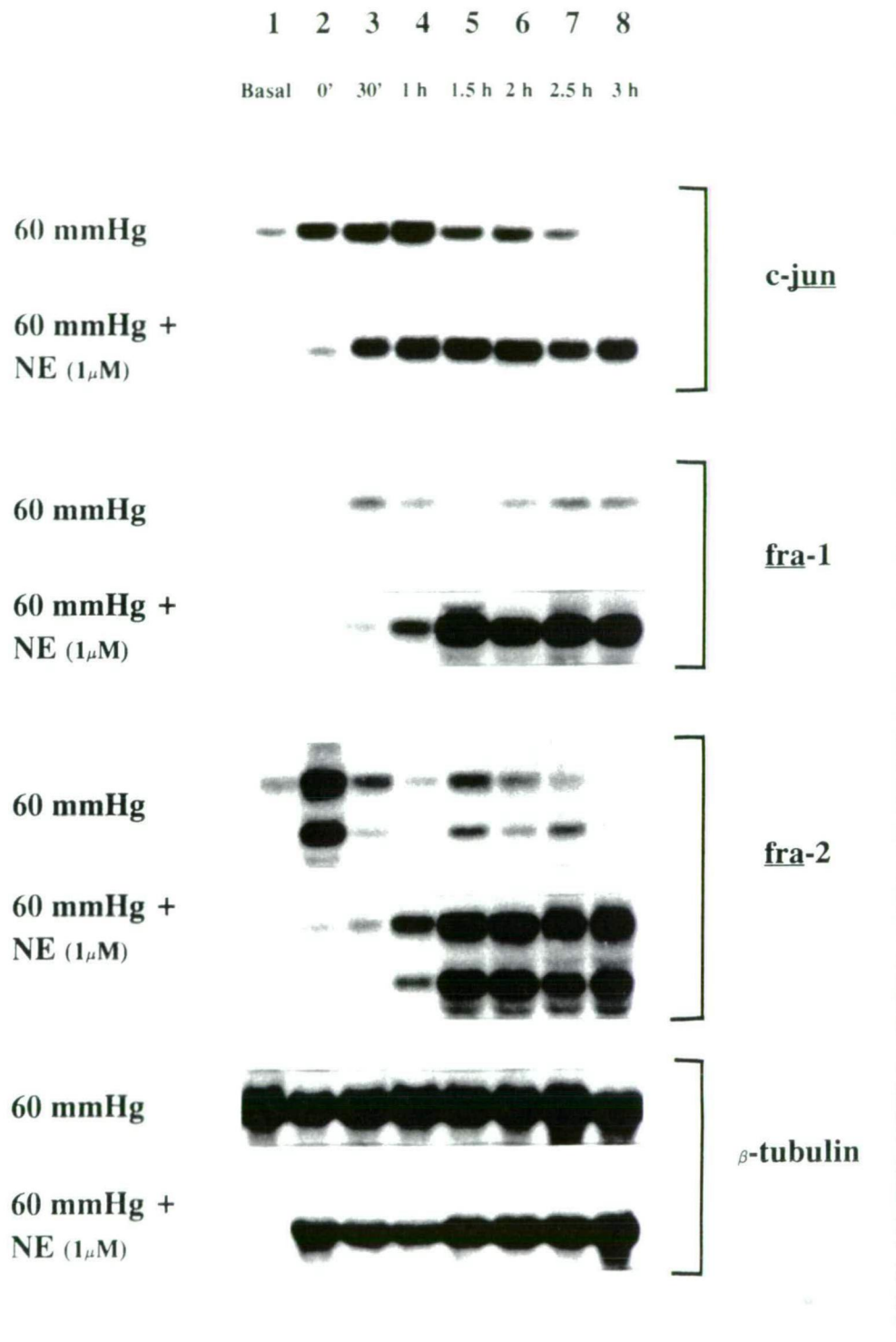

Fig 5.4 Cardiac expression of c-*fos* like genes and c-*jun* in the perfused rat heart in response to norepinephrine.

Total RNA was extracted from rat hearts directly after removal from the animal (Basal, Lane 1) or at the various times indicated following constant perfusion at 60 mmHg with Krebs-Henseleit buffer alone (Upper panel) or with buffer containing norepinephrine (1 μM, lower panel). c-*jun*, *fra*-1, *fra*-2 and β-tubulin transcripts were detected as previously described.

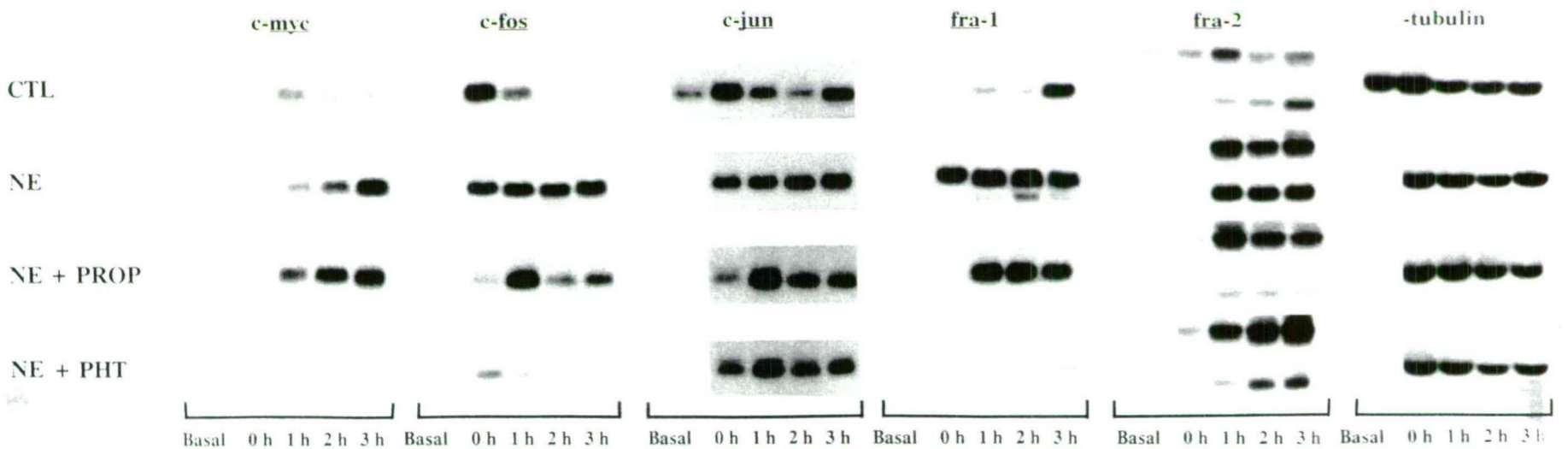

Fig 5.5 Contribution of the α- and β-components of norepinephrine action to early response gene expression in the perfused rat heart.
Total RNA was extracted from rat hearts directly after removal from the animal or at the various times indicated following constant perfusion at 60 mmHg with Krebs-Henseleit buffer alone (CTL) or with buffer containing norepinephrine (NE, 1 μM), norepinephrine and propranolol (NE, 1 μM + PROP, 20 μM) or norepinephrine and phentolamine (NE, 1 μM + PHT, 10 μM). c- *myc*, c-*fos*, c-*jun*, *fra*-1, *fra*-2 and β-tubulin transcripts were analyzed as described previously.

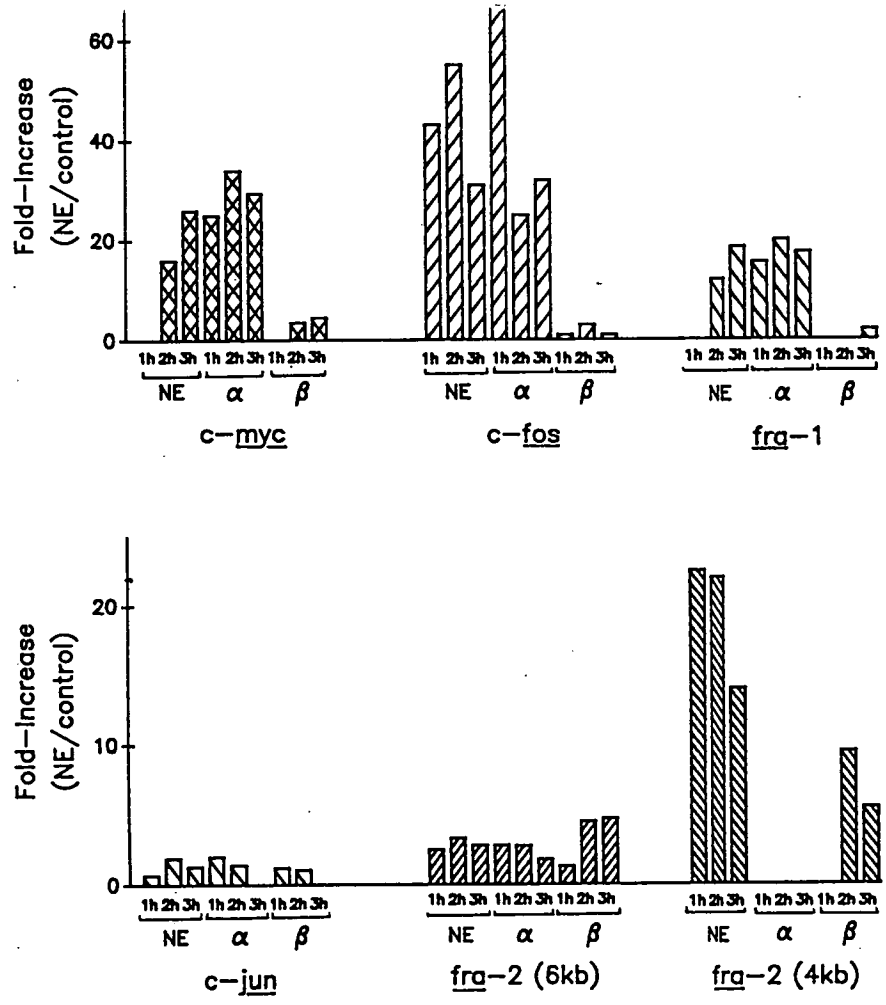

Fig 5.6 Quantification of the α- and β-components of norepinephrine action on early response gene expression in the perfused rat heart.

The hybridization signals generated by northern blotting in figure 5.5 were quantitated via densitometry and, after standardization to β-tubulin levels in each track, were expressed as the fold increase over control (basal) mRNA levels observed in hearts from untreated rats. NE: norepinephrine (10^{-6} M); α: NE (10^{-6}M) + propranolol ($2x10^{-5}$ M); β: NE (10^{-6}M) + phentolamine (10^{-5} M).

α–adrenergic stimulation whilst increased *fra*-2 mRNA levels were observed following both α- and β-stimulation. Interestingly, the pattern and timing of induction of the two *fra*-2 transcripts differed with respect to each other. The higher molecular weight transcript (6.0 kb) was responsive to both α- and β-adrenergic agents, rising to a peak at 1 and 3 h respectively. In contrast the lower molecular weight transcript (4 kb) was only sensitive to β-adrenergic stimulation and exhibited maximal expression at 3 h. Once again, relative to hearts perfused with buffer alone, α– and β-adrenergic agonists had little if any additional effect on c-*jun* expression. Similar perfusion with either α- or β-agonists (phenylephrine and isoproterenol respectively) produced similar expression of the early-response genes as the analogous experiments with NE + α or β blockers presented here (data not shown).

The mechanism or mechanisms which might couple the α-adrenergic receptors to modification of early-response gene expression in the heart are unknown. However recent studies in isolated neonatal myocytes indicate that α_1-adrenergic agents stimulate PKC and that stimulation with PMA, a potent activator of PKC, reproduces certain effects of α_1-stimulation on myocyte growth and gene expression, including induction of c-*myc* (Starksen *et al.*, 1986). Accordingly then, hearts were perfused in the presence of PMA to determine whether activation of PKC-dependent pathways might be linked to induction of c-*myc* in the adult heart (Fig. 5.7, Lanes 1-3). Hearts perfused at a constant pressure of 60 mmHg in the presence of PMA (20 nM) exhibited increased cardiac levels of c-*myc* mRNA with respect to hearts perfused with the biologically inactive phorbol ester, 4a phorbol 12b 12a-didecanoate (PDC: Fig. 5.7, Lanes 4-6). Although translocation of PKC was not directly measured this has been shown to occur in rat cardiac tissue following adrenergic stimuli (Allo *et al.*, 1992; Henrich and Simpson, 1988). Accordingly, these results provide indirect evidence that α_1-induction of c-*myc* occurs via activation of PKC associated pathways. The expression of other α_1-sensitive early-response genes was not examined in response to PMA.

Similarly, pathways which might link β-adrenergic receptor cellular occupation to early-response gene transcription in the heart are not known. However

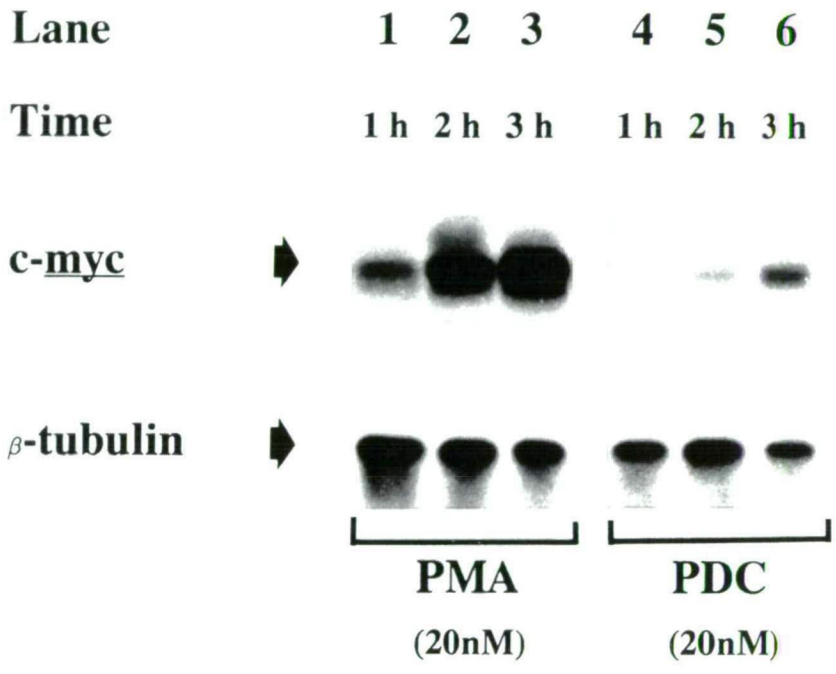

Fig 5.7 Cardiac expression of c-*myc* in response to PMA or PDC.
Total RNA was extracted from rat hearts removed at the various times indicated following 60 mmHg perfusion with modified Krebs-Henseleit buffer containing phorbol myristic acid (PMA, 20 nM; Lanes 1-3) or the inactive phorbol ester 4a-phorbol 12b 12a-didecanoate (PDC, 20 nM; Lanes 4-6). c- *myc* and β-tubulin transcripts were analyzed as described previously.

β-adrenergic agents increase cardiac levels of cAMP and augment protein synthesis and transcription of α-MHC. Furthermore, agents such as forskolin which also increase cAMP, lead to increased protein synthesis in the perfused heart (Xenophontus *et al.*, 1989). Accordingly, it was of interest to determine whether β-mediated induction of *fra*-2 was regulated by pathways linked to cAMP. Hearts perfused at a constant pressure of 60 mmHg in the presence of forskolin (2 μM) exhibited increased both *fra*-2 mRNA transcript levels (Fig. 5.8, Lanes 1-3) with respect to hearts perfused with buffer alone (Fig. 5.8, Lanes 4-6). The observed response was specific for the β-sensitive gene *fra*-2 since similar treatment did not increase expression of the α-sensitive gene c-*fos* (results not shown). Although cardiac cAMP levels were not directly measured, this study provides indirect evidence that *fra*-2 is activated by pathways which may involve cAMP.

5.4.1 The Effect of Increased Perfusion Pressure on Cardiac Early-Response Gene Expression

Pressure overload of the heart *in vivo* following aortic stenosis is accompanied by expression of a panel of early-response genes, quantitative and qualitative alterations in isocontractile protein synthesis and cardiac hypertrophy. Recent *in vitro* experiments have suggested that a parameter most related to increased protein synthesis in the perfused heart was increased stretch of the ventricular wall, as a consequence of increased aortic pressure (perfusion pressure) (Kira *et al.*, 1984). In light of this it was of interest to determine whether increased perfusion pressure might also augment expression of the early-response gene program in the perfused heart in absence of changes in humoral factors, and if so, to compare this response with the qualitative and quantitative changes that are observed following perfusion in the presence of NE.

When the hearts were perfused at constant pressure of 120 mmHg, cardiac c-*myc* and c-*fos* mRNA levels were significantly increased with respect to hearts perfused with buffer at 60 mmHg (Fig. 5.9). The level of c-*myc* expression was directly dependent on the perfusion pressure with 120 mmHg resulting in greater induction than 90 mmHg. The time of maximal c-*myc* expression occured after 2-3 h of perfusion at at

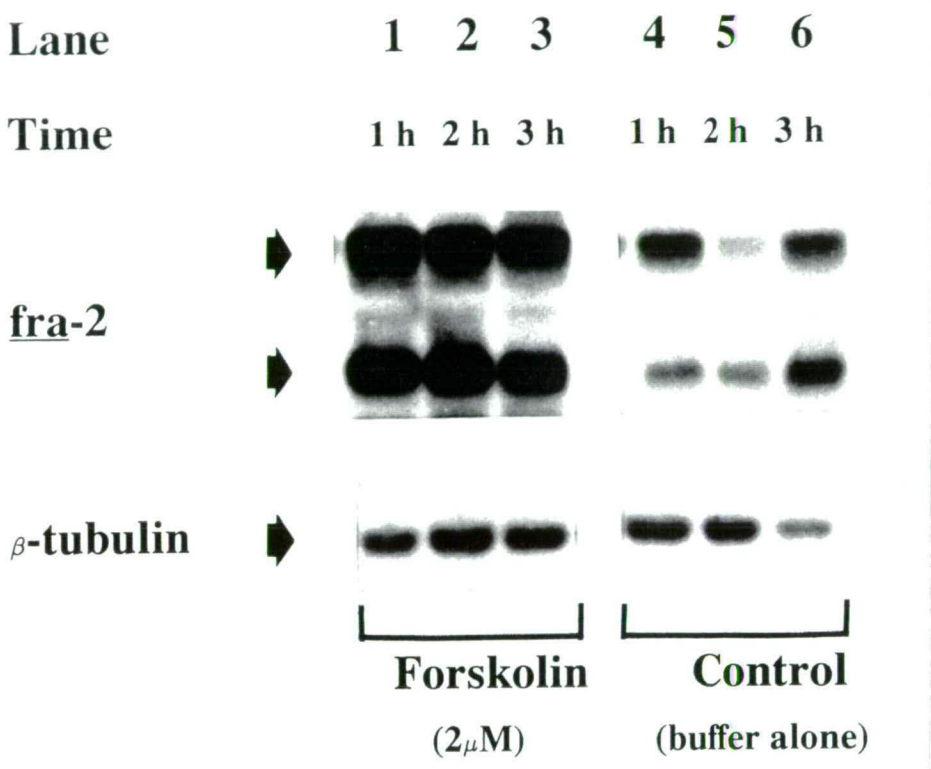

Fig 5.8 Cardiac expression of *fra*-2 in response to forskolin.
Total RNA was extracted from rat hearts removed at the various times indicated following 60 mmHg perfusion with Krebs-Henseleit bicarbonate buffer alone (Lanes 4-6) or buffer containing forskolin (2 μM, Lanes 1-3). *fra*-2 and β-tubulin transcripts were analyzed as described previously.

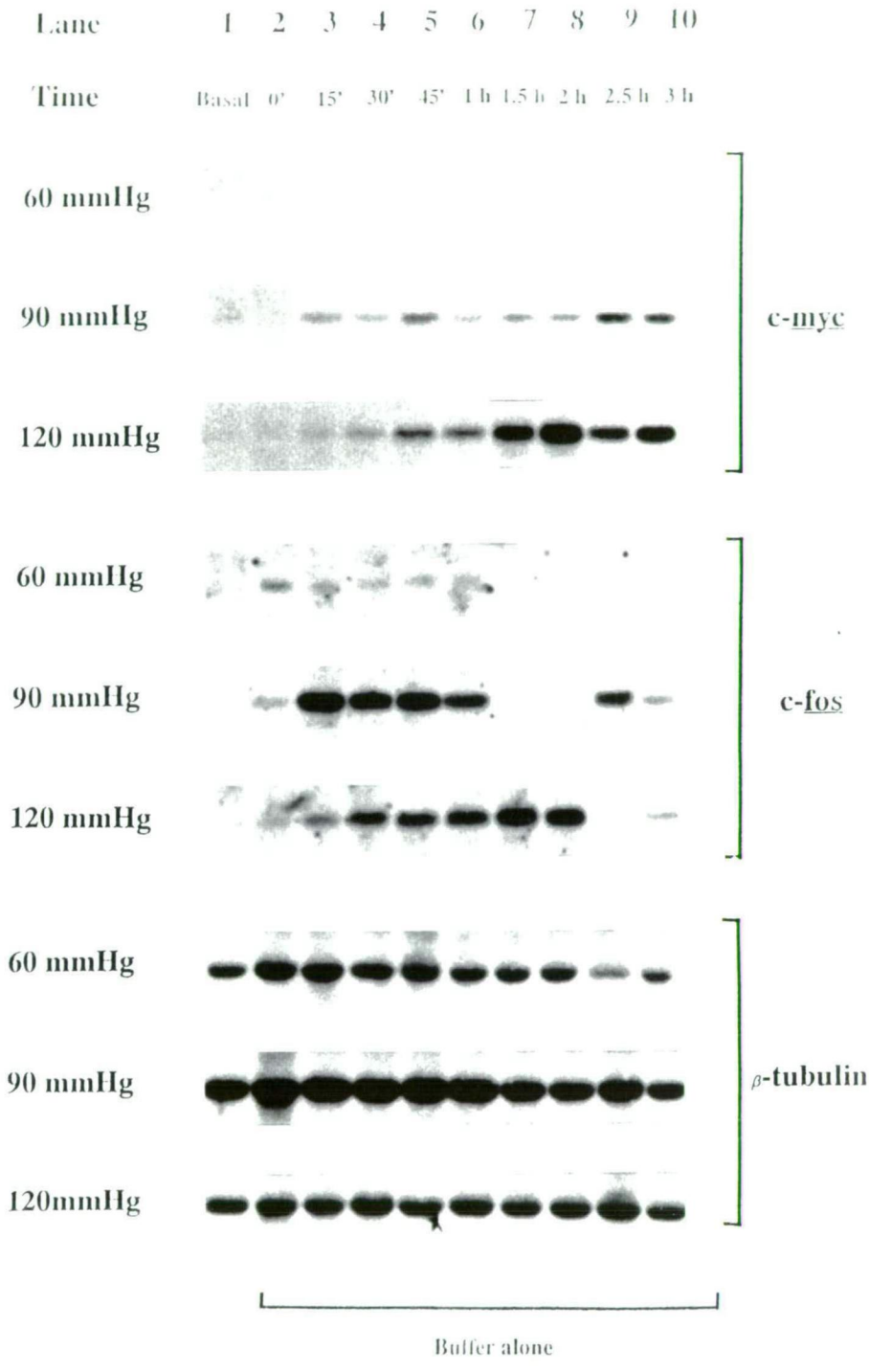

Fig 5.9 Effect of elevated coronary perfusion pressure on c-*myc* and c-*fos* expression in the rat heart. Total RNA was extracted from rat hearts directly after removal from the animal or at the various times indicated following perfusion at a constant pressure of 60, 90, or 120 mmHg. c-*myc* and c-*fos* transcripts were analyzed as described previously.

pressures of 90 and 120m although further studies demonstrated that mRNA levels of this gene remain elevated above basal perfusion levels for as long as the pressure load was maintained (see chapter 6, Fig. 6.1). In contrast the level of c-*fos* expression was approximately equivalent in response to perfusion at 90 or 120 mmHg, however the exact timing of maximal induction varied at each pressure: appearing maximal between 15-45 min for 90 mmHg and 1-2 h for 120 mmHg. In both cases the response was transient although expression of this gene was still slightly above baseline levels during extended perfusion at 120 mmHg for up to 6 h (see chapter 6, Fig. 6.1). Comparison of the relative abilities of pressure load and NE to modulate c-*myc* and c-*fos* expression indicates that c-*myc* is more responsive to aortic pressures equivalent to 120 mmHg than to NE (1 μM: Fig. 5.10), although removal of the β-component of NE raises levels of this gene to near those observed in response to 120 mmHg (comparative results not shown). In contrast c-*fos* appears to be equally responsive to both pressure (120 mmHg) and adrenergic stimulus (1 μM: Fig. 5.10).

Similarly, the expression of c-*jun*, *fra*-1 and *fra*-2 was investigated during elevated perfusion pressure (Fig. 5.11, autoradiograms not shown). c-*jun* expression was not altered significantly when perfusion pressure was adjusted to 120 mmHg with respect to hearts perfused at 60 mmHg, although this lack of relative induction may be due to the fact that c-*jun* was already induced to high levels during perfusion at 60 mmHg with buffer alone. Similarly *fra*-1 expression was not altered by the higher perfusion pressure and this is in accordance with observations *in vivo* that this gene is not pressure responsive (Rockman *et al.*, 1991). In contrast the α/β-responsive transcript of *fra*-2 (6.0 kb) was elevated by perfusion at 120 mmHg to levels similar to those observed in response to NE. However the β-responsive transcript of this gene (4.0 kb) was not significantly altered by this treatment.

5.4.2 Regional Localization of c-*myc* and c-*fos* Expression in the Perfused Heart

Determination of the regional expression of early-response genes in the rat heart *in vivo* (see chapter 4) following NE administration is potentially complicated by the hemodynamic effects of this hormone which can independently modify cardiac early-response gene expression (Mulvagh *et al.*, 1987; Komuro *et al.*, 1987; Komuro *et*

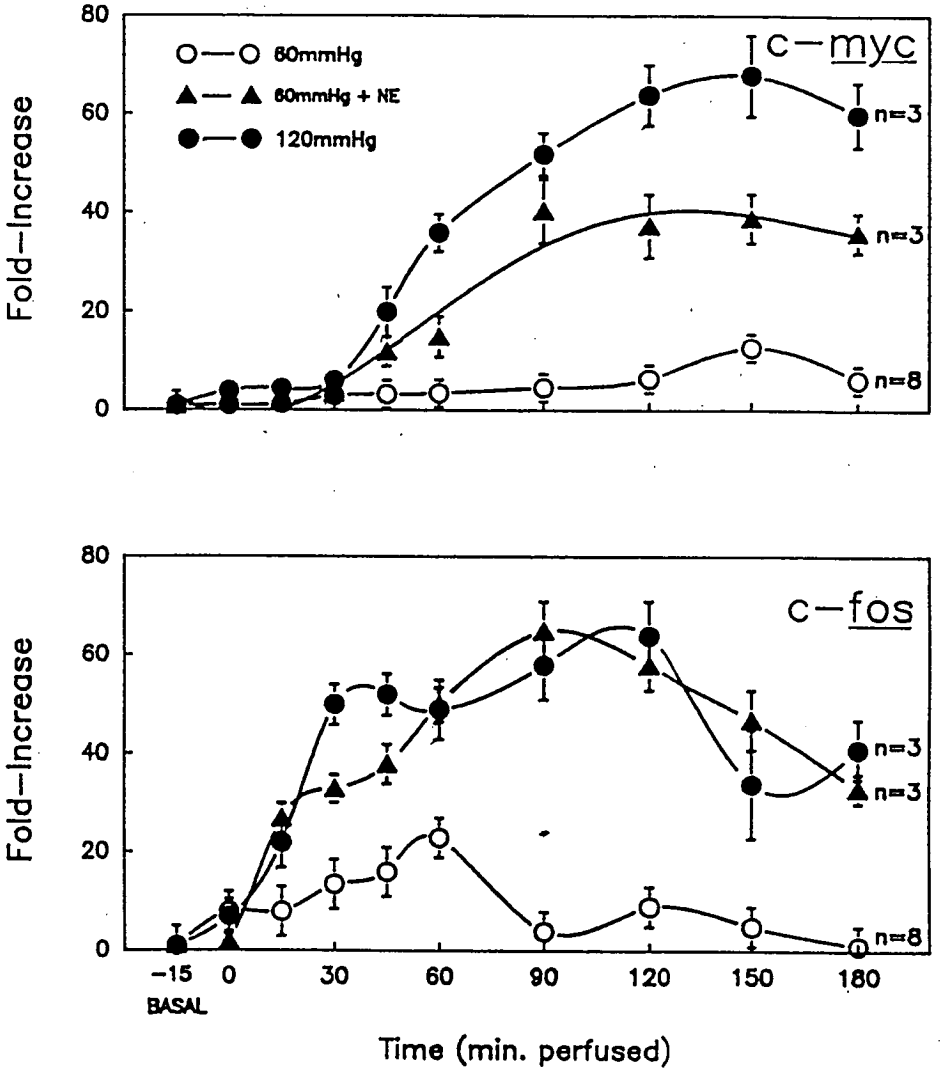

Fig 5.10 Quantification of c-*myc* and c-*fos* expression in response to elevated perfusion pressure or norepinephrine in the rat heart.

The hybridization signals generated by northern blotting in figures 5.1, 5.3 and 5.9 were quantitated via densitometry and, after standardization to β-tubulin levels in each track, were expressed as the fold increase over control (basal) signals observed in hearts perfused at 60 mmHg. Vertical bars indicate standard error mean (S.E.M.).

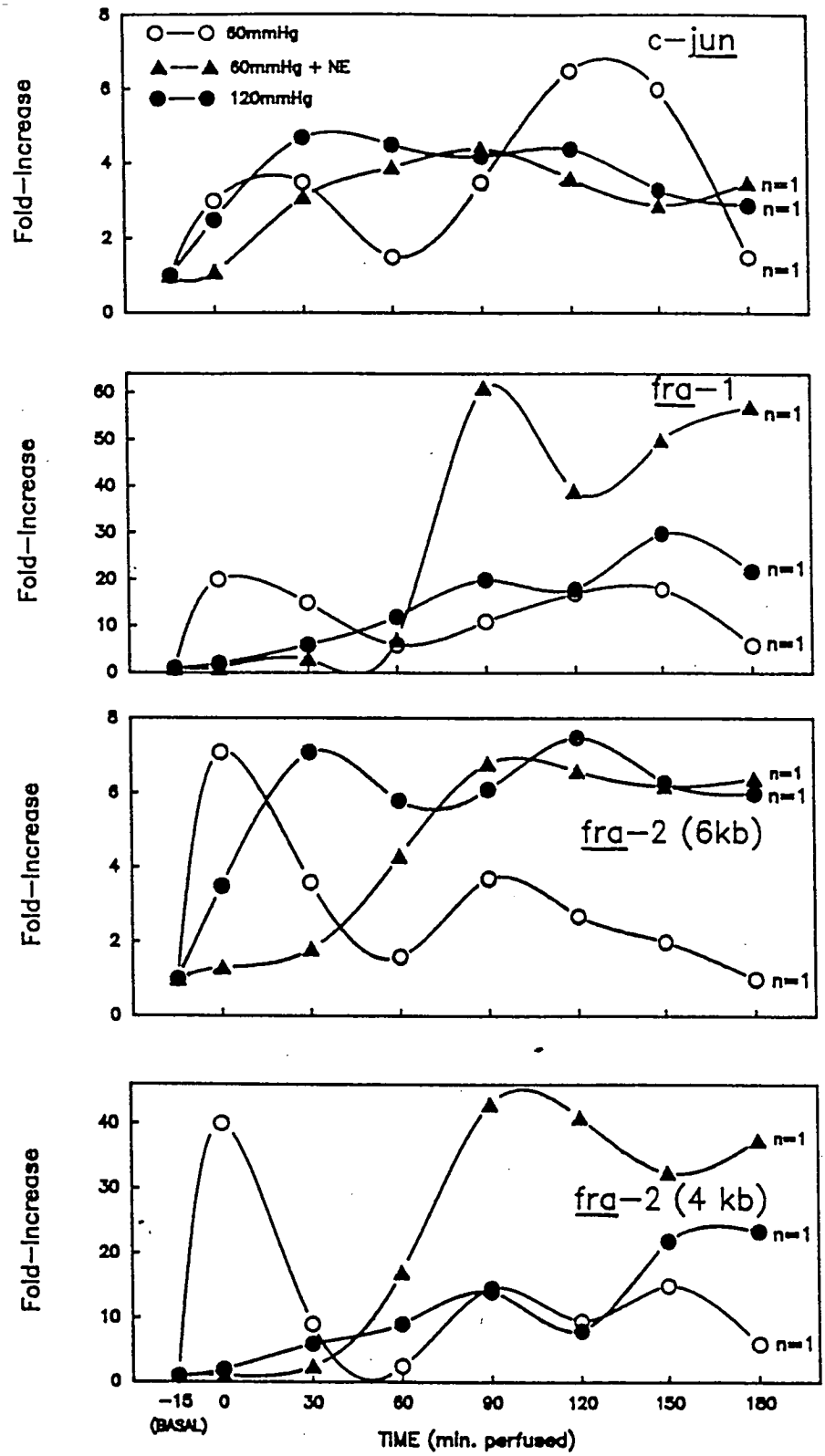

Fig 5.11 Quantification of c-*fos* like and c-*jun* gene expression in perfused rat heart in response to norepinephrine and elevated pressure.
The RNA hybridization signals observed by northern blotting in figure 5.4 and results not shown were quantitated via densitometry and, after standardization to β-tubulin levels in each track, were expressed as the fold increase over control (basal) signals observed in hearts perfused with Krébs buffer alone at 60 mmHg.

al., 1991). Accordingly, it was of interest to analyse separately the effect of NE and pressure on regional localization of these genes.

Perfusion at 60 mmHg resulted in greatest expression of c-*myc* mRNA after 3 h and this was located mainly to the combined atrial sample and the septum. Similarly, maximal c-*fos* expression was observed in the combined atrial sample and also the right ventricle after 1 h of perfusion and then subsequently, but at lower levels, in the septum after 3 h (Fig. 5.12). These autoradiograms were over-exposed relative to autoradiograms obtained from hearts perfused with NE (1 μM) or at 120 mmHg, in order that the regional expression could be better assessed.

Perfusion at 60 mmHg with NE (1 μM) resulted in maximal expression of c-*myc* and c-*fos* mRNA after 2 h and 1-2 h respectively and this was mainly localized to the left and right ventricle with considerably less expression in the septum (Fig. 5.12) and combined atrial samples. By the third hour of perfusion in the presence of NE, c-*myc* and c-*fos* mRNA were only observable in the left and right ventricle (Fig. 5.12).

Elevation of the perfusion pressure from 60 mmHg to 120 mmHg resulted in maximal c-*myc* expression after 3 h and this response was approximately of the same intensity in each chamber of the heart for each time point (Fig. 5.12). Similarly, c-*fos* mRNA levels were augmented by this treatment appearing maximal between 1 and 2 h however, whilst the response was approximately equal in the left and right ventricle and septum, little or no c-*fos* expression was observed in the combined atrial sample (Fig. 5.12).

These results are typical of three separate experiments and are not an artifact of loading as demonstrated by equal intensity of bands following hybridization to the control probe β-tubulin.

5.5 DISCUSSION

Cardiac hypertrophy is a complex process which is initiated and promoted by both hemodynamic and hormonal factors, but resolution of these can be difficult in whole animal models. For instance, pressure-overload (Komuro *et al.*, 1988; Mulvagh *et al.*, 1987) and NE administration (this thesis) have been shown to cause cardiac hypertrophy in adult rat hearts and elevate mRNA coding for a number of

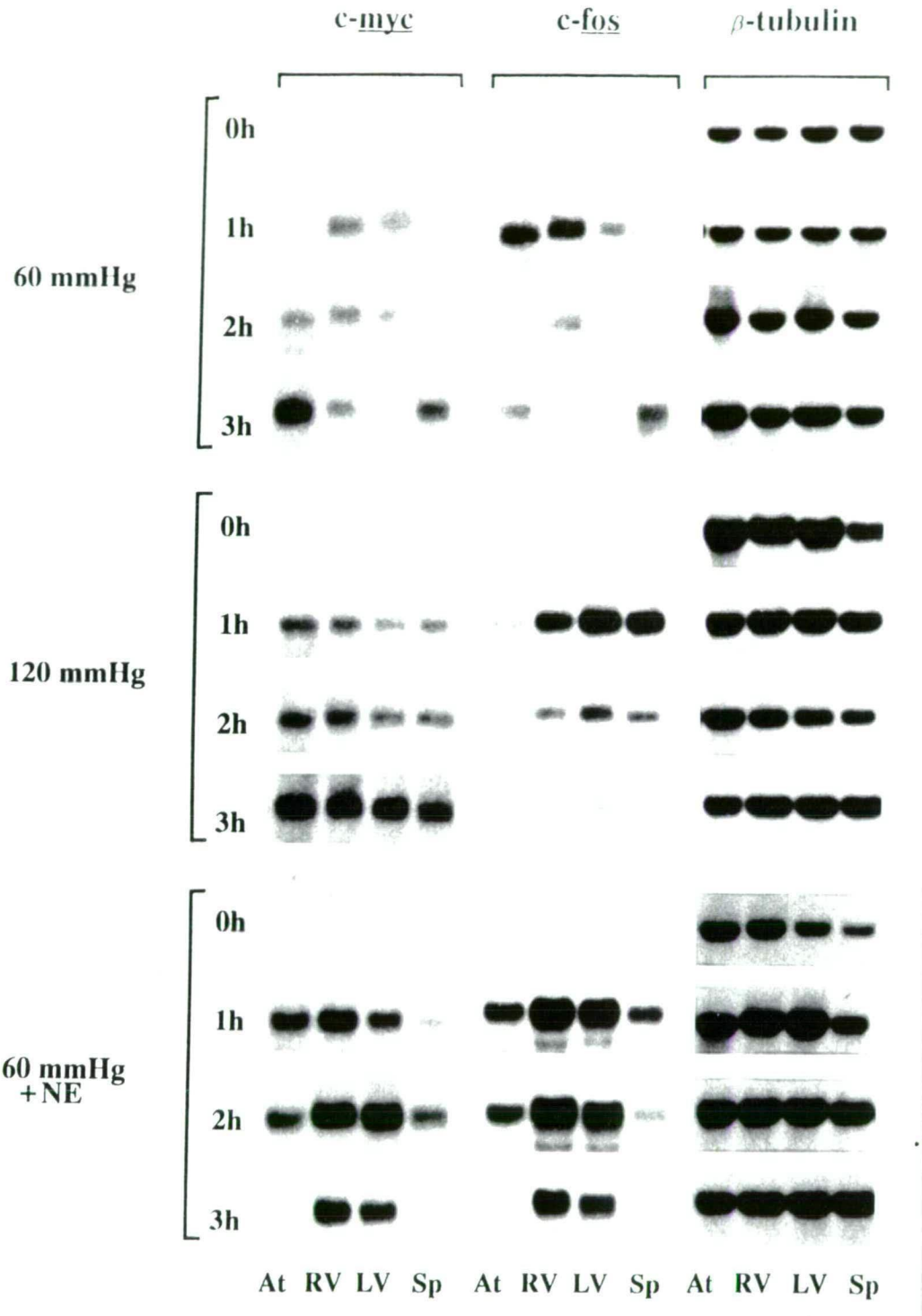

Fig 5.12 Regional expression of c-*myc* and c-*fos* in response to elevated perfusion pressure or norepinephrine in the perfused heart.

Total RNA was extracted from rat heart chambers (At: atria, RV: right ventricle, LV: left ventricle, Sp: septum) removed at the various times indicated following 60 mmHg perfusion with Krebs-Henseleit bicarbonate buffer alone at 60 mmHg or 120 mmHg or with buffer containing norepinephrine (1 μM) at 60 mmHg. c-*myc*, c-*fos* and β-tubulin transcripts were analyzed as described previously.

early-response genes. However, elevated pressure load and increased activation of the adrenergic system appear to be interrelated processes, for example, pressure overload following aortic stenosis has been associated with up regulation of cardiac-adrenergic receptors whilst administration of moderate to high doses of NE *in vivo* can lead to pressure overload of the heart. Accordingly the work in this chapter has attempted to dissociate the effect of some potential growth signals on cardiac early-response gene expression by use of the isolated, Langendorff perfused heart system. This model has the advantage over *in vivo* studies that it is possible to examine separately the effects of NE or pressure-load on some of the events in cardiac hypertrophy, such as increased protein synthesis and early-response gene expression, whilst still maintaining the potentially critical cell-cell interactions within this organ.

The coronary perfusion pressure for control hearts was selected at 60 mmHg since previous studies have indicated that this pressure results in sufficient coronary flow rates to ensure adequate oxygenation of isolated rat hearts by the relatively simple perfusion medium (Krebs-Henseleit buffer) used in these experiments (Williamson, 1964). Interestingly this treatment alone was sufficient to elevate cardiac mRNA of c-*myc*, c-*fos*, c-*jun*, *fra*-1 and *fra*-2 . Increased c-*myc* expression rose steadily during perfusion whilst c-*fos, c-jun, fra*-1 and *fra*-2 exhibited more complex profiles reaching a number of peaks during the perfusion period. These responses in the apparent absence of stimulatory factors are puzzling but may simply be due to "stress" associated with removal of the heart from its *in situ* surrounds and perfusion *in vitro*. For instance, surgery itself may lead to altered catecholamine and other neurotransmitter release (Manders and Vatner, 1976; Vatner and Braunwald, 1975; Vatner and Smith, 1974), whilst general anesthesia has been shown to depress most aspects of autonomic reflex control (Vatner *et al.*, 1971) reduce myocardial contractility and increase baseline heart rate (Manders and Vatner, 1976) in mammals. It is possible that any of these factors either alone or in combination could initiate early-response gene expression which would be observed during subsequent perfusion. The possibility of surgery leading to increased catecholamine activity was tested in these experiments by means of prior treatment of the animal with both α-and β-adrenergic blockers before heart removal, but this did not attenuate the expression of any of the genes examined. An alternative explanation for these results might be that the expression of these putative transcription

factors, a process which appears to be tightly regulated *in vivo*, may become deregulated during perfusion *in vitro* in the absence of possible serum-derived regulatory factors. For example, basal expression of c-*fos* appears to be at least partially regulated by binding of a serum response factor (SRF) to a serum response element (SRE) in the 5' control region of the c-*fos* gene (Curran, 1991; Angel and Karin, 1991) In order to test this hypothesis, hearts were perfused with Krebs buffer supplemented with freshly isolated bovine serum (10%), however no change in the expression profiles or intensity of any of the genes examined was observed with respect to hearts perfused with buffer alone. A further possibility is that particular regions of the heart are relatively poorly perfused at the basal aortic pressures used here (60 mmHg) and subsequent ischemia of these areas may lead to stress-related early-response gene expression. This is not unreasonable since c-*fos*, at least, is rapidly induced in tissue surrounding the infarct zone following experimental regional ischemia of the heart (author's personal observation). One possibility is that cardiac interstitial edema caused by perfusion with Krebs buffer may lead to microvascular compression and thus non uniform perfusion of the heart. Indeed during perfusion at 60 mmHg regional expression of c-*myc* and c-*fos* mRNA levels were not uniform but restricted mainly to the atria and right ventricle. However further studies are required to ascertain whether or not these regions are compromised during Langendorff perfusion at a pressure of 60 mmHg. This possibility could perhaps be tested by examining cardiac early-response gene expression in perfused hearts in which edema is minimized for instance, by increasing the density of the perfusate with substances such as dextran sulfate. Finally, it is entirely possible that the observed responses are simply due to stress associated with the higher than normal coronary flow rate associated with this *in vitro* model since the *in vivo* coronary flow rat in rat hearts is 2-4 ml/min/g compared to 6-8 ml/min/g in isolated hearts perfused under 60 mmHg of pressure.

i) induction of early-response genes in the perfused heart by NE

Although hearts exhibited increased expression of a number of early-response genes during perfusion at 60 mmHg of pressure with buffer alone, significantly greater cardiac expression of many of these genes was observed during perfusions in the presence of stimuli associated with cardiac hypertrophy *in vivo* and increased protein synthesis *in vitro* (Morgan *et al.*, 1991). For example, perfusion at a

constant pressure of 60 mmHg with Krebs buffer containing NE (1 nM to 1 μM) resulted in a significant increase the cardiac mRNAs of c-*myc*, c-*fos*, *fra*-1 and *fra*-2 with respect to hearts perfused at 60 mmHg with buffer alone. Quantitatively, these responses were of similar intensity to those observed following a single injection of NE *in vivo*. Interestingly, c-*jun* levels were not significantly altered during perfusion with NE with respect to hearts perfused with buffer alone. It is possible that this gene is already maximally stimulated by perfusion with buffer alone or that c-*jun* does not respond specifically to NE but rather is simply a marker of cellular stress. Certainly the difference in response of this gene with respect to the functionally related gene c-*fos* is interesting and warrants further investigation.

At least part of the observed early-response gene expression *in vivo* following NE treatment is likely to be attributable to secondary pressure loading following vasoconstriction of the peripheral vasculature. In contrast, in these *in vitro* experiments NE-mediated induction of early-response genes cannot simply be ascribed to pressure loading of the heart since pressure load was held constant at 60 mmHg. Furthermore the responses cannot be due to local vasoconstriction of the coronary vasculature since, as the result of local metabolic affects, NE does not mediate constriction of the cardiac vasculature (Vatner, 1992 and references there in). It is possible, however, that the pronounced effects that NE has on cardiac inotropy and chronotropy may indirectly modulate cardiac expression of these putative trans-activating factors. In argument against this, studies from this laboratory have shown that significant reduction of cardiac contractility by perfusion with buffers containing low Ca^{2+} levels, does not significantly alter NE-mediated early-response gene expression in the perfused heart (unpublished data). Thus taken together with the previous results, these experiments provide the first evidence that NE can activate the early-response gene program in adult myocytes (c-*fos*, c-*jun* and related genes) and in cardiac non myocyte cells (c-*myc*) independent of the changes in cardiac hemodynamic parameters that this hormone causes. Since similar perfusion of hearts with adrenergic agents has been shown to augment protein synthesis and ribosomal formation (Morgan *et al.*, 1992), the present studies provide further evidence implicating early-response genes in the initiation of cardiac hypertrophy *in vivo*.

The regional localization of c-*myc* and c-*fos* mRNA during perfusion with NE revealed that both early-response genes were expressed to approximately the same extent in the left and right ventricle. These results are not unexpected since both ventricular chambers should receive equal exposure to the drug, however this distribution contrasts significantly with *in vivo* studies (chapter 4) in which both genes were expressed predominantly in the left side of the heart following a single injection of NE. It is probable that the significant physiological differences in stimuli to which the myocardium is exposed in each of these two model systems is responsible for the differing spatial distributions. For instance, pressure overload of the left side of the heart following administration of NE *in vivo* most probably contributes to the increased early-response gene expression in this region and such an affect would be absent *in vitro*.

Interestingly, expression of c-*myc* and c-*fos* in the atria and septum of hearts perfused in the presence of NE, is significantly less than in the left and right ventricles. Such differences suggest that the pathway(s) linking occupation of the adrenergic receptors to nuclear expression of c-*myc* and c-*fos* in these regions are less responsive than in the ventricles. It is possible that this simply reflects tissue distribution of adrenergic receptors, or alternatively, the atria and septum may be poorly perfused and therefore receive less exposure of NE than regions which might be better perfused such as the ventricles. Further studies are required to verify these possibilities.

ii) α -adrenergic response

in vivo studies presented in this thesis indicate that a significant proportion of NE-mediated cardiac early-response gene expression can be reproduced by stimulation of the α-adrenergic receptors. These findings are supported by the present *in vitro* studies since perfusion in the presence of NE and the β-adrenergic blocker propranolol or perfusion with the α-adrenergic agonist phenylephrine led to significant elevation of c-*myc*, c-*fos*, *fra*-1 and *fra*-2 mRNA levels above those observed following perfusion with buffer alone. α_1-adrenoreceptor stimulation is thought to stimulate phosphoinositide-hydrolysis of membrane phosphatidylinositols which stimulate the production of diacylglycerol and inositol phosphate (reviewed in Berridge, 1987). Diacylglycerol in turn stimulates protein kinase C to phosphorylate target proteins

whilst inositol triphosphate increases cytoplasmic Ca^{2+} concentration by stimulating release of calcium from intracellular stores. Both of these signaling pathways are likely transducing mechanisms of α-adrenergic-mediated early-response gene expression since both calcium and PKC regulatory elements have been identified in the promoter regions of a number of early-response genes. In these studies α_1-adrenergic induction of c-*myc* could be reproduced by perfusing hearts in the presence of PMA, a potent activator of PKC, however no similar response was observed following treatment of hearts with the inactive phorbol ester PDC. Although translocation of PKC was not directly studied in these experiments, this event has been demonstrated to occur in the heart and isolated myocytes following adrenergic stimulation (reviewed in Simpson, 1990). Moreover, these results are in close agreement with those of Simpson's group who have demonstrated in neonatal myocyte cultures that agents which activate PKC also increase myocyte expression of c-*myc* and cardiac hypertrophy (Starksen *et al.*, 1986). Thus considered together these studies are indirectly suggestive that cardiac expression of c-*myc* by NE, in both the adult heart and in neonatal myocytes, is at least partially regulated via PKC dependent pathways. Similarly, although not tested in these studies, it is likely that other α-adrenergic inducible early-response genes such as c-*fos* are also regulated via PKC pathways in the adult heart, since induction of these genes has also been correlated with activation of this second messenger system in neonatal myocyte cultures. Further studies will need to determine whether PKC induction is a necessary prerequisite for early-response gene expression in the heart following α_1-adrenergic stimulus and if so, to determine which specific isoforms of PKC are translocated under these conditions. It appears that at least some of these points are currently being investigated in neonatal myocyte cultures (Simpson, 1991).

iii) β *-adrenergic response*

In contrast to α-adrenergic stimulation, perfusion of hearts in the presence of β-adrenergic agents resulted in increased expression of *fra*-2 only, whilst cardiac mRNA levels of other genes examined following this treatment were either unchanged (c-*jun*) or even lower (c-*myc*, c-*fos*, and *fra*-1) than observed following perfusion with buffer alone. These results are not however, in entire agreement with those from *in vivo* studies (this thesis; Barka *et al.*, 1987). For instance in this thesis, treatment of intact

animals with β-adrenergic agents resulted in small increases in the levels of c-*fos* c-*jun* and *fra*-1 and much greater elevation in c-*myc* and *fra*-2 with respect to the levels of these genes observed in response to α_1-stimulus alone or NE. Similarly Barka's group demonstrated cardiac expression of one early-response gene, c-*fos* following administration of both α– and β–adrenergic agents (Barka *et al.*, 1987). A number of interpretations of these results are possible. Firstly, given the ability of β-adrenergic agents to elevate *fra*-2 expression in the heart both *in vivo* and *in vitro* it is likely that this putative transactivator is a *bona fide* β-adrenergic responsive gene in the adult heart. In contrast c-*fos*, c-*jun* and *fra*-1 which exhibited no expression *in vitro* and only low expression *in vivo* following β-agents, are probably not directly regulated by β-receptor occupation in target cardiac cells. Rather, *in vivo* induction of these genes is more likely to be the result of cardiac α–adrenergic stimulation following β-mediated release of NE from presynaptic nerve terminals (see chapter 1 for a more in-depth discussion). In contrast to the heart *in vivo*, isolated perfused hearts are denervated and thus are not subject to this presynaptic release.

It is more difficult to explain the apparent inconsistencies with c-*myc* given the intensity of the β-adrenergic response of this gene *in vivo* and the absence of induction *in vitro*. Interestingly this *in vitro* response is in agreement with neonatal myocyte culture studies of Simpson which also failed to link β-adrenergic activation to c-*myc,* albeit in neonatal myocyte cultures (Starksen *et al.*, 1986). It is possible then, that c-*myc* is indirectly stimulated by non-cardiac derived humoral factors following β-stimulation *in vivo* and consequently cardiac cells would not be exposed to these factors during perfusion *in vitro* or during cell culture. This notion might be tested by comparing c-*myc* expression in cultured cardiac cells following incubation with serum derived from normal rats or rats previously treated with β-adrenergic agents. Further studies are clearly warranted to resolve this matter.

In the heart β-adrenergic agonists alter a number of ionic and second messenger systems and among these responses probably the strongest mechanistic link is a rapid increase in cAMP levels and this preceeds such events as cardiac growth. For example, Morgan's group has demonstrated that agents such as forskolin that raise levels

of cAMP in the perfused heart (Xenophontos *et al.*, 1989), also increase protein synthesis and ribosome formation in that tissue whilst other studies have isolated a number of cAMP-regulated genes that are responsive to growth factors/hormones (Roesler *et al.*, 1988). Furthermore, many early-response genes including those of the c-*fos* family contain cAMP response elements in the 5' control regions. Thus taken together, cellular transduction pathways involving cAMP are likely mechanisms by which β-adrenergic agents affect *fra*-2 expression in the heart. Indeed the present studies support this notion since perfusion of hearts with forskolin, a drug known to increases cellular cAMP levels, resulted in increased cardiac expression of *fra*-2, and this response was quantitatively and qualitatively similar to that observed in response to β-adrenergic agents. Furthermore the observed induction was specific for the β-responsive gene *fra*-2 since similar treatment did not augment cardiac levels of the α-responsive gene c-*fos*. Thus these results demonstrate that cAMP is a possible second messenger system linking β-adrenergic receptor stimulation to *fra*-2 expression in the rat heart. Once again further studies will need to determine whether elevation of cAMP levels is required for, or merely accompanies β-adrenergic induction of *fra*-2 in the heart and this could perhaps be achieved using specific inhibitors of cAMP formation or addition of the cAMP analogue 8-bromo-cAMP.

iv) the effect of increased perfusion pressure on cardiac early-response gene expression

Recent reports have demonstrated that experimental aortic stenosis in rodents leads to a rapid rise in the cardiac levels of a number of early-response genes and that this event is followed by increased protein synthesis and cardiac hypertrophy (Mulvagh *et al*, 1987; Izumo *et al*, 1988; Komuro *et al*, 1988). However because such studies have been conducted *in vivo* it has not been possible to determine whether increased load itself is directly coupled to increased gene transcription and protein synthesis or whether in fact these responses are mediated via secondary release of humoral factors either from within the heart (paracrine or autocrine) or from non cardiac tissue (endocrine). Accordingly, part of the work in this chapter has sought to discount the possible contribution of circulating humoral factors to the initial stages of pressure-load induced cardiac hypertrophy by examining early-response gene

expression in isolated hearts perfused with a serum-free medium (Krebs-Henseleit buffer) under increasing aortic pressure loads (60-120 mmHg). This model was specifically chosen since Morgan's group using this model have been able to demonstrate that increased stretch of the ventricular wall as a direct consequence of elevated aortic pressure leads to activation of early events in hypertrophy such as increased ribosome formation and protein synthesis (Kira *et al.*, 1984; Morgan *et al.*, 1987; McDermott and Morgan, 1989).

When the aortic perfusion pressure was increased from 60 mmHg to 90 or 120 mmHg a significant increase in cardiac expression of c-*myc*, c-*fos* and *fra*-2 was observed with respect to hearts perfused at 60 mmHg. The induction of c-*myc* and c-*fos* was more rapid than observed *in vivo* following aortic stenosis (Izumo *et al.*, 1988; Mulvagh *et al.*, 1987; Komuro *et al.*, 1988) and this may indicate that the stimulus *in vitro* is greater or more consistent than observed in the *in vivo* preparation. Interestingly the response of c-*myc* and c-*fos* to the increased load differed with respect to each other. For instance, c-*myc* expression is proportionally greater at 120 mmHg than 90 mmHg whist elevation of aortic pressure from 90 to 120 mmHg had no additional effect on c-*fos* expression. One interpretation of these results is that c-*fos* has a lower threshold of inducibility by pressure than c-*myc* and is thus already maximally stimulated during perfusion at 90 mmHg. During the course of these studies Swynghdauw's group published data complimentary to that presented here in which they demonstrated that c-*myc* and c-*fos* mRNA levels increase in the isolated perfused heart in direct proportion to the increase in coronary flow (Bauters *et al.*, 1988). Since coronary flow and coronary perfusion pressure are directly related in non-working Langendorff preparations, these two studies provide preliminary evidence that increased coronary perfusion pressure and/or coronary flow is sufficient stimulus to increase cardiac early-response gene levels.

The cardiac expression of these two genes during elevated perfusion pressure was further characterized at the level of each cardiac chamber. Elevation of perfusion pressure resulted in an equivalent induction of c-*myc* in all chambers of the heart and this probably indicates that these regions are subjected to similar loading via the coronary vasculature system. Regional distribution of c-*fos* was similar to c-*myc*

with the exception of the atria where mRNA levels of this gene were extremely low during elevated perfusion pressures. These results indicate that following pressure load, prior expression of c-*fos* is not a necessary prerequisite for the induction of c-*myc,* at least in the atria. Thus pathways linking pressure load and c-*fos* induction are either absent or down regulated in atrial tissue and given the inducibility of this gene in the atria via NE, indicates that pressure load and NE activate c-*fos* via alternate mechanisms.

In addition to c-*fos* and c-*myc* these studies demonstrated for the first time that *fra*-2, a gene structurally related to c-*fos*, is inducible in the heart following increased mechanical load and considered with the results presented previously, they establish this gene as a further member of the early-response gene family responsive to contrasting types of hypertrophic stimulus (e.g. NE and aortic stretch). However not all genes structurally or functionally related to c-*fos* appear to be responsive to increased aortic pressure, since neither c-*jun* nor *fra*-1 mRNA levels were increased in the heart when aortic pressure was raised from 60 mmHg to 120 mmHg. The failure to observe increased expression of *fra*-1 correlates well with the observation of Chein's group who reported that aortic stenosis in mice does not increase cardiac expression of *fra*-1, although this treatment led to increases in cardiac c-*fos*, and c-*jun* levels (Rockman *et al.*, 1991). Taken together these results provide further evidence of the differential responsiveness of c-*fos* and related families of early-response genes during hypertrophic stimulus and strengthens the notion that differential expression of these genes is a means to ensure diversity and specificity of cellular responses to extra-cellular stimuli, thus allowing for target gene selectivity. In contrast, the lack of responsiveness of c-*jun* to increased aortic pressure is puzzling especially considering that other groups have reported induction of this gene both *in vivo* and more recently in an *in vitro* study similar to this, in which it was shown that increasing left ventricular systolic force gave rise to increased ventricular mRNA levels of c-*fos* and c-*jun* (Schunkert *et al.*, 1991). Such discrepancies might be explained by the differences in the nature of the load stimulus between different experiment systems. In the present studies increased early-response gene expression can most probably be correlated with the increase in passive systolic stretch to which these chambers are exposed. For example, elevation of

perfusion pressure has direct effects on the ventricular wall (and presumably the atrial wall) to elevate intracoronary blood volume by as much as 60% (Morgenstern et al., 1973), to increase sarcomere length by 10% and to stretch and thicken the ventricular wall (Olsen *el al.,* 1981). In contrast, in the studies of Schunkert *et al.,* (1991) hearts were subjected to an acute increase in systolic wall stress by means of inflatable balloons in the ventricular space. It was concluded that active systolic tension, but not passive stretch was the major factor controlling increased cardiac early-response gene levels in this isolated heart model. Obviously the models used in the present studies and those of Schunkert and Swynghdauw do not fully mimic events which take place during pressure overload *in vivo.* Clearly further studies are needed to determine the relative contribution of active and passive stretch to increased cardiac early-response gene expression and left ventricular hypertrophy *in vivo* following aortic stenosis. In this respect however it is worthy to note that the experiments of Morgan's group established that intraventricular pressure development, cardiac contraction, oxygen consumption, glucose 6-phosphate production, energy availability and coronary flow could be dissociated from the stimulatory effect of higher aortic pressures on protein synthesis. Rather, they suggested that passive stretch of the ventricular wall as consequence of increased aortic pressure, could be the mechanical parameter most closely related to the observed increased protein synthesis (Kira *et al.*, 1984).

Despite the minor differences described above, the *in vitro* studies described here and those of others are supportive of the idea that increased wall stress in general is a potent signal for the induction of a subset of early-response genes.

The results presented here have demonstrated that increases in circulatory levels of non-cardiac derived growth factors such as NE are not required for pressure mediated increases in cardiac early-response gene expression. Recent studies have demonstrated that direct stretching of neonatal myocytes in culture is sufficient to induce c-*fos* expression independent of humoral factors (Komuro *et al.*, 1989; Komuro *et al.*, 1991) and therefore it is likely that stretching of adult myocytes as a consequence of increased aortic pressure is the primary and direct stimulus for the observed early-response gene expression in the present experiments. However, as discussed previously, evidence is accumulating to implicate non-myocyte derived cardiac growth factors as major regulators of cardiac gene expression and growth. For example

endothelin derived from endothelial cells and a number of fibroblast-derived growth factors are capable of inducing early-response gene expression and hypertrophy in cultured myocytes (Neyses *et al.*, 1991; unpublished data in Parker *et al.*, 1991). Further studies will be needed to ascertain whether such paracrine factors are important mediators of early-response gene expression and cardiac hypertrophy in adult myocytes following pressure overload.

The intracellular pathways by which mechanical stimulus might directly or indirectly stimulate early-response gene expression in the perfused heart were not studied in this work although results from others have implicated a number of candidate transducing mechanisms including PKC, cAMP, ion fluxes and direct mechanical connections between external and nuclear membranes. However the results from such studies have been less than definitive and further studies are required to fully understand the mechanisms of mechanical force on cardiac growth.

v) summary

The studies in this chapter have made use of an isolated perfused heart system in order to dissociate the direct and indirect affects of adrenergic hormones on cardiac early-response gene expression. With this model it was demonstrated for the first time that treatment with NE increases mRNA coding for a number of early-response genes in the adult heart, and that this response can be dissociated from the systemic hemodynamic effects often associated with this hormone *in vivo*. These results are in agreement with the *in vivo* studies presented earlier since they confirm that the majority of these responses are mediated by the α-adrenergic receptors. However one gene, *fra*-2 was also responsive to β-adrenergic stimulation, although the significance of this is yet to be determined.

In a complimentary series of experiments it was also shown that increased aortic pressure load in hearts perfused in the absence of NE and other growth factors, also elevated cardiac levels of a number of early-response genes. However, the exact subset of genes and the temporal, spatial distribution of the responses differed to that observed following treatment with NE. Taken together, and with those of previous chapters these studies clearly illustrate that adrenergic hormones and pressure load can independently regulate early-response gene expression in the adult heart. Accordingly then it is possible to speculate that particular subsets of these nuclear acting genes may

serve as common transcription factors in cellular pathways connecting adrenergic stimuli and mechanical activity with cellular enlargement and alterations in phenotype in adult cardiac myocytes and other cell types *in vivo*.

CHAPTER 6

EXPRESSION OF PHENOTYPIC MARKERS OF CARDIAC HYPERTROPHY IN THE ISOLATED PERFUSED HEART.

6.1 INTRODUCTION

It was demonstrated in the previous chapter that adrenergic agents and pressure overload can independently increase early-response gene expression in the isolated perfused heart. Previous studies have shown that these trophic stimuli also increase rDNA transcription and cardiac ribosome content in the perfused heart and taken together they suggest that the isolated perfused heart is a suitable model system in which to study at least the initial stages of cardiac hypertrophy. However, cardiac hypertrophy is also marked by qualitative changes in the expression of specific contractile, and non-contractile genes whose products play important roles in cardiac structure and function. Such changes in gene expression appear to be characteristic for a particular hypertrophic stimulus. For example, α–SkA and ANP mRNA levels are rapidly down regulated in ventricular tissue following birth but can be restored to levels similar to those observed in the neonate following imposition of pressure-overload *in vivo* (Izumo *et al.*, 1988; Schwartz *et al.*, 1986) or α_1-adrenergic stimulus in the adult animal (this thesis; Schwartz *et al.*, 1986) or rat ventricular myocardial cells (Bishopric *et al.*, 1987). In contrast, during thyroid hormone-induced hypertrophy the expression of fetal-specific genes are not altered whilst the relative expression of contractile genes associated with the adult phenotype are even further upregulated (e.g. α-MHC: Morkin *et al.*, 1983). Consequently it has been suggested (for a more detailed discussion see chapter 1) that such alterations in gene expression (cardiac plasticity) might be used as phenotypic markers of the presence and extent of particular cardiac pathologies observed in response to different hypertrophic stimuli. Studies in this chapter have sought to determine whether it is possible to detect such changes in the expression of hypertrophic "marker" genes including ANP, α–SkA and α-MHC in the isolated perfused rat heart in response to pressure-overload, adrenergic agents and thyroid hormone. If so, then this might establish the perfused heart as a suitable *in vitro* model

in which to study pathways that link the immediate responses to hypertrophic stimuli, for example, early-response gene expression, to secondary events in this process including qualitative changes in contractile and non-contractile genes.

6.2 METHODS

6.2.1 *in vitro* Coronary Perfused Hearts

Hearts were isolated and perfused in the same manner as described in chapter 5. NE and T_3 were prepared as described in chapter 3 and when used, where included in the perfusion medium as described below. Hyperthyroidism was induced in rats by treating animals with T_3 (0.2 mg/kg/day) for two weeks as described in chapter 3.

6.3 EXPERIMENTAL PROTOCOLS

6.3.1 Effect of NE and Perfusion Pressure on ANP and α-SkA Expression

To determine whether NE alone or in combination with elevated perfusion pressure could modulate expression of ANP and α-SkA, hearts were perfused from 15 min to 6 h at pressures of 60 mmHg and 120 mmHg with buffer alone or, alternatively, at 60 mmHg and 120 mmHg in the presence of NE (1 μM). Following perfusion hearts were frozen in liquid nitrogen and processed for northern analysis as described in chapter 2.

6.3.2 Effect of T_3 and NE on α-MHC Expression

To assess the effect of T_3 and NE on α-MHC expression, hearts were perfused at 60 mmHg of pressure from 30 min to 6 h with perfusion media containing T_3 (1 nM) or a combination of T_3 (1 nM) and NE (1 μM). Following perfusion hearts were processed for northern analysis as described above.

6.4 RESULTS

6.4.1 α-SkA and ANP Expression

In the first series experiment hearts were perfused with Krebs-Henseleit buffer under a coronary perfusion pressure or 120 mm Hg for 15 min to 6 h and the subsequent changes in expression of c-*myc*, c-*fos*, ANP and α-SkA measured by northern analysis (Fig. 6.1). As shown previously (see chapter 5) elevating the perfusion pressure from 60 mmHg to 120 mmHg increased cardiac mRNA levels of the early-response genes c-*myc* and c-*fos*. Increased c-*myc* mRNA levels were maximal after 4-5 h of perfusion (Fig. 6.1) and this relatively sustained response is similar to that observed for c-*myc* in pressure overloaded hearts *in vivo* (Mulvagh *et al.*, 1987). c-*fos* mRNA expression was more transient with maximal levels observable after 2 h and near basal by 6 h of perfusion (Fig. 6.1). However, in direct contrast, elevation of perfusion pressure from 60 to 120 mmHg did not alter mRNA levels of the cardiac isocontractile gene α-SkA or the atrial naturetic gene ANP (Fig. 6.1). These results differ from *in vivo* studies in which acute pressure overload has been shown to markedly increase ventricular mRNA levels for both of these genes (Izumo *et al.*, 1988; Schwartz *et al.*, 1986).

In a second experiment, to establish whether NE might modulate α-SkA mRNA levels, hearts were perfused for 15 min to 6 h at either 60 or 120 mmHg in the presence of NE (1μ M). As shown in Fig. 6.2 hearts perfused at 120 mmHg with NE (1μ M) did not exhibit increased cardiac α-SkA mRNA levels relative to the zero time control although either treatment results in significant induction of the early-response gene program (see chapter 5). Similarly, perfusion at 60 mmHg in the presence of NE did not modulate α-SkA mRNA levels (results not shown). These results contrast with the *in vitro* studies of Simpson group who showed that cultured neonatal cardiomyocytes treated with NE (1 μM) exhibit a 3-4 fold increase in mRNA levels of this gene within 3-4 h (Long *et al*,, 1989). The effect of NE on ANP mRNA levels was not studied.

6.4.2 α-MHC Expression

It is well established that thyroid hormone can directly regulate transcription of α-MHC *in vivo* (Mahdavi *et al.*, 1989; Nadel-Ginad and Mahdavi, 1989) and consequently it was of interest to determine whether this might also be observable in the isolated perfused heart. In the first experiment the basal expression of α-MHC mRNA was assessed in hearts perfused at 60 mmHg with Krebs-Henseleit buffer alone (Fig.

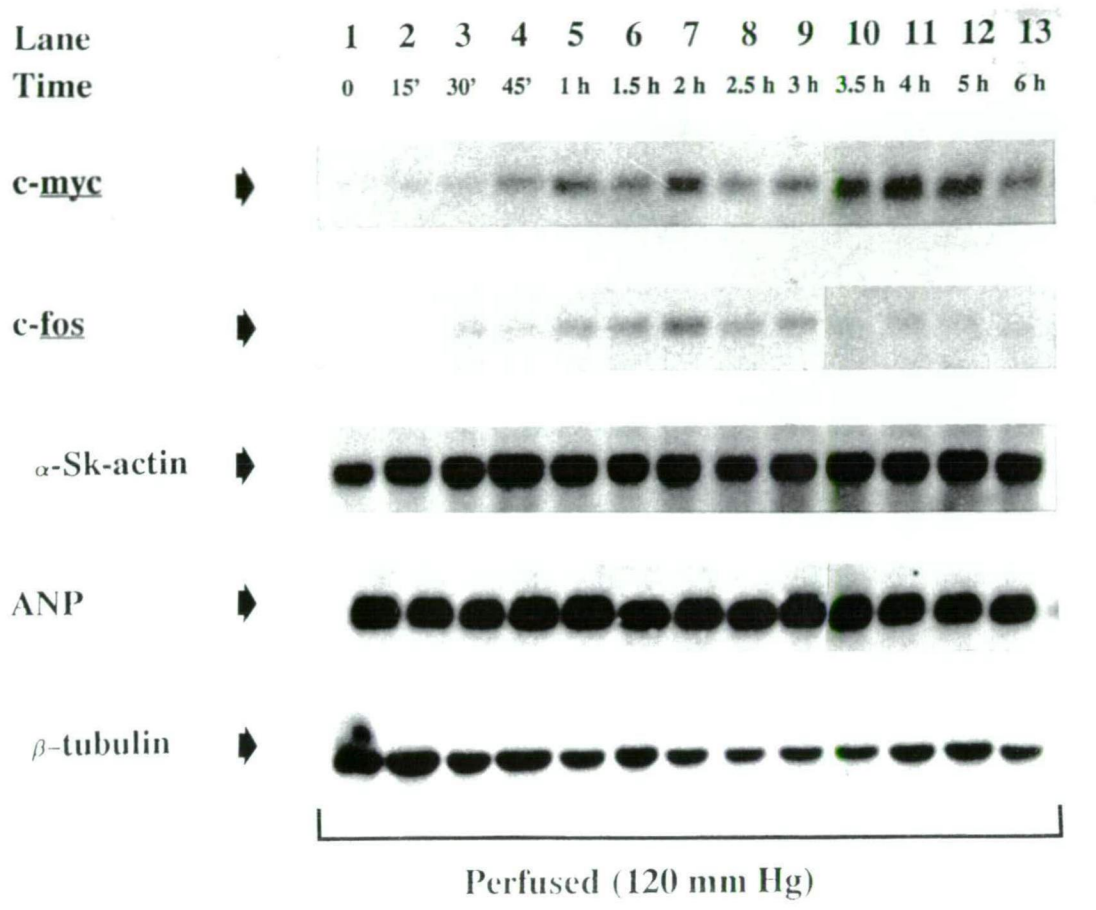

Fig 6.1 Expression of c-*myc*, c-*fos*, α-skeletal actin (α- Sk-actin) and atrial naturetic peptide (ANP) in rat hearts perfused at a constant coronary pressure of 120 mmHg.
Total RNA was extracted from rat hearts at the various times indicated following perfusion at constant pressure of 120 mmHg (Lanes 1-13) with modified Krebs-Henseleit buffer. After electrophoresis and northern blotting, the RNA (50 μg) was hybridized to c-*myc*, c-*fos*, α-skeletal actin, ANP and β-tubulin.

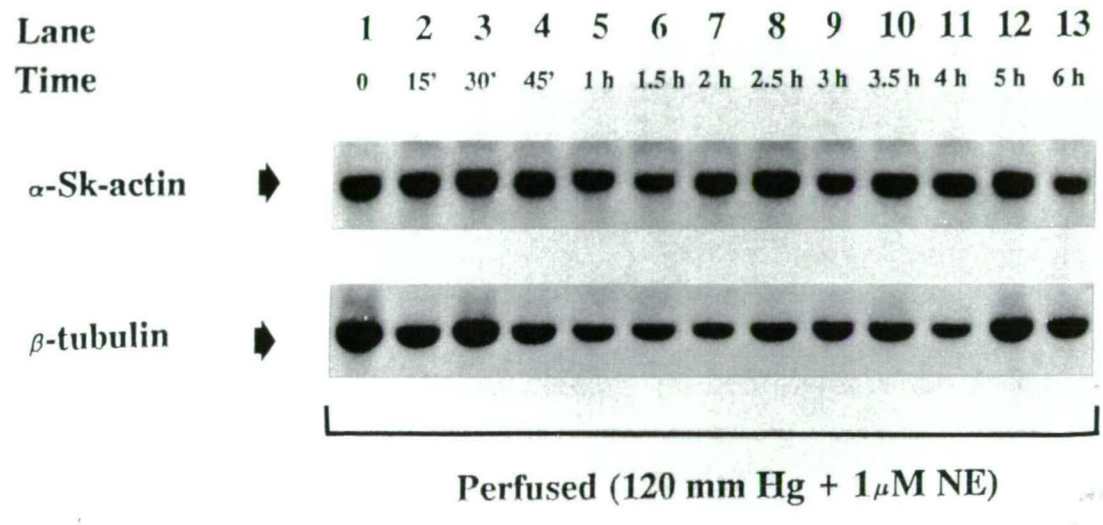

Fig 6.2 Expression of α-skeletal actin (α-Sk-actin) in perfused rat heart in response to norepinephrine. Total RNA was extracted from rat hearts at the various times indicated following perfusion at constant pressure of 120 mmHg with Krebs-Henseleit buffer containing 1 μM norepinephrine. After electrophoresis and northern blotting, the RNA (50 μg) was hybridized to α-skeletal actin and β-tubulin.

6.3, upper tracks). Hearts perfused in this manner for up to 6 h exhibited a progressive reduction in α-MHC expression during the course of the perfusion (5-10 fold less after 6 h, Fig. 6.4). This was not part of a general down regulation of cardiac isocontractile gene expression since α-SkA mRNA levels were not similarly reduced (results not shown). However, hearts perfused at the same pressure with the inclusion of T_3 (10 nM) in the perfusate did not exhibit decreased α-MHC levels, rather this treatment caused a transient increase (2 fold) in α-MHC mRNA levels between 2-4 h of perfusion (Fig. 6.3, lower tracks and Fig. 6.4). Further experiments established that levels of T_3 as low as 0.1 nM were sufficient to prevent a decrease in α-MHC mRNA levels during perfusion (results not shown). These experiments demonstrate regulation of α-MHC mRNA levels in the isolated perfused heart by thyroid hormones for the first time .

In contrast to T_3, the effect of adrenergic agents on α-MHC expression is less than clear. Studies with cultured adult myocytes have been inconclusive with some investigators demonstrating a positive effect of NE and β-adrenergic agonists on α-MHC expression whilst others have reported no changes (Dubus *et al.*, 1990; Rupp *et al.*, 1991). However, when hearts were perfused in the presence of NE (1 μM) alone a progressive reduction in α-MHC mRNA was still observed (results not shown) indicating that NE alone is insufficient to prevent down regulation of cardiac α–MHC mRNA in the absence of T_3. Since any potential NE-mediated regulation of α-MHC in the perfused heart may be obscured by the down regulation of this gene observed during the absence of T_3, hearts were subsequently perfused with NE (1 μM) in the presence of T_3 (1 nM) and α-MHC mRNA expression compared to hearts perfused with T_3 alone (perfusion control). As before, hearts perfused with T_3 exhibited a moderate, transient increase in α-MHC mRNA levels (2 fold) after 1-2 hours of perfusion (Fig. 6.5, Lanes 2-7 and Fig. 6.6). However when NE was also included in the perfusate α-MHC mRNA levels were rapidly and transiently increased to levels markedly above those observed in response to T_3 alone but returned to basal by 3 h of perfusion (Fig. 6.5, Lanes 8-13 and Fig. 6.6). The level of maximum expression (5-8 fold) was similar to that observed in hyperthyroid rats (Fig. 6.5, Lane 1). Perfusion with T_3 did not elevate mRNA levels of c-*fos* (Fig. 6.4, Lanes 2-7) or c-*myc* (results not shown) with respect to

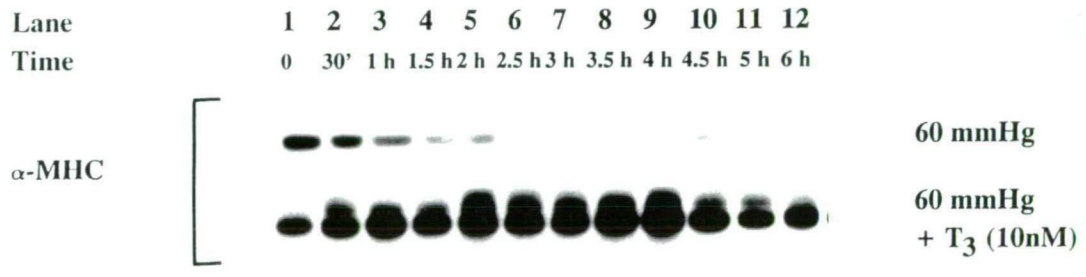

Fig 6.3 Expression of α-myosin heavy chain (α-MHC) in perfused rat heart in response to triiodo-L-thyronine.

Total RNA was extracted from rat hearts at the various times indicated following perfusion at constant pressure of 60 mmHg (upper track) with Krebs-Henseleit buffer or buffer containing 10 nM triiodo-L-thyronine (lower track). After electrophoresis and northern blotting, the RNA (50 μg) was hybridized to an α-myosin heavy chain probe .

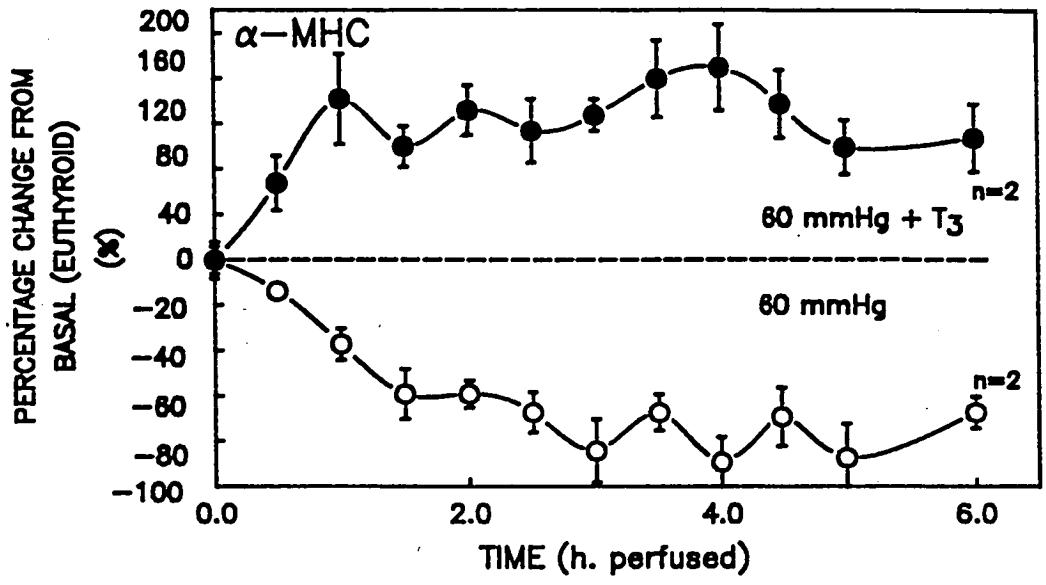

Fig 6.4 Quantification of α-myosin heavy chain (α-MHC) mRNA levels in the perfused heart in response to triiodo-L-thyronine (T_3).
The hybridization signals observed by northern blotting in Fig. 6.3 were quantified via densitometry and, after standardization to β-tubulin levels (not shown) in each track, were expressed as percentage change from basal levels of RNA observed in euthyroid animals. Vertical bars indicate standard deviation.

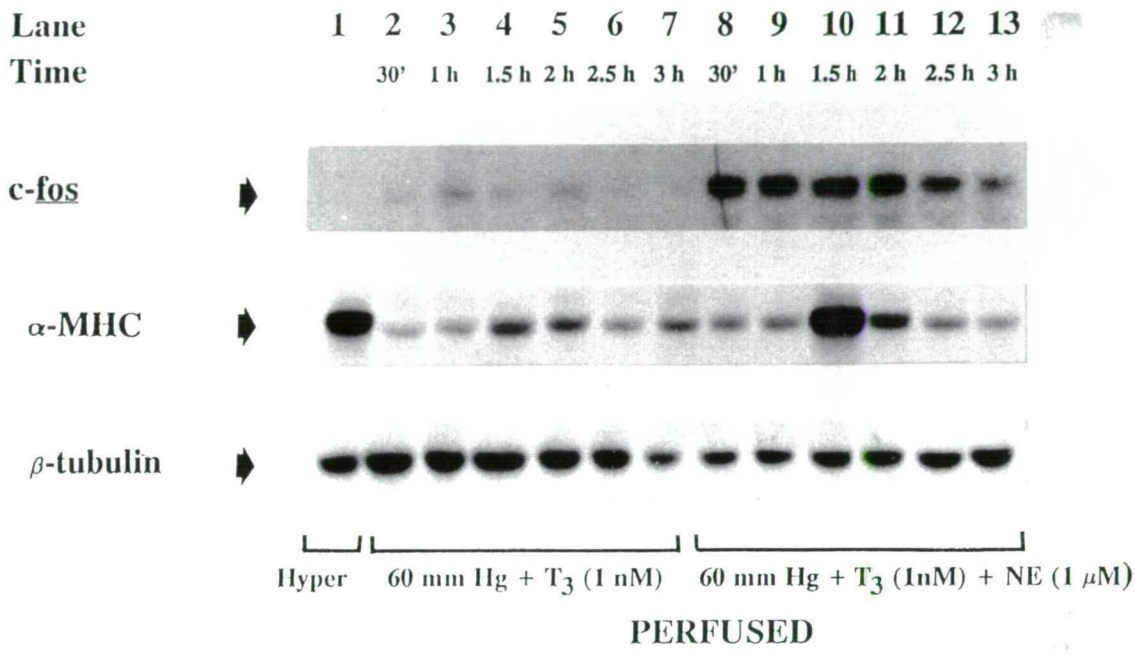

Fig 6.5 Expression of c-*fos* and α-myosin heavy chain mRNA in perfused rat heart in response to triiodo-L-thyronine alone and in combination with and norepinephrine.
Total RNA was extracted from rat hearts at the various times indicated following perfusion at constant pressure of (60 mmHg) with Krebs-Henseleit buffer containing triiodo-L-thyronine (1 nM) alone (Lanes 2-6) or with triiodo-l-thyronine (1nM) and norepinephrine (1 mM, Lanes 8-13) . Lane 1 contains RNA extracted from a hyperthyroid rat as the result of treatment with 2 μg/kg of T_3 for 7 days. After electrophoresis and northern blotting, the RNA (50 μg) was hybridized to c-*fos*, α-myosin heavy chain and β-tubulin.

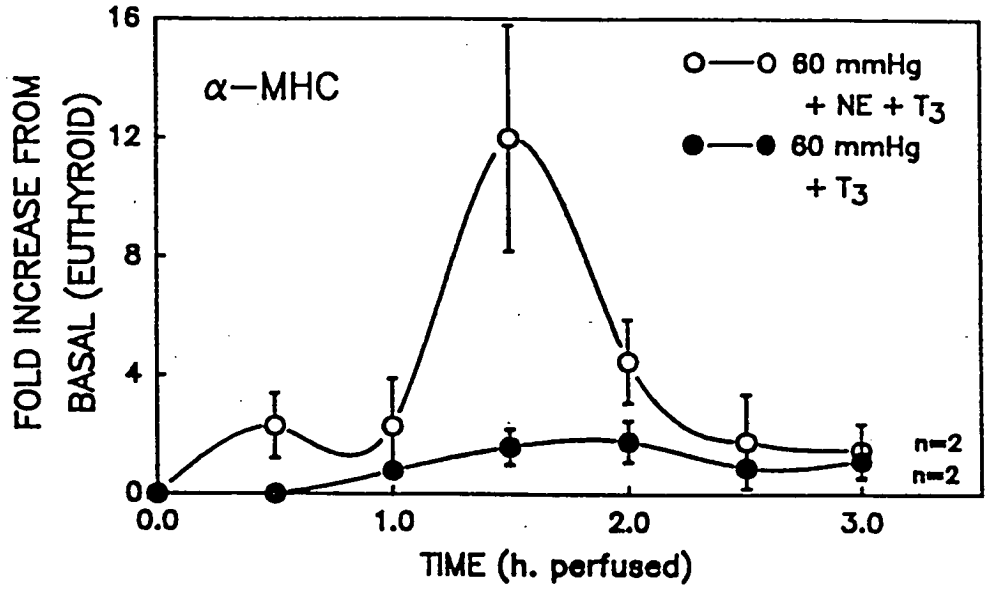

Fig 6.6 Quantification of α-myosin heavy chain expression in perfused hearts in response to triiodo-L-thyronine alone and in combination with norepinephrine.
The hybridization signals generated by northern blotting in Fig. 6.5 were quantified via densitometry and, after standardization to β-tubulin levels (not shown) in each tract, were expressed as fold induction of equivalent signals observed in euthyroid animals. Vertical bars indicate standard deviation.

hearts perfused with buffer alone and this is in accordance with *in vivo* observations presented earlier (see chapter 3). However, T_3 was not able to prevent increase in the early-response gene program elicited by NE since c-*fos* levels (Fig. 6.5, Lanes 8-13) were significantly elevated within 30 min of co-perfusion with T_3 and NE and were still above basal after 3 h. The expression of other early-response genes during this treatment was not studied.

These results indicate that the continuous administration of NE (1 mM) in the presence of T_3 (1 nM) in the isolated perfused heart leads to a rapid and transient accumulation of α-MHC mRNA with respect to hearts treated with T_3 alone. Furthermore, the increased contractile gene expression is preceded by induction of the early-response gene program. Further studies are require to determine which component of NE action (i.e. α or β) is responsible for induction of α-MHC and whether the prior induction of the early-response gene program is a necessary prerequisite .

6.5 DISCUSSION

Cardiac hypertrophy observed in response to administration of adrenergic agents, thyroid hormones or following pressure overload appears to be a coordinate process involving both quantitative and qualitative changes in protein expression. Protein products that might have pivotal roles in regulating these processes include those of the early-response gene families and the induction of some of these putative trans-activating factors following hypertrophic stimuli have been clearly demonstrated in the proceeding chapters both *in vivo* and in an isolated perfused heart system. The latter model has the distinct advantage in that the complex systemic interactions associated with many hypertrophic stimuli can be dissociated, however, it is yet to be formally demonstrated whether qualitative changes in expression of cardiac structural or functional genes can be observed in this system following treatment with hypertrophic agents. Accordingly the studies in this chapter have examined the acute expression of a number of structurally or functionally important cardiac genes including ANP, α-SkA and α-MHC,

in the isolated perfused heart system in response to NE or T_3 administration or elevated coronary perfusion pressure.

i) expression of ANP and α -SkA in response to pressure load and/or NE

ANP and α-SkA mRNA are normally expressed at low levels in the left ventricle of untreated adult rat hearts however their mRNAs rapidly accumulate in this chamber following imposition of aortic stenosis. α-SkA and ANP mRNA levels have also been shown to be rapidly upregulated in neonatal ventricular myocyte cultures (Knowlton *et al.*, 1991; Chien, *et al.*, 1991) and in whole hearts *in vivo* in response to chronic administration of NE (this thesis). However in these experiments elevation of perfusion pressure from 60 mmHg to 120 mmHg did not increase mRNA levels of either ANP or α-SkA at any time point with respect to zero time control. Similarly inclusion of NE in the perfusion buffer for periods up to 6 h did not elevate ANP or α-SkA mRNA levels (ANP levels not shown) above those observed in control hearts.

A reason for this lack response for these two genes in this experimental system is not clear although a number of plausible explanations exist. For instance it is possible that the length of perfusion time (6 h) was insufficient to allow for any appreciable increase in mRNA to be observed. However, other groups have demonstrated that α-SkA transcripts, at least, are observable within 1-3 h following the initiation of acute pressure overload or following NE treatment (Black *et al*, 1991). It is important to note though, that the α_1-adrenergic mediated induction of α-SkA observed by Simpsons group was studied in a neonatal myocytes culture system, the responses of which may differ greatly with myocytes in the adult heart *in vivo* (for further discussion see chapter 1). Alternatively the apparent lack of induction may be due to possible quantitative and qualitative differences in hypertrophic stimuli experienced by the heart perfused *in vitro* with respect to hearts *in vivo*. For example it is possible that pressure- or NE-induced expression of ANP or α-SkA in adult hearts *in vivo* requires the presence of a permissive serum derived factor that would be absent during *in vitro* perfusions. Finally, since the northern blots presented here represent RNA extracted from whole hearts including the atria, the constitutively high level of expression of ANP in atria may mask any subsequent changes in expression of this gene in the ventricles. Further studies will need to examine the expression of these genes at a regional level either by

gross dissection and northern analysis or alternatively by *in situ* hybridization or immunohistochemistry.

ii) expression of α -MHC in response to NE and/or T_3

A large body of evidence now exists implicating thyroid hormone in the direct regulation of α-MHC transcription (see chapter 1) however the role of adrenergic agents in modulation of this gene is not known. Part of this lack of understanding probably stems from the close interrelationships between these two hormones since recent studies have demonstrated that thyroid hormones can directly up regulate β-adrenergic receptors at the level of transcription. Results from cell culture have been conflicting and do not always agree with *in vivo* studies (Buttrick *et al.,* 1993). For instance, it has been reported that activation of adenyl cyclase accompanies α-MHC mRNA (Gupta *et al.* 1991) but these studies are contrary to the earlier work of Gustafson (Gustafson *et al.,* 1987). Furthermore, contractility has been reported to regulate cardiomyocyte MHC content yet many studies are performed variously with beating or arrested cells. Thus it would appear that the outcome of such experiments in neonatal and adult myocyte cell cultures are dependent in large part on differences in culture conditions. In the present experiments the effect of T_3 and NE on α-MHC mRNA levels was examined in isolated perfused hearts. When hearts were perfused in the absence of T_3 and NE a rapid decrease in α-MHC mRNA was observed during the perfusion time course. However the reduction in α-MHC mRNA levels could be prevented by the inclusion of T_3 alone but not NE alone, in the perfusate. These findings underline the importance of T_3 in the regulation of basal α-MHC levels in the rat heart and demonstrate that NE by itself is insufficient to maintain α-MHC expression in the absence of thyroid hormone. Interestingly, when hearts were perfused with T_3 and NE together a transient rise in α-MHC was observed above that seen in response to T_3 alone. These results indicate that NE is able to potentate the effect of T_3 on α-MHC expression and agree with recent work of Leinwands group (Buttrick *et al.,* 1993) suggesting that the thyroid hormone response element is not sufficient for complete regulation of α-MHC *in vivo*. Further studies will need to determine which component of NE action (α or β) is responsible for the observed induction of α-MHC and to identify potential cis- and trans-acting

elements involved in this process. This might be achieved by transfecting myocytes in culture with appropriate vectors or direct gene transfer into adult rat hearts (Kitis *et al.*, 1991; Buttrick *et al.*, 1993). In addition it will also be important to assess the significance of the transient α-MHC mRNA induction since presumably a more sustained elevation of this transcript is required in order to significantly affect cardiac α-MHC protein levels and subsequent cardiac performance.

iii) Summary

α-SkA and ANP are rapidly inducible in the rat heart *in vivo* following aortic stenosis or NE treatment, however no change in expression in either of these genes was observed in these experiments in isolated hearts perfused under elevated pressure or in the presence of NE. The reason for this apparent conflict in results is not known but it may be due to experimental design or temporal restrictions pertaining to the perfused heart system. Further studies are required in which the expression of these two genes are studied at a regional level either by *in situ* hybridization or immunohistochemistry. In contrast α-MHC mRNA levels rapidly diminish during perfusion with Krebs-Henseleit buffer but can be restored to levels similar to control animals *in vivo* by including T_3 in the perfusate. NE alone had no effect on α-MHC expression but when used in conjunction with T_3 a rapid and transient increase in α-MHC mRNA was observed. The mechanism by which NE potentated the effect of T_3, on α-MHC expression is not know but warrants further investigation. These preliminary studies provide the first evidence that the isolated perfused heart system can be used to assess not only the primary responses to hypertrophic stimuli (e.g. early-response gene expression) but also changes in expression of structural genes which are often used as end point markers of different hypertrophic phenotypes.

CHAPTER 7

GENERAL DISCUSSION

Despite the recent advances with investigative techniques the molecular mechanisms which regulate the development and growth of the mammalian heart remain poorly understood. This is due in part to the fact that cardiac biogenesis is a complicated process involving cell proliferation, cell commitment, cell differentiation and chamber diversification. Furthermore, at each of these stages the heart exhibits an array of different phenotypes due to alterations in expression of genes thought to be critical to cardiac structure and function.

In contrast to embryonic development in which the myocytes undergo proliferative growth (hyperplasia), the major contributor to postnatal enlargement of the mammalian heart is an increase in size (hypertrophy) of a fixed number of myocytes. In addition, the adult heart can hypertrophy above that normally expected, in response to a diverse range of physiological and pathological stimuli including increased pressure and mechanical load or in response to changes in hormone levels and following ischemia. In the initial stages, the growth is generally seen as an adaptive response of the organism, however, during latter stages it may be insufficient to cope with the increased demand or is associated with pathological changes, such that the heart ultimately fails. The exact reasons why growth of the heart changes from one of compensatory hypertrophy to a stage of decompensation are not completely understood although it is known that this transition is accompanied by further changes in cardiac phenotype. For example, hypertrophy mediated by vasoactive hormones or pressure-overload is characterized by re-expression in the left ventricle of a group of genes including ANP, α-SkA and Ca^{2+} ATPase whose expression is normally restricted to the fetal or neonatal heart (de la Bastie *et al.*, 1990; Izumo *et al.*, 1988; Schwartz *et al.*, 1986) It is thought that changes in the expression of these so called "phenotypic marker genes" are at least partly responsible for the different metabolic, contractile and electrochemical properties of this organ observed during various cardiac hypertrophic diseases (Reviewed in van Bilsen and Chien, 1993). It follows then that the elucidation of the molecular signals which regulate these phenotypic changes should allow for a better understanding of the general

mechanisms which regulate cardiac growth and development and have far reaching implications for clinical treatment of heart disease. Accordingly, the work embodied in this thesis has examined the expression of a group of nuclear-acting early-response genes including c-*myc* and c-*fos* whose actions have been suggested to be pivotal to the initiation and maintenance of cardiac hypertrophy and the accompanying phenotypic changes, often by analogy to their involvement in growth and differentiation in other tissues. The effect of two hypertrophic hormones, NE and T_3 on cardiac nuclear-acting early-response expression was studied since these agents have been shown to cause hypertrophy which differs both quantitatively and qualitatively from each other suggesting that alternative mechanisms of action may be involved in modulating each growth response.

The results presented in this thesis have demonstrated that c-*myc,* c-*fos* and genes structurally or functionally related to c-*fos* including *fra*-1, *fra*-2 and c-*jun* are rapidly and transiently induced during NE mediated cardiac hypertrophy. By use of the isolated perfused heart system to model the initial stages of adult cardiac growth it was also shown that NE can elevate expression of these genes in the absence of elevated pressure load, thus supporting results derived from neonatal cell culture studies which have demonstrated that adrenergic hormones can effect cardiac growth and early-response gene expression independent of mechanical stimuli (for review see Simpson *et al.*, 1991). Increased pressure load and NE stimulation of adult hearts resulted in the induction of both distinct and similar sets of early-response genes indicating that these stimuli may share some pathways coordinating cardiac growth whilst others may be more specific for each stimuli. Interestingly the induction of these genes in response to NE and pressure load showed regional, temporal, cellular and receptor mediated specificity thus providing a possible mechanism by which these putative transcription factors might coordinate hypertrophic signals at the cell surface to the complex array of long term changes observed during cardiac hypertrophy. For example, the observation that c-*fos* expression following NE/pressure stimulation was localized mainly to the myocyte nuclei of the left ventricle is in good correlation with the observation that myocyte growth and re-expression of the fetal gene program is also greatest in this chamber (reviewed by Chien *et al.*, 1991) and supports the hypothesis that this gene may

be important in regulating the genetic reprogramming of myocytes following NE mediated hypertrophy.

In direct contrast, c-*myc* expression was restricted mainly to non-muscle cell types following NE administration and pressure overload thus reducing the likelihood that this gene plays a direct role in orchestrating the growth and phenotypic changes of adult myocytes. It is possible that this gene is involved in coordinating the mitogenic growth of non-muscle cell types such as fibroblasts which occurs concomitant with myocyte hypertrophy during cardiac growth. It is worthy to note however, that myocytes are not the only cardiac cell type which exhibits specific alterations in gene expression following trophic stimulation. For example, cardiac fibroblasts can acquire certain properties characteristic of the myocyte phenotype such as expression of sarcomeric actin and muscle-specific actin filaments following stimulation with peptide growth factors and it is possible that both c-*myc* and c-*fos* may be involved in regulating such gene reprogramming (Eghbali *et al.*, 1991A). Indeed the importance of non-myocyte cell types to the regulation of cardiac structure and function is indicated by recent studies implicating transforming and fibroblastic growth factors including TGFβ, aFGF and bFGF released from fibroblasts in the regulation of cardiac myocyte hyperplasia, differentiation and hypertrophy (for review see Cummins *et al.*, 1993). More over, one of these growth factors, $TGF\beta_1$, appears to be critical to the regulation of collagen biosynthesis, the inappropriate accumulation of which, has been implicated in hypertrophic myocardial fibrosis (Eghbali *et al.*, 1991B). It remains to be seen as to the role of early-response gene expression in regulating this and other non cardiac myocyte cell functions although one recent report indicates that collagen biosynthesis in hypertrophying hearts may be independent of the fibroblast response to transcription factors such c-*fos*, and c-*jun* (Eghbali *et al.*, 1991 B).

Multiple factors have been shown to cause cardiac hypertrophy *in vivo* including administration of thyroid hormones (Sanford *et al.*, 1978; Clarke and Ward, 1983) Similarly in this study chronic treatment of rats with thyroid hormone resulted in significant hypertrophy within 2-3 days. In contrast to adrenergic administration however, thyroxine treatment was not accompanied by increased c-*myc*, c-*fos*, c-*jun* or *fra*-1 levels. In agreement with this the recent study by Green also failed to detect

increased c-*myc* expression following T_3 administration (Green *et al.*, 1991) . Taken together these results indicate that induction of this particular set of early-response genes is not an absolute requirement for the initiation and maintenance of cardiac hypertrophy and that alternative pathways must link the thyroid receptor with nuclear events regulating myocyte growth and gene transcription. For example, thyroid hormones have been shown to directly affect the transcription of a number of genes (e.g. α–MHC, β-adrenergic receptor) by interaction of the thyroid receptor with cis-acting elements in their 5' regions (Bahouth, 1991; Nadal Ginard and Mahdavi, 1989) and it is possible that this hormone may affect transcription of other genes important to the cardiac hypertrophic response by a similar mechanism. Unexpectedly, and in contrast to other early-response genes investigated, administration of thyroxine led to a gradual rise in *fra*-2 mRNA. Whether this early-response gene is a permissive factor for thyroid hormone induced hypertrophy is not known and the ability of thyroxine to positively modulate expression of members of the c-*fos* gene family has not been previously reported although recent studies suggest that the thyroid receptor can interact with AP-1 to affect its transcription activity (Lopez *et al.*, 1993). Further studies are required to determine if this is a direct transcriptional effect of the activated thyroid receptor on *fra*-2 expression or whether increased *fra*-2 levels are secondary to T_3 mediated alterations in cardiac contractility or activity of hormonal factors.

Thyroid hormone mediated cardiac hypertrophy is phenotypically different to pressure/NE mediated growth in that it is not accompanied by re-expression of the fetal genes and this is entirely constant with the hypothesis that re-expression of this fetal gene program requires prior expression of a certain panel of early-response genes such as Fos and Jun. Alternatively, increased early-response gene expression following NE treatment or pressure overload may be a coincidence unrelated to myocyte growth or qualitative alterations in gene expression. Indeed the evidence presented here and those of others implicating a role for Fos and Jun in cardiac myocyte growth and genetic reprogramming following NE administration or pressure-overload has come from correlative studies. Future studies will need to formally demonstrate both *in vitro* and *in vivo* that these transcription factors can directly alter cardiac myocyte gene expression. With respect to this, some recent evidence supporting such a function has been obtained

using neonatal myocyte cultures. In the studies of Bishopric *et al.*, 1992 expression vectors encoding c-*fos* and c-*jun* were used to analyze the role of these early-response genes in mediating the transcriptional induction of α-SkA by adrenergic stimulation. They were able to demonstrate that over expression of Fos/Jun trans-activated the fetal isoform α-SkA promoter 5 fold but did not affect transcription of the α-MHC promoter, an isoform associated with the adult heart. These results suggest a direct role for these genes in regulating α-SkA expression and more generally in cardiac actin isoform switching. However not all transfection studies with neonatal myocytes cultures support a positive role for early-response genes in trans-activating the fetal gene program. For example McBride *et al.*, (1993) demonstrated in transient co-transfection assays that Jun, Fos, Fra-1, Fos-B and v-Fos trans-repress the ANP promoter 5-10 fold in both quiescent and α_1-adrenergic stimulated atrial and ventricular myocytes. Deletion analysis indicated that repression did not require typical AP-1 binding sites or serum response elements but was targeted at a cardiac specific element within the ANP promoter. Furthermore, repression by Jun occurred via the N-terminal activation domain and did not require the DNA binding domain suggesting that AP-1 repression involved interaction with one or more limiting cardiac-specific factors. Thus, although stimulation of neonatal cardiomyocytes with α_1-adrenergic agents induces cellular hypertrophy and increased AP-1 activity and ANP expression, induction of ANP in this model must result from activation of a Fos/Jun independent pathway. The above studies have provided the first evidence indicating that Fos and structurally or functionally related genes can have both positive and negative effects on the transcription of the cardiac gene program. A similar ability of Fos/Jun to both trans-repress and activate has been observed in other systems (Shaw *et al.*, 1989; Schule *et al.*, 1990; Gius *et al.*, 1990; Lucibello *et al.*, 1990). It remains to be seen however, whether these transcription factors are functional in other models of neonatal myocyte hypertrophy (ie: stretch or growth factor induced) or indeed if similar mechanisms of action are conserved in the more relevant adult myocytes *in vivo*. As a first step in addressing these questions it is important that similar transient transfection assays to those performed in the neonatal myocyte system be performed in primary adult myocytes cultures, although the greater inherent problems in maintenance of the more mature cells dictates that these

experiments will be considerably more difficult. Even so, recent studies have demonstrated that recombinant adenovirus can efficiently transfer reporter gene constructs into adult ventricular myocytes although the appropriate experiments examining the effects of putative regulators have yet to be undertaken (Kirshenbaun *et al., 1993*). Even if such transfection experiments are fruitful it will still be necessary to determine their relevance to the physiological and pathological growth of the cardiac myocyte *in vivo*.

One possible alternative model in which to close the gap between the observations of molecular and cellular biologists *in vitro* and the pathophysiological effects of growth pathways on cardiac hypertrophy *in vivo* is the use of transgenic animals (reviewed by Sigmund, 1993). To achieve this it will be necessary to fuse the gene of interest, in this case early-response genes such as c-*fos*, to transcriptional control elements which confer myocyte-specific expression. Following integration of this construct into the genome of the animal, it will be possible to examine the effect of hypertrophic growth stimulation on the expression and function of the target gene in the context of the whole heart. To date a diverse panel of cardiovascular genes whose regulatory sequences confer tissue and/or inducible expression to a heterologous protein have been identified (for reviews see Hunter *et al., 1993*; Sigmund, *1993*).

Gene "knockout" experiments may also prove to be a powerful approach to the study of cardiac hypertrophy. For example, recently mice lacking a functional c-*fos* gene have been created using embryonic stem cells targeted by homologous recombination at the c-*fos* locus (Johnson *et al.*, 1992). Interestingly this mutation is not lethal, rather it causes a wide range of phenotypic deficiencies with some of the homozygous mutant animals surviving for over 7 months. Such animals would be ideal models in which to determine whether c-*fos* is important in the regulation of genes thought to be structurally or functionally important to the hypertrophying heart. Similarly mice lacking functional *fra*-1 or *fra*-2 genes may become available and together these mouse models should become increasingly important in the search for regulators of cardiac development, growth and disease in the future.

One difficulty with the models outlined above is their high cost and excessive time required to generate the new animal lines. A possible novel alternative to these

systems is the direct injection of expression/reporter constructs into adult hearts *in vivo* (Buttrick *et al.*, 1992; Kitsis *et al.*, 1991; Acsadi *et al.*, 1991; Lin *et al.*, 1990). Indeed preliminary reports already exist demonstrating the utility of direct injection technique in mapping cis- and trans-acting factors involved in cardiac specific gene expression (Buttrick *et al.*, 1993; Kitsis *et al.*, 1991). Using this technique it may be feasible to determine the importance of early-response gene expression to the structure and function of cardiac myocytes *in vivo*.

The studies in this thesis and the majority of those in the current literature have focused on the molecular signals linking trophic stimuli to the altered expression of specific subsets of phenotypic marker genes seen as being important to the myocyte structure and function during hypertrophy. However, for a number of reasons caution has to be used in extrapolating the significance of these results to the human situation. Firstly, it is now becoming evident that the phenotypic changes in the adult human heart following hypertrophic stimuli are not necessarily the same as those in small rodents. For example in rats α-SkA is present in extremely low levels in the normal adult left ventricle but following pressure overload the mRNA and protein levels of this gene rise dramatically (Schwartz *et al.*, 1986) . In contrast α-SkA remains expressed at quite high levels in the human left ventricle throughout life (Gunning *et al.*, 1983; Vandekerckhove *et al.*, 1986). Such observations underline the fact that cardiac hypertrophy is qualitatively different between species and raises the question as to the relevancy of delineating pathways leading to increased α-SkA in the rat heart in terms of cardiac hypertrophy of the adult human heart. Secondly, the use of certain genes traditionally seen as being prototypical markers of pathological hypertrophy may have to be revised. For example, decompensated hypertrophy associated with chronic pressure overload in rats is characterized by re-expression of ANP, α-SkA and Ca^{2+} ATPase in the left ventricle (Feldman *et al.*, 1993). However when hypertrophy of the left ventricle is reduced to a level similar to that of the normal heart by use of levels of angiotensin converting enzyme inhibitors which do not reduce the pressure load on the heart, only the level of Ca^{2+} ATPase is reduced (Lorell *et al.*, 1993). Such findings indicate that Ca^{2+} ATPase closely correlates with decompensated hypertrophy whilst ANP and α-SkA may simply be markers of pressure load *per se*. These studies clearly

demonstrate the importance of defining exactly which sets of genes are important in contributing to the altered performance of the heart during physiological and pathological hypertrophy so that the ability of putative transcription factors such as c-*fos* and c-*jun*, to modulate their expression can be tested in the appropriate model systems.

In addition to the qualitative changes in gene expression discussed above, postnatal cardiac growth is also characterized by a general increase in myocyte protein synthesis and it is this trophic process which accounts for the increase in myocyte size (reviewed by Morgan *et al.*, 1987). Increases in general protein synthesis have been shown to occur as the result of raised myocyte cellular capacity to produce protein viz., an increase in cellular ribosome content (McDermott and Morgan, 1989; Siehl *et al.*, 1985). In turn increased ribosome biogenesis is largely due to elevated ribosomal DNA (rDNA) transcription and accelerated rates of transcription can be observed within 12 h following hypertrophic stimuli (McDermott *et al.*, 1991 and references there in). The mechanisms by which rDNA transcription is controlled are relatively poorly understood although recent studies indicate that at least two transcription factors, UBF and SL-1 in addition to polymerase 1 are required for efficient transcription from vertebrate rDNA promoters (reviewed by Xie and Rothblum, 1993). It is feasible then that early-response genes such as c-*fos* may be involved in coordinating this response by directly binding to the promoter region of the 45S gene and interacting with the initiation factors to modulate transcription. Indeed, putative AP-1 consensus sequences have been identified in the promoter regions of the 45S gene (Rothblum, L. I., pers. comm.). Alternatively early-response genes may be involved in trans-inducing the rDNA transcription factors themselves. In support of this UBF mRNA and protein is rapidly induced in response to hypertrophic stimuli such as norepinephrine in neonatal myocyte cultures and this induction is preceded by c-*fos* expression (Rothblum, L. I., pers. comm.). Further studies are require to identify the molecular mechanisms involved in regulating rDNA transcription and to determine possible roles of nuclear acting early-response genes in this process. It will be interesting to determine whether cardiac rDNA transcription and qualitative changes in gene expression are regulated through similar or distinct pathways during NE-mediated cardiac hypertrophy. In contrast, the results presented here indicate

that c-*fos* and c-*jun* are probably not involved in regulating general protein synthesis during T_3 mediated cardiac hypertrophy.

In conclusion this thesis has demonstrated that c-*fos, c-jun* and related proteins of the AP-1 complex fullfill the temporal, spatial and cellular-specific requirements necessary for transcription factors proposed to be active in the initiation and maintenance of NE mediated cardiac hypertrophy *in vivo*. These physiologically relevant studies are of increasing importance in light of recent *in vitro* neonatal myocyte experiments demonstrating that c-*fos* and c-*jun* can directly regulate the expression of genes thought to be structurally and functionally important to the hypertrophic response of the heart following NE administration and pressure overload. Future studies will need to examine the ability of the heart to initiate cardiac hypertrophy following inhibition of c-*fos* and/or c-*jun* expression using transfection studies *in vitro* or by transgenic and direct injection techniques *in vivo*. In contrast the prototypical early-response gene, c-*myc* is localized to non-myocyte cell types following NE administration making it unlikely that this gene has a direct role in regulating adult myocyte hypertrophy. Further studies are required to determine if this gene is involved in potentiating the growth and function of non-myocyte cell types which occurs concomitant with cardiac myocyte hypertrophy.

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