GENE EXPRESSION IN MUSCLE TISSUE AND CELLS

by

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A thesis submitted to the faculty of graduate studies and research, McGill University, in partial fulfillment of the requirements for the Master of Science degree

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ABSTRACT

Cellular differentiation is accompanied by the modulation of gene expression. I have compared the expression of various genes, using the slot-blot technique, in two different systems. First, the level of expression of a wide variety of genes was analyzed in the hypertrophied heart of transgenic mice expressing the polyomavirus large T-antigen gene, and compared to normal control heart. I have shown that most changes in gene expression occurred mainly during early stages of heart hypertrophy. These genes code for proteins known to play a role in signal transduction, and transcriptional and growth control. The latter stages of cardiac hypertrophy are accompanied by changes in the expression of genes that are mostly involved in stress responses. Second, we analyzed the expression of various genes in three mouse myogenic cell lines undergoing differentiation in several culture conditions. The adult (C2C12) and fetal-derived (G7 and G8) myoblast cell lines were exposed to either retinoic acid, dimethyl sulfoxide, or transforming growth factor ß. These three molecules are known to have profound effects on cellular growth and differentiation. I have shown that these treatments result in significant changes in expression of a wide variety of genes. Interestingly, all three cell lines differed considerably in their pattern of gene expression. Results from the analysis of these two systems demonstrate that differentially induced morphological changes of muscle cells, result in cell type specific changes in expression of a variety of genes.

RESUME

La différentiation cellulaire est accompagnée de la modulation de l'expression de plusieurs gènes. J'ai comparé le niveau d'expression de différents gènes de deux systèmes cellulaires indépendants, à l'aide de la technique *slot-blot*. Premièrement, le niveau d'expression d'une variété de gènes a été analysé chez les coeurs atrophiés d'une lignée de souris dont les cardiomyocytes exprime le transgène PVLT, comparativement aux cardiomyocytes des coeurs contrôles provenant de souris normales. J'ai démontré que les différences majeures dans l'expression de certains gènes se produisent au début du stade de l'atrophie cardiaque. Les gènes dont le niveau d'expression est modifié chez les coeurs atrophiés, codent pour des protéines impliquées dans le contrôle de la transcription et de la croissance cellulaire, ainsi que dans la transduction des signaux cellulaires. Les gènes qui ont un patron d'expression modifié lors des derniers stades de l'atrophie cardiaque codent surtout pour des protéines impliquées dans la réponse au stress cellulaire. Deuxièmement, j'ai analysé l'expression d'une variété de gènes de trois lignées de myoblastes qui ont subit différents traitements en culture. Les lignées adultes (C2C12) et foetales (G7 et G8) de myoblastes de souris ont été exposées soit à l'acide rétinoique, ou DMSO, ou au facteur de croissance transformant ß (TGFß). Ces trois molécules sont caractérisées par leurs rôles dans le contrôle de la croissance et de la différentiation cellulaire. J'ai démontré que ces traitements résultent en la modulation d'une grande variété de gènes. Les trois lignées cellulaires diffèrent considérablement dans leur patron d'expression génique lorsqu'elles sont exposées à ces molécules. Ces résultats démontrent que, lorsque'on induit de différentes façons des changements morphologiques à des cellules musculaires, il en résulte une réponse génique spécifique aux différents types cellulaires.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

The development of the slot blot multigene screening technique represents an original contribution to scientific knowledge. This method allows the analysis of any desired set of mRNA species within a tissue, or population of cultured cells.

Work presented in this thesis is currently being prepared for publication.

ACKNOWLEDGEMENTS

I would like to thank Dr. Lorraine Chalifour for friendship, patience, advice, and motivation during the pursuit and preparation of this thesis.

I am grateful to every member of our laboratory who offered support and counsel during good times and bad. Thank you Ramez, Sheida, Harvey, Tracey, Jeanne, Dao, Joelle, Lunjun, Hashem and Ala-Eddin, for everything. Also thanks to Stephen for translating the abstract.

Lastly, but certainly not least, I would like to thank Mum and Dad and Dave for unflagging support and encouragement during my studies.

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CHAPTER ONE

INTRODUCTION

Modification of gene expression, directing active transcription, inhibition, or expression at a basal level, is a cell's method of responding to its environment. For example, if the cell is exposed to increased temperatures, heat shock genes will be transcribed, in order to repair damage and allow the cell to regain normal function (Beinz and Pelham, 1987). Other methods of changing the protein content of the cell is to activate latent forms of proteins, or degrading mRNAs encoding proteins unhelpful to the situation before they can be translated.

De novo transcription is one of the most important methods a cell has to respond to a situation. Therefore analysis of the expression profile of a tissue or cells undergoing a certain challenge will provide insight as to how cells cope with these situations.

The goal of my research project was to examine patterns of gene expression in tissues and cells undergoing developmental and morphological changes. I have examined the pattern of gene expression in two cases.

1) Normal versus transgenic hypertrophied murine heart
2) Embryonic and adult skeletal myoblasts, treated with inducers of proliferation and differentiation

Both of these systems were observed to change in response to a challenge. The transgenic mice developed a cardiac hypertrophy due to the expression of the transgene Polyomavirus Large T-antigen. Skeletal myoblast lines demonstrated changes in myotube formation and proliferative capacity when treated with retinoic acid, dimethylsulfoxide, or transforming growth factor ß.

Gene products must interact with each other in order to maintain cellular homeostasis in times of stress. Thus examination of expression of many genes at one time will be more informative than restrictive analysis of a single gene. Genes

analyzed in this work included proto-oncogenes, growth factors, and genes encoding proteins forming striated muscle contractile apparatus.

1.1 Transgenic animals

Transgenic techniques allow genetic manipulation of intact animals and transmission of this genetic change to their progeny. This technology involves the delivery of a novel gene into the germ line of an animal. (For review, see Chalifour 1991). The novel DNA is known as the transgene. A transgene typically has promoter sequences (directing tissue or organ specificity of expression) followed by a structural gene coding region. Sequences may originate from different species, thus a human enhancer/promoter may direct expression of a bacterial gene in a transgenic mouse. Mating of transgenic animals leads to the generation of transgenic lines. Within a transgenic line, the transgene copy number, integration site, level of expression, and genetic background are all constant. Mice are the most popular vehicles for transgenes, due to high reproductive capabilities, relative ease and low cost of care, and availability of characterized inbred lines. Trangenic technology, however, has now spread to the use of domestic livestock, resulting in transgenic cows, pigs, sheep, rabbits, goats, rats, and even fish.

Transgenic animals are invaluable in experiments studying control of development, molecular interactions, the action of oncogenes, and gene expression and regulation. Transgenic animals are also useful models of certain diseases such as cardiomyopathy, many cancers including cancers of the central nervous system, liver, heart, bone, kidney, mammary gland, lens, skin and others, and can be used in drug and therapy testing. Transgenic livestock may also be used as living bioreactors (Pursel et al., 1989, Van Brut, 1988) producing large quantities of engineered proteins harvested from milk or blood.

There are three main methods of generating transgenic animals (Hogan et al., 1986, Jaenisch, R., 1988).

1) microinjection of transgene sequences into the fertilized egg
2) direct delivery of transgene into the blastocoel cavity
3) retroviral infection or transfection of transgene sequences into an embryonic stem cell

Microinjection is the most widely used method of generating transgenic animals.

The main advantage of transgenic techniques is the analysis of pathology development *in vivo*. The trangene interacts with homeostatic and protective mechanisms leading to more realistic and applicable experiments than those involving *in vitro* systems. Disadvantages of transgenic techniques include the inability to control the site of transgene integration, copy number, and level of expression within the tissue. Transgenic technology advanced in the 1980s, mainly due to two factors;

1) Advances in technology permitting the successful manipulation and implantation of animal embryos to result in live progeny.
2) The advent of recombinant techniques facilitating the manipulation of DNA to be used as a trangene.

The combination of these factors allowed progression in the field of transgenics to its current advanced state. Our laboratory has developed a transgenic mouse line expressing polyomavirus Large T-antigen in the heart.

1.2 Polyomavirus

Polyomavirus (PV) is a small double stranded virus of the papovavirus family. PV encodes three early genes; PV small T (PVST), PV middle T (PVMT), and PV large T (PVLT). These genes

are expressed early during the normal lytic course of infection of susceptible mouse cells. The early genes function in viral DNA replication, host transcriptional modulation, and induction of viral late proteins. In a non-permissive cell, the virus takes a different course and integrates into the host cell genome. This integration may result in host cell immortalization or transformation. The biochemical activities of polyomavirus which results in an altered cellular phenotype are not yet completely characterized.

1.2.1 Early functions; PVST, PVMT, PVLT

PVMT is a plasma membrane protein involved in the transformation of immortalized cells. It has been found to be phosphorylated by cellular tyrosine kinase pp60 c-SRC (Courtneige and Smith, 1983). PVST is a cytosolic protein, however, its role in the viral life cycle and effect on the host cell is unclear (Tooze 1981).

PVLT is a DNA-binding nuclear phosphoprotein capable of immortalizing primary cells. It has ATPase and helicase activities and its presence is imperative for correct viral DNA replication and transcriptional regulation. PVLT binds with high affinity to several sites within both viral promoters, controlling the transcription of both early and late genes. This site also contains the origin of viral replication. PVLT is known to bind to Rb, an antiproliferative protein possibly removing a block to proliferation, and altering cell growth characteristics.

PVLT alone confers an immortalized phenotype in rodent primary cells (Glaichenhaus et al., 1985, Rassoulzadegan et al., 1982, 1983). Most of these immortalization studies were performed on fibroblast-like cells. PVLT may potentially influence many different cell types, as a wide variety of cells are affected following PV infection into susceptible cells (Dawe et al., 1987, Eddy et al., 1982, Freund et al., 1987, Gross et al., 1970,

Rochford et al., 1990,). PVLT's combined characteristics of a less severe effect on cells (immortalizing not transforming) and its potential to act in many cells, makes it an ideal choice as a transgene.

1.2.2 Polyomavirus early gene transgenic mouse models

PVLT Transgenic mice

Transgenic mouse models using PVLT as a transgene have been described by several groups. Expression of PVLT has resulted in inhibition of the differentiation of lens tissue (Griep et al (1989). Polyomavirus promoter-driven PVLT synthesis resulted in PVLT expression in pituitary gland and the testes. A testicular phenotype was not observed, however, extremely latent pituitary tumours resulted (Bautch et al., 1987). Cardiomyocyte hypertrophy (Chalifour et al., 1990) and Leydig cell hyperplasia (Chalifour et al., 1992) have also been described in metallothionein driven PVLT synthesis. None of the PVLT effects were malignant except in the case of the pituitary gland, where PVLT was oncogenic only after extended latent period.

These results suggests that PVLT acts early in the phenotype as a 'first hit', and that further phenotypic change may be the result of cellular events modifying the effect of PVLT expression. The limited effect of PVLT on target tissues may be because its immortalizing rather than transforming activity, only minimally disrupts cell growth.

PVMT Transgenic mice

Transgenic mice with PVMT as the transgene develop early aggressive tumors. Guy et al, (1992) describe PVMT-transgenic mice with mammary tumors and further metastasis to the lung. A model described by Bautch et al, (1987) developed multifocal

lethal hemangiomas in vascular endothelium. Affected transgenic endothelium was transplanted into histocompatible acceptor mice. Acceptor mice died of hemangioma at the transplant site, demonstrating that the PVMT transgene had transformed an endothelial cell which could proliferate and cause tumors in other animals.

Chalifour et al., (1990), describe a model in which PVLT was linked to a metallothionein promoter known to be active in most tissues. This allowed examination of the effect of PVLT in an extended range of target tissues. In one line of transgenic mice made with this construct, the transgene was expressed in both heart and testes. The mice developed a cardiac hypertrophy where the heart increased in weight about 3.3 fold over normal heart. Affected animals died on average at 160 days due to heart failure. One of my projects was a study of gene expression of these affected hearts compared to non-transgenic unaffected mice.

1.3 Striated muscle

1.3.1 Origins of cardiac and skeletal muscle

Vertebrate development involves the generation of three primordial tissues in the embryo (Gilbert, 1988); the ectoderm, the endoderm, and the mesoderm. All muscle originates from mesodermally derived tissue. The mesoderm of an embryo is divided into 5 regions, of which dorsal mesoderm, and lateral plate mesoderm are relevant. The dorsal region of the mesoderm gives rise to connective tissue, such as cartilage and dermis, as well as bone and skeletal muscle. The lateral plate mesoderm gives rise to vessels and cells of the circulatory system, as well its major component, the heart.

Dorsal mesoderm and skeletal muscle development

Blocks of mesoderm along the embryonic neural fold develop into a segmented pattern, each segment termed a somite. Cells in the middle area of the somite form the myotome, which contains the progenitors of striated muscle of both back and limbs (Gilbert, 1988). Myogenic cells colonize structures such as the limbs leaving founder populations that will generate the significant muscle mass of the adult. Myotomal muscles appear as repeated segments composed of mononucleated myocytes. Myotomes are transient and are replaced by multinucleate musculature. It is unclear whether the early myotomal myocytes fuse to become multinucleate, or die and the area is recolonized by more mature myogenic cells capable of fusing to form multinucleate myotubes (Sassoon, D. A., 1993). Skeletal muscle myotubes may contain up to 100 nuclei. Upon fusion into myotubes, contractile proteins become highly expressed, and the cells differentiate to a contractile phenotype. At or around birth almost all the skeletal muscle cells are in the form of differentiated myotubes. Some myoblasts do, however, continue to exist in adult muscle as satellite cells. These cells can proliferate to a limited degree and play a part in repair of muscle after injury. Skeletal muscle exists in multiple forms; slow twitch, fast twitch and a super-fast version located in the eye. Fast twitch muscles contract and fatigue quickly. ATP needed for muscle contraction is generated by immediate, but inefficient anaerobic glycolysis of glycogen and glucose. These are the types of muscles used in fast activity like sprinting. Slow twitch skeletal muscles contract and fatigue slowly and contain myoglobin and many mitochondria. ATP needed for muscle contraction is generated by steady aerobic catabolism of glucose and fats. Slow twitch muscles are used for activities requiring endurance. Twitch is determined by innervation, and is not intrinsic to the muscle cell.

Lateral plate mesoderm and cardiac muscle development

The circulatory system is the first functional unit in the embryo, and the heart is its first functional organ. Heart progenitor cells migrate from the lateral sides of the mesoderm at a very early stage in embryonic development (Gilbert, 1988). The cells move independently and unlike skeletal muscle progenitors, do not derive from somites. Once the precursor heart cells have reached their target site, they differentiate into endocardium (which becomes the inner lining of the heart) and the epimyocardium (which becomes the layer of heart muscles). Epimyocardial cells differentiate into cardiomyocytes which remain mononuclear or more rarely fuse once to give rise to a binucleated cell. Cardiomyocytes branch erratically unlike skeletal muscle fibres which are arranged into uniform bundles.

The proliferative and differentiated form of the cardiomyocytes are morphologically very similar, distinguished only by contractility of mature cells. Skeletal myoblasts, on the other hand, must stop proliferating and fuse into large myotubes to be able to assume differentiated contractile duties.

1.3.2 Sarcomeric contractile proteins

Cardiac and striated muscle fibres are both made up of myofibrils which contain sarcomeres as a basic repeating unit. Sarcomeres are composed of thick and thin filaments which slide past each other as the muscle contracts. The thick filament is made up of myosin, a complex of a heavy chain (MHC) and two light chains (MLC). The thin filaments are made up of actin helices. Tropomyosin is a complex of several molecules which fits into the major groove of the actin helix. Each tropomyosin pair is associated with a trimeric complex of troponin (TN) composed of TN I, TN C, and TN T. TN C is sensitive to calcium concentration. At high concentrations, TN C releases the TN I-tropomyosin

inhibition of actin-myosin ATPase activity, allowing contraction to occur. (Fora review, see Darnell et al., 1990)

1.3.3 Gene expression during cardiac development

In mammals, cardiac myoblastb proliferation occurs before or soon after birth. Unlike skeletal muscle, where proliferation and differentiation occur at different times during development, cardiac muscle can perform differentiated functions as it proliferates in fetal and early neonatal life. After birht and arrest of cellular proliferation, increase in cardiac mass occurs solely by hypertrophy of pre-existing cardiac cells (Springhorn et al, 1992)

It has been noted that terminally differentiated heart cells responding to stress, recapitulate the transcriptional profile of a fetal proliferating cardiac cell (Nadal-Ginard and Mahdavi, 1989, Parker et al., 1991). A change toward a more fetal/neonatal morphology and transcriptional profile has also been noted upon long term *in vitro* culture of adult ventricular muscle cells (Claycomb et al., 1980, Eppenberger-Eberhardt et al., 1990).

Cardiac growth is attained in one of two ways; hyperplasia of fetal heart, or hypertrophy of post-natal heart. Contractile gene expression in the heart reflects its developmental stage. A recent review has described the expression levels and isoforms of important structural and contractile proteins during cardiac development (Lompre et al, 1991).

Contractile proteins in normal heart development

Myosin Heavy Chain (MHC) is the major component of the myosin thick filament and has ATPase activity. In mammalian heart two MHC isoforms are present. The α and ß isoforms are coded by separate genes clustered on chromosome 14 in mouse. The α MHC is cardiac specific and is not detected in other tissues, while ßMHC is expressed in both heart and skeletal muscle

(Lompre et al., 1984, Mahdavi et al., 1984, Sinha et al., 1984). In mice and rats, the α isoform is upregulated immediately following birth and predominates throughout the rest of life. ßMHC is detectable in trace amounts in young animals. In older animals, ßMHC reappears and the levels increase with age.

Each MHC is associated with two light chains: either one of an alkali MLC1 or MLC3, and a phosphorylatable regulatory MLC2 to form a functional muscle filament. These light subunits are necessary for the regulation of myosin ATPase.

MLC1/3, or alkali light chains are transcribed from at least three genes. The first gene is expressed only in fast skeletal muscle and gives rise to two isoforms generated by differential splicing; ($MLC1_F$, $MLC3_F$). The second is expressed in cardiac ventricle and slow skeletal muscle ($MLC1_V$ = $MLC1_S$) The third is expressed in cardiac atria and embryonic skeletal muscle ($MLC1_A$ = $MLC1_{emb}$).

MLC2 is also encoded by several genes. One gene is expressed in fast skeletal muscle ($MLC2_F$). Another is expressed in cardiac ventricle and slow skeletal muscle ($MLC2_V$ = $MLC2_S$); and yet another in cardiac atria ($MLC2_A$).

In fetal human heart, the ventricle co-expresses the fetal skeletal isoform $MLC1_{emb}$ (the isoform also found in adult atria) with the ventricular isoform $MLC1_V$. In both rat and human, after birth the ventricular MLC isoform is downregulated in the atria and the atrial MLC isoform is downregulated in the ventricle. (Price et al., 1980, Whalen et al., 1982)

There are three types of actin; α, ß and γ, each encoded by a separate gene. Actin monomers aggregate and twist into a double helix to form the thin filament. Alpha cardiac actin and α skeletal actin are both expressed in the fetus, but α cardiac actin is the main isoform expressed in adult heart. The skeletal muscle actin

isoform has been detected in young rat heart (Mayer et al, 1984), however, the presence of the skeletal isoform decreases as the animal ages from neonate.

Regulatory proteins of the contractile apparatus

Tropomyosin (TM) is a rod-like protein of two subunits (α TM and ß TM), associated with the thin filament. Its role is in regulating calcium activation of the actomyosin complex. The ß subunit is slightly more negatively charged than the α subunit. Both subunits have been detected in fetal rat ventricle, however, the adult ventricle expresses only the αTM subunit.

The Troponin (TN) complex is made up of three different molecules; TN I, TN C, and TN T.

TN T binds directly to TM. In rat, two genes have been detected, a cardiac and a fast skeletal gene. An immuno-histochemical analysis revealed the presence of two TN T isoforms; a 42.5 KDa species present in fetal heart, and a 41 KDa form present in adult (Sabry and Dhoot, 1989, Saggin et al, 1988).

TN I is a specific inhibitor of the ATPase of actomyosin. TN-I's binding to actin interferes with the formation of actomyosin. Little is known about the TN I genes except that there are multiple isoforms in both rabbit and chicken. The cardiac isoform differs from the skeletal counterpart by amino acid substitutions within the TN I sequence. Recently a fetal to adult switch of TN I isoform expression has been described in heart (Sabry and Dhoot, 1989), however the relationship and dynamics of the fetal and adult isoforms remain to be elucidated.

TN C confers calcium concentration sensitivity to the myofibrils. TN C binds 3 to 4 moles of Ca++ per mole of TN C in both skeletal and cardiac muscle (Van Eard and Takahashi, 1975). Calcium binding initiates muscle contraction. No information of developmental isoforms of this gene is available.

Summary of gene expression in cardiac development

Most contractile proteins experience a switch in isoforms around birth. In the ventricle of a rat model, ßMHC is replaced with αMHC, although the ß isoform may reappear in later life. Alpha skeletal actin expressed in fetal heart is turned off leaving only the continued expression of cardiac actin. The developmental form of TN I is replaced by the adult isoform, and ß TM is replaced by αTM. In the atrium, the predominant isoform expressed throughout both pre- and post-natal life is αMHC, however, ßMHC is detected toward the end of gestation, and increases during post-natal development (reviewed in Lompre et al, 1991).

1.3.4 Gene expression during cardiac hypertrophy

There is more than one model of cardiac hypertrophy in mammals (Lompre et al, 1991). One model describes the cardiac response to thyroid hormone (TH), and associated molecules T3 (triiodothyronine) and T4 (tetraiodothyronine) treatment. Other models describe chronic hemodynamic overload obtained by increasing pressure on the heart by mechanical blockage of afferent blood vessels.

Thyroid hormone induction of cardiac hypertorphy

Cardiac growth may be obtained by treatment with thyroid hormone (TH). TH increases the size of the myocyte, and the amount of total RNA and protein found within it, although DNA replication and cellular proliferation remain unaffected. The cardiomyocyte is reorganized, and a change in contractile performance and ion transport have been noted. TH plays a role during normal heart development in maturation and regulation of gene expression.

Witholding TH from the adult cardiac ventricle increases the amount of fetal ßMHC detected at the expense of αMHC. TH

replacement restores the adult phenotyope (Lompre et al., 1984). TH has minimal influence on αMHC expression in the atria. T3 increases the level of α cardiac actin in rat cardiomyocytes *in vitro* (Gustafson et al, 1986). Effects on α skeletal actin were not observed. In a TH-induced hypertrophy of rat myocardium, administration of thyroid hormone T3 resulted in an increase in both left and right ventricle weight (Greene et al, 1993). Analysis of gene expression in the right ventricle showed an increase in the mRNA of *c-myc*, *c-fos*, and ßMHC. No change in mRNA levels were detected in the left ventricle.

In summary, response of the ventricle to low levels of TH is a decrease of αMHC, a concurrent increase of ßMHC, and induction of expression of *c-myc* and *c-fos*. TH seems to have little effect in atria. In vitro, T3 induces an increase in expression of α cardiac actin in cultured rat cardiomyocytes, although no effect is observed on tropomyosin or troponin expression. Other genes were not examined in this model.

Chronic hemodynamic overload (CHO)

One of the responses of the heart to a sustained increased workload is hypertrophy. This is observed in human diseases which put stress on the heart such as chronic hypertension, valvulopathies, coronary atherosclerosis and response to physical training and hormonal treatment. Two models of hemodynamic overload have been generated.

1) Constriction of pulmonary artery in rabbit (Nagai et al., 1987).

2) Coarctation of the aorta in rat (De la Bastie et al., 1987, Ray et al., 1987).

Constriction of the abdominal aorta results in a 10 - 20% hypertrophy within 2-4 days after operation (Izumo et al., 1987, Mercadier et al., 1989). Constriction of the ascending aorta is more effective, resulting in 40 % hypertrophy at day 2 (Umeda et

al., 1987). This increased effectiveness may be explained by a more immediate mechanical load on the left ventricle, leading to a more severe adjustment on the part of the heart. Maximal ventricular hypertrophy peaks at the end of the 2nd week post-op and does not progress further, suggesting that the heart has reconciled its processes to its new load. Total RNA in the cardiomyocytes peaks at day 2 - 4 following aortic constriction, and decreases back to normal levels by the end of the second week. Protein and RNA turnover rates also increase and peak at days 2 - 4. Similar results for proteins were observed in the rabbit model. Translational efficiency increased by a factor of 2 in the rat hypertrophy model, and by a factor of 1.4 in the rabbit model. Taken together, these observations suggest the existence of transcriptional effects in the generation of cardiac hypertrophy.

Contractile gene expression during CHO

In the rat, hemodynamic overload results in the accumulation of the ßMHC (fetal) isoform with a coordinated decrease in the α (adult) isoform (Gorza et al., 1981, Lompre et al., 1979, Martin et al., 1985, Mercadier et al, 1981). In rat, where αMHC predominates in adult heart, there is the potential for a complete reversal of MHC phenotype in the overloaded chamber (left ventricle). In cases of severe overload, labelling of rat left ventricles with anti-ßMHC demonstrate the expression of the fetal isoform throughout the entire ventricular wall, illustrating that ßMHC is expressed in all of the myocytes. In less severe hypertrophy, application of an anti-ßMHC antibody reveals only sporadic labelling of the left ventricle (Gorza et al., 1981). It has been suggested that this phenomenon of non-homogeneous staining is related to wall stress, or stretch of the cardiomyocytes. Increased stress may lead to the expression of the fetal MHC isoform.

Treatment of hypertension in these rats restores αMHC expression, even if the hypertrophy does not diminish (Dussaule et al., 1986). Interestingly, hypertrophy resulting from intense physical training produces an overexpression of αMHC (Rupp, 1981, Scheuer et al.,1982). Physical training also restores expression of the correct adult MHC isoform in an animal with artificially induced hypertrophy (Scheuer et al., 1982). This suggests that MHC isoform switching and hypertrophy are not directly related, as different methods of hypertrophy induction results in different MHC isoform accumulation.

Most of the studies in MLC expression during hypertrophy have been performed in humans. In a study of 83 human patients, The regulatory light chain MLC2V accumulated in all samples. Severe atrial overload resulted in the accumulation of MLC1V (Cummins, 1982). Spontaneously hypertensive rats showed in increase in the mRNA encoding a ventricular form (MLC2V) in their atria before observable cardiac overloading had begun (Kumar et al., 1988). In a human study, Hirzel at al (1985) report of an increase in an atrial alkali light chain (MLC1A) in ventricle, although the severity of the overload was not clarified.

In the rat CHO model, a rapid increase in α skeletal actin mRNA was detected, peaking during the first week of overload, but subsiding to normal levels during the second week. The skeletal actin isoform was expressed in all myocytes throughout the affected ventricular wall (Schiaffino et al, 1989b).

The ß (fetal) form of tropomyosin accumulates in response to aortic coarctation, with simultaneous accumulation of both striated and non-muscle ßTM mRNAs. The expression of the abnormal isoforms is less prominent in chronic than acute stages of the hypertrophy.

Non contractile genes expressed during hypertrophy

In the pressure-overload rat model of hypertrophy, (Mulvagh et al., 1987) observed an induction of *c-myc* in the left ventricle and atrium. The atria sustained an expression of *c-myc* longer than the ventricle, and *c-myc* expression was more evident in older animals than younger ones. In a study of spontaneously hypertensive rats, Negoro et al., (1988) noted an increase in *c-myc* in the heart. Izumo et al, (1988) saw a transient induction of both *c-fos* and *c-myc* occurring within an hour of pressure overload, preceding expression of fetal isoforms of contractile proteins. Haegerty (1989) noted a stimulation of *c-myc*, *c-fos* and *Ha-ras* expression at 3 days post-op which had subsided by day 9. It was noted that, *c-fos* ,and *c-myc* (Komuro et al, 1988) as well as hsp 70 (Delcayre et al., 1988) had rapid transient expression, while *Ha-ras* was more gradual and sustained. This study also noted that the expression of *c-erbA*, *c-erbB*, *c-sis*, and *c-myb* mRNA remained unaffected by pressure overloaded hypertrophy.

Stretching of isolated rat ventricular cardiomyocytes caused α to ß MHC transition and a transient induction of *c-fos* (Yazaki and Komoro, 1989) suggesting that induction of these genes occurs upon increased ventricular wall stress. Perfusion and stretching of whole hypertrophied hearts isolated from 10 week post-op rats showed a decrease in the level of *c-fos* and *c-jun* mRNA. The authors suggested, that as the hypertrophied hearts were 10 weeks post-op, the decreased proto-oncogene expression might have been due to the adaptation of the heart to sustained stress. Transient increases of gene expression at 2 - 3 weeks may have subsided after 10 weeks.

TGFß increased transiently in surrounding rat myocytes bordering experimentally induced infarct (Thompson, 1988), perhaps involved in compensatory hypertrophy of the surviving myocardium.

A model involving growth hormone treated rats (Turner et al., 1988), showed an increase in heart size of 93%. Whether this is relevant is questionable, as the body weight also increased 112%. However, mRNA encoding Insulin-like Growth Factor I (IGF 1) and IGF II increased 3-4 fold. Other tissues did not show an appreciable increase in IGF I mRNA.

A study determining the level of EGR-1 expression in cardiac cells during early and end-stages of differentiation was performed by Sukhatme et al (1988). A northern analysis of normal heart RNA illustrated a high basal level of EGR-1 expression. Treatment of the cell line P19S1801A1 (P19) with 0.5% DMSO induced a cardiac phenotype of visibly beating cells. Northern analysis of *EGR-1* mRNA after treatment of P19 cells with DMSO, demonstrated an increase of expression during differentiation.

Summary of gene expression in CHO-generated hypertrophy

In summary, chronic hemodynamic overload results in an increase in ßMHC, with concomitant decrease of αMHC. The normal balance of these isoforms can be restored by exercise or treatment of hypertension. A ventricular isoform of MLC appears in atria while an atrial MLC isoform appears in ventricle. α–skeletal actin appears; peaks, and then disappears after the second week of hypertrophy. ßTM, *c-fos*, *c-myc*, *Ha-ras*, hsp 70, EGR-1, IGF I, and IGF II expression have all been shown to increase in various experiments. It seems that some fetal genes are re-expressed during hemodynamic overload-generated hypertrophy.

Cardiac hypertrophy generated by TH treatment or physical training does not result in expression of many genes associated with a fetal phenotype, therefore, a fetal program is not obligatory for all cardiac hypertrophies.

1.4 Skeletal muscle cell lines

1.4.1 Myoblasts and myotubes

Muscle arises from myoblasts, the cells destined for myogenesis. Myoblasts are capable of proliferation and differentiation. Upon contact and recognition of another myoblast and decreased serum levels, spontaneous fusion occurs leading to formation of a multinucleated myotube. Cessation of myoblast proliferation is necessary prior to initiation of fusion. The myotube, representing the end-mitotic stage of the muscle cell, will no longer divide or synthesize DNA, but produces large amounts of muscle-specific proteins. These include contractile apparatus and cell surface components necessary for neuromuscular transmission.

Myoblast cell lines used in this study

Embryonic skeletal muscle derived G7 and G8 cell lines, as well as the adult skeletal muscle derived C2C12 cell line were analyzed. G7 and G8 cells are clones which arose spontaneously from a culture of cells removed from hind limb muscle of a fetal Swiss Webster mouse. The G8 clone is non-tumorigenic, or simply immortalized, whereas the G7 clone is tumorigenic and has become transformed (Christian and Nelson, 1977).

The myoblast cell line C2C12 is a subclone derived by H. Blau (1985) of C2 cells, originally isolated by Yaffe and Saxel (1977). The C2 parent line was isolated from normal adult C3H mouse hind limb muscle. The myoblasts described above proliferate in culture to confluency, and may form some spontaneous myotubes. C2C12 cells have greater rate of spontaneous fusion than G7 and G8 cells. Decreasing serum concentration from 10% to 2% induces fusion of all 3 myoblast lines to the terminally differentiated stage of myotubes.

Contractile gene expression

Transcription of contractile genes has been studied in cultures of dividing myoblasts and fused myotubes (Devlin and Emerson 1978). Dividing myoblasts were transferred into medium containing low serum to stimulate differentiation. The progression of fusion, as well as the transcriptional status of various contractile proteins were observed from 0 to 64 hours. It was noted that synthesis of MHC, MLC1, MLC2, TN T, TN C, TM1 and TM 2 was initiated simultaneously at the time of myoblast fusion. A marked increase in transcription was observed 20 hours after induction of differentiation, followed by a new higher steady state level of transcription at 30 hours. In this study MLC3 was not activated during myotube induction.

A study by Bains et al (1984) detected an extremely high level of cardiac actin mRNA during myotube differentiation. Within 12 hours of initiation, cardiac actin was detectable in C2C12 cells. The cardiac transcript peaked at levels 6 times the detectable amount of skeletal transcript at that time, and then declined to levels lower than skeletal actin mRNA after 3 days. Kinetics of cardiac actin transcription followed closely to the observed rate of muscle cell fusion. Fusion was maximal at 24 - 36 hours, and essentially complete at 3-4 days. The results obtained from culture were in sharp contrast to those obtained from adult skeletal muscle tissue which showed only trace amounts of cardiac actin, and high levels of skeletal actin by northern analysis. The authors also note that the embryonic forms of many contractile proteins are expressed in cultured cells, even if derived from adult skeletal muscle.

Proto-oncogene expression in myoblasts and myotubes

Dot blot analysis of proto-oncogene expression of the rat myoblast cell line L6a1 was conducted (Leibovitch et al, 1986). Induction of L6a1 myoblasts into terminally differentiated

myotubes resulted in no obvious change in expression of *c-abl*, *c-myb* and *Ha-ras*. However, *N-ras* transcripts increased and *c-src*, *c-fes*, *c-fgr*, *Ki-ras*, *c-fos*, *c-myc*, *c-sis*, *c-fms*, *c-erb A*, and *c-erb B* were all markedly reduced. Interestingly, when the L6a1 cultures approach confluency, *c-fos* transcription increases abruptly to 3-4 fold its normal level, and then rapidly declines when contractile protein transcripts start to accumulate, becoming undetectable in resulting myotubes (Leibovitch et al, 1987). This suggests that regulation of *c-fos* is involved in initiation of the myogenic process.

Transcriptional regulation in skeletal muscle is influenced by the presence or absence of basic helix-loop-helix muscle specific factors such as MyoD, myogenin/myf 4, myf 5, and mrf 4/herculin/myf 6. A change in expression of any of these factors will result in alteration of regulation of muscle-specific genes. Expression of MyoD in hearts of transgenic mice led to the expression of skeletal muscle-specific regulatory (myogenin) and structural genes (skeletal actin) (Miner et al., 1992), and was lethal to the mice. Activity of these regulatory proteins has not been detected in cardiomyocytes, appearing to be exclusive to skeletal muscle. They are thought to control each others expression, production of muscle-specific structural and contractile proteins, and events during organogenesis in the embryo (Weintraub et al., 1991, Sassoon, D., 1993).

1.4.2 Inducers of myoblast proliferation and differentiation

The myoblast cell lines G7, G8, and C2C12 were treated with retinoic acid (RA), transforming growth factor-β_1 (TGFß), and dimethyl sulfoxide (DMSO) (Al Mustapha et al., submitted).

Retinoic acid (RA) is known to profoundly enhance cellular differentiation. RA induces differentiation of mouse embryonal carcinoma cells in vitro, and suppresses malignant transformation, both *in vitro* and *in vivo* (DeLuca, L G., 1991,

Merriman and Bertman, 1979, Lotan, R., 1980). Exogenously supplied, RA caused dramatic effects on pattern formation during limb development and regeneration in the chick embryo and newt (Brockes, J., 1989). RA was expected to induce differentiation of the myoblasts into myotubes.

TGFß is a 25 kDA di-sulfide linked dimer which exists in multiple forms; $TGF\beta_{1,2,3}$, and $_4$ (Sporn et al., 1992). Of these, $TGF\beta_1$ is the most widely characterized and is available commercially. TGFß is present in developing somites, active in repair of myocardial infarction, and is stored and secreted as a latent protein by cardiomyocytes. TGFß in general inhibits the differentiation of myogenic cell lines (Massague, J., 1990). TGFß also contributes to the process of tissue development and repair in vivo, including fetal myogenesis; activities in which induction of differentiation is necessary. TGFß3 is highly expressed and secreted by C2C12 myoblasts and was shown to inhibit C2C12 myoblast fusion (Lafyatis et al., 1991). Treatment of myoblasts with TGFß was expected to result in proliferation arrest.

DMSO causes P19 teratocarcinoma cells to stop proliferating, and differentiate into a muscle phenotype with visibly striated cells (McBurney at al., 1982). G7, G8 and C2C12 myoblasts were expected to react in a similar fashion upon exposure to DMSO.

1.5: Aims and summary

Genes activated during pathogenesis, or developmental alteration, are likely to be involved in cell surface signal reception, signal trasnduction, transcriptional control, and proliferative or differentiated functions of the cell. Bearing in mind these considerations, we created a list of genes to be analyzed (Figure 2, Methods)

A technique was developed to examine the expression of specific genes among any set of mRNAs isolated from a tissue or cells. This technique was employed to analyze the expression

profile of two systems responding to challenge. PVLT-transgenic mice developed enlarged hearts. Cardiac hypertrophy in some cases is associated with a re-emergence of fetal gene expression. Conversely, G7, G8 and C2C12 myoblasts were induced to differentiate into a more adult phenotype of myotubes. I screened the gene expression in these systems undergoing switches between fetal undifferentiated and adult differentiated phenotypes.

CHAPTER TWO

MATERIALS

2.1: Plasmids harboring cDNA/gDNA of interest

The following plasmids were obtained from the American Type Culture Collection (ATCC). The ATCC identification number follows each gene in parentheses. EGR-1 (63027), v-Ha-ras (41013), v-Ki-ras (41027), c-jun (63026), c-jun-B (63025), c-jun-D (63024), v-abl (41034), v-raf (41022), v-erb-A (41018), Rb-1 (57450), p53 (57254), HSP70 (57494), Myosin Light Chain 1/3 alkali (59702), Creatinine Kinase M (57678), Endothelial Cell Growth Factor ß (53335), TGFα (59950), TGFß1 (59954), myf-5 (59914), c-sis (57050), wnt-1 (63013).

The following plasmids were generously donated by; GAPDH, Dr. David Ann, U. Minnesota; Proliferating Cell Nuclear Antigen (PCNA), Dr. Renato Baserga, Thomas Jefferson U.; human androgen receptor (sv-ARo) Dr. Leonard Pinsky, McGill University; metallothionein (hMTIIA/9), Dr. Philippe Gros, McGill; c-fos, Dr. John Hiscott, McGill University; MYO D, Dr. Harold Weintraub, Fred Hutchinson Cancer Research Center, Seattle; pCC1 (human ß cardiac myosin heavy chain cDNA), Dr. Michael Sole, U. of Toronto; pATA2 (human cardiac actin) and pZI9 (skeletal actin), Dr. Michael McBurney, U. of Ottawa; LK295 (human skeletal actin 3' untranslated region), LK300 (human cardiac actin 3' untranslated region), and LK650 (rat troponin 1), Dr. Howard Prentice, U.S.C. at Los Angeles; pRTO3.10 (ß-actin) Dr. Denis Banville, Biotechnology Research Institute, Montreal; 4C (cTN1, cardiac troponin 1) and PCR-6 (sTN-1, skeletal troponin 1), Dr, Anne Murphy, Washington U., St. Louis; IGF-1 and IGF-2, Dr. Peter Rotwein, Washington U. School of Medicine, St. Louis, and c-myc, Dr. Marie Trudel IRCM, Montreal.

2.2: Enzymes and equipment

Molony Murine Leukemia virus RNase H-minus Reverse Transcriptase was purchased from Canadian Life Technologies. DNA polymerase I, Klenow fragment, oligo dT, random primers, Sephadex G50, and guanidine isothiocyanate were purchased

from Pharmacia. Taq polymerase and RQ1 DNAse were purchased from Promega. Gene Screen Plus was obtained from NEN/Dupont, and slot blot manifolds were purchased from Tyler Research Instruments, Edmonton, Canada. Hybridization ovens and bottles were obtained from Bellco Glass Co. A DNA Thermal Cycler was purchased from Perkin Elmer Cetus. Other necessary chemicals and reagents were purchased either from BDH or Sigma.

CHAPTER THREE

METHODS

A flow chart of the method can be found in Figure 1.

3.1: Preparation of Plasmids

DNA from plasmids was isolated using standard maxi-preparation techniques and purified from bacterial DNA after centrifugation through an ethidium bromide/cesium chloride gradient (Ausubel et al., 1992). Each DNA sample was verified as correct by restriction endonuclease digestion and examination of the DNA fragment pattern after electrophoresis through an agarose gel and subsequent ethidium bromide staining. DNA samples were quantified and purity confirmed by ultraviolet spectrophotometry.

3.2: Preparation of Slot Blot Membranes

Gene Screen Plus was cut to the correct size, soaked for at least 1 hr in 0.4 M Tris HCl pH 7.4, and prepared for blotting by following manufacturers instructions. DNA samples were diluted to 250 ng, 25 ng and 2.5 ng per 20 ul in 0.125 N NaOH, 0.125 X SSC (0.375 M NaCl, 0.037 M $Na_3C_5H_5O_7 \cdot 2H_2O$). Genes used in this assay are listed in Figure 2. DNA was applied to the membrane with light suction, the membrane was then removed from the manifold, allowed to air dry, and the DNA cross-linked to the membrane by UV (50 mJoules, 50 seconds). Membranes were prepared in batches and stored in plastic bags between sheets of filter paper.

3.3: Preparation of RNA

RNA was isolated essentially by the method of Chirgwin et al., (1979). Tissues were homogenized in 4 M guanidine isothiocyanate/ 1M sodium citrate (pH 7.0/ 0.1 M ß-mercaptoethanol) using a Brinkman homogenizer. The guanidine-tissue solution was centrifuged at 45,000g for 16 hours at 20°C in a Beckman SW60 rotor through a 5.7 M cesium chloride/0.1M

EDTA cushion and the RNA pelleted. The RNA pellet was resuspended in DNAse buffer (40 mM Tris HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 0.1 M $CaCl_2$), and any residual DNA removed by addition of 1 ul RQ1 RNAse-free DNAse (1 U/ul).

Figure 1; Flow chart of Slot Blot technique

Total RNA was prepared from a tissue or cell homogenate as described in the methods. Reverse transcription of messenger RNA was accomplished with oligo-dT primers and RNAse H-minus reverse transriptase. The first strand cDNA was purified and used as template in a random priming reaction including ^{32}P-labelled dCTP. Resulting radiolabelled double stranded cDNA probe was hybridized to membranes containing bound DNA encoding genes of interest. Each screening included all genes listed in figure 2. Probe and membrane were hybridized at 57^{o}C for 16 hours. The membranes were washed and exposed to X-OMAT X-ray film. Resulting autoradiographs were scanned with a linear laser densitometer and quantitation was performed as described in the methods.

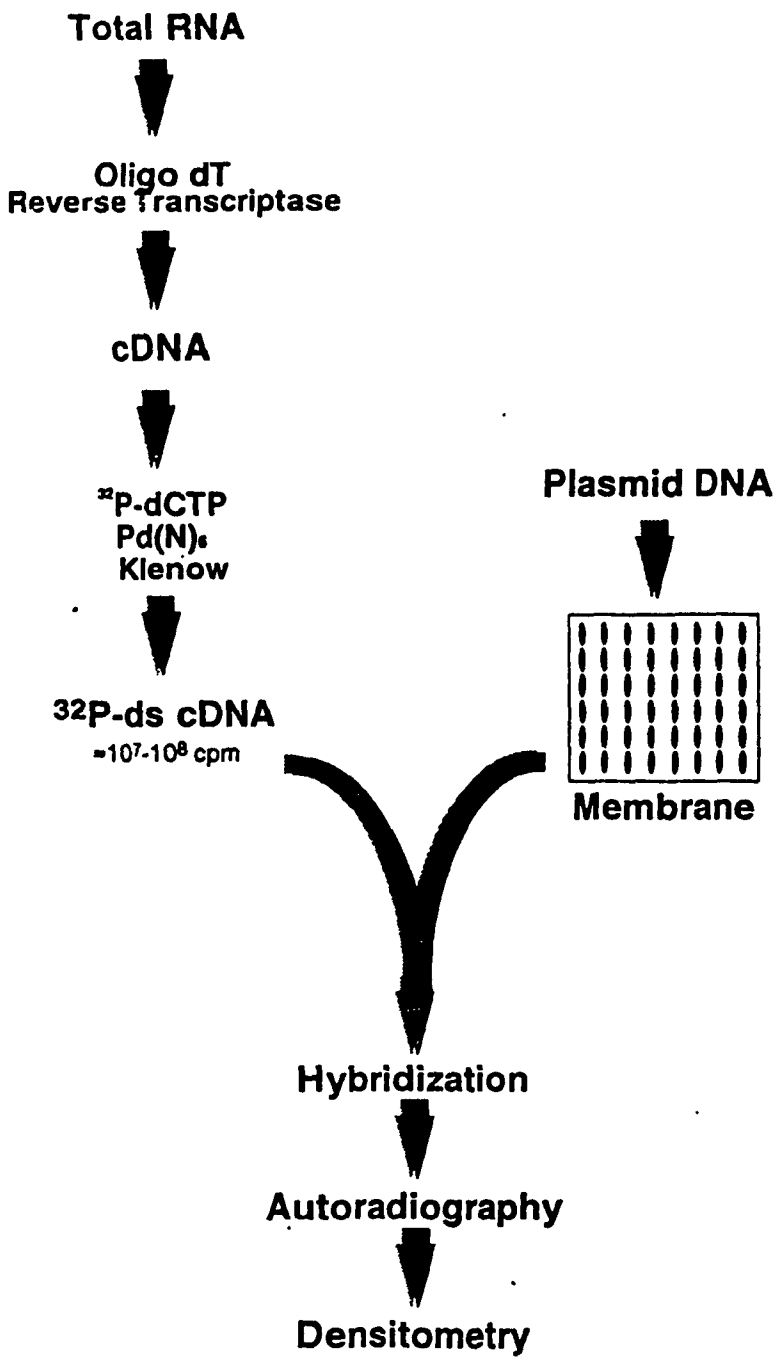


Figure 1: Flow Chart of Slot Blot Technique

Figure 2: Genes analysed with slot blot method

These proto-oncogenes, muscle specific genes, growth factors, anti-oncogenes and heat shock proteins were used in the screening assay. The genes were chosen for their roles in growth and transcriptional control, damage repair, stress response, signalling, and muscle specificity. Genes of interest were supplied in expression vectors and were not purified from vector sequences prior to application to membranes. The negative control in this assay was a pBR322 plasmid devoid of insert. pBR322, due to its bacterial origin, should not hybridize specifically to mammalian-derived cDNA probe. The negative control vector has sequences similar to the vectors containing the genes of interest, and will detect cross-hybridization of probe to vector backgrounds. The positive control is glyceraldehyde phospho-dehydrogenase (GAPDH), a housekeeping gene assumed to be transcribed at relatively constant levels in all cells and tissues.

Figure 2; Genes analyzed with slot blot method

proto-oncogenes

c-myc
c-fos
c-sis
c-neu
int-1
PCNA
v-abl
Ha-ras
Ki-ras
c-jun
junD
junB
v-erbA
EGR-1

muscle specific genes

human card. actin (pata2)
murine sk. actin (pZ19)
human sk. actin (LK295)
human card. actin (LK300)
ß actin (pRT03)
card. troponin I (CTN I)
sk. troponin I (STN I)
human ß card. MHC (pCC-1)
myoD
myf 5
muscle creatinine kinase (MCK)
myosin light chain (MLC)

growth factors

IGF I
IGF II
TGFa
TGFß

stress genes and antioncogenes

hsp 27
hsp 70
hsp 90
Rb
p53

controls

pBR322 (-)
GAPDH (+)
MTPVLT (transgene)

Proteins were removed with a phenol/choloroform extraction and the DNA-free RNA was collected by ethanol precipitation. The quantity and quality of the RNA was determined spectrophotometrically and by examination of an aliquot after electrophoresis through an ethidium bromide stained agarose gel (Figure 3, panel A). The total RNA was stored at -70°C as an ethanol (EtOH) pellet.

3.4: Preparation of Radiolabelled Double Stranded DNA Probe

Five micrograms of total RNA were removed, spun down in a 0.5 ul eppendorf tube for 20 minutes in a benchtop centrifuge, washed twice with 75% EtOH, evaporated to dryness in a Savant Automatic speedvac (model AS180), and resuspended in 9 ul of distilled water in preparation for reverse transcription. Two ul of oligo dT (0.5 ug/ul) were added to the RNA resulting in a total volume of 11 ul The tube was heated at 80°C for 10 minutes, then chilled on ice for 5 minutes. To each tube was added 4 ul of reverse transcription buffer, 2 ul of 0.1 M DTT (both reagents supplied with the enzyme), and 1 ul of dNTP mix (10 mM each of dCTP, dTTP, dATP, dGTP). One ul of RNAse H-minus reverse transcriptase was added and the reaction was incubated at 37°C for 1 hour. In order to visualize first strand product, 1 uCi of 3000Ci/mmol ^{32}P dCTP was added with the 10 mM dCTP during the reverse transcription. A small aliquot of the product was subjected to agarose gel electrophoresis and exposed to X-ray film (Figure 3, panel B). Three reverse transcription experiments were performed, using five, ten and twenty micrograms of total RNA. It was determined that 5 ug of total RNA was sufficient to generate adequate amounts and length of first strand cDNA.

Following reverse transcription, 30 ul of TE was added and the reaction mixture was spun through through a sephadex G50 spin column (2 minutes at 1000 g) to remove unincorporated

nucleotides. The column was washed with an additional 50 ul of TE to result in a final volume of 100 ul containing the first strand of cDNA. The sample was evenly divided into 2 tubes and evaporated to dryness. One of these aliquots was used in the second strand reaction. The other tube was stored at -20°C for later reactions.

Radioactive probe was generated by a random priming reaction (Asubel et al 1992) using the first strand cDNA product as template. Thirteen microlitres of distilled water and 2 ul of random primers (pdN6, 4.2 ug/ul, Pharmacia) were added to to the tube containing the dessicated first strand cDNA product. The tube was heated at 80°C for 10 minutes and then chilled on ice for 5 minutes. A reaction mix containing 2.5 ul of dNTPs (0.5 mM dGTP, dATP, dTTP), 2.5 ul of Klenow buffer (supplied by manufacturer), 5 ul of ^{32}P-dCTP (3000 Ci/mM) and 1 ul of Klenow per reaction was prepared and 11 ul of the mix was added to each tube to yield a final volume of 25 ul. The random priming reaction was allowed to proceed at 37°C for 2 - 4 hours, or left overnight. The reaction was stopped by addition of 75 ul of TE, and the reaction mix applied to a sephadex G50 spin column to remove unincorporated radiolabelled nucleotides from the cDNA probe (Asubel et al, 1992). The DNA probe was denatured at room temperature by the addition of 0.1 volumes of 1 N NaOH and incubation for 5 minutes, followed by neutralization with an equal volume of 1 N HCl. Probe was stored at -20°C and used within one week of synthesis.

Figure 3: Example of RNA quality and first strand cDNA synthesis

Panel A: Aliquots of RNA prepared with protocol described in materials and methods. Lanes contain 5 and 2.5 ug of total RNA respectively.

Panel B: Autoradiograph of single stranded cDNA product generated by increasing amounts of oligo dT-primed total RNA template. Lane a; Lambda/Hind III markers with arrows indicating 2 kb and 0.5 kb markers. Lanes b, c, and d contain radiolabelled first strand cDNA generated from 5, 10 and 20 ug of total RNA respectively. Note that the first strand cDNA ranges in size from 200 nucleotides to over 2kb.

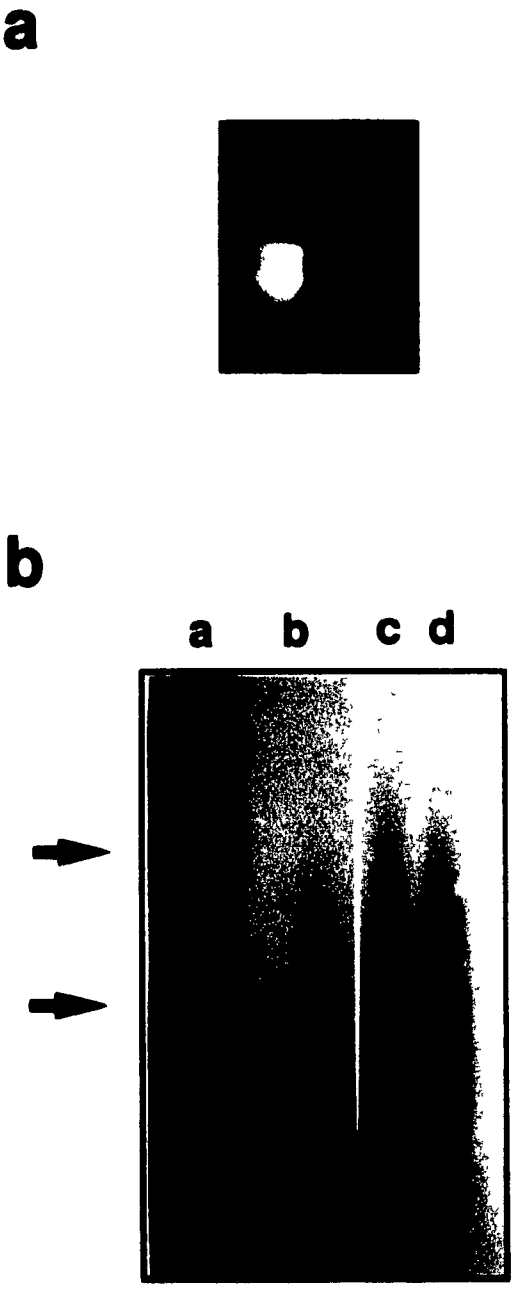


Figure 3: Example of RNA Quality and First Strand cDNA Synthesis

Alternatively, radiolabelled second strand was synthesized using random primers in a Polymerase Chain Reaction (PCR). As the probe was generated with equal efficiency by either PCR or Klenow random priming (as determined by scintillation counting of the resulting probe) it was decided that Klenow based synthesis would be the method of choice for synthesis of the second strand due to cost, ease, and availability of equipment.

3.5: Hybridization

Membranes were incubated with radiolabelled probe in glass tubes in a hybridization oven. Membranes (6.2 x 6.8 cm) were prehybridized in 10 mls of 1% SDS / 1 M NaCl /10% dextran sulphate at 58°C for 2-4 hours. The prehybridization buffer was removed and replaced with 5 mls of hybridization buffer containing 1% SDS, 1 M NaCl, 10% dextran sulphate, 2 x 10^6 cpm radioactive cDNA probe, and 100 ug of sheared denatured herring sperm DNA per ml of hybridization buffer. The membranes were hybridized overnight at 57°C. The following day, the hybridization buffer was removed, and the membranes washed twice with 1 X SSC, 0.1% SDS for 20 minutes at room temperature, followed by two washes of 0.1 X SSC, 0.1% SDS at 50°C (20 X SSC is 3 M NaCl/0.3 M Na citrate). The damp membranes were exposed to X-OMAT film at -70°C and developed using an automatic X-OMAT film processor.

As soon as possible after exposure, the damp membranes were stripped of the hybridized probe by boiling in 0.1 X SSC/ 2% SDS for 20 minutes. The stripped membranes were exposed to X-OMAT film for a week to verify that the membranes were free of radioactive probe. Stripped filters were then rinsed in 2 X SSC, air-dried, and stored as previously described for re-use in

further experiments. Membranes may be stripped and re-used twice without losing significant amounts of bound DNA.

3.6: Quantitation;

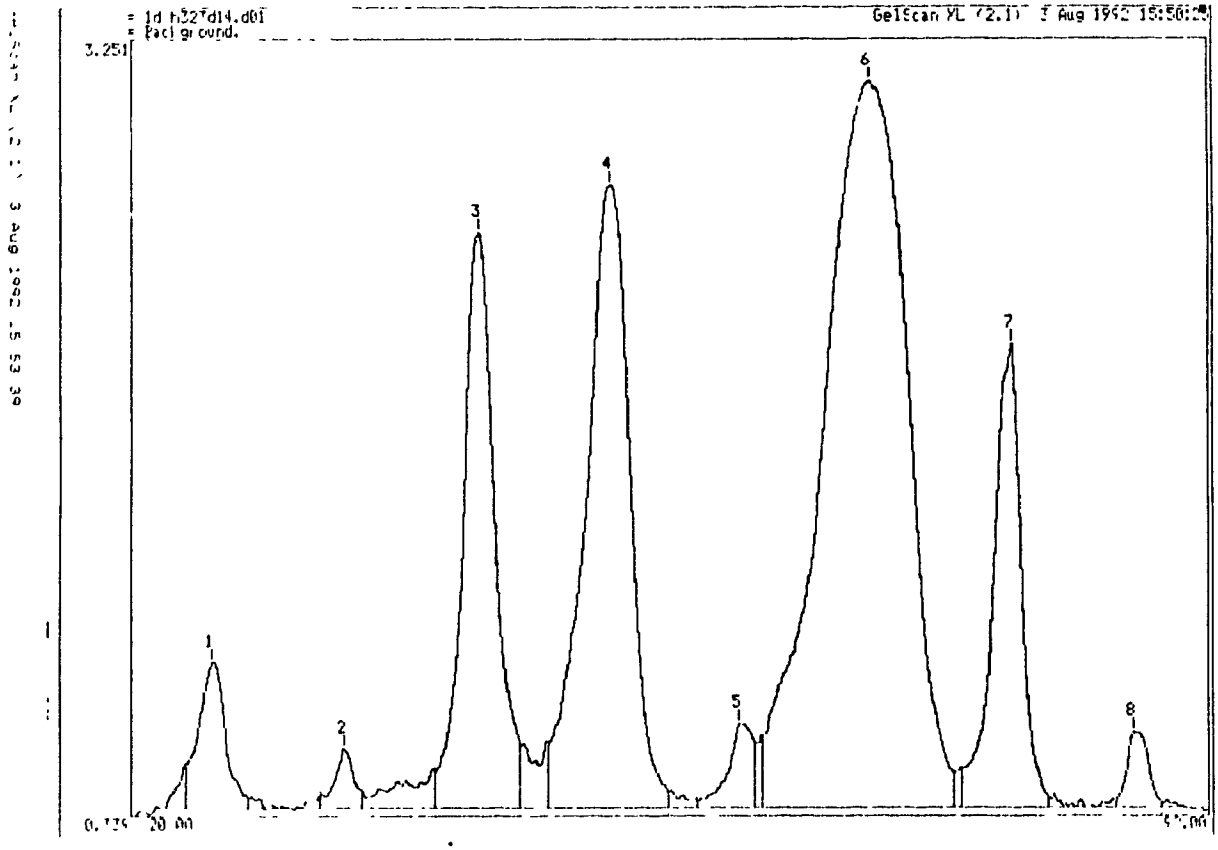
Each membrane contained genes at 2.5 ng per slot, 25 ng per slot and 250 ng per slot. Densitometry using an LKB Ultroscan XL laser densitometer was performed on the 250 ng slots, which were the clearest, and the area under each peak was noted. An example of a densitometric scan of an autoradiograph is provided in Figure 4. Calculations were as follows; the corrected area (area generated by each gene minus the area generated by the negative control background vector; either PGEM4Z or PBR322) was divided by the corrected area obtained for GAPDH to give an intensity value. The intensity of each gene is therefore expressed relative to GAPDH. When comparing gene expression of affected tissue or cells to unaffected counterparts, affected values are described as fold increase or decrease of the normal expression value of that gene.

Figure 4: An example of a densitometric scan

Densitometry data (Panel A) was generated from a linear scan of the autoradiograph in Panel B. The autoradiograph used affected transgenic heart cDNA probe hybridized to membrane-bound DNA sequences. Exposed areas and their corresponding densitometric peaks are identified as follows: Peak 1: PVLT, 2: int-1, 3: junD, 4: c-jun, 5: c-myc, 6: ECR-1, 7: c-fos, 8: v-raf.

Note that peak 1 corresponds to the transgene PVLT, which is expressed in transgenic hearts. Peak 6 corresponds to EGR-1, a gene detected in at abnormally high levels in all stages of PVLT-induced hypertrophy. junD (peak 3), c-jun (peak 4), and c-fos (peak 7) are also expressed at high levels in this particular heart.

A



B

Figure 4: An Example of a Densitometric Scan

CHAPTER FOUR

RESULTS

Coordination of expression of many genes is necessary during cellular proliferation, development, and differentiation. We have examined gene expression in two models of development and differentiation. Cardiac hypertrophy in PVLT-transgenic mice represents a model of abnormal growth and development. Skeletal muscle myoblast fusion is a model of normal proliferation and differentiation. It has been shown that artificially induced cardiac hypertrophy (Lompre et al, 1991) and myoblast fusion (Leibovitch et al., 1986) result in altered gene expression. Due to these reports, we hypothesized;

1) PVLT-induced cardiac hypertrophy would result in detectable changes in gene expression,

2) Exposure of G7, G8, and C2C12 skeletal myoblasts to chemicals known to affect proliferation and differentiation such as retinoic acid, dimethyl sulfoxide and transforming growth factor ß, should cause appreciable differences in gene expression.

In order to test these hypotheses, a screen of gene expression was performed with the slot blot technique described in the methods section (Chapter 3, Figure 1). Figure 2 shows a list of all genes screened by this assay. Data is presented separately for the transgenic hearts and the myoblasts.

4.1: PVLT-Transgenic mouse hearts

4.1.1: Macro and Microscopic appearance of cardiac tissue

Expression of the PVLT transgene in cardiomyocytes caused a hypertrophy of 1.5 - 5 fold (Figure 5). The increase in cardiac mass was caused by cardiomyocyte hypertrophy, rather than hyperplasia (Chalifour et al., 1990). Figures 6 to 9 illustrate histological changes in PVLT-transgenic hearts as the pathology progresses.

Figure 5: Comparison of heart size between a non-transgenic and an affected PVLT transgenic mouse.

Panel A: Opened chest cavity showing the position of the organs of the chest and upper abdomen of an age-matched non-transgenic mouse. The white arrow points to the heart.

Panel B: Opened chest cavity of an affected PVLT transgenic mouse. Note the large size of the heart and the distortion of the chest cavity into the upper abdomen.

Panel C: Comparison of excised hearts. Left; excised heart from affected animal from panel B. Right; excised heart from non-transgenic animal from panel A. The scale is in centimeters.

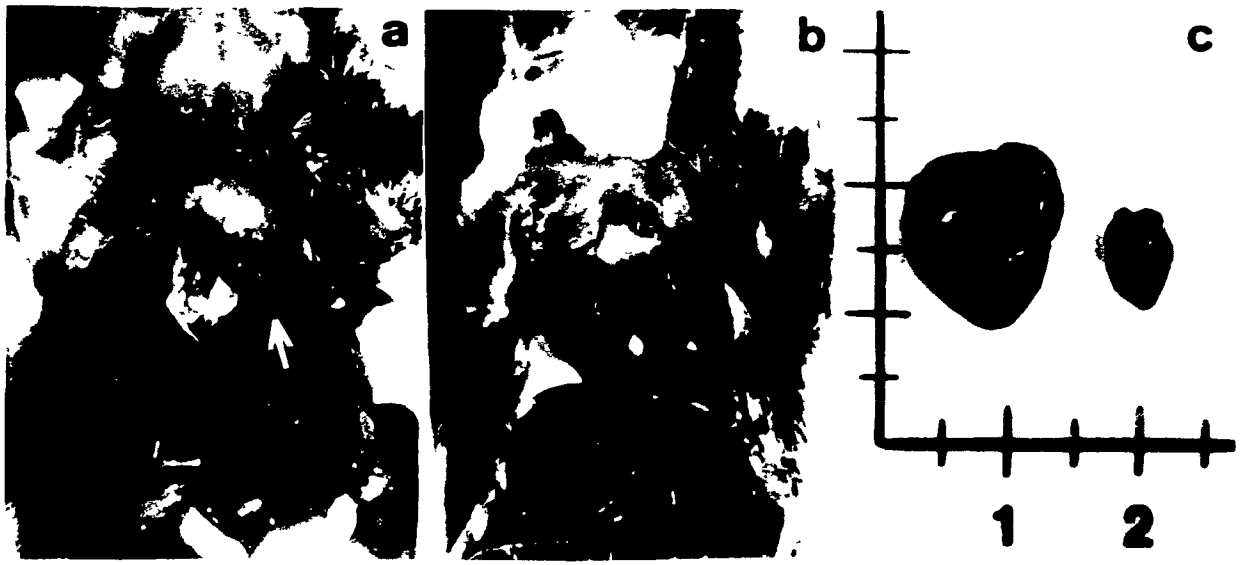


Figure 5: Comparison of Heart Size Between a Non-transgenic and an Affected PVLT Transgenic Mouse.

Figure 6 depicts the histology of a normal non-transgenic mouse heart of approximately 150 mg. Note that the heart is composed of both cardiomyocytes and non-muscle cells (interstitial cells, endothelium, etc.). Cardiomyocytes are larger than the interstitial cells, a fact easily visualized by comparing their nuclei and cytoplasm as indicated in figure 6 panel B.

Figure 7 shows the histology of an affected PVLT-transgenic heart tissue at an early stage of hypertrophy. Although heart weight has increased to 244 mg, cardiomyocytes do not appear significantly larger than those of the normal heart in figure 6.

A heart weight of 457 mg is considered to be representative of mid-stage hypertrophy (figure 8). The cardiomyocytes are noticeably larger than normal (compare figure 8 panel B to figure 6 panel B). Many but not all cardiomyocytes are enlarged at this stage. This indicates heterogeneity of hypertrophy at a cellular level.

Histology of late stage hypertrophy is illustrated by a heart weighing 728 mg (figure 9). Note the vary large, densely staining cardiomyocyte nuclei. This heart displays a histology very different from the normal non-transgenic sample. It is interesting to note that the disease progression does not correlate well with age, nor with transgene dosage. Homozygotes and heterozygotes for the transgene develop equally severe pathology. We wondered if a change in gene expression accompanied the histological differences observed during pathology development in PVLT-trangenic hearts

Figure 6: Histological appearance of normal non-transgenic heart

Hematoxylin and eosin staining of normal 119 day old mouse heart (150 mg).

Panel A: 100 X magnification

Panel B: 200 X magnification
Arrow denotes cardiomyocyte nuclei, arrowhead denotes interstitial cell nuclei.

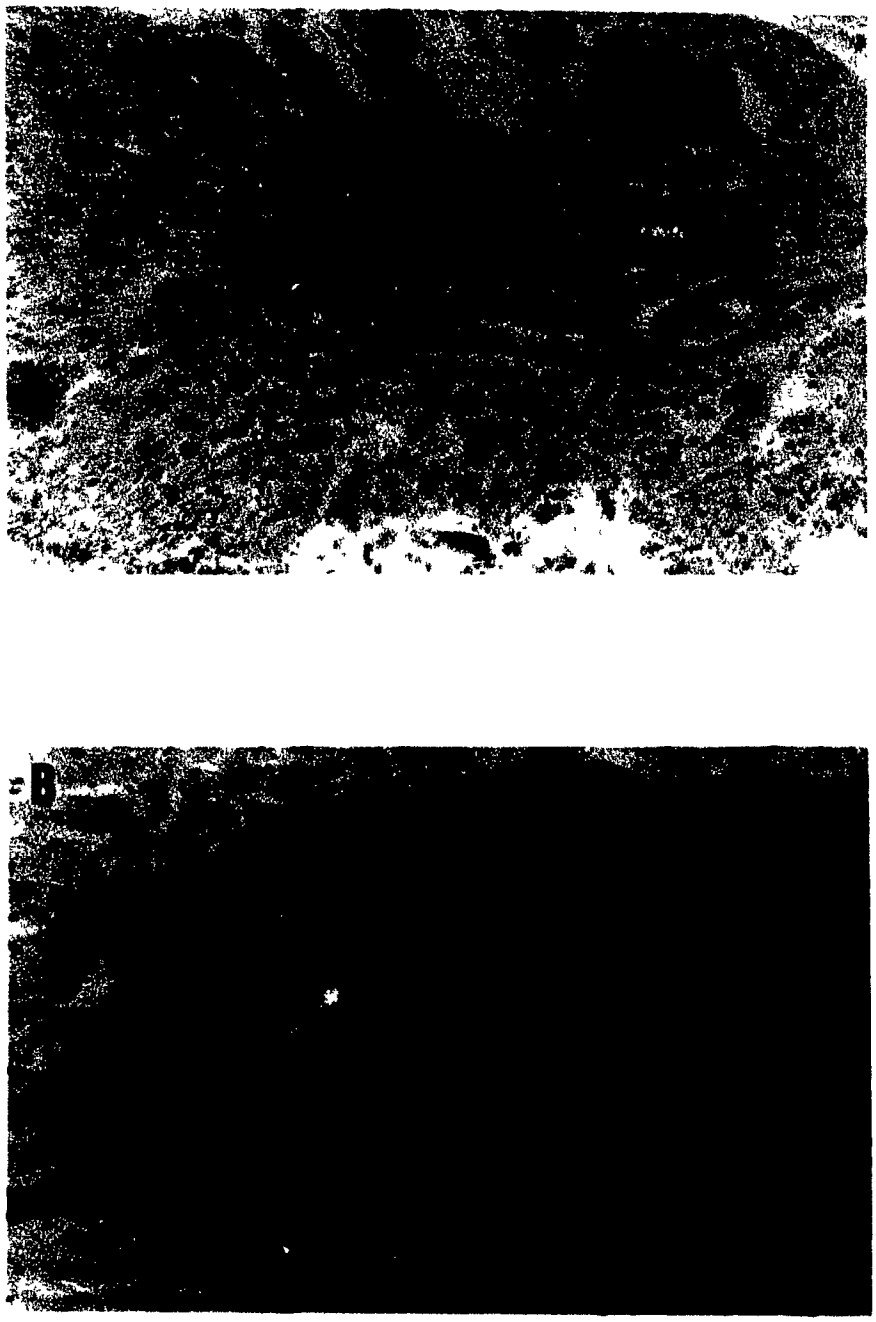


Figure 6: Histological Appearance of Normal Non-transgenic Heart

Figure 7: Histological appearance of transgenic early stage hypertrophied heart

Hematoxylin and eosin staining of small (244 mg) early stage hypertrophied heart from an 82 day old mouse.

Panel A: 100 X magnification

Panel B: 200 X magnification
Arrow denotes cardiomyocyte nuclei, arrowhead denotes interstitial cell nuclei.

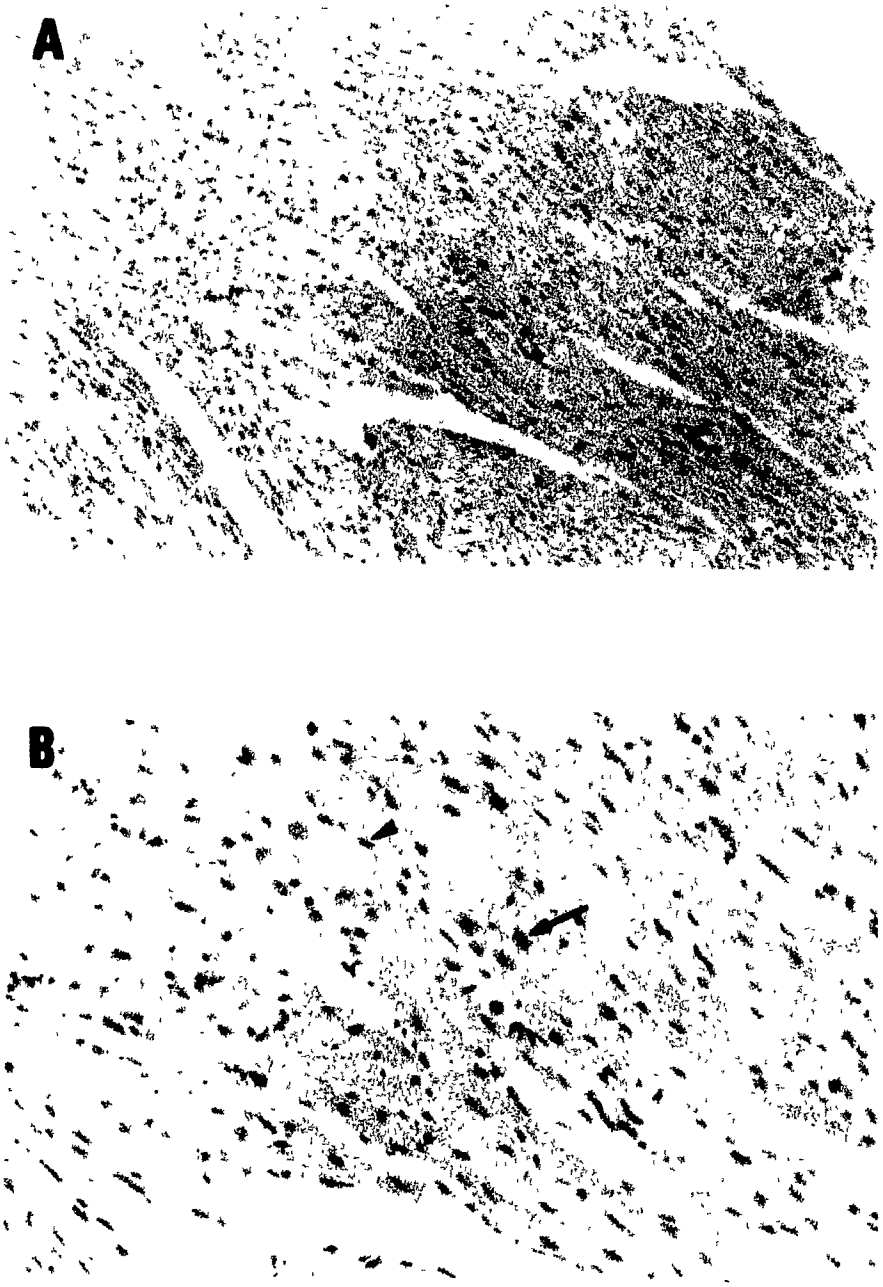


Figure 7: Histological Appearance of Transgenic Heart in an Early Stage of Hypertrophy

Figure 8: Histological appearance of transgenic mid-stage hypertrophied heart

Hematoxylin and eosin staining of medium-sized (457mg) hypertrophied heart from a 170 day old mouse.

Panel A: 100 X magnification

Panel B: 200 X magnification
Arrow denotes cardiomyocyte nuclei, arrowhead denotes interstitial cell nuclei. Presence of enlarged cardiomyocytes is more noticeable than in normal or small stage hypertrophy.

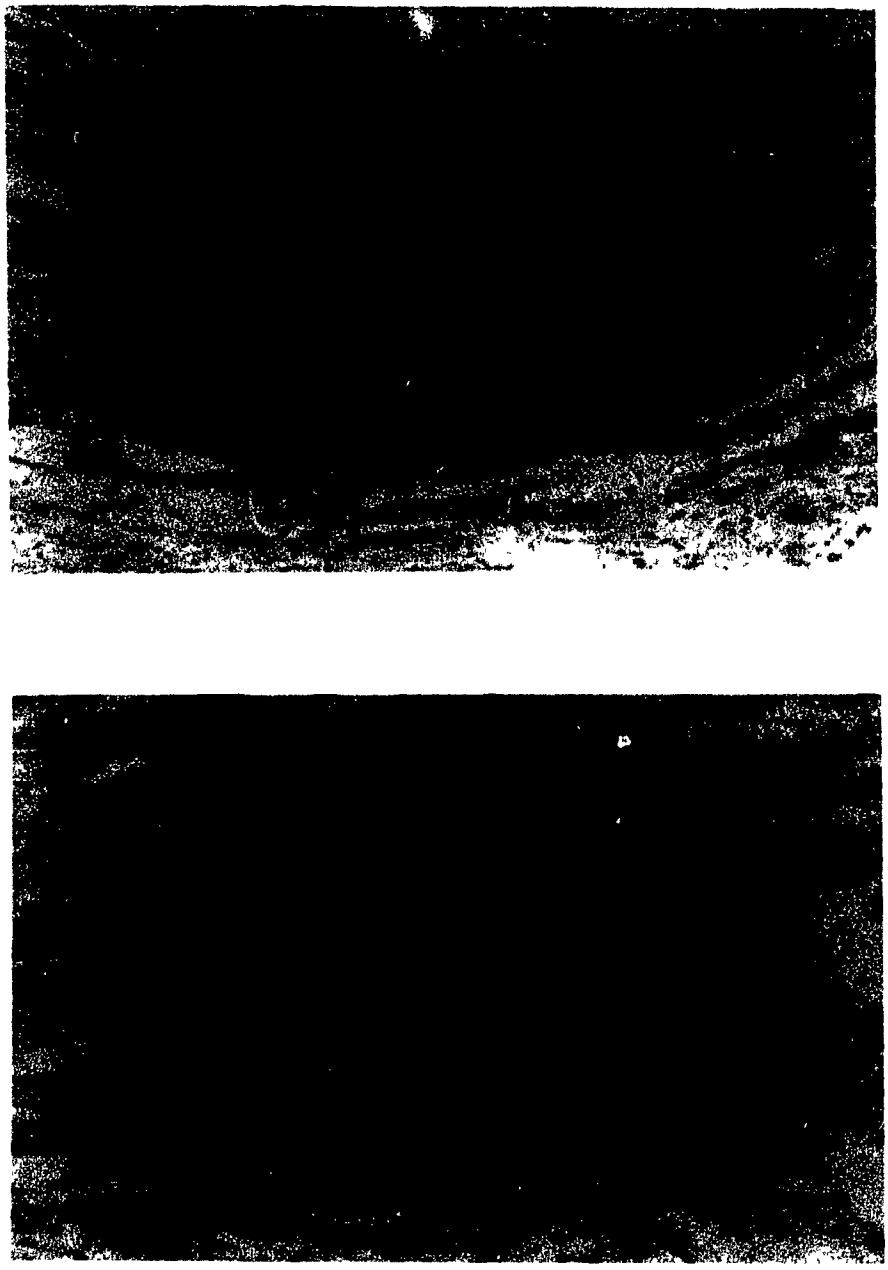


Figure 8: Histological Appearance of a Transgenic Heart at an Intermediate Stage of Hypertrophy

Figure 9: Histological appearance of transgenic late stage hypertrophied heart

Hematoxylin and eosin staining of large (728mg) hypertrophied heart from a 147 day old mouse.

Panel A: 100 X magnification

Panel B: 200 X magnification

Arrow denotes cardiomyocyte nuclei, arrowhead denotes interstitial cell nuclei. Note that enlarged cardiomyocytes predominate.

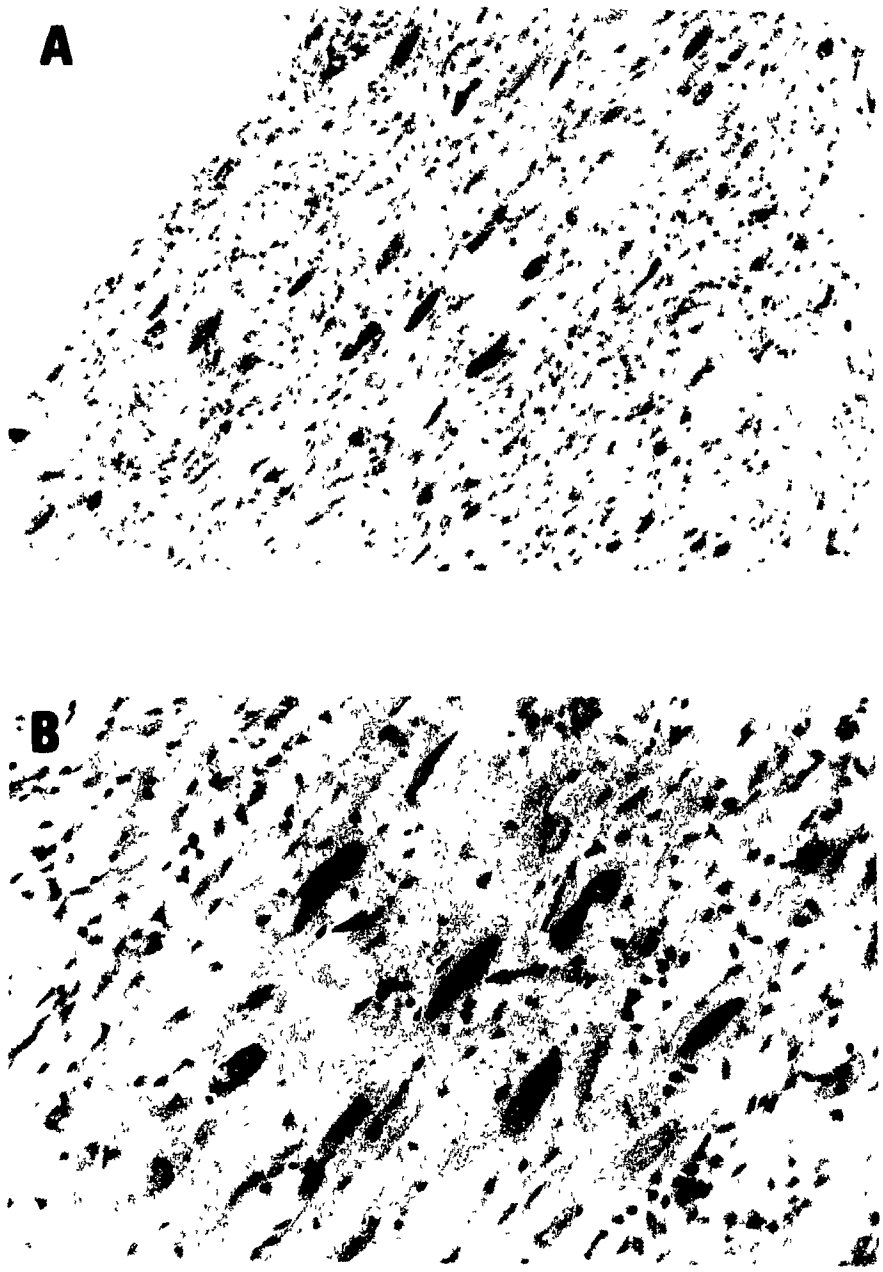


Figure 9: Histological Appearance of a Transgenic Heart at a Late Stage of Hypertrophy

4.1.2: Alterations in gene expression during hypertrophy

Using a technique useful for screening patterns of gene expression (Chalifour et al, submitted), we compared proto-oncogene, stress protein, and growth factor mRNA levels in normal heart (mass = 240 g) versus three affected transgenic hearts (mass = 323 g, 556 g, 666 g) representative of each stage. Figure 10 illustrates the difference of hybridization intensities between normal and affected hearts. RNA from a normal non-transgenic heart is displayed on the left of the figure and the pattern from RNA of a late stage hypertrophied heart is shown to the right. Included on each membrane was a representative plasmid vector pBR322 to allow subtraction of cross-hybridization to vector rather than insert sequences in calculations. Each membrane also contained the house-keeping gene glyceraldehyde phospho-dehydrogenase (GAPDH), which served as a baseline in calculations.

Table 1 respresents the quantitation and organization of intensities generated by transgenic and non-transgenic hearts. Altered gene expression in PVLT trangenic heart was classified by three hypertrophy stages; early (minimal hypertrophy), middle (mid-sized hypertrophy) and late (extreme hypertrophy).

Early stage hypertrophy;

Similar to the results of pressure overload, increased *c-jun*, *c-myc*, EGR-1, and *Ha-ras* expression were detected as well as that of TGFß. *junD* and *c-fos* expression are altered during both early and mid stages, but are expressed at normal levels in the late stage heart. *junD* and *c-fos* are therefore required beyond the initial remodelling stage of hypertrophy.

Mid-stage hypertrophy

HSP 70 and 27, IGF-1, p53 and *c-neu* were expressed only in mid-stage hypetrophied heart.

Figure 10: Slot Blot hybridization intensities of genes of interest in normal and affected transgenic hearts.

Slot blots were prepared as described in materials and methods. Three increasing amounts of DNA encoding each gene were applied to the membrane; 2.5 ng, 25 ng, and 250 ng. The membranes were hybridized with labelled cDNA probe, washed and exposed to X-ray film. Densitometry of the exposed areas of the film permitted evaluation of the intensity of expression of each gene. The figure is arranged such that the gene of interest at the left of the table is accompanied by a representative slot blot of both a normal and a transgenic hybridization experiment.

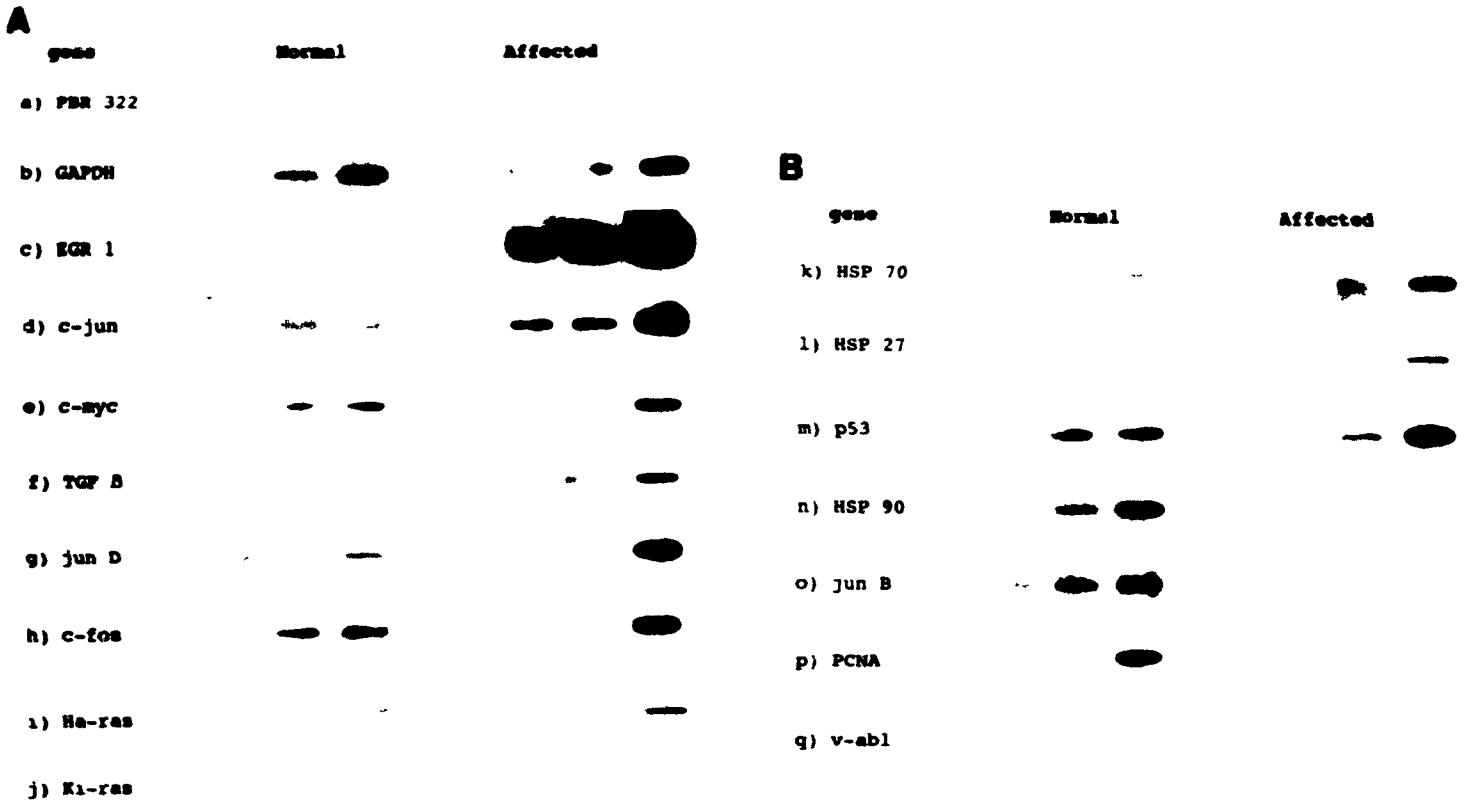


Figure 10: Slot Blot Hybridization intensities

Table 1: Gene expression changes in small, mid-sized and large transgenic hearts.

The table includes data generated from 4 normal, and a representative small, mid-size and large transgenic hearts. Their weight in milligrams can be found directly below the sample. The genes of interest are found to the left of the table followed by the intensity of expression in normal heart (average value +/- standard deviation of all normal hearts), and data generated by transgenic hearts. The trangenic heart data is recorded as fold increase or decrease (indicated by up or down arrows) of the normal intensity value. Insignificant deviation from the normal intensity value is shown as "N".

Table 1: Gene expression changes in small, mid-sized and large transgenic hearts

	Normal	Transgenic Hearts Small	Mid-size	Large
Size (mg)	240±25	323	556	666
Continuously altered				
EGR-1	0	60↑	7.3-10↑	5↑
Altered early stages only				
c-jun	1.5±1.3	18.4↑	N	N
c-myc	0.8±1	2.6↑	N	N
TGF-β	0.06±1	4↑	N	N
Ha-ras	0.56±0.4	5↑	N	N
Altered early and mid-stage				
junD	0.135±0.16	138↑	7↑	N
c-fos	0.53±1	25↑	4↑	N
Altered mid-stage only				
HSP 70	0.35±0.5	N	22↑	N
HSP 27	0.23±0.24	N	7↑	N
IGF-1	0.4±1.2	N	2↑	N
p53	1.06±0.4	N	6↑	N
c-neu	0.53±0.3	N	10↑	N
Altered mid-stage and late-stage				
HSP 90	4.3±1.2	N	0	20↓
TGF-α	0	0	0	0.27↑
Ki-ras	0.64±0.3	N	25↓	2.5↓
PCNA	4±3.1	0	0	33↓
Altered late-stage only				
junB	2.5±2	N	N	10↓
Unaltered at any time				
abl, Rb, IGF-2, erbA				

Late stage hypertrophy

HSP 90, *Ki-ras*, Proliferating Cell Nuclear Antigen (PCNA) and *junB* all showed lower than normal expression in late stage hearts. TGFα showed only a very slight decrease in expression. Expression of all other genes analyzed were not detectably altered from non-transgenic values, thus transcriptionally, the late stage hypertrophied heart was not radically different from a non-transgenic unaffected heart.

PCNA was decreased uniformly throughout hypertrophy. It was not detectable by the screening method except at very low levels in the late stage heart.

Summary

PVLT-induced hypertrophy resulted in gene expression changes. Interestingly, it is possible to associate these changes with stages of pathology. Genes with expression modified during early stages of pathology are mainly transcription factors. Later stages of disease include alterations in the expression of growth control or stress management genes. Examination of more samples may further substantiate a possible time course of transcriptional changes during PVLT-induced hypertrophy.

4.2: Myoblast cell lines treated with RA, DMSO, and TGFß

Previous experiments with RA, DMSO and TGFß suggest that they induce muscle phenotypes. Treatment of embryonal carcinoma cells with DMSO induced differentiation into both cardiac and skeletal phenotypes (McBurney et al., 1982). Retinoic acid plays a major role in many developmental systems including muscle (Halevy et al., 1993). Treatment of cardiac fibroblasts with TGFß resulted in a striated muscle phenotype (Eghbali et al., 1991). Addition of TGfß to L_6E_9 rat skeletal myoblasts in mitogen rich medium causes terminal differentiation into myotubes (Zentella and Massague, 1992)

These experiments prompted investigation of the effects of these inducers on an *in vitro* muscle development system. Established cell lines of murine embryonic skeletal muscle origin; G7 and G8, and murine adult skeletal muscle origin; C2C12, were treated with the above inducers (Al Mustapha et al, submitted). 10^5 cells were incubated for four days with or without inducer (DMSO, $TGF\beta_1$, RA). After this period, cells were analyzed to determine the effects of the inducers on cellular proliferation, myotube formation and gene expression.

4.2.1: Morphology of treated and untreated cells

Incubation with 1% dimethyl sulfoxide (DMSO) had a slight inhibitive effect on proliferation and myotube production in G7, G8 or C2C12 cells, whereas higher concentrations (2 - 5%) were lethal. TGFß mildly increased cellular proliferation in all three myoblast lines with a concomitant small decrease in their ability to differentiate and form myotubes. Retinoic acid (RA), at 10^{-4} to 10^{-7} M slightly increased the percentage of cells able to form myotubes in the G7 and G8 lines, but myotube formation was increased four-fold in RA-treated C2C12 cells. These effects are summarized in Table 2.

Figures 11 and 12 depict the appearance of these myoblasts in culture. Figure 11 is composed of three photographs of G8 cells. Panel A shows a non-confluent culture of proliferating G8 myoblasts. Depletion of serum in the media causes fusion into myotubes depicted in panel B. Treatment of G8 cells with RA and TGFß had little or no effect on myotube induction. Both chemicals resulted in morphology similar to that shown in panel A. DMSO at 1% inhibited G8 proliferation (panel C) resulting in sparse cell populations. G7 myoblasts reacted similarly to G8 cells upon exposure to the inducers, therefore G7 photographs are not shown.

Table 2: Compilation of the effects of dimethyl sulfoxide (DMSO), transforming growth factor-ß (TGFß), or retinoic acid (RA) on G7, G8, and C2C12 myoblast cell lines.

Table 2: Effects of DMSO, TGFß and RA on G7, G8 and C2C12 myoblast cell lines

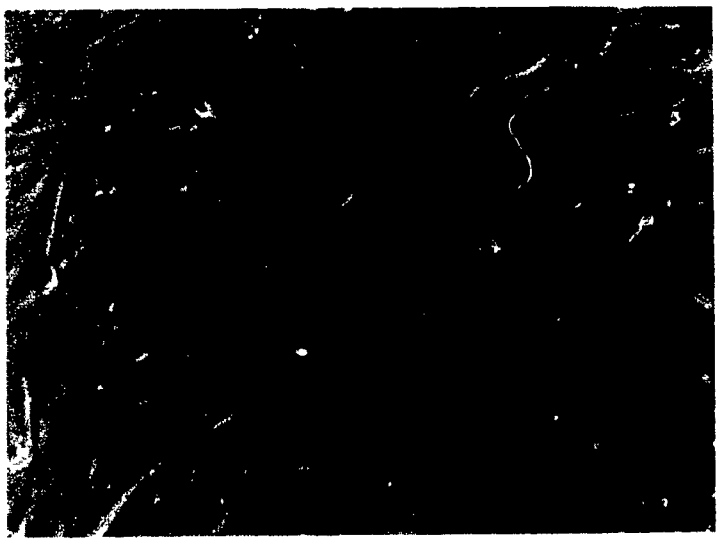
cell type	DMSO	TGFß	RA
G7	- slight inhibition of proliferation, - decreased myotube formation	- slight increase in proliferation, - decreased myotube formation	no visible effect on myotube formation
G8	- slight inhibition of proliferation, - decreased myotube formation	- slight increase in proliferation, - decreased myotube formation	no visible effect on myotube formation
C2C12	- slight inhibition of proliferation, - decreased myotube formation	- slight increase in proliferation, - decreased myotube formation	- 4-fold increase in myotube number - myotubes are larger, contain more nuclei and appear 2 days earlier than controls

Figure 11: G8 myoblast and myotube morphology

Panel A: Proliferating G8 myoblasts in 10% serum
Magnification = 200X

Panel B: Terminal differentiation of cell into myotubes caused by removal of serum. Arrow indicates a myotube. Magnification = 200 X

Panel C: DMSO treatment of G8 myoblasts causes inhibition of proliferation. Spherical particles are dead cells. Maginification = 200X



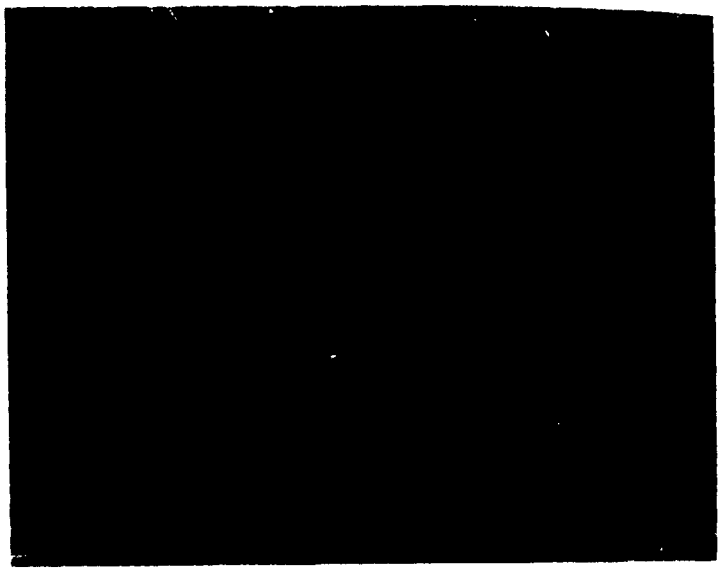


Figure 12 contains photographs of C2C12 cells. Panel A depicts C2C12 myoblasts approaching confluence. Serum depletion resulted in fusion of the myoblasts into myotubes (Figure 12, panel B). The arrow in this panel indicates a normal C2C12 myotube. Note that C2C12 myotubes are larger than G8 myotubes (compare figure 11, panel B to Figure 12, panel B).

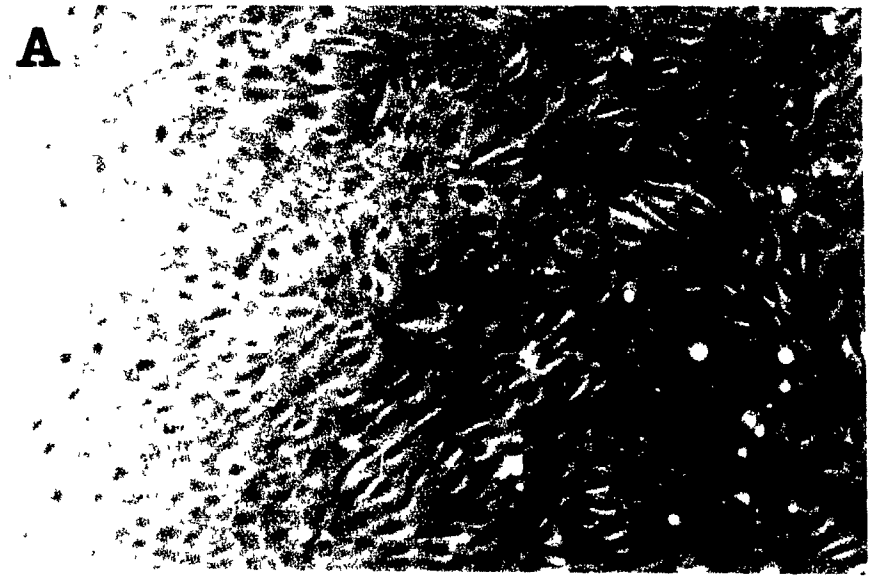
Treatment with TGFß and DMSO had similar effects on C2C12 cells as on G7 and G8s, therefore data is not shown. Incubation of C2C12 cells with RA, however caused early induction of larger, myotubes, containing a greater number of nuclei (Figure 12, panel C). The arrow in this panel indicates a giant myotube caused by RA treatment.

Figure 12: C2C12 myoblast and myotube morphology

Panel A: A confluent culture of C2C12 myoblasts.
Magnification = 200X

Panel B: Deprivation of serum results in fusion of myoblasts into myotubes. Arrow indicates a myotube.
Magnification = 200 X

Panel C: Treatment of C2C12 myoblasts with retinoic acid results in accelerated and more pronounced myotobe formation. Arrow indicates large myotube. Tubes form two days earlier than in untreated cultures, contain greater numbers of nuclei and are larger overall (compare myotube size in panel B to panel C).
Magnification = 200X


A


B

4.2.2: Inducer effects on muscle cell transcription profile

G7 transcription profile (Table 3)

RA-treated G7 cells showed a decrease in expression of proliferation-associated genes *c-myc* (6-fold) and *c-jun*. (2-fold). Significant transcriptional changes were not observed upon DMSO-treatment of these cells. A screen of gene expression of TGFß-treated G7 cells showed that only the myf5 muscle regulatory factor (MRF) was appreciably affected, its expression decreasing 2.5-fold. The expression of another MRF, myoD was found to be unaltered. PCR analysis of myf-6 expression in RA-treated cells by Sheida Bonyadi in our laboratory found that expression was not altered in neither untreated control G7 cells, or in RA-treated G7 cells (data not shown).

G8 transcription profile (Table 4)

Transcriptionally, RA-treated G8 cells showed expression of an actin increased from undetectable in untreated G8 cells, as well as a 2.2-fold increase of c-myc expression. Upon screening the mRNA present in DMSO-treated G8 cells, it was found that actin leve s were increased from zero as was the transcription of *junD*. Gene expression of TGFß-treated G8 cells, demonstrated increased actin levels as well as both c-jun (2.75-fold) and junD (increased from 0).

C2C12 transcription profile (Table 5)

The screening procedure detected increases in the expression of p53 (4.6-fold), hsp 27, (3-fold) and erbA (6.3-fold) in RA-treated C2C12 cells. Upon exposure to DMSO C2C12 cells increased expression of p53 (24-fold), Ki-ras (22-fold), c-neu (19-fold), and hsp 27 (11-fold). Changes in gene expression of TGFß-treated C2C12 cells were unremarkable except that of v-erbA which increased 30-fold.

Table 3: Alteration of gene expression in untreated control G7 cells and G7 cells exposed to RA, DMSO, and TGFß.

The genes listed in the far left hand column showed significant alteration of expression following treatment with RA, DMSO, or TGFß. The level of expression of these genes in control untreated G7 cells are listed in the next column. The upper value represents the mean of three separate experiments and below, in parentheses is the range of expression of the three samples from lowest to highest. Levels of expression from G7 cells treated with RA, DMSO, or TGFß are organized as follows; The mean of three experiments is shown, followed in parentheses by the fold increase (indicated by an up arrow) or decrease (down arrow) from the conrol mean. The range of expression from lowest to highest is provided below the mean in parentheses. A letter "N" means that the expression did not differ significantly from the control value. A significant difference is recognized if the mean of the three treated sample falls outside the range of the control values.

Table 3: Gene expression in treated and untreated G7 myoblasts

gene	G7 mean (low → high)	G7 + RA mean (fold ↑ ↓) (low → high)	G7 + DMSO mean (fold ↑ ↓) (low → high)	G7 + TGFß mean (fold ↑ ↓) (low → high)
myoD	1.25 (0.66 → 1.59)	N	N	0.52 (2.4↓) (0.22 → 0.88)
c-myc	0.84 (0.25 → 1.44)	0.15 (6↓) (0.05 → 0.24)	N	N
c-jun	2.92 (2.4 → 3.37)	1.66 (2↓) (1.37 → 1.96)	N	N

Table 4: Alteration of gene expression in untreated control G8 cells and G8 cells exposed to RA, DMSO, and TGFß.

The table is organized as described in the legend to table 3. RA treatment of G8 cells resulted in an increase in expression of skeletal actin and *c-myc*. Treatment with DMSO resulted in elevated expression of both cardiac and skeletal actin as well as *junD*. DNA encoding both the cardiac and skeletal actin isoforms included the last exon, an area of extensively shared homology. The actin data is therefore not specific, and should be regarded as total actin. Exposure to TGFß caused an increase in actin, *c-jun* and *junD* expression

Table 4: Gene expression in treated and untreated G8 myoblasts

gene	G8 mean (low → high)	G8 + RA mean (fold ↑ ↓) (low → high)	G8 + DMSO mean (fold ↑ ↓) (low → high)	G8 + TGFß mean (fold ↑ ↓) (low → high)
c actin	0	N	0.77 (0.14 → 1.26)	0.72 (0.46 → 1.206)
sk actin	0	0.65 (0.49 → 0.78)	1.37 (1.15 → 1.6)	1.49 (1.03 → 1.85
c-myc	0.93 (0.68 → 1.11)	2.02 (2↑) (1.26 → 3.55)	N	N
c-jun	0.89 (0.87 → 1.2)	N	N	2.45 (3↑) (1.19 → 4.79)
jun D	0	N	0.52 (0.024 → 1.29)	0.15 (0 → 0.3)

Table 5: Alteration of gene expression in untreated control C2C12 cells and C2C12 cells exposed to RA, DMSO, and TGFß.

The table is organized as described in the legend to table 3. RA treatment of C2C12 cells resulted in increased expression of p53, hsp 27 and v-erbA, while decreasing expression of an actin. Exposure of C2C12 myoblasts to DMSO increased expression of Ki-ras, p53, c-neu and hsp 27. TGFß treatment caused increased expression of v-erbA.

Table 5: Gene expression in treated and untreated C2C12 myoblasts

gene	C2C12 mean (low → high)	C2C12 + RA mean (fold ↑ ↓) (low → high)	C2C12 + DMSO mean (fold ↑ ↓) (low → high)	C2C12 + TGFß mean (fold ↑ ↓) (low → high)
c actin	0.95 (0.28 →2.24)	0.16 (6↓) (0 → 0.31)	N	N
Ki-ras	0.11 (0 → 0.44)	N	2.41 (22↑) (2.3 → 2.5)	N
p53	0.16 (0 → 0.31)	0.74 (5↑) (0.39 → 1.1)	3.88 (24↑) (1.1 → 6.66)	N
c-neu	0.2 (0 → 0.33)	N	3.92 (19↑) (1.1 → 6.82)	N
hsp 27	0.48 (0.02 →1.16)	1.41 (3↑) (1.13 → 1.67)	5.54 (11↑) (1.69 → 9.34	N
v-erbA	0.14 (0 → 0.55)	0.89 (6↑) (0.69 → 1.08)	N	4.25 (30↑) (1.03 → 9.44)

4.2.3; Effects on gene expression in G7, G8 and C2C12 myoblast cell lines arranged by inducer

The data may also be grouped by inducer to analyze the overall effect of each chemical on the myoblast lines. Gene expression altered in all treated samples, and two out of three treated samples have been included. These data are compiled in Tables 6 - 8.

Effects of TGFß on gene expression (Table 6)

Changes in gene expression mediated by TGFß was analyzed in triplicate, or in some cases, duplicate samples. The average level of expression of a gene was calculated from three similarly treated samples. If this average fell outside the range of the 3 untreated controls, it was considered significant in 3/3 samples as indicated by the superscript a. These cases included actin in G8 cells, and verbA in C2C12 cells.

In some cases, only two of the three TGFß-treated samples fell beyond the range of control sample values. This less stringent selection indicated alteration on gene expression of *c-jun, junD,* and *c-neu* in TGF-treated G8 cells, *myoD, EGR-1,* and *c-neu* in G7 cells, and *c-neu, Rb, p53, hsp 27,* and *glut4* (a muscle-specific glucose transporter) in C2C12 cells, in two out of three treated samples.

Effects of DMSO on gene expression (Table 7)

Three out of three DMSO-treated G8 cell samples showed increased actin and *junD* expression, whereas two out of three showed increased *c-myc* and *c-fos* expression. Two out of three DMSO-treated G7 samples showed increased skeletal actin and EGR-1, and decreased c-neu expression. Only two samples of gene expression for c-neu, Ki-ras, p53, and hsp 27 were available in

DMSO-treated C2C12 cells, however, theses genes demonstrated increased expression in both samples. Two out of three DMSO-treated C2C12 samples showed increased skeletal actin and junD, and decreased MCK expression.

Effects of RA on gene expression (Table 8)

All samples of RA-treated G8 cells showed increased skeletal actin and *c-myc* expression. All RA-treated G7 samples demonstrated decreased *c-fos, c-myc,* and *c-jun* expression, and all RA-treated C2C12 cells showed decreased cardiac actin and ßMHC, and increased *hsp 27* and *v-erbA* expression. Two out of three RA-treated G8 samples showed increased cardiac actin, ßMHC, TGFα, and *c-myc* expression, while two out of three RA-treated G7 samples showed decreased *myf5* expression.

Table 6: Effect of TGFß in gene espression in G7, G8, and C2C12 myoblast cell lines

Genes shown to have differentiated expression are listed in the far left column. Their expression was increased (upwards pointing arrows) or decreased (downwards pointing arrow), from the normal range. Genes whose expression did not differ greatly from normal values are indicated by "N".

3/3[a]; gene expression altered in three out of three samples examined

2/3[b]; gene expression altered in two out of three samples examined

2/2[c]; gene expression altered in two out of two samples examined

Actin, c-neu, c-fos, c-jun, and junD expression increased in TGFß-treated G8 cells, while EGR-1 expression was increased in TGFß - treated G7 cells. c-neu and myoD expression decreased in the treated G7 cells. C2C12 cell showed increased hsp 27, glut 4, verbA, and c-neu expression.

Table 6: Effects of Transforming Growth Factor ß on gene expression in G7, G8, and C2C12 myoblasts

gene	G8 cells	G7 cells	C2C12 cells
pata 2	↑ (3/3)[a]	N	N
pZ19	↑ (3/3)	N	N
myoD	N	↓ (2/3)[b]	N
myf5	N	↓ (3/3)	N
c-myc	N	↓ (2/2)[c]	N
c-jun	↑ (3/3)	N	N
jun D	↑ (2/3)	N	N
EGR 1	N	↑ (2/3)	N
c-neu	↑ (2/3)	↓ (2/3)	↑ (2/3)
p53	N	N	↑ (2/3)
hsp 27	N	N	↑ (2/3)
glut 4	N	N	↑ (2/3)
verbA	N	N	↑ (3/3)

Table 7: Effect of DMSO in gene espression in G7, G8, and C2C12 myoblast cell lines

Table 7 is arranged as described in the legend of Table 6. Expression of total actin and transcription factors c-myc and c-fos was elevated in DMSO-treated G8 cells. DMSO-treated G7 cells demonstrated an increase in EGR-1 and a decrease in skeletal actin and c-neu transcription. C2C12 cells showed and increase in expression of hsp 27, Ki-ras, c-neu, junD, and skeletal actin expression upon treatment with DMSO.

Table 7: Effects of Dimethyl Sulfoxide on gene expression in G7, G8, and C2C12 myoblasts

gene	G8	G7	C2C12
pata 2	↑ (3/3)[a]	N	N
pZ19	↑ (3/3)	N	N
LK295	N	↑ (2/3)[b]	↑ (2/3)
c-myc	↑ (2/3)	N	N
c-fos	↑ (2/3)	N	N
jun D	↑ (3/3)	N	↑ (2/3)
EGR 1	N	↑ (2/3)	N
c-neu	N	↓ (2/3)	↑ (2/2)[c]
Ki-ras	N	N	↑ (2/2)
p53	N	N	↑ (2/2)
hsp 27	N	N	↑ (2/2)

Table 8: Effect of RA in gene espression in G7, G8, and C2C12 myoblast cell lines

Table 8 is arranged as described in the legend for Table 6. G8 myoblasts treated with RA demonstrated increased expression of total actin, ßMHC, TGFα, and c-myc. Treated G7 cells resulted in decreased expression of myf5, and the transcription factors c-fos, c-myc, and c-jun. RA-treated C2C12 myoblasts showed decreased expression of actin and ßMHC, and increased expression of hsp 27, and verbA.

Table 8: Effect of Retinoic Acid on gene expression in G7, G8 and C2C12 myoblasts

gene	G8	G7	C2C12
pZ19	↑ (3/3)[a]	N	N
pata 2	↑ (2/3)[b]	N	↓ (2/2)[c]
myf5	N	↓ (2/3)	N
pcc1	↑ (2/3)	N	↓ (2/2)
TGF α	↑ (2/3)	N	N
p53	↑ (2/3)	N	N
c-fos	N	↓ (2/2)	N
c-myc	↑ (3/3)	↓ (2/2)	N
c-jun	N	↓ (2/2)	N
hsp 27	N	N	↑ (2/2)
verbA	N	N	↑ (2/2)

Summary

Exposure of myoblast cells lines to RA, TGFß and DMSO did result in changes in gene expression. Morphologically, the three myoblast lines reacted similarly to the inducers, except in the case of RA. In this case, the adult-derived C2C12 myoblast line demonstrated accelerated differentiation with larger, more nucleated myotubes. G7 and G8 differentiation paths were not unusually affected by RA. A general screen of transcription revealed that the cells did not respond similarly to the inducers. G7 cells seemed to decrease transcription of certain genes while G8 and C2C12 cells responded by increasing transcription of others.

CHAPTER FIVE

DISCUSSION

The pattern of genes expressed in a tissue or cell is reflective of its developmental state and environment. For example, fetal cells, in general, express genes more associated with proliferation, and mature non-dividing cells, in general, express genes more associated with their differentiated function. Cells undergoing phenomena such as differentiation, development, or induction by a certain chemical, will alter their transcriptional profile in order to transcribe the necessary genes. In this study, the expression profile of two systems undergoing change were examined.

1) Cardiac hypertrophy in murine heart caused by expression of a PVLT transgene.
2) Embryonic and adult skeletal murine myoblasts treated with inducers of proliferation and differentiation

5.1: Gene Expression in PVLT-transgenic hearts

Expression of the PVLT transgene in mice caused a cardiac hypertrophy (Figure 4). The increase in cardiac mass was caused only by an increase in cardiomyocyte size, or hypertrophy, not an increase in cell number. Thus, even during this cardiac pathology, anti-proliferative control was maintained. As the phenotype takes several months to develop, and as hypertrophy was evident before pathological symptoms were observed, it is unlikely that PVLT alone was responsible for the hypertrophy. We hypothesized that the effect of PVLT was to affect expression of other cellular genes, possibly those responsible for growth control such as proto-oncogenes and growth factors. Findings from other hypertrophy models (reviewed in the introduction, pages 12 - 17) suggest that the pattern of gene expression in pressure overloaded heart, but not thyroid hormone-treated heart, resembled that of proliferating fetal cardiomyocytes. The reversion to a fetal type of gene expression may be necessary to allow cardiomyocytes to return to a rapid synthetic state, permitting hypertrophy. In the rat hemodynamic overload model, after 2 weeks, the hypertrophy stabilized and the expression of

some genes such as α skeletal actin, *c-fos*, and *c-myc*, were back at normal levels, suggesting that continued expression of these genes were no longer required once the heart reached its new size.

We wondered if PVLT-induced hypertrophy would be more similar to pressure overload hypertrophy, evidenced by the re-expression of proliferative genes, or thyroid hormone-induced hypertrophy, which does not involve their re-expression.

Altered gene expression in PVLT transgenic heart was classified by three hypertrophy stages, early, middle and late, with corresponding gene expression changes compiled in table 1. Early stage hypertrophy includes hearts of 200 - 350 mg weight. Minimal histological change is observed in early stage hearts. Mid-stage hypertrophy includes hearts of 350 - 500 mg weight with some enlarged cells present. Late stage hypertrophy includes hearts of 500 mg weight or greater with high numbers of enlarged cardiomyocytes.

5.1.1: Gene Expression during early stage hypertrophy

Increase in EGR-1 expression was greatest in the smallest heart, and decreased as the heart enlarged. EGR-1 (also known as Krox 24, TIS8, NGF1-A or zif-268) stimulates expression of ßMHC *in vitro* (Gupta et al., 1991). ßMHC is the fetal isoform absent in normal heart, but present in cardiac hypertrophy models. These facts argue that EGR-1 has a possible role in induction of fetal isoforms of muscle-specific proteins in the adaptive response to hypertrophy. EGR-1 expression was elevated in all stages of PVLT-induced hypertrophy suggesting that its presence is necessary not only for initiation, but also maintenance of the hypertrophy gene expression pattern.

TGFß is involved in wound repair (Sporn and Roberts, 1992) and embryonic formation of the heart (Akhurst et al., 1990).

Other studies have demonstrated a transcriptional response of TGFß to cardiac hypertrophy (Villareal and Dillman, 1992). It is found in a latent form in cardiomyocytes, in prime position to direct heart repair due to abnormal cardiac growth or tissue damage. Its expression in early stages of hypertrophy may direct activities involved in tissue remodelling.

c-myc belongs to a family of short-lived DNA-binding nuclear phospho-proteins (Meek and Street, 1992). Many cancers show evidence of genetic alteration of this locus usually leading to constitutive high levels of *c-myc* expression. *c-myc* is one of the genes to be rapidly induced upon treatment of quiescent cells with mitogens. Presumably, *c-myc* serves a function in growing cells not required by their quiescent counterparts. Over-expression of *c-myc* can replace requirement for PDGF in cell culture (Armelin et al., 1984). Thus, *c-myc* may function within a signalling pa hway, or further downstream as a transactivator of transcription.

Elevated *c-myc* expression during the early stages of hypertrophy may indicate a switch of the cardiomyocyte from quiescence in to a more dynamic state. Although usually associated with proliferation, c-myc may prepare the cardiomyocyte for increased growth.

The products of *c-fos* and *jun* genes form homodimer and heterodimer AP-1 transcriptional regulators. The presence of multiple c-jun-related genes such as junB and junD allow a variety of possible heterodimers (Abate and Curran, 1990). The three jun proteins are almost identical at the C-terminal region necessary for dimerization and DNA binding, however, their N-termini, responsible for transcriptional activation, diverge. c-jun and jun B are considered to be "immediate early" genes, activated directly following stimulation to proliferate, but junD is only weakly activated (Castellazzi et al, 1991). In resting cells, junD expression was found to be higher than c-jun or junB (Ryder et

al., 1989). *junD*, *junB*, and *c-jun* have different target genes, therefore preferential expression of one jun species over another will result in specific activation of certain genes.

Normal heart appears to express primarily *c-jun* and *junB* transcripts, with a smaller amount of *junD*, thus c-fos/c-jun and c-fos/junB heterodimers predominate compared to c-fos/junD heterodimers. PVLT-hypertrophied heart has increased mRNA levels of c-jun and junD with decreased junB in later stages. Affected hearts would then contain more c-fos/c-jun and c-fos/junD heterodimers in earlier stage hypertrophy and less than the normal numbers of c-fos/junB heterodimers in late stage hypertrophy.

junD and *c-fos* expression are altered during both early and mid stages, but are expressed at normal levels in the late stage heart. These genes are may not be required beyond the initial remodelling stage of hypertrophy.

5.1.2: Gene expression during mid-stage hypertrophy

HSP 70 and 27, IGF-1, p53 and c-neu transcripts were detected exclusively in the mid-stage heart. Heat shock genes are activated in response to stress and are thought to be involved in the repair of damaged tissue (Bienz and Pelham, 1987) Delcayre et al. (1988) noticed an increase in the expression of HSP 70 following hemodynamic overload. Heat shock promoter regions are also sensitive to c-fos and c-myc (Kingston et al.,1984), and both genes are increased in early and middle stages. The function of HSPs in the heart, however, is unknown.

c-neu and IGF-1 expression was increased in PVLT-induced cardiac hypertrophy. IGF-I (or insulin-like growth factor-1) is found in normal heart (Long et al., 1990), and showed increased expression in a model of skeletal muscle hypertrophy (Turner et al., 1988). Expression of IGF-1 is regulated by growth hormone

(Mathews et al., 1986), and GH has been shown to cause cardiac hypertrophy (Penny, et al., 1985). In the normal rat heart examined by Mathews et al., (1986), exposure to increased levels of growth hormone only minimally increased IGF-1 expression. In the PVLT-induced hypertrophy, this pathway may be activated to a greater degree due to the abnormal growth situation, or IGF-1 expression may be induced by other factors not identified by the assay.

C-Neu or erbB-2 is member of the Epidermal Growth Factor receptor family (Slamon et al., 1989). C-Neu is a transmembrane protein with an intracellular region containing a tyrosine kinase domain. Despite its similarity to the EGF receptor, it has been demonstrated that C-Neu does not bind EGF, thus appearing to be a receptor for a yet unidentified ligand (Bargmann et al., 1986). Amplification of *c-neu* has been detected in breast carcinoma and other tumors (Naber et al., 1990), and is therefore associated with abnormal growth conditions. The C-Neu receptor may be associated with the atypical growth of the cardiomyocytes.

P53 is a nuclear DNA-binding phospho-protein with anti-proliferative and transcription factor properties (Montenarh, M., 1992). Its presence in mid-sized hearts may play a role in maintaining the proliferation-arrested state of the cardiomyocytes, counteracting the activities of proliferation-associated genes found to be over-expressed during cardiac hypertrophy. Interestingly, another anti-proliferative protein Rb, does not show elevated levels of expression. Rb is either not involved in growth arrest of these cardiomyocytes, or is not present in detectable levels.

5.1.3: Gene expression during late stage hypertrophy

Expression of HSP 90, *Ki-ras*, Proliferating Cell Nuclear Antigen (PCNA) and *junB* was detected at decreased levels in late

stage hearts. TGFα showed only a very slight decrease in expression.

The decreased expression of PCNA (a gene involved in proliferation) is not surprising in late stage hypertrophied heart. A decrease in *junB* with normal levels of *c-jun* and *junD* would favor the expression of *c-jun* and *jun D* targeted genes over *jun B* targeted genes as was hypothesized in the early stage heart. Which genes are targeted by which jun species is as yet unknown.

HSP 70 and 27 were increased in the mid stage and HSP 90 is decreased in late stage, suggesting that these heat shock proteins have differing activities in stressed heart. Increased expression of genes involved in the stress response during the early stages suggest that more remodelling is occurring, with an increased need for damage repair. Later stages do not have abnormal levels of these stress proteins, suggesting that late stage hypertrophied hearts have adjusted and have a fairly stable phenotype.

5.1.4: Summary

The majority of the molecular remodelling of the PVLT-hypertropied heart takes place in the early and middle stages of phenotype development. The primary genes involved are transcription factors (EGR-1, *c-fos, c-myc, c-jun, junD*). The preponderance of altered transcription factor expression may indicate the activation of a new pattern of muscle-specific and non-muscle genes necessary to deal with the ongoing hypertrophy. Genes found to be affected during later stages are involved with repair and stress management (HSP 70, HSP 90, HSP 27, IGF-I) or are necessary to maintain the proliferation arrested state (p53). Most genes are re-expressed at normal or near-normal levels again in the late stage hypertrophied heart, indicating that the phenotype has stabilized and no longer requires the presence of transcripts involved in active tissue

remodelling. Early stage hypertrophy resembles the chronic pressure-induced hemodynamic overload model with elevated expression of proliferative genes. The similarity subsides as pathology progresses to later stages. A greater number of affected hearts must be analyzed in order to make direct conclusions about the gene expression in PVLT-induced cardiac hypertrophy. Limitations of the screening system do exist and will be discussed later in this chapter.

5.2: Effects of inducers of proliferation and differentiation RA, DMSO, and TGFß on gene expression in G7, G8 and C2C12 myoblasts

Tables 3, 4, and 5 organize data of genes with altered expression in three out of three samples from inducer-treated myoblasts. The tables are arranged by cell line and indicate fold increases or decreases of average control gene expression. Tables 6, 7, and 8 are arranged by inducer and include data of alterations in gene expression in three out of three, and two out of three treated samples. The data indicate how many samples increased or decreased gene expression compared to the control range.

5.2.1: Effects of TGFß on G7, G8 and C2C12 cells (Table 6)

Originally associated with transformation, TGFß is now known to have both positive and negative effects on cellular proliferation and differentiation. Its activity is highly cell-type specific and is modulated by other growth factors present in serum (Massague, 1990).

TGFß binds to three specific receptors at the cell membrane (>250 kDa, 85 kDa, and 65 kDa) through which it is coupled to intracellular signalling mechanisms. L6 rat myoblasts do not possess the largest (>250 kDa) TGFß receptor found on many

other cell types, and it appears that the smaller (85 and 65 kDa) receptors mediate activities caused by TGFß. Binding assays and cell counts revealed that approximately 18000 TGFß receptors were present on each L_6 myoblast (Ewton et al, 1988).

Zentella and Massague (1992) reported induction of L_6E_9 rat skeletal myoblast differentiation upon inclusion of 5 - 50 pM $TGF\beta_1$ or $TGF\beta_2$ in mitogenic media (20% fetal calf serum, 10% bovine calf serum). Myotubes appeared within three days of TGFß addition, and were maximal at 6 days. Differentiation was accompanied by an immediate decrease of *c-myc* expression, and an increase of myogenin expression and MCK activity. In this model, TGFß not only arrested proliferation, but actively induced differentiation and accumulation of muscle specific proteins.

Olson et al. (1986), studied the effect of TGFß (5 ng/ml) in low serum, fusion-promoting media on C2 skeletal myoblasts. Although proliferation was arrested, the presence of TGFß did not allow differentiation, as MCK and acetylcholine receptor expression, and myotubes were not observed. TGFß effects on the expression of genes other than MCK and actylcholine receptor were not examined.

Our studies show a slight increase in proliferation of G8, G7, and C2C12 murine myoblasts upon exposure to TGFß (10 ng/ml) in high serum media. The disparity of these three experiments reinforces the pleiotropic aspect of TGFß activity, and its sensitivity to the presence of other factors in serum.

Effects of TGFß on gene expression in G8 cells (Table 6)

Treatment of G8 myoblasts with TGFß caused elevation of expression of *c-jun* (2.75-fold), *junD* (increased from 0), and actin (increased from 0) (numerical data from Table 4), in all three samples, as well as an increase in *c-fos* and *c-neu* expression in two out of three samples.

Studies by Li et al., (1990) on the effect of TGFß (5 ng/ml) on differentiated BC_3H1 smooth muscle cells, described the induction of *jun B* and *c-jun* mRNA. TGFß treatment of differentiated cells caused an abrupt 20-fold increase of *jun B*, and a less immediate 2.5-fold increase in *c-jun* mRNA. It is important to note that Li and our experiments involve different muscle cell types (smooth vs skeletal), developmental stages (differentiated vs proliferating), and treatment times (2 hours vs 4 days). However, in both cases, *jun* species were induced, therefore, a brief aside on the characteristics of the AP-1 transcription factor is in order.

Jun-Fos heterodimers, or Jun-Jun homodimers form the AP-1 transcription factor, which binds to a responsive enhancer seququence (TRE) and regulates expression of AP-1-responsive genes. *c-jun*, *junB* and *junD* have similar dimerization and DNA-binding capabilities, but differ in their activating regions (de Groot et al., 1991). The three *jun* species are differentially regulated, have different biological activities, and dimerize in various combinations with themselves and Fos to create a variety of AP-1 dimers (Castelazzi et al.,1991). These characteristics allow for great diversity of transcriptional regulation of AP-1-responsive genes.

Quiescent fibroblasts stimulated to re-enter the cell cycle demonstrate rapid and transient activation of *c-jun* and *junB*. *junD* is only slightly activated by serum growth factors and is expressed at a higher level in quiescent 3T3 fibroblasts than in proliferating fibroblasts (Ryder et al., 1989). Thus, *junD* seems to be regulated differently than *c-jun* and *junB*. In co-transfection experiments, expression with *c-fos* allowed *c-jun* and *junD* greater transactivator activity, with *junD* showing a greater cooperativity (17-fold increased activation activity) compared to *c-jun* (only 4-fold). Since high levels of *junB* and *junD* expression are detected in non-proliferative tissues such as the brain, both factors must activate other target genes in addition to those

associated with proliferation (Hirai et al., 1989). Particular target genes for each *jun* species have not yet been identified, however, it is known that dimers containing c-Jun and JunD activate genes with single TREs in their enhancer/promoter region, while dimers containing JunB repress single, but activate genes with multiple TREs (de Groot et al., 1991).

We detected increased expression of *c-jun* and *junD* in three out of three samples, and *c-fos* in two out of three samples of TGFß-treated G8 cells. Genes containing single TREs in their enhancers should be activated, while JunB-sensitive genes controlled by multiple TREs should remain unaffected in these cells. Slight proliferation was induced, therefore *c-jun* may be activating some of its targets involved in proliferation.

Elevated total actin and *c-neu* expression was also detected in some TGFß treated G8 cells. Zentella and Massague (1992) noted expression of muscle specific proteins, but only following arrest of proliferation. In TGFß-treated G8 cells, proliferation is slightly boosted, yet we detect elevated expression of actin. Its presence in the proliferating G8 cells is not understood.

c-neu encodes a 185 kDa glycoprotein related to the epidermal growth factor receptor (EGFR), and is part of the *erbB* family of receptor tyrosine kinases. Neu oncogene activation is achieved by either a single point mutation, or gene amplification or over-expression of the *c-neu* product (McKenzie, S. J., 1991). The c-Neu tyrosine kinase may be involved in the signalling pathways activated by TGFß binding to its receptor.

Effect of TGFß on gene expression in G7 cells (Table 6)

A screen of gene expression of TGFß-treated G7 cells showed that only the *myf5* *m*uscle *r*egulatory *f*actor (MRF) was appreciably affected, its expression decreasing 2.5-fold, although still detectable (numerical data from Table 3). Of other MRFs,

myoD was found to be unaltered, and in RT-PCR assays, Sheida Bonyadi in our laboratory demonstrated that *myf6* was undetectable in both untreated, and TGFß-treated G8 cells.

Somites, and axial and forelimb muscles in the fetal mouse show *myf5* expression until approximately day 13 of gestation, when expression falls to undetectable levels (Ott et al., 1991). Conversely, *MyoD* expression is not detectable in these tissues until approximately day 11 and continues to be expressed into adulthood (Sassoon et al., 1989), therefore *myf5* and *myoD* are inversely expressed in these tissues. The *myf5* :*myoD* mRNA ratio becomes reduced upon treatment of G8 cells with TGFß, mimicking the end-gestation situation. The treated cells are maturing, but are not yet ready to exit the proliferative fetal programme which requires a further decrease of *myf5* expression to undetectable levels.

The expression of *EGR-1* is increased and that of *c-neu* decreased in two out of three experimental samples. Decrease of expression of the tyrosine kinase *c-neu* may indicate that the signalling pathway in which it functions is not necessary in TGFß-treated G7 cells. *EGR-1*, also known as *zif268*, *TIS-8*, *Krox24*, *nur77*, and *NGFI-A*, is involved in transcriptional regulation. It is also known to be a serum-inducible, early growth response gene with similar kinetics to *c-fos* (Christy and Nathans, 1989). Target genes of *EGR-1* other than cardiac MHC in rats (Gupta et al, 1991), have not been identified, but it is probable that some of its target genes are involved in the proliferative response due to *EGR-1*s inducibility by growth factors. G7 cells treated with TGFß experience a slight boost in proliferation, a phenomenon which may involve *EGR-1*-targeted, proliferation-associated genes.

Effect of TGFß on gene expression in C2C12 cells (Table 6)

C2C12 cells treated with TGFß reacted similarly to G7 and G8 cells. A slight prolongation leading to postponement of myotube formation was observed. Olson et al., (1986) also describe differentiation inhibition by TGFß, but not by prolonging proliferation. Olson et al. speculated that TGFß directly inhibited the differentiation programme of C2 skeletal myoblasts. In G7, G8, and C2C12 cells, differentiation seems to be postponed by continued proliferation, suggesting that TGFß affects differentiation both directly and indirectly.

Changes in gene expression in TGFß-treated C2C12 cells included *verbA*, which increased 30-fold, calculated from the average taken from 3 samples (numerical data from Table 5). *verbA* expression of TGFß-treated cells showed great variability, from 1.03 to 9.44 within three samples. The calculated 30-fold increase is probably an overstatement, although TGFß induced *v-erbA* expression was significantly (if not very precisely) increased above untreated C2C12 controls (values range from 0 to 0.55). *v-erbA* is a thyroid hormone receptor analog with tyrosine kinase activity. It may be involved in cell signalling pathways responding to TGFß treatment. Other genes which showed increased expression in two out of three samples include *p53*, *c-neu*, hsp 27, and glut4. p53 and hsp 27 are involved in growth and damage control, glut4 is a muscle-specific glucose transporter, and *c-neu*, a tyrosine kinase. These four genes may be involved in management of the cells reaction to the inducer.

Summary of effects of TGFß

TGFß caused slight proliferation enhancement in all three myoblast lines without evidence of differentiation. TGFß treatment caused expression of a muscle-specific gene and transcription factors in G8 cells, and expression of genes involved

in growth and damage control in C2C12 myoblasts. Transcription in G7 cells was the least affected by TGFß.

5.2.2: Effects of DMSO on G7, G8, and C2C12 cells (Table 7)

Treatment of pluripotent P19 embryonal carcinoma cells with DMSO caused differentiation towards a striated phenotype (McBurney et al., 1982). In our study, DMSO inhibited growth and differentiation of G7, G8, and C2C12 myoblasts. DMSO is lipid soluble and does not depend on receptors to enter a cell.

Effects of DMSO on gene expression in G8 cells (Table 7)

Upon screening the mRNA present in DMSO-treated G8 cells, it was found that total actin and *junD* levels were increased from undetectable in all samples (numerical data in Table 4)

As actin (a muscle-specific gene) and *junD* transcription increased together in DMSO-treated cells, it would be interesting to know which muscle genes, if any, are activated by the AP-1 transcription factor. The enhancer/promoter regions of the following genes has been analyzed; ßMHC (Shimizu et al., 1992), human cardiac actin (Sartorelli et al., 1992), myosin light chain 2 (Qasba et al.,1992), muscle creatinine kinase (Johnson et al., 1989), cardiac troponin T (Mar et al., 1988), Atrial Natriuretic Factor (Seidman et al., 1988), and skeletal actin (Lee et al., 1991), but no TRE sequences were detected. The increase in actin expression following DMSO treatment of G8 cells is probably not directly related to the concomitant increase in expression of *junD*. Muscle-specific gene expression usually follows proliferation arrest in the pathway towards myotube formation. Actin expression may be increased to indicate the step following proliferation arrest has begun. The cells were harvested four days following treatment, perhaps too prematurely to observe

myotube formation in DMSO-treated G8 cells. Untreated G8 cells were observed to fuse (although minimally) at 6 days.

Expression of *c-myc* and *c-fos* were also elevated in two out of three samples. These genes are usually elevated during response to growth stimulus and have also been implicated in apoptosis (Buttyan et al., 1988). The cell death programme can be initiated by glucocorticoid, irradiation, and withdrawal of growth factors, and can be prevented by RNA and protein synthesis inhibitors. It has been proposed that gene transcription and translation are required in this phenomenon (Colotta et al., 1992). Therefore, it is interesting to note the expression of genes involved in apoptosis in DMSO-inhibited G8 cells.

Effect of DMSO on gene expression of G7 cells (Table 7)

DMSO treatment of G7 myoblasts resulted in a increased expression of skeletal actin and EGR-1, and decreased expression of *c-neu* in two out of three samples. As gene expression was never altered in all three samples together, DMSO does not have consistant effects in the G7 myoblast experiments.

The increase in skeletal actin expression was specific, as only the 3' untranslated region of the gene was used in the assay. Detection of skeletal actin-specific expression indicates possible differentiation towards a muscle phenotype. As DMSO inhibited proliferation, skeletal actin may be the vanguard of muscle-specific genes expressed prior to and during myotube formation. The *c-neu* tyrosine kinase and *EGR-1* transcription factor may be involved in signalling pathways and transcription of genes in response to DMSO treatment. *EGR-1* has been associated with cell proliferation, therefore its expression here may indicate its involvement as an activator of other targets uninvolved in cell growth.

Effect of DMSO on gene expression in C2C12 cells (Table 7)

Interestingly, striking transcriptional changes were detected in C2C12 cells upon DMSO treatment, although no great effect on cellular morphology was observed. Screening of the transcriptional state of DMSO-treated C2C12 cells revealed an increase in expression of *p53* (24-fold), *Ki-ras* (22-fold), *c-neu* (19-fold), and hsp 27 (11.5-fold) in all samples (numerical data from Table 5). *p53* induction may be correlated with the inhibition of proliferation seen upon DMSO treatment of the C2C12 cells. Although the morphological change was much less marked upon DMSO treatment than upon RA treatment, increase in *p53* expression was much greater (24-fold after DMSO treatment compared to 4.6-fold after RA treatment). Hsp 27 may have been increased due to the environmental challenge provided by exposure to DMSO. The tyrosine kinase activity of c-neu may be required in cellular signalling during response to DMSO treatment.

The mammalian family of *ras* genes consists of 3 proto-oncogenes; *Ha-ras*, *Ki-ras*, and *Nras*. *ras* genes code for 21 - 24 kDa proteins which are associated with the inner cell membrane by the post-translational addition of palmitic acid. *ras* proto-oncogenes share considerable homology to G-proteins, and are thought to participate in cellular signal transduction pathways. (Barbacid, M, 1987). Microinjection of oncogenic Ki-Ras into rat embryo fibroblasts caused transient *c-fos* expression (Gauthier-Rouviere et al., 1990). *c-fos* expression was specifically inhibited by co-injection of serum response element (SRE) sequences known to be present in the *fos* promoter. SRE co-injection also inhibited DNA synthesis, indicating that *ras*-induced proliferation acts through SRE-regulated genes. Anti-calcium phospholipid-dependant protein kinase (PK C) antibody or a PK C inhibitor as co-injectant with Ki-Ras, also caused prevention of *c-fos* induction and DNA synthesis. Thus, the ras signalling pathway leading to *c-*

fos induction and DNA synthesis involves PK C and serum responsive factor activity.

Increased *Ki-ras* expression upon DMSO treatment of C2C12 cells may signify the involvement of a cell signalling pathway in response to DMSO. Elevated *c-fos* expression was not detected, however, a transient increase would have been missed, as the cells were harvested 4 days after treatment, long after such a response would have subsided.

Expression of *junD* was elevated in two out of three DMSO-treated C2C12 samples, suggesting that activation of genes with single TRE-containing promoters may occur. As DMSO inhibits the proliferation of C2C12 cells, it is interesting to note the expression of the quiescence-associated *junD* in preference to the growth-associated *c-jun* and *junB*.

Summary of effects of DMSO

DMSO inhibited growth and differentiation of all three myoblast lines. G8 cells demonstrated expression of muscle-specific genes and transcription factors when treated with TGFß. This suggests that exposure to TGFß and DMSO may activate similar pathways, although DMSO is lipid soluble and TGFß functions through a receptor. Cellular mechanisms shared by TGFß and DMSO must diverge at some point, as the response to the two inducers is quite different. Of the three cell lines, G7 cells, as with TGFß, again show the least transcriptional reactivity. G7 cells show gene expression changes of skeletal actin, *c-neu* and *EGR-1* in only two out of three treated samples, the rest of the genes analyzed showing similar expression to untreated G7 cells. C2C12 demonstrated activation of growth and damage control genes as well as components of signalling pathways.

5.2.3: Effects of RA on G7, G8 and C2C12 myoblasts (Table 8)

Retinoic acid (RA) is a vitamin A derivative which exhibits major effects on biological processes such as differentiation and embryonic development. RA manifests its activity through nucleic retinoic acid receptors; RAR-α, RAR-β, RAR-γ, and RXR which function as ligand-inducible transcriptional enhancer factors (Talmage and Lackey, 1992). Halevy et al., (1993), studied the effects of RA on mouse adult C2 myoblasts, the parental line of the C2C12 subclone. Addition of 10^{-8} M RA to C2 myoblasts decreased DNA synthesis and induced differentiation after 48 hours of RA exposure. Myogenin mRNA and MCK activity were elevated in the RA-treated C2 cells compared to controls, and these increases correlated with myotube formation. All three characteristics were clearly detectable at 48 hours.

10^{-7} M RA did not greatly affect cell growth or differentiation of fetally derived G7 of G8 cells. However, adult-derived C2C12 cells were induced to form myotubes within 48 hours. Induced C2C12 myotubes contained more nuclei and were larger than untreated C2C12 myotubes.

Effects of RA on gene expression in G8 cells (Table 8)

10^{-7} M RA did not effect the growth of fetally-derived G7 cells. Increased expression of total actin, as well as *c-myc* was detected (numerical data in Table 4). Increased actin expression probably reflected the uninterrupted course of events in RA-treated G8 cells following 4 days in culture. Mitogens and serum are depleted, and the cells are nearing confluency, both normal signals for proliferation arrest, and muscle-specific gene expression. Untreated control cells, however, did not demonstrate an increase in actin expression following four days of culture.

A 2.2-fold increase in expression of *c-myc* was detected in RA-treated G8 cells. Despite many studies associating the short-lived nuclear phospho-protein *c-myc* to transcription factor-like activities, its direct mechanism of action remains to be elucidated. Studies of other nuclear regulatory proteins have revealed structural homologies with *c-myc* such as the "leucine zipper", helix-loop-helix, and basic region motifs which suggest dimerization and sequence specific DNA binding capabilities. *In vitro* studies with *c-myc* fragments have demonstrated specific binding to a palindromic recognition site called the "E box" (Prendergast et al., 1991). The leucine zipper region of c-Myc, and its palindromic recognition site suggest dimerization to similar or identical proteins.

In mice, a protein termed "Myn" was found to interact with Myc, and shared the same DNA recognition site (Prendergast et al, 1991). In humans, c-Myc dimerizes with a homologous protein called "Max" and these heterodimers bind the E box motif (Blackwood and Eisenman, 1991). As with the *jun* family, target genes of c-Myc/Max have not been identified. c-Myc transcription is inducible by growth factors, yet its unknown targets make it difficult to associate c-*myc* with activation of only proliferation-related genes. Also, c-Myc and Max have different transcriptional activation activities. Over-expression of *c-myc* activates, whereas over-expression of *max* represses E box-associated transcription of a reporter gene (Kretzner et al., 1992). Therefore the ratio of Myc to Max in a cell is important in determining whether target promoters are activated or repressed.

RA treatment of G8 cells resulted in an increase of *c-myc* expression, perhaps increasing the normal Myc:Max ratio when compared to untreated cells. Activation of genes regulated by E box sequences will be favored in RA-treated G8 cells if *max* expression is not altered. *Max* expression, however, was not analyzed in this study.

Two out of three RA-treated samples showed increased expression of ßMHC, TGFα and *p53*. As G8 cells are not greatly influenced by RA, and the progression towards confluency may necessitate the expression of *p53* and its growth control functions. As G8 myoblasts approach confluency and fusion, the expression of a muscle-specific gene like MHC is expected. TGFα was originally isolated from transformed cells, and has great homology to epidermal growth factor (Lyons and Moses, 1990). Its expression may be associated with cell to cell signalling in preparation for, and during fusion.

Effects of RA on gene expression in G7 cells (Table 8)

Screening mRNA isolated from RA-treated G7 cells revealed a decrease in expression of *c-myc* and *c-jun*. These genes are usually associated with cells entering the proliferative stage of the cell cycle. Their decreased expression in RA-treated G7 cells may reflect the slight inhibition of proliferation that these cells experience. A study of proto-oncogene expression during myoblast fusion by Leibovitch et al., (1986) also described a decrease in *c-myc* expression, although *c-jun* was not included in their study. Decreased *c-myc* expression will decrease the normal c-Myc/Max ratio causing repression of E box controlled genes (if *max* expression is not altered).

Decreased *c-jun* expression will alter the balance of Jun species within RA-treated G7 cells such that more dimers containing JunD and JunB are present. A relative increase in activation of multiple-TRE, JunB-sensitive genes may occur. A study by Nicholson et al., 1990), demonstrated that RA and its associated nuclear receptor RAR, repressed transcriptional activity of the stromelysin gene, and an artificial construct, each containing TREs in their promoters. Evidence of direct physical interaction with the TRE or components of the AP-1 complex (c-Fos, c-Jun), however, was not found. As physical interaction is

lacking, it is possible that RA-RAR inhibits the expression of AP-1 dimer components, thus removing TRE activators. This theory is consistent with the RA-mediated decrease in *c-jun* expression detected in G7 cells.

myf5 expression was decreased in two out of three samples of RA-treated G7 cells. An RT-PCR assay did not detect expression of another muscle regulatory factor, *myf6*, in either G7 controls, or RA-treated G7 cells. Decrease of myf5 expression may reflect the transformed nature of these cells, however RA usually acts to re-differentiate transformed cells (Hong et al., 1990). The decrease in *myf5* expression detected in RA-treated G7 cells is not understood.

Effect of RA on gene expression in C2C12 cells (Table 8)

Radical myotube induction, similar to reports of RA treatment of C2 cells by Halevy et al., (1993), were observed in RA-treated C2C12 cells. This is consistent, as the C2 cells used in Halevy's experiments are the parent cells of the C2C12 subclone used in our studies. The mRNA screening procedure did not register striking changes in muscle-specific gene expression except a 6-fold decrease in actin expression. This is surprising, as normal myoblast fusion is usually accompanied by increased muscle-specific gene expression (Buckingham et al., 1986, Caravatti et al., 1982, Devlin and Emerson, 1978). It is possible that the screening process is not sensitive enough to register these changes. Another possibility, and a more probable one is that the variability of the control values made it difficult to determine the validity of values generated by RA-treated cells.

The screening procedure did however, detect increases in the expression of *p53* (4.6-fold), hsp 27, (3-fold) and *verbA* (6.3-fold) (numerical data from Table 5). An increased expression of the anti-proliferative factor *p53* can be correlated with the abrupt arrest of proliferation of the RA-treated C2C12 cells in

order for premature fusion to occur. Hsp 27 belongs to a larger family of "stress proteins". These proteins are induced in various combinations to respond to environmental challenges and developmental transitions (Hickey et al., 1986). Exposure to a differentiation agent such as RA could be termed an environmental challenge, or accelerated myoblast fusion into myotubes, a developmental transition. Therefore, it is not surprising that hsp 27 is induced by RA-treatment of C2C12 cells.

v-erbA is an oncogene of the avian erythroblastosis virus. It was found to have a cellular counterpart, *c-erbA*, an altered version of the thyroid hormone receptor (Damm et al., 1989). In many tissues, thyroid hormone stimulates maturation, causing differentiation of some tissues, and apoptosis of others, removing fetal characteristics in order to develop adult counterparts (Gilbert, S., ed, 1988). Maturation into myotubes may involve the expression of the thyroid hormone receptor, or its analog; *verbA*. RA is known to increase the activity or expression of elements of classical signal transduction pathways, such as protein kinase C (Plet at al., 1982), protein kinase A (Kraft and Anderson, 1983), and platelet-derived growth factor α-receptor (Wang et al., 1990). RA may regulate the thyroid hormone receptor (*v-erbA*) in a similar manner by increasing the expression of *v-erbA*, a thyroid hormone receptor analog.

Summary of effects of RA

RA prematurely induced myotube formation in the adult C2C12 myoblast population, but not in the fetally derived G7 and G8 myoblasts. Expression of transcription factors was inhibited in RA-treated G7 cells. G7 cells are transformed, and RA has been shown to reduce tumor formation by promotion of a more differentiated state. A decrease in expression of transcription factors associated with proliferation may be an RA-induced step toward diminishing the transformed phenotype, although the effect was not visible in their morphology. G8 cells showed

increased transcription of growth control and muscle-specific genes, and C2C12 cells demonstrated induction of damage control and signalling genes.

Only in the case of RA treatment did the adult skeletal muscle-derived C2C12 cells differ in morphological/developmental response to the embryonically derived G7 and G8 cells. Screening the mRNA of all cell types indicated transcriptional differences not reflected in their morphology. This suggests that fetal and adult-derived myoblasts may follow separate paths toward myotube formation.

5.2.4 Future Experiments

It would be interesting to analyze a time course of G7, G8, and C2C12 myoblasts harvested at 1, 2, 3, and 4 days following inducer treatment, perhaps revealing an interplay between signalling, transcriptional, and structural gene expression. Observation of inducer treatment on differentiated myotubes of these cell lines may prove instructive, as TGFß-treatment of differentiated muscle cells (Li et al., 1990) resulted in alteration of *junB* expression.

5.2.5 Conclusions

In general, G7 myoblasts responded to all three inducer by decreasing transcription, suggesting that all three inducers modify the G7 transformed phenotype. G8 responded to inducers by upregulating expression of transcription factors such as *c-myc*, *c-fos* and *c-jun*, while C2C12 myoblasts tended to increase components of signal reception and transduction pathways such as K*i-ras*, *c-neu* and *verbA*. These observations indicate that although G7 and G8 myoblasts physically respond similarly to the inducers, their patterns of transcription do not mimic each other. C2C12 cells, which respond strikingly to RA, has a transcriptional pattern which is dissimilar to the two fetal-derived cell lines.

Thus, the three myoblasts lines respond to the various inducers with unique trancriptional patterns.

5.3: Limitations of the screening system

The slot blot technique is a general screening procedure, designed only to give an idea as to whether a difference exists in gene expression between control and experimental systems (ie; normal vs transgenic hearts or untreated vs treated cells). A rough quantitation was obtained by standardizing gene expression to the positive control (GAPDH) on each membrane and expressing values as fold increases or decreases from values generated by untreated or unaffected cells or tissues. More precise quantitation is not possible with this method, however, it gives a general idea of whether the expression of a gene is altered in the experimental system compared to the control.

This screening indicates which genes should be analyzed in more detail with more fastidious methods. For example, screening of the transgenic hearts indicate that further analysis of EGR-1, *c-jun, junD, hsp 70, hsp 90, Ki-ras* and *PCNA* may be interesting. These genes all seem to experience a radical change in expression when compared to normal hearts. Further analysis should be carried out by northern blots or reverse transcription followed by polymerase chain reaction (RT-PCR) with gene-specific primers to confirm results obtained by the primary screening. Of the two procedures, northern blots are more reliable in terms of quantitation of the message, whereas RT-PCR is a more sensitive method of message detection.

If the untreated control values vary to a great degree, it is not possible to make direct conclusions about the experimental values. For example, three samples of RA-treated G7 cells demonstrated uniformly low values of c-fos expression (0 --> 0.063) compared to the mean of 3 samples of untreated G7 cells

(0.32). However, the large variation of *c-fos* expression in the untreated samples (0 --> 0.76) will not allow the conclusion that c-fos levels are decreased in RA-treated G7 cells. In all cases, only mean experimental values which fall outside the range of control values from the screening were considered to be significant.

Variability within sets of samples may be overcome by simply increasing the number of samples screened from a particular population, or by scrutinizing the technique with the intention of locating and minimizing possible sites of variation.

To minimize variability in the original total RNA preparation, cells should be prepared meticulously. Manipulations such as feeding, counting, and chemical treatment performed quickly and if possible always by the same person and at the same time of the day. This was performed to the best of our ability. Five micrograms of total RNA was used in the reverse transcription. It is possible that 5 ug is not a representative sample of all mRNA species expressed in a cell. Northern analysis requires between 5 to 20 ug of total RNA (Ausubel et al, 1992) for adequate detection. Amounts of RNA in these experiments fall within, if at the low end, of this spectrum. Figure 3 demonstrated that 5 ug of RNA was enough to generate sufficiently long first stranded cDNA.

The reverse transcription reaction is probably the most variant step during probe generation. The oligo-dT primer binds within the poly-A tail an unknown distance from the actual 3' coding region. Primers bound far away from the coding region risk having the reverse transcriptase fall off before reaching the appropriate region. This problem can be curtailed by using anchored primers. These primers contain a stretch of 11 thymidine residues followed by one of two other nucleotides. In this way, reverse transcription will always be initiated at the immediate 3' end of the mRNA transcript. Reverse transcriptase is also error-prone and sensitive to reaction conditions, and thus

is a very likely candidate for generating variability within the experiment. Random priming and hybridization manipulations are not likely to generate great variability. Thus, variation within the protocol may cause the wide range of expression that was observed in almost all sets of samples. It is therefore important that the mean of the combined experimental samples falls outside the range of the control values in order to recognize a significant result. Genes detected by this method to have differential expression should be followed up by more fastidious methods of confirmation and quantitation such as RT-PCR and Northern analysis.

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