Construction of Recombinant Adenoviruses Encoding Skeletal Troponin C Protein and Expression Analyses in Transduced Cardiac Myocytes

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Bismillahir - Rahmanir - Raheem

In the Name of Allah, the Most Beneficent, the Most Merciful.

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

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List of Abbreviations

β-gal -	beta-galactosidase
$[Ca^{++}]_i$ -	Intracellular Calcium
$[Na^{+}]_i$ -	Intracellular Sodium
°C	Degrees Centigrade
µg	Micrograms
^{32}P -	Radioactive Phosphorus-32 Isotope
AAV -	Adeno-associated Virus
Ad5 -	Adenovirus type5
AMP -	Adenosine Monophosphate
AP -	Alkaline Phosphatase
Ap^r -	Ampicillin Resistance Marker
ATP -	Adenosine Triphosphate
BME -	Basal Medium (Eagle)
BrdU -	Bromodeoxyuridine
BSA -	Bovine Serum Albumin
Ca^{++} -	Calcium ion
cAMP -	Cyclic Adenosine Monophosphate
Cap -	Capsid gene
CBFHH -	Calcium and Bicarbonate-Free Hanks with HEPES
cfu -	Colony Forming Unit
CIP -	Calf Intestinal Phosphatase
CMV-IE -	Cytomegalovirus Immediate Early
cpe -	Cytopathic Effect

CsCl - Cesium Chloride

cTnC - Cardiac Troponin C

dCTP - Deoxyribose Cytosine Triphosphate

DEPC - Diethylamine Pyrocarbonate

DMEM - Dulbecco's Modified Minimal Essential Medium

dNTP - Deoxyribonucleotide Triphosphate

DOTAP - (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate)

E1 - Adenovirus Early Region 1

EPO - Erythropoietin

EtBr - Ethidium Bromide

FCS - Foetal Calf Serum

FGF - fibroblast growth factor

H^+ - Hydrogen ions (protons)

H_2O_2 Hydrogen peroxide

HA - Influenza Virus Hemagglutinin Antigen

HA-tag - Influenza Virus Hemagglutin Antigen Epitope Tag

HBS - HEPES-Buffered Saline

HCA - Human Cardiac Actin

HEK - Human Embryonic Kidney

HIF-1 - Hypoxia Inducible Factor-1

HSP - Heat Shock Protein

HSV - Herpes Simplex Virus

HVJ - Hemagglutinating Virus of Japan

Ig Immunoglobulin

ITR - Inverted Terminal Repeats

K^+ - Potassium ion

LMP - Low Melting Point

LTR - Long Terminal Repeats

LTU - Late Transcription Unit

MCS - Multiple Cloning Site

MEM - Minimal Essential Medium

mg Milligrams

Mg^{++} - Magnesium ion

MHC - Myosin Heavy Chain

µl Microlitres

ml. Millilitres

MLC - Myosin Light Chain

MLC-2v - Myosin Light Chain-2 ventricular Isoform

MLP - Major Late Promoter

Mn-SOD - Manganese Superoxide Dismutase

MoMuLV - Murine Moloney Leukemia Virus

Na^{+} - Sodium ion

ORF - Open Reading Frame

PBS Phosphate-Buffered Saline

PCR - Polymerase Chain Reaction

PDE - Phosphodiesterase

pfu - Plaque Forming Units

P_i - Inorganic Phosphate

PKC - Protein Kinase C

Poly A - Polyadenylation Signal Sequence

Rep - Replication gene

RT-PCR - Reverse Transcribed, Polymerase Chain Reaction

SERCA Sarco-endoplasmic Reticulum Ca^{++} ATPase

SkAct - Skeletal α-actin

SR - Sarcoplasmic Reticulum

sTnC - Skeletal Troponin C

sTnCΔ8 - Truncated skeletal Troponin C with last 8 codons deleted

sTnCΔ12 - Truncated skeletal Troponin C with last 12 codons deleted

sTnCΔ9 - Truncated skeletal Troponin C with last 9 codons deleted

SV40 - Simian Virus 40

TBS - Tris-Buffered Saline

TCA - Trichloroacetic Acid

TnC - Troponin C

TnI - Troponin I

TnT - Troponin T

TRITC - Tetramethylrhodamine Isothiocyanate

T-Tag - SV40 Large T-antigen Epitope Tag

UTR - Untranslated Region

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ABSTRACT

In ischemia, the decreased blood flow in the ventricle creates a pathophysiological condition which often lead to a progressive contractile failure in the heart. Currently available treatment options for heart failure, comprising pharmacological and surgical interventions, are less than satisfactory and often associated with various adverse effects. Since the levels of calcium are rarely changed in ischemic myocardium, evidence now suggests the reduction in contractility is due to the decrease in responsiveness of the myofilaments to available calcium. This reduced responsiveness is thought to be a consequence of desensitization of myofilaments to calcium, caused by alterations in the intracellular conditions such as pH and metabolite accumulation.

Troponin C is a regulatory protein of the myofilament which binds to calcium to trigger the process of contraction. This protein exists in two isoforms, skeletal and cardiac, which are spatially and temporally regulated. Protein isoform substitution experiments in cardiac and skeletal muscle fibres have shown that the skeletal isoform of Troponin C is more resistant to adverse intracellular conditions such as changes in pH, than its cardiac isoform. Thus, sTnC is a good candidate for manipulation to alter the calcium sensitivity of myofilaments during ischemia.

Work in this project builds the primary stage of a long-term project, for using gene transfer method to overexpress the skeletal isoform of Troponin C in cardiomyocytes. The long-term aim is to achieve complete or partial substitution of the native cardiac isoform and study the effects on contractile force produced, in normal and ischemic cardiomyocytes, both *in vitro* and *in vivo*.

This project has involved designing, constructing and analyzing expression of adenoviral gene transfer vectors overexpressing the sTnC isoform. Several

adenoviral vectors were generated with the wild type sTnC gene under the control of muscle-specific promoters. To facilitate analysis of protein expression and its subcellular localization, the sTnC protein was tagged with epitope tags and adenovirus generated, with this gene under the control of constitutive (CMV) and cardiac-specific (HCA) promoters. Epitope-tagged adenoviruses were expressed *in vitro* using mouse fibroblast (NIH3T3) cells and analyzed by western blot analysis, showing successful constitutive expression. Recombinant adenoviruses containing epitope-tagged-sTnC under the control of the human cardiac actin promoter showed cardiac-specific expression in cultured cardiomyocytes, *in situ*, using immunocytochemistry. The constitutively-expressing sTnC adenoviral vector showed successful expression in cardiomyocytes in culture, using northern blot analysis.

A range of adenoviral vectors have been successfully generated, and constitutive and tissue-specific expression has been established for some of these vectors. Successes attained in this project have established the initial requirements to achieve the long-term goal to alter calcium sensitivity of myofilaments, by overexpression of sTnC isoform in cardiomyocytes, both *in vitro* and *in vivo*.

Chapter 1

Introduction

1.1. Heart Failure

Cardiac failure follows from an interaction of complex pathophysiological conditions, causing a progressive deterioration of pump function of the heart. The compromised contractility results in inadequate blood flow which fails to meet the body's essential metabolic needs (Hongo *et al.,* 1997). The failing myocardium shows several cellular and sub-cellular aberrations which may include abnormal levels of intracellular Ca^{++} (Lee *et al.,* 1988; Morgan *et al.,* 1990; Morgan *et al.,* 1992; Denvir *et al.,* 1995), alterations in cytoskeleton proteins (Ganote and Vander Heide, 1987), functional and quantitative decrease of β-adrenergic receptors (Bristow *et al.,* 1982), myocyte hypertrophy (Hongo *et al.,* 1997), and even myocyte death (Davies *et al.,* 1996).

The most commonly accepted cause for ventricular contractile failure is partial or complete occlusion of the coronary artery (Hongo *et al.,* 1997). Such obstructions to the myocardial blood supply may lead to reversible or irreversible loss of myocyte function (Jennings and Reimer, 1991) or even complete myocyte death (Davies *et al.,* 1996) causing a condition called *Myocardial Ischemia* (Lee and Allen, 1991; Anversa *et al.,* 1993; Davies *et al.,* 1996). However, after a brief period of obstruction, and subsequent re-establishment of blood supply to the tissue can result in reversal of the detrimental effects. This process is known as *Reperfusion*. Tissue damage or necrosis is also thought to occur as a consequence of the reperfusion process and as such is termed *Reperfusion Injury* (Engler *et al.,* 1983; Werns *et al.,* 1986; Downey, 1990).

1.1.1. Myocardial Ischemia

Ischemic heart disease is a complex clinical syndrome, resulting from shortage of blood circulation in the myocardium (Anversa *et al.,* 1993). In general, coronary artery blockage is temporary, resulting in a deficiency of oxygen supply to the cardiac myocytes, hence making the intracellular environment hypoxic. This scarcity of blood flow leads to a reversible episode

of contractile dysfunction with no cell death, termed as *Myocardial Stunning* (Braunwald and Kloner, 1982; Katz, 1992). However, if the episode of coronary artery blockage is prolonged, myocardial tissue injury may be irreversible (Opie, 1996). Such injury generally comprises an entire loss of myocyte function or even death of myocytes, either scattered "diffusely" throughout the heart wall as found in Ischemic Cardiomyopathy, or distributed segmentally as observed with Myocardial Infarction (Anversa *et al.,* 1993).

Prolonged hypoxia is seen as the principal component of irreversible ischemic injury, but the effects of hypoxia itself are not as devastating as the broader range of pathologies associated with ischemia (Katz, 1992). The most fundamental outcome of ischemia is the reduction in developed tension in ventricular muscle cells. This effect has far reaching consequences at the intra-organ level. Altered developed tension is the outcome of contributory factors which are of metabolic, mechanical and electrochemical nature (Allen and Orchard, 1987).

The metabolic changes occurring at the onset of ischemia in the myocardium. fall into two broad categories; 1) the reduction in the O_2 supply and substrate availability to the tissue and 2) the accumulation of the metabolic products (Jennings and Reimer, 1991; Braunwald and Sobel, 1992; Katz, 1992). Ischemia can occur in varying degrees of severity, ranging from slight reduction in blood flow (low-flow ischemia), to complete absence of perfusion (total ischemia where both substrate delivery to and extrusion of metabolic products from cells is greatly diminished (Jennings and Reimer, 1991).

1.1.1.1. Energy Status of Cardiomyocytes in Ischemia

With the onset of severe ischemia myocardial O_2 becomes exhausted within 8 to 10 seconds (Jennings and Reimer, 1991). ATP remains relatively constant for the first few minutes of ischemia (Allen and Orchard, 1987) while cellular creatine phosphate is depleted and aerobic glycolysis switches over to anaerobic glycolysis (Jennings and Reimer, 1991). Due to the unavailability of an exogenous glucose supply, the glycogen stores become the main substrate for glycolysis. However, aerobic glycolysis is transient and soon

ceases due to lack of ATP (Jennings and Reimer, 1991), and NAD (Katz, 1992). Lactate, an end-product of anaerobic glycolysis accumulates in the ischemic tissue in addition to certain glycolytic intermediates such as α-glycerol phoshphate (Jennings and Reimer, 1991). Other metabolites that accumulate include protons (H^+), inorganic phosphate (P_i) and potassium (Allen and Orchard, 1987; Jennings and Reimer, 1991). These metabolic changes are contributing factors in myocyte dysfunction and subsequent contractile failure (Jennings and Reimer, 1991).

1.1.1.2. Metabolite Accumulation by Heart Cells in Ischemia

The intracellular metabolism in ischemic myocytes generates several catabolic products which accumulate due to absence of wash-through by the blood supply. Many products such as protons (H^+) are directly toxic and others may contribute to increasing the osmotic load of the myocytes (Jennings *et al.,* 1986). The catabolic products of ATP produces several smaller molecules such as ADP and AMP where the latter is further degraded to adenosine (ADO) and P_i and then to Inosine (INO). ADO and INO can diffuse from the myocytes to the extracellular space. The INO is further degraded to hypoxanthine and xanthine and are retained within the ischemic tissue (Jennings and Steenbergen, Jr. 1985), and contribute to free radical generation (Chambers *et al.,* 1985). In addition, lactate is also being produced (Jennings *et al.,* 1985; Jennings *et al.,* 1986), adding to the increasing osmotic load of the myocytes. The myocytes attempt to adjust the increased osmotic load by exporting K^+ to the extracellular fluid (Kleber, 1984; Jennings and Reimer, 1991); the passive leakage of K^+ from cells is known to be a common response to osmotic swelling of myocytes and other cells (Pine *et al.,* 1982).

1.1.1.3. Intracellular Acidosis

The hydrolysis of the high-energy phosphate compounds ATP and phosphocreatine generates large quantities of inorganic phosphate (P_i), a weak acid which liberates protons (H^+) (Katz, 1992). Lactate, another weak acid, will add to the H^+ content in the ischemic myocytes. As a consequence,

protons accumulate during ischemia in the myoplasm and cause a decline in the pH. The accumulation of lactate is also considered to be a major contributor to the acidosis of myoplasm (Allen and Orchard, 1987).

1.1.1.4. Ion Balance in Cardiomyocytes

Ischemia disrupts ionic balance of the myocytes, possibly by perturbations of ion transport system in the sarcolemmal membrane (Tani, 1990). Calcium (Ca^{++}), Sodium (Na^{+}), protons (H^{+}) and potassium (K^{+}) are the major ions with the levels of each being inter-dependent, for maintaining the ionic balance needed for normal cell functions.

Disorders of ion channel regulation have far reaching consequences on the electrophysiology and contractile performance of the heart. An increase in the extracellular potassium $[K^{+}]_o$ is seen at the onset of ischemia and hypoxia due to K^{-}-efflux from the myocytes with a concomitant fall in intracellular K^{+} (Kleber, 1984). This anomaly of K^{+} levels across the membrane leads to depolarization of the cell membrane (Carmeliet, 1978), with crucial consequences on slowing down conduction of action potential and production of cardiac rhythm disorders termed as *Arrhythmias* (Allen and Orchard, 1987). Calcium accumulation, on the other hand is seen as the main cause of irreversible myocardial injury or infarction.

Alterations of intracellular sodium ($[Na^{+}]_i$) levels, indirectly effect levels intracellular calcium ($[Ca^{++}]_i$), through the action of the Na-Ca exchange mechanism present in the plasma membrane. This exchanger is potentially reversible, with the direction of flow determined by the gradient of each ion and membrane potential (Eisner and Lederer, 1985). Under normal conditions, Na^{+} entry is accompanied by H^{+} or Ca^{++} efflux from the cell (see table 1.1). While the Na^{+} can be removed by the Na^{+}/K^{+} ATPase, the Ca^{++} concentration within the cell will remain stable (Jennings and Reimer, 1991), but during Na pump inhibition (as in ischemia, usually due to low ATP), the Na^{+}-Ca^{++} exchanger may reverse, allowing Ca^{++} entry in exchange for Na^{+} efflux (Allen and Orchard, 1987). This behaviour of the Na^{+}-Ca^{++} exchanger is specially important in ischemia, when intracellular acidosis causes the Na^{+}-H^{+}

exchanger to expel excess H^+ from the cell, in exchange for extracellular Na^+, hence increasing the $[Na^+]_i$. Higher $[Na^+]_i$ levels thus produced, lead to a passive exchange of the increased intracellular Na^+ for extracellular Ca^{++} by the reversed Na^+-Ca^{++} exchanger, resulting in elevated $[Ca^{++}]_i$ (Neely and Grotyohann, 1984).

Intracellular accumulation of calcium ions (Ca^{++}) is often implicated as the primary event in irreversible myocardial injury and cell necrosis (Tani, 1990). High levels of $[Ca^{++}]_i$ are toxic for myocytes and inhibit oxidative phosphorylation. Lipolytic and proteolytic enzymes also become activated in response to high Ca^{++} and damage the myocyte membrane (Jennings and Reimer, 1991; Lee and Allen, 1991). A rapid uptake of extracellular Ca^{++} immediately after reperfusion has been observed (Lee and Allen, 1991), which may precipitate muscle damage by a variety of routes (Allen and Orchard, 1987). Ca^{++} overload is not seen in myocardium that is not irreversibly injured (Jennings *et al.,* 1985), which implies a significant role of elevated cytosolic free Ca^{++} in causing irreversible damage to the myocardium.

Reperfusion of ischemic myocardium with oxygenated blood, results in the formation of highly reactive cytotoxic free radicals which are postulated to add to the injury of myocardium (Downey, 1990). These oxygen-derived free radicals are produced by activated neutrophils that infiltrate the ischemic reperfused myocardium (Lucchesi, 1990). The oxidants produced include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl anion (Kloner *et al.,* 1989). Sources of such radicals include Hypoxanthine released from the ischemic heart, which is oxidized by xanthine oxidase upon reperfusion, producing both superoxide and hydrogen peroxide (Chambers *et al.,* 1985). Free radicals have the ability to react with and damage membrane phospholipids, proteins and nucleic acids, which cause structural changes in myocytes leading to reversible or irreversible cell damage.

1.1.1.5. Calcium Homeostasis in Ischemic Cardiomyocytes

Intracellular Ca^{++} homeostasis plays a central role in regulating excitation-contraction coupling in the vertebrate heart. Systolic contraction of the heart depends upon the availability of free cytosolic Ca^{++} to the myofilaments (Brixius *et al.,* 1997), whereas diastolic relaxation requires the $[Ca^{++}]_i$ to return to the resting levels Resting state $[Ca^{++}]_i$ is approximately 10^{-7} M which is raised momentarily to 10^{-5} M in response to excitation, allowing Ca^{++} interaction with myofilaments and triggering the contractile process (Allen and Lee, 1997). This transitory increase in $[Ca^{++}]_i$, which takes place in response to action potential, and is known as a *calcium transient*.

Several studies have reported little or no difference in levels of Ca^{++}-transients in control and failing hearts (Gwathmey *et al.,* 1987; Gwathmey *et al.,* 1990; Vahl *et al.,* 1994). However, vacillations in Ca^{++} homeostasis during ischemia, have wide ranging and sometimes detrimental effects on the contractile performance of myocardium Such abnormalities of $[Ca^{++}]_i$ handling, rather than decreased $[Ca^{++}]_i$, in acute and chronic heart failure, are suggested as the cause underlying systolic and diastolic dysfunction (Perreault *et al.,* 1990; Liao *et al.,* 1994). Moreover, measurements of $[Ca^{++}]_i$ in ischemia have clearly demonstrated that the fall in contractile force of the myocardium is not due to failure of Ca^{++} transients, but due to reduced sensitivity of the myofilament proteins to respond to Ca^{++} (Lee and Allen, 1991). Hypoxia, which is an essential component of ischemia is found to decrease the myofibrillar responsiveness to calcium in the myocardium (Hajjar and Gwathmey, 1990).

1.1.2. Myocardial Remodelling

Extended duration of ischemia can lead to myocyte cell death and formation of an infarct, which is a group of necrotic and non-contracting myocytes or regions of scar tissue. As myocytes are terminally differentiated non-dividing cells, the infarct cannot be repaired. Loss of a part of the myocardium, redistributes the load of contraction to the remaining viable myocardium (Anversa *et al.,* 1993). The myocardium adapts to the increased workload by

an increase in cell volume and altered expression of specific genes (Komuro and Yazaki, 1993). The contractile proteins myosin heavy chain and alpha-actins are selectively induced during myocardial hypertrophy. β-Myosin Heavy chain gene transcription is elevated while its alpha isoform RNA is downregulated, possibly as a means for efficient contraction. Skeletal α-actin, a developmentally regulated gene expressed during the fetal stage, is expressed again in hypertrophy in equal quantity to the cardiac α-actin isoform (Schwartz *et al.,* 1986). Growth factors (Parker *et al.,* 1992), hormones (Nadal-Ginard and Mahdavi, 1989), catecholamines (Bishopric *et al.,* 1987; Bishopric and Kedes, 1991) are seen as the stimuli responsible for initiating hypertrophy. Mechanical stress, such as stretch (Komuro and Yazaki, 1993) or pressure overload are also postulated as possible inducers of hypertrophy.

1.1.3. Drug Therapies for Failing Heart

Heart failure remains a lethal condition with treatment options which are less than satisfactory. Pharmacological therapies aimed at supporting the pump function of the heart are expected to have greater impact on treatment of heart failure (Lee and Allen, 1997). Inotropic drugs are widely used which aim at improving the contractile function of the failing heart. *Digitalis*, a cardiac glycoside, has been in used for about two centuries and works by inhibiting the Na^{+}-Ca^{++} exchange, thereby increasing intracellular Ca^{++} concentration (Webster *et al.,* 1996). Though beneficial effects have been obtained, the side-effects produced by the drug are significant. *β-adrenergic agents* and *phosphodiesterase (PDE) inhibitors* also act by indirectly increasing the $[Ca^{++}]_i$. The β-agonists act by occupying the β-adrenergic receptor causing a rise in cyclic AMP *via* the action of coupled G protein and adenyl cyclase, whereas PDE inhibitors reduce the activity of the cAMP breakdown. The cAMP causes the phosphorylation of Ca^{++}-channel proteins of the sarcolemma, causing increase Ca^{++} entry and increase sarcoplasmic reticulum loading. Thus both β-adrenergic agents and PDE cause increase in force of contraction by elevating $[Ca^{++}]_i$. In addition, higher cAMP may affect

other proteins such as Troponin I and Phospholamban, and cause acceleration in the relaxation rate of myocytes (Zhang *et al.,* 1995), which may contribute to overall efficiency of contractile function.

Elevated $[Ca^{++}]_i$ is known to cause a state of "*Calcium overload*", where increases in $[Ca^{++}]_i$ beyond a certain level causes a decline in force (Allen *et al.,* 1985), and can also cause spontaneous release of Ca^{++} from the sarcoplasmic reticulum, when its contents become excessive (Lakatta, 1992). The conventional positive inotropes which act by elevating $[Ca^{++}]_i$, are likely to cause reduced contractility, aftercontraction, and also trigger arrhythmias due to sudden elevation of $[Ca^{++}]_i$ (Lee and Allen, 1997). An excess of arrhythmias is a serious problem associated with most positive inotropic agents used to date (Allen and Lee, 1997). In addition, elevated intracellular $[Ca^{++}]$ may result in unwanted consumption of energy, as Ca^{++} recycling uses more energy to bring Ca^{++} levels down, by SR-uptake and expulsion from the cell. The myosin ATPase activity is of course already increased, due to increased cross-bridge cycling, consuming the larger part of the ATP store (Gibbs, 1978; Ruegg and Morano, 1989). Hence, such a strategy seems clinically ineffective for treatment of diseased myocardium which is already deficient in energy (Allen and Lee, 1997; Lee and Allen, 1997).

Pharmacological agents which can increase the calcium responsiveness of myofilaments, have recently come under focus, for use as positive inotropic drug for treatment of heart failure (Lee and Allen, 1997; Strauss *et al.,* 1997). *Calcium sensitizers* are compounds that increase the force of contraction without increasing the $[Ca^{++}]_i$ levels, by increasing the responsiveness of myofilaments to Ca^{++} (Lee and Allen, 1991; Lee and Allen, 1997). These compounds can produce a positive inotropic effect, without provoking the dangerous side-effects, associated with drugs which elevate $[Ca^{++}]_i$. Currently, a number of Ca^{++}-sensitizers are under investigation, and agents such as EMD 53998 have been found to increase calcium sensitivity by influencing the myosin-actin interaction (Lee and Allen, 1991; Haikala *et al.,* 1995). Another drug, Pimobendan achieves increased efficiency of Ca^{++}-binding by increases

the affinity of Troponin C for Ca^{++} (Lee *et al.,* 1989). In order to further elucidate the mode of action of such drugs, their interaction with the myofilament components is also under investigation.

The search for an ideal therapy for progressive heart failure is being vigorously pursued. It has become evident from the currently available treatments that an ideal treatment should be able to restore the decreased contractile function, without elevating the $[Ca^{++}]_i$. In addition, the therapeutic intervention should not impose excessive demands of energy on the ischemic myocardium, already short of energy reserves.

1.1.4. Gene expression in Ischemia

On reperfusion, the reversibly injured myocardium shows a period of contractile dysfunction termed as myocardial stunning (Braunwald and Kloner, 1982). Myocardium, which has undergone brief episodes of reversible ischemia, is found to have the ability for tolerating a subsequent, more prolonged episode of ischemia (Murry *et al.,* 1990; Jennings and Reimer, 1991). This phenomenon is called *Ischemic Preconditioning* (Murry *et al.,* 1986). The mechanism of such a protective effect is speculative. Adenosine has been linked to preconditioning possibly by opening of ATP-sensitive K^+ channel (Speechly-Dick *et al.,* 1995), or activation of protein kinase C (Ytrehus *et al.,* 1994). Investigations into such adaptive responses could offer new molecular targets for therapeutic modulation of heart failure.

Ischemic stress in myocardium induces expression of genes such as the immediate early genes *c-fos*, *c-jun*, *jun*-B and *Egr-1* (Brand *et al.,* 1992; Wechsler *et al.,* 1994). The products of these genes *trans*-activate other genes which underlie the repair process in a reversibly injured myocardium. Stress related genes, such as Heat-shock Protein (HSP) 27, 70 and 89 and heat-shock transcriptional factor 1 (HSF-1) are induced by repeated ischemia and reperfusion episodes (Wechsler *et al.,* 1994; Nishizawa *et al.,* 1996). Antioxidant genes such as Manganese-Superoxide dismutase (Mn-SOD) and peroxisomal Catalase genes are also induced in ischemia and the enzyme levels of these free radical-scavenging enzymes are raised with successive

ischemia and reperfusion episodes (Das *et al.,* 1993). β-Adrenergic receptors play an important role in myocardium stimulation by catecholamines, leading to increased contractility acting *via* the receptor-adenylate cyclase system. There is an increase in the number of β-adrenergic receptors in the plasma membrane, despite the release of large and desensitizing levels of endogenous catecholamines (Ihl-Vahl *et al.,* 1995).

Ischemia induces an intracellular environment with accumulated metabolic products, hypoxia, low glucose and altered redox status. All these factors contribute to the pathophysiology of the ischemic myocardium. Some of these components of ischemia are found to trigger transcriptional activation of genes which may play a role in aiding the failing myocardium to adapt to stressed conditions. Hypoxia is an essential component of ischemia and is known to up-regulate genes including transcription factors, metabolic enzymes and growth factors such as Erythropoietin (EPO). Expression of growth factor is stimulated in hypoxia. *Cis*-acting enhancer element in the 3′ flanking DNA sequence of the EPO gene is found to bind a *trans*-acting hypoxia-inducible factor (HIF-1) (Semenza and Wang, 1992). Several hypoxia-inducible genes are known to contain HIF-1 binding sites including glycolytic enzymes (Semenza *et al.,* 1994). HIF-1 appears to play a central role in the hypoxia signalling by activating hypoxia-responsive genes at the transcriptional level.

1.1.5. Models of Heart Disease

Even though a wealth of information exists about clinical aspects of ischemia, the molecular controls and processes and cellular responses are poorly understood. This is partly due to non-availability of an animal model of heart failure and also to the technical and ethical limitations of carrying out analyses on whole animals or humans (Webster *et al.,* 1996). Furthermore, the ischemic injury depends upon the duration and degree in reduction of coronary flow and variable restoration of cardiac function is achieved on reperfusion (Jennings and Reimer, 1991). Ischemia, being a multifactorial process, lowers the possibility of finding an animal model to mimic the

pathophysiology of human ischemia. A number of *in vivo.* and *in vitro.* models of ischemia have been developed in the three categories of chronic, transient and permanent ischemia.

1.1.5.1. Chronic Ischemia Model

This model involves reduction of blood flow to the myocardium, yet enough for sustaining the cells and keeping them viable. Clinically, such a condition may arise by partial occlusion of a coronary artery by an atherosclerotic plaque or a blood clot. Insufficient perfusion of a region of myocardium, supplied by the clogged artery, causes a deficit in meeting the metabolic demands of a contracting heart. Chronic ischemia is marked by decreased contractility due to waste metabolite build-up, and energy depletion.

Chronic myocardial infarction can be induced in rats by coronary artery ligation. It is a well-established model of chronic heart failure and compares to myocardial infarction in humans (Hongo *et al.,* 1997).

1.1.5.2. Reperfusion Model for Transient Ischemia

Temporary interruption of coronary blood flow to the myocardium followed by reperfusion exposes the myocytes to two extreme conditions of stress. First, the myocardial tissue experiences hypoxia-related stress conditions during a period of no-flow and then oxidative stress on reperfusion. Ischemia with duration of less than 15 minutes generally does not result in myocyte death or necrosis (Jennings and Reimer, 1983). Restoration of blood flow induces formation of reactive oxygen intermediates, which damages the cell components directly. In addition, the cardiac function may not return to normal for hours and the contractile function remains severely compromised during the early stages the reperfusion. The transient models of ischemia are particularly helpful for identifying the molecular processes involved in the adaptive response to stress and preconditioning (Brand *et al.,* 1992).

Rat models for transient ischemia are produced by 15 to 60 minutes of coronary artery occlusion, followed by 7 days of perfusion. No cell death is observed after 15 minutes while 60 minutes occlusion produces myocardial necrosis (Prentice *et al.,* 1996). These models have been used to assess

gene transfer by adenovirus (Leor *et al.,* 1996) and retrovirus and direct DNA injection (Prentice *et al.,* 1996).

1.1.5.3. Permanent ischemia or Infarct Model

This model simulates the cellular environment produced in the myocardium where a coronary artery branch supplying the region is occluded permanently. Such a level of occlusion approximates the clinical condition of myocardial infarction (Ytrehus and Downey, 1993). Due to the presence of collateral blood flow, different areas of the affected myocardium will be perfused to varying degrees. Tissues not accessible by the collateral flow become necrotic, while areas with low-flow become permanently ischemic. The necrotic, non-contracting myocytes affect the overall synchronised contractility of the myocardium (Ytrehus and Downey, 1993). The necrotic tissue responds differently to a signal to contract due to compromised impulse conduction, sometimes causing regions of the ventricles to contract independently of the normal conduction system. The uneven pattern of electrical excitability in the myocardium is known as *arrhythmia* (Allen and Orchard, 1987).

A number of studies on the infarct model have led to the understanding of the phenomenon of myocardial remodelling, which is an adaptive mechanism for the diseased heart to overcome stress (Parker and Schneider, 1991). The main feature of remodelling is hypertrophy, whereby the terminally differentiated cells of the myocardium increase in size to adapt to increased workload (Parker, 1993). Infarct models have contributed to the understanding of changes in pattern of gene expression which occur during cardiac remodelling (Komuro and Yazaki, 1993).

1.1.5.4. Cell Culture Model

Isolated myocytes in culture have also provided valuable insight into the cellular response of cardiac myocytes to stress conditions. Established cardiac cell lines are not available, but primary neonatal and adult myocytes have been successfully isolated, and conditions for culture of these cells have now been optimized (Simpson and Savion, 1982; Bugaisky, 1988; Lokuta *et al.,* 1994). Moreover, healthy primary cardiocytes are able to contract

spontaneously in culture, making them useful for contraction studies. Conditions such as pO_2, pH, glucose and metabolite levels can be monitored and altered to simulate an ischemic environment. Techniques such as patch clamp, and video edge detection analysis (Chen *et al.,* 1996), can be carried out on single cells. Due to versatility of this model by providing unlimited control of the environmental variables, it can be used to mimic the chronic, transient and permanent ischemia models.

Cell culture models have been widely used in studying stress responses (Webster and Bishopric, 1992; Janero *et al.,* 1993; Shyu *et al.,* 1995) ,preconditioning (Webster *et al.,* 1995), acidosis (Acosta and Li, 1980; Chen *et al.,* 1996), Ca^{++}-flux (Murphy *et al.,* 1987) and other variables which occur in cardiocytes during ischemia.

1.1.6. *Ex vivo* Models for Assessing Contractile Performance

The main parameter of interest in ischemia and heart failure is contractility. Isolated papillary muscles, trabeculae and myocardial muscle fibres are extensively used for investigating force generation and shortening produced in cardiac muscle, under a given set of conditions. The isolated tissue is set up in an apparatus, integrating a force transducer, to measure force production with the muscle fibre immersed in a small bath containing buffers (Strauss *et al.,* 1997). Full heart preparations are also used in some cases to study outcomes of inotropic intervention produced by drugs or diseased conditions of myocardium (Edes *et al.,* 1995; Sia *et al.,* 1997).

1.1.6.1. Skinned Muscle Fibres

A useful model in contractile force measurement is the demembranated or "skinned" preparation of cardiac muscle. Detergents, such as Triton X-100, are used to dissolve the membrane, leaving the contractile apparatus functionally intact. Selective detergents such as saponin are also used for partial permeabilization, when required. Skinned fibres are bathed in buffers which mimic the intracellular environment where conditions of pH, ATP, and ion concentrations can be varied as desired. These models have been used for studying sarcoplasmic reticulum and myofilament function (Palmer and Kentish,

1994; Denvir *et al.,* 1995; Fuchs and Wang, 1996), and effects of varying intracellular conditions (Solaro *et al.,* 1988; Brixius *et al.,* 1997) and drugs on contractility. Several calcium sensitizing compounds have been evaluated using the skinned fibre system (Edes *et al.,* 1995; Haikala *et al.,* 1995).

Much of the work contributing to the understanding of muscle contraction has been carried out on skinned fibres. However, these preparations are unusable with drugs acting *via*membrane receptors or in experiments involving membrane depolarization due to the non-existence of the membrane.

1.1.6.2. Isolated Papillary Muscle and Trabeculae

Ventricular papillary muscle and specialized fibres known as trabeculae are also used in contractility studies of the heart. These muscle fibres are isolated from diseased or normal myocardium and immersed in appropriate buffers. Parameters such as myofilament Ca^{++}-sensitivity (Lab *et al.,* 1984; Akella *et al.,* 1997), Ca^{++}-flux (Lee and Allen, 1992), acidosis (Solaro *et al.,* 1988) or the effect of drugs (Lee and Allen, 1991; Edes *et al.,* 1995) are studied under varying intracellular conditions by altering buffer components, which can simulate conditions of ischemia and/or reperfusion (Lee and Allen, 1992).

Analyses on trabeculae and papillary muscle include force measurements using a force transducer, and may involve electrical stimulation. Ca^{++} flux studies are carried out by injecting the muscle with fluorescent indicators such as aequorin (Lee and Allen, 1992).

1.1.7. Transgenic Models

Various models for simulating hypertrophy and myocardial ischemia have been developed using transgenic technology, and characterized for phenotype. Transgenic mice overexpressing single genes involved in cardiac physiological and mechanical functions have also been developed. Transgenics expressing contractile proteins such as skeletal Troponin C (Metzger *et al.,* 1993), β-tropomyosin (Mariappan *et al.,* 1995), and membrane associated proteins like A1 adenosine receptor (Matherne *et al.,* 1997) and Na^{+}-Ca^{++} exchanger (Adachi-Akahane *et al.,* 1997), in heart have been developed and effects of these genes in normal and ischemic conditions

studied. In addition, "knock out" mice with a specific gene "silenced", such as for the SR regulatory protein phospholamban have been constructed (Grupp *et al.,* 1995) and outcome of absence or down-regulation of this protein studied. Cardiac physiology of such transgenic mice can be studied *in vivo.* using miniaturized techniques on whole hearts or *in vitro.* using single isolated cells. These studies allow dissection of interactions between various factors which may lead to heart disease, and provide the insight for development of novel therapeutic strategies (Christensen *et al.,* 1997).

1.2. Ultrastructure and Physiology of Contractile Myocytes in the Heart

The process of contraction in skeletal and cardiac muscle is carried out by muscle cells called *myocytes*. Myocytes are specialized cells with a sophisticated and powerful intracellular apparatus made up of several structural proteins. Under the light microscope, both types of muscle show striations or banded appearance which is a demonstration of the highly organized contractile protein machinery accommodated intracellularly.

In heart, the contractile proteins form a major part of cardiomyocytes. The cardiac myocytes comprise only one-third in number of all cells present, but occupy 75% of the volume of myocardium. Ventricular myocytes are organized into groups called *myofibers*, held together by collagen connective tissue. The individual cardiomyocytes are roughly cylindrical in shape with the atrial myocytes being smaller than ventricular myocytes. The larger ventricular myocytes measure about 17-26 microns in diameter with length measuring between 60 to 140 microns. Both atrial and ventricular myocytes are branched and attached to adjacent cells in a end-to-end manner (see fig. 1.2.1).

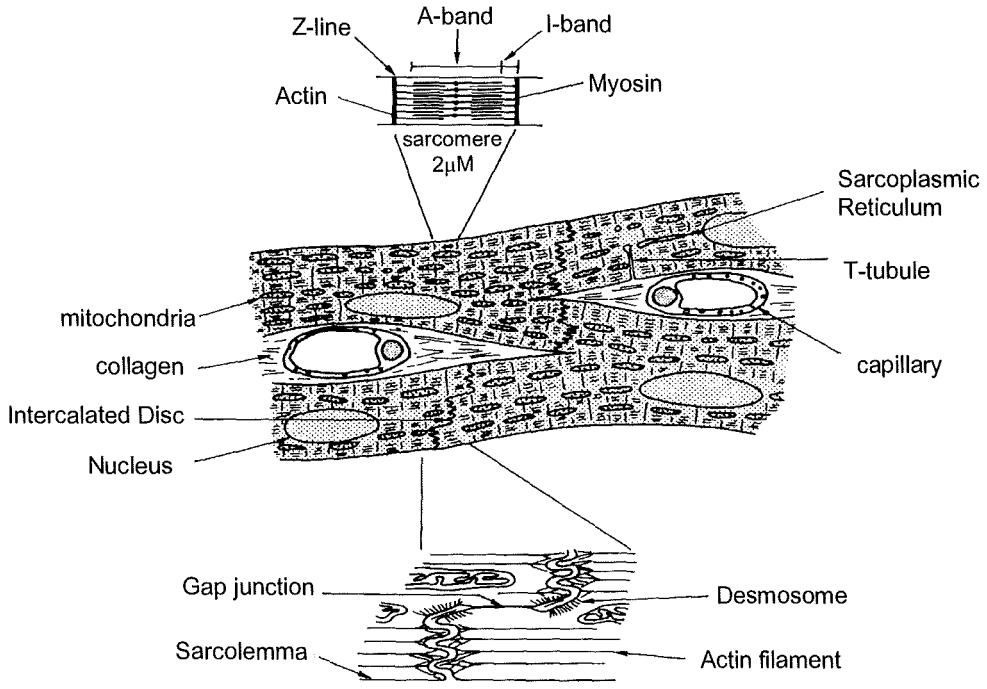


Fig 1.2.1: Illustration of a section of myocardium, based on electron microscopic studies. Organization of sarcomeres in the myocytes, and gap junctions between the latter are magnified to show details of the structure (see text). (J.R. Levick; An Introduction to Cardiovascular Physiology, 2nd ed., Butterworth-Heinemann, UK.

Each cardiomyocyte is bounded by a cell membrane called the *sarcolemma* constituting a bilipid layer and several ion channels. Myocytes comprise cylindrical structures called *myofibrils* which constitute the elementary contractile components (see fig. 1.2.2). Myofibrils are generally 1 micron in diameter, often spanning the whole length of the myocyte, made up of repeating structural units called the *sarcomere*. Sarcomeres are the basic functional unit of contractility comprising a hexagonally arranged lattice of interlocked thick and thin filaments (see figs 1.2.2 and 1.2.3).

The sarcolemma of myocytes invaginates into the cell forming an extensive tubular network known as *Transverse tubules* (or *T-tubules*), which is seen as an extension of extracellular space into the cell interior. The nucleus is often centrally located with some myocytes having more than one nuclei. Mitochondria are interspersed between the myofibrils and mainly located immediately beneath the sarcolemma, provide the ATP needed for contractile functions and maintenance of ion gradients. Of the other sub-cellular organelles, the *Sarcoplasmic Reticulum* (*SR*) stands out in importance for its role in Ca^{++} handling during the contraction cycle (see section 1.1.1.5).

The SR is a network of tubules and flattened vesicles spreading throughout the myocytes, interspersed among the myofibrils. Parts of the SR lie very close to the T-tubules where it expands into bulbous sacs, often lying along the inner surface of the sarcolemma or wrapped around the T-tubules. Such regions of the SR are called *terminal cisternae*, and contain the calcium release channels also referred to as *Ryanodine receptors*. The longitudinal or network SR is made up of branching tubules and performs the function of calcium uptake into the SR by an ATP-requiring Ca^{++} pump, called *Sarcoendoplasmic Reticulum* Ca^{++}*-ATPase* (*SERCA*). Calcium taken up by the latter is stored in the SR at high concentrations and released when required *via* the ryanodine receptors.

1.2.1. Contractile Apparatus

The principal role of cardiomyocytes lies in the contraction-relaxation cycle, which brings about the unified contraction of the ventricle muscles.

Contraction of myocytes is brought about by the shortening of its sarcomeres, the basic contractile unit. Major molecules involved in the process are the thin (actin) filaments and the thick (myosin) filaments of the sarcomere.

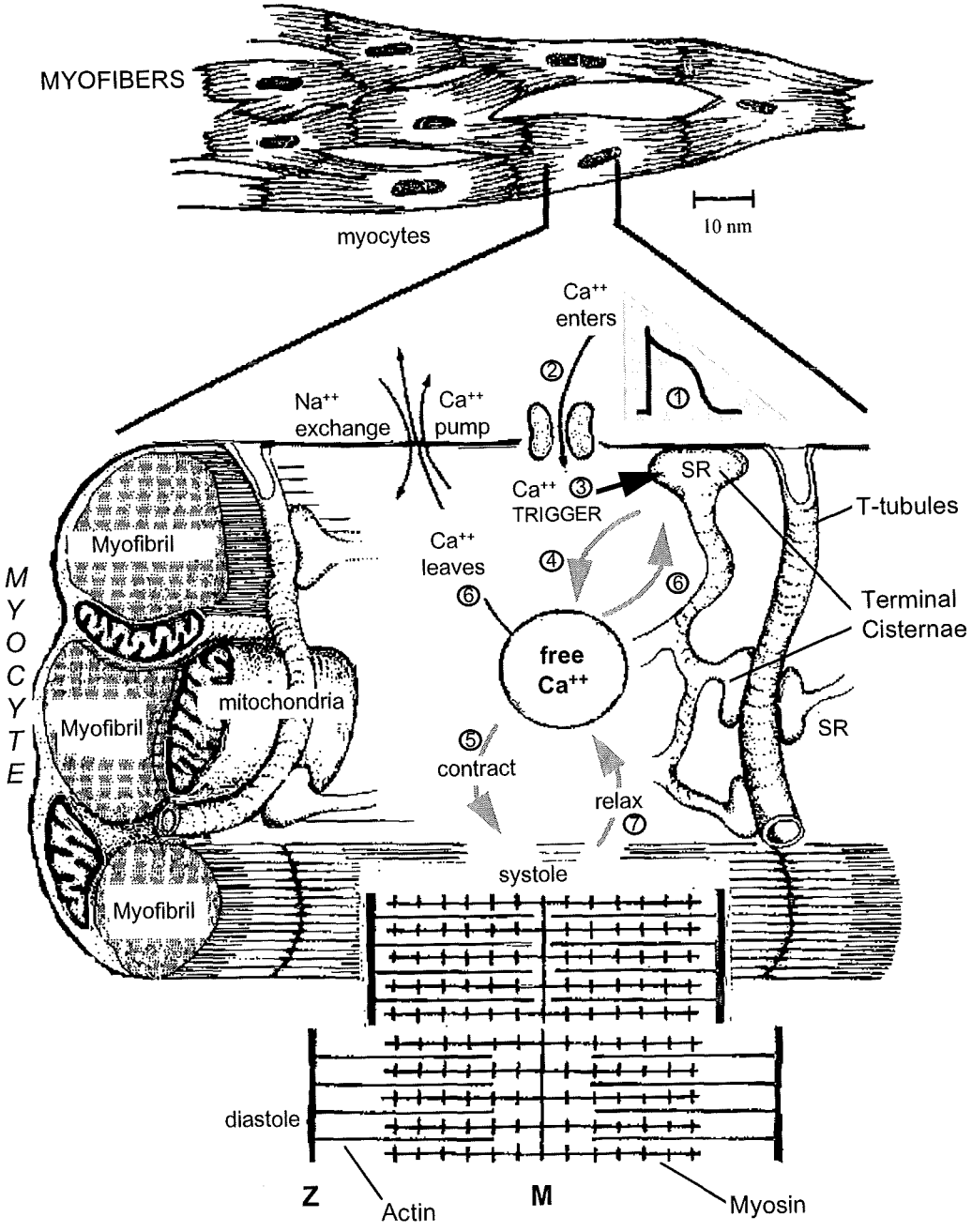


Fig 1.2.2: Illustration of organization of cardiac myofibers including subcellular structures in the cardiac myocytes, down to the level of sarcomeres. The movement of Ca^{++} in the excitation-contraction cycle, and the actin-myosin overlap during systole and diastole is also shown. Steps involved in calcium movement are numbered, and discussed in section 1.2.5.2. (modified from Opie, L.H.; Mechanics of Cardiac Contraction and Relaxation. In: Heart Disease: A Textbook of Cardiovascular Medicine, edited by Braunwald, E., :W.B. Saunders, USA, 1997).

1.2.1.1. The Sarcomere

The sarcomere is a unit of the contractile apparatus, occurring as repeating units in a myofibril. The banded appearance of the myofibres is attributed to this typical modular organization of the sarcomeres (Alberts *et al.,* 1983). Electron microscopy reveals that the sarcomere being demarcated by the *Z-line* or more appropriately the *Z-disc*, which is the anchor-site for *actin* (thin) filaments. The *myosin* (thick) filament, on the other hand, extends in either direction from the center of the sarcomere toward the Z-line, but not reaching it. The myosin and actin filaments overlap and the extent depends upon the contractile state of the myocyte. The myosin filament is however secured to the Z-line by a protein called *Titin* which itself is anchored to the Z-line. Titin, a slender myofibrillar protein, is the largest protein molecule yet described. It has two distinct segments; a non-extendible portion which interacts with myosin and an extendible region which can stretch as the sarcomere shortens (Opie, 1997). The thick and thin filaments are arranged in interlocking lattice within the sarcomere and bring about the contraction of muscle without the individual actin or myosin molecules changing in length. This is achieved by the two filaments sliding over each other, bringing the boundaries (Z-discs) of sarcomere closer. With several sarcomeres undergoing this process, the cardiomyocyte contracts.

1.2.1.2. Thick filaments and Myosin

The molecular structure of the myosin molecule is now well understood. It is composed of two heavy chains (*myosin heavy chains* or *MHC*) and two pairs of light chains (*myosin light chains* or *MLC-1* and *MLC-2*). The main body (sometimes referred to as tails) of the myosin molecule consists of two MHC's intertwined with each other into a coil, with each MHC ending in a head at the amino terminal. (Fig. 1.2.3). Light chains are associated with each of the myosin head regions which also possess ATPase activity. MHC has two isoforms, α and β, with the β isoform predominant in adult humans. Both isoforms have the same molecular weight, but differ in their ATPase activity. MLC-1, also known as *essential* MLC, appears to be involved in interaction with myosin, while MLC-2, known as the *regulatory* MLC, is a potential site for

phosphorylation, which is postulated to increase the affinity of myosin for actin.

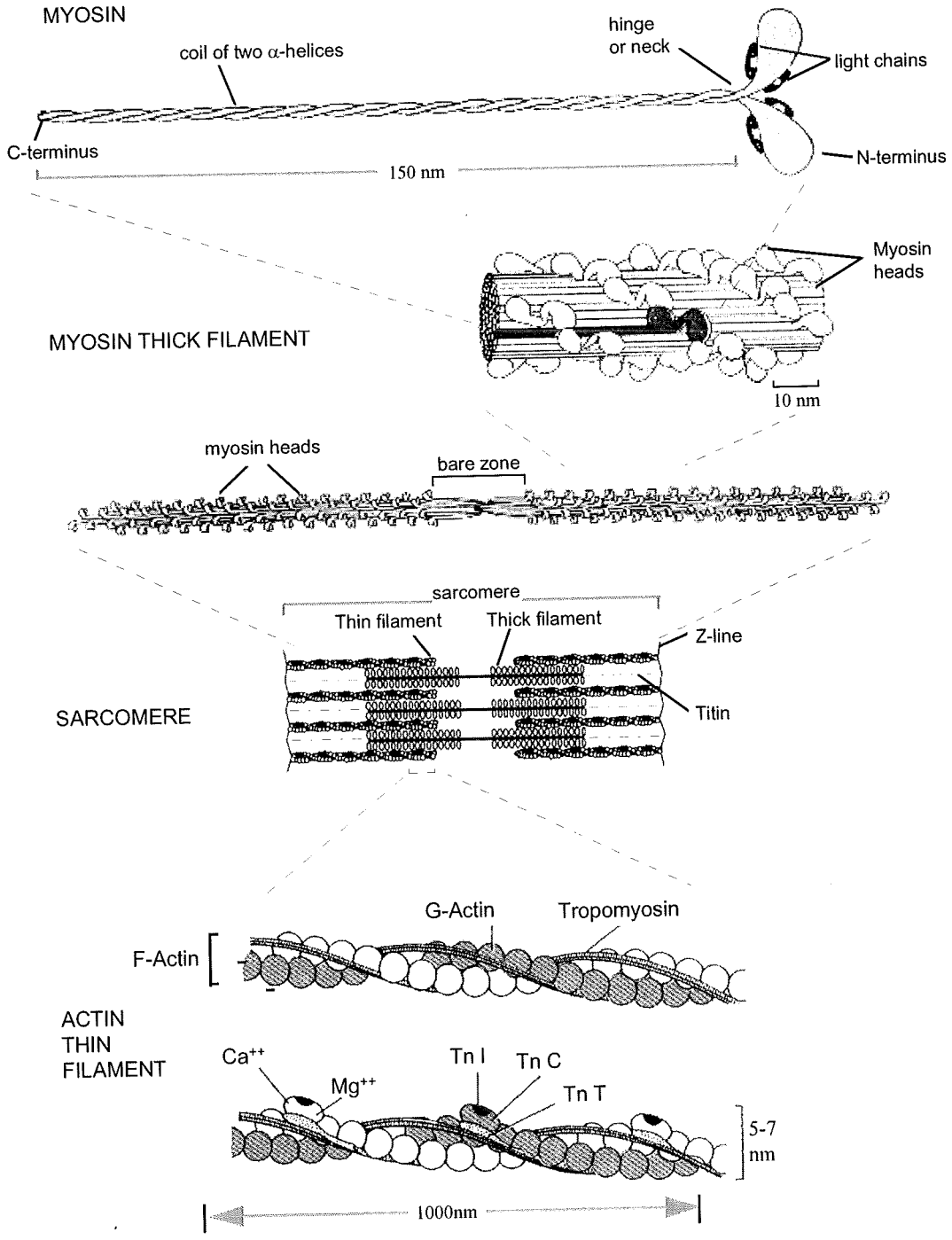


Fig 1.2.3: Ultrastructure of sarcomere showing components of myosin thick filaments and of actin thin filaments, including actin, tropomyosin and the Troponin complex. (adapted from Alberts, B., Bray, D., Lewis, J., Raff, M., Keith, R., and Watson, J.D: Molecular Biology of the Cell, Garland Publishing Inc. Conneticut, USA. 1983)

Spatially, myosin assembles a filament with the coiled tail oriented towards the center of the filament and a globular flexible head projecting to the side. The thick filament thus appears as possessing tiny side arms or oar-like "cross-bridges" extending about 13 nm to make contact with the thin filaments. The base of the head referred to as the "neck", is a region of the myosin which changes in configuration in the contractile cycle. The myosin heads have "nucleotide pockets" which bind ATP and its breakdown products, and is in close proximity of the myosin ATPase.

1.2.1.3. Thin filaments

The thin filaments are composed mainly of actin and in addition contain the regulatory apparatus for controlling the process of contraction. The thin filament regulatory proteins comprise Tropomyosin protein and the Troponin complex.

The *globular actin* (*G-actin*) monomers aggregate to form the *fibrillar actin* or *F-actin* molecule. F-actin is intertwined in a helical pattern with the *Tropomyosin*, which is a linear molecule, lying in the F-actin groove and acting as a backbone for the actin helix. The *Troponin complex* consists of three proteins with different but interactive functions, positioned at a regular interval of 38.5nm along the twisting F-actin molecule (Fig. 1.2.3). *Troponin T* mediates attachment of the whole troponin complex to the tropomyosin and actin. *Troponin I* is the inhibitory protein which inhibits interaction between actin and myosin: Under conditions of low cytosolic Ca^{++} , Troponin I positions tropomyosin on the thin filaments such that sites of actin-myosin interaction on the actin molecule are unavailable for cross-bridge formation. *Troponin C* is the calcium-binding protein which is in fact the regulatory "switch" protein in this complex: It binds calcium on its release in high amounts from the SR, changing in conformation and relieving the inhibition of myosin-actin interactions imposed by Tropomyosin and Troponin I.

1.2.2. Nature of Actin-Myosin Interaction or Cross-bridges.

Myosin heads interact with specific sites on the actin filament forming cross-bridges which have been postulated to exist in either a strong or weak binding

state. In the resting condition, the cross-bridges are attached in a weak state. This weak state of binding is due to the masking of myosin interaction sites by tropomyosin as described in the previous section. On elevation of calcium levels in the cytosol, a series of events known as "*excitation-contraction coupling*" are initiated. Calcium interacts with Troponin C, disengaging the inhibitory effect of Troponin I and Tropomyosin, and establishes a strong binding state of the cross-bridges. This binding state persists until high Ca^{++} levels are present, however, continuous presence of such a state is undesirable as no relaxation is possible. Therefore, it is proposed that ATP binding to the myosin head brings the cross-bridges into a weak binding state even at high calcium concentrations (Brenner, 1988). Hydrolysis of ATP to ADP and P_i reverses the state of strong binding. A conformational change in the myosin head also occurs at the myosin head which brings about the relative movement of the thick and thin filaments, described in fig. 1.2.4.

1.2.3. Molecular Model of Muscle Contraction

The molecular model of muscle contraction is based on A.F. Huxley's model and has been elaborated at the molecular level (Ruegg, 1990). This model is also called the *sliding filament* model, as the filaments are propelled past each other by repeated altering of cross-bridge binding states.

Muscle contraction can be seen as a repetitive process where the myosin heads act as force generating, or "stroke" producing structures, by changing conformations in between states of ATP association, breakdown by myosin-ATPase and subsequent release from the head. The model has been described in fig. 1.2.4.

Cross-bridge cycling is continuous process which should occur provided there is sufficient intracellular Ca^{++} to bind to Troponin C. Thus, at a given moment, myosin heads may be at different states of flexion. Some will be flexed and some preparing to flex, with others extending, attached to or detached from actin. Numerous cross-bridge cycles spanning only a few milliseconds, move the thin filament towards the central region of the thick filaments, resulting in a

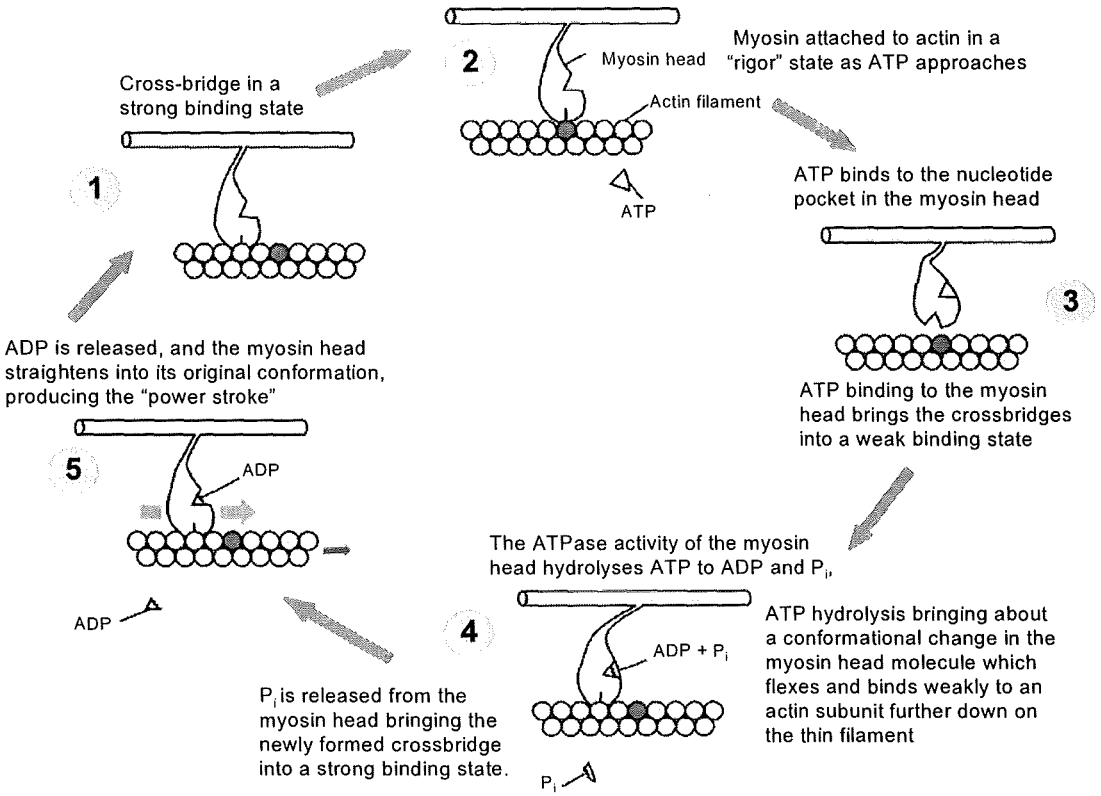


Fig 1.2.4: Schematic presentation of the cross-bridge cycle in myocytes according to A.F. Huxley's *sliding filament* model. The cyclic process involves strong and weak binding of the cross bridges in presence of high Ca^{++} concentrations. Conformational changes in the angle of myosin head bring about the "stroke" action, causing sliding of the myosin thick filaments relative to the actin thin filaments. Cross-bridge cycling proceed during the whole period of a calcium transient with several cross-bridges cycling simultaneously, causing shortening of the sarcomere. (modified from Lodish, H. *et al.*, Molecular Cell Biology, W.H. Freeman & Co. USA).

shortening of the sarcomere. The number of cross-bridges formed at a given time is important in force production by the myocytes. The sum total of all shortening sarcomeres leads to *systole*, the contraction phase of the cardiac cycle. At low $[Ca^{++}]_i$ abundance, the Troponin C is not occupied by Ca^{++} and no cross-bridge cycling occurs and the *diastole* part of the cardiac cycle begins to set in.

1.2.4. Ion Pumps of the Sarcolemma

Ion channels of the sarcolemma have a critical role in maintaining the ionic balance and consequently the electrical status of a myocyte. Ions such as K^+, Na^+ and Ca^{++} can permeate, or are gated through these channels by exchange or by ATP dependent mechanisms. These channels maintain ion

concentration differences across the cell membrane producing electrical potential differences of up to -60mV to -90mV in resting myocytes. K^+ intracellular concentrations are 35 times higher than the extracellular concentration with potassium having greater permeability than other ions. Outward diffusion of K^+ ions, down its gradient, through K^+-channels creates a separation of charge compared to the cell exterior. Ion channels maintain this resting potential and also bring the cell back to the resting potential after an action potential. The main ion pumps of the sarcolemma and their functions are shown in table 1.1.

Table 1.2.1: Main Ion Channels of Myocyte Sarcolemma

Ion Channel	Function	Activated by	ATP required	Inhibited by	overall effect on cell
Na+-K+ ATPase	3 Na^+ out 2 K^+ in	membrane potential	Yes	ouabain	depolarization; sustaining K^+ and Na^+ concentrations
Na^+-Ca^{++} exchanger	3 Na^+ in Ca^{++} out reversible	membrane potential Ca^{++} and Na^+ gradients	No		responsible for expulsion of 3/4 of the cell calcium in resting cardiomyocytes; the direction of the exchanger reverses and Ca^{++} are carried into the cells, albeit in smaller amounts, during action potential; sustains late Ca^{++} plateau
Na^+-H^+ exchanger	1 H^+ out 1 Na^+ in reversible	high extracellular Na^+ high intracellular H^+	No	amiloride and its derivatives	maintaining intracellular pH.

Levels of intracellular calcium are critical for efficient contractility of myocytes. In addition to the membrane bound ion channels of the sarcolemma, $[Ca^{++}]_i$ levels in myocytes are regulated by two main channels present in the sarcoplasmic reticulum. Calcium ions are taken up to accumulate in the SR by

the calcium-pumping ATPase, also called *SERCA*. The stored Ca^{++} is released in large amounts into the cytosolic space *via* a calcium release channel (also called the Ryanodine receptor) in an indirect response to electrical stimulus.

1.2.5. Calcium Fluxes in Contraction-Relaxation Cycle

Intracellular calcium level increase in myocytes and subsequent contraction, is associated with electrical stimulation of myocyte membrane or *excitation*, which occurs on arrival of an action potential. This phenomenon is called *excitation-contraction coupling*. In intact heart, the Ca^{++} level rises in 10 to 20 milliseconds (ms) and within 200 to 400 milliseconds, the calcium is removed from the cytoplasm (Allen and Kurihara, 1980), and maintained at the resting stage levels, until the next excitation. Thus high Ca^{++} concentration in the cytoplasm is transient and amplitude of this pulse of Ca^{++} is referred to as the *calcium transient*. Calcium transients are produced and maintained by a complex system tightly regulated by a range of membrane bound ion channels of the sarcolemma and the sarcoplasmic reticulum. Some of these ion gates are ATP-dependent or gradient-propelled while others may respond to an electrical stimulus and are known as electrogenic.

1.2.5.1. Calcium Handling

The cellular mechanisms involved in calcium ion fluxes during contraction and relaxation, and their association with wave of excitation are not fully clear. However, a working model based on Ca^{++} release from the SR has been conceptualized (Fabiato, 1983; Nabauer *et al.,* 1989). The model is based on observations that the SR releases relatively large amounts of Ca^{++} in response to a much smaller amount entering the myocyte during each cardiac cycle. The model links entry of small amounts of extracellular Ca^{++} into the cells, to the release of larger amounts of Ca^{++} by the SR into the cytosol, and is known as, the theory of *calcium-induced calcium release*. The SR Ca^{++} release is known to raise the $[Ca^{++}]_i$ up to ten-fold. Calcium levels return to normal at the termination of excitation, by uptake of excess cytosolic Ca^{++} by the sarcoplasmic reticulum.

The theory of calcium-induced calcium release is now widely accepted after characterization of the ion channels of the sarcoplasmic reticulum (SR) involved in Ca^{++} release (Opie, 1997). The myocytes have an elaborate system of Ca^{++} handling gated by sarcolemmal ion channels which maintain the normal 10,000-fold trans-sarcolemmal gradient of Ca^{++}. Within the cell, the sarcoplasmic reticulum serves as a store of Ca^{++}, which is released in response to an action potential. The SR is gated by SR-Ca^{++} ATPase, (SERCA) which during resting stage, maintains the cytosolic Ca^{++} at resting levels, by pumping any extra Ca^{++} back into the SR lumen. The total amount of Ca^{++} stored in this organelle (termed the *SR Ca^{++} load*), and the fraction of load released by Ca^{++} entry, become important determinants of the amplitude of the Ca^{++}-transient and subsequent contractions (Backx *et al.,* 1989; Mubagwa, 1995). Stored Ca^{++} is released from the Ca^{++}-release channels known as ryanodine receptors.

1.2.5.2. Calcium Release from Sarcoplasmic Reticulum

The calcium release channels of the SR is a part of a complex structure known as the ryanodine receptor. These channels lie in close anatomical proximity to the sarcolemmal Ca^{++} channels located in the invaginated sarcolemma structures, the T-tubules. Each of these sarcolemmal Ca^{++} channels can induce a number of SR calcium release channels. Calcium enters the myocyte when the voltage-sensitive calcium channel opens in response to depolarization of the T-tubule (fig. 1.2.2-(1) & (2)) (Fabiato, 1983). The calcium ions induce changes in molecular configuration of the ryanodine receptor, opening the SR-calcium release channels (fig. 1.2.2-(3)). Hence the stored SR calcium is discharged into the cytosol increasing the $[Ca^{++}]_i$ up to 10-fold (fig. 1.2.2-(4)). Free cytosolic Ca^{++} causes activation of the myofilaments in the sarcomere (fig. 1.2.2-(5)).

The rise in cytosolic Ca^{++} stops with passing of the wave of excitation. There is no entry of Ca^{++} into the myocyte or any Ca^{++} release by the sarcoplasmic reticulum at this stage and Ca^{++} concentration starts to fall to its resting level, initiating the relaxation of myocyte (fig. 1.2.2-(7)). The fall in cytosolic Ca^{++}

level is brought about by uptake and re-storing of free cytosolic Ca^{++} by the SR, and mainly by balancing of $[Ca^{++}]_i$ by the sarcolemmal ion channels such as the Na^{+}-Ca^{++} exchanger (fig. 1.2.2-(6))(Schwartz *et al.,* 1992).

1.2.5.3. Calcium Uptake and Storage into Sarcoplasmic Reticulum

Calcium ions are taken up by the SR for storage by the SR calcium-pumping ATPase or SERCA. The cardiac isoform of this pump is known as SERCA-2a (MacLennan *et al.,* 1997). For each mole of ATP hydrolysed, two Ca^{++} ions are taken up to accumulate in the sarcoplasmic reticulum. SERCA is regulated by a protein called phospholamban, which when in unphosphorylated state, inhibits the pump. High cytosolic Ca^{++} and β-adrenergic stimulation are known to phosphorylate phospholamban, activating the uptake of Ca^{++} by SERCA.

1.2.6. Myoplasmic Calcium Concentration and Force Generation

The resting or non-contracting muscles are maintained at a Ca^{++} level where no tension is generated in the myofibrils. During the excitation-contraction phase, the myoplasmic Ca^{++} concentration increases and excess Ca^{++} becomes available to bind the low affinity sites of Troponin C and triggers contraction in the myofibers. Hence, higher $[Ca^{++}]_i$ or efficient binding of Ca^{++} to TnC is seen to influence force of contraction directly. Furthermore, there is an upper limit, above which Ca^{++} concentration becomes so large that the Troponin C is saturated and unable to generate any additional force (discussed in section 1.2.6.1). However, force is known to increase beyond this Ca^{++} threshold, possibly by better thin and thick filament interactions.

A force-Ca^{++} relationship can be presented as a curve plotted between force and myoplasmic calcium concentration. This force-calcium relationship is able to reflect the nature of interactions taking place under normal physiological conditions or those resulting from intervention by inotropic agents (fig. 1.2.5). The resting $[Ca^{++}]$ is 1-2 x 10^{-7} mol/litre, lying just below the tail of the curve and activation of contraction beginning at approximately 5 x 10^{-7} mol/l and saturates between 10^{-5} mol/l and 10^{-4} mol/l of Ca^{++}, as demonstrated in

studies on skinned muscle fibres (Fabiato and Fabiato, 1978; Kentish, 1984; Pan and Solaro, 1987).

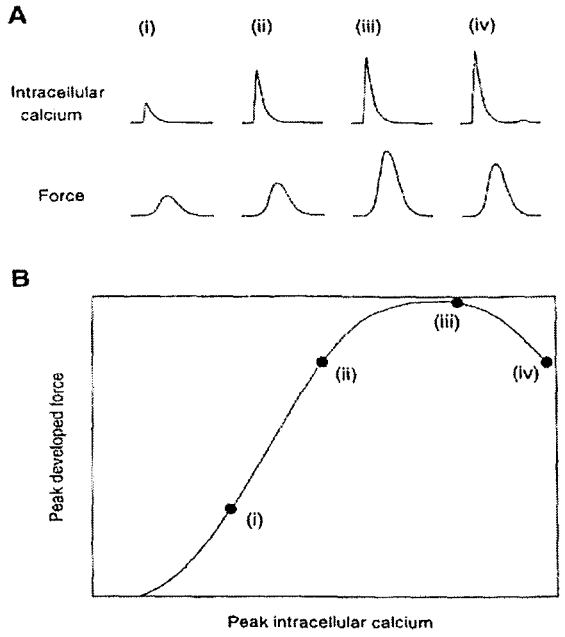


Fig 1.2.5: Relation between $[Ca^{++}]_i$ and force in intacrt heart muscle during inotropic intervention. **A**- intracellular calcium transients and the force produced in intact heart muscle at four levels of extracellular calcium. **B**- shows a typical relationship curve between peak $[Ca^{++}]_i$ and peak force. Points marked in roman numerals correspond to the muscle activation levels shown in A. Note that at the highest concentration of $[Ca^{++}]_i$, the force developed begins to decline (adapted from Lee, J.A. and Allen, D.G.; Cardiovascular Research 36:10-20, 1997).

In normal conditions, a twitch is induced well down the force-Ca^{++} relation curve, i.e. much before the point where myofilaments are saturated with Ca^{++}. An inotrope which acts by increasing contractile force by elevating the concentration of $[Ca^{++}]_i$, will produce a curve similar to shown in fig.1.2.5-B. An agent able to produce a more efficient response to a given calcium concentration by increased occupancy of Troponin C (such as calcium sensitizers), manifests itself in the force-Ca^{++} curve as shown in fig. 1.2.7(b), showing a parallel leftward shift of the entire force-Ca^{++} relationship curve. Similarly, an increase in force produced at the threshold where Ca^{++} induced response saturates under normal conditions, is also known to occur. This is postulated to happen due to better actin-myosin or cross-bridge interactions and alters the force-Ca^{++} relationship as shown in fig 1.2.6 (b).

An inotropic intervention brings about certain characteristic changes in the force-Ca^{++} curve, as discussed above. Force-pCa relationship curves which help in determining the molecular mechanism or mechanisms responsible for bringing about this change in contractility. Such relationship curves are useful method of determining the mechanism of action for any positive or negative inotropic intervention.

1.2.6.1. Altering Maximum Ca^{++}-activated Force

Force generation in muscle can be manipulated by changing the maximum Ca^{++}-activated force. This concept aims at modifying the force produced per cross-bridge, the number of active (force-generating) cross-bridges under a given condition, or the number of cross-bridges employed to produce a "contractile

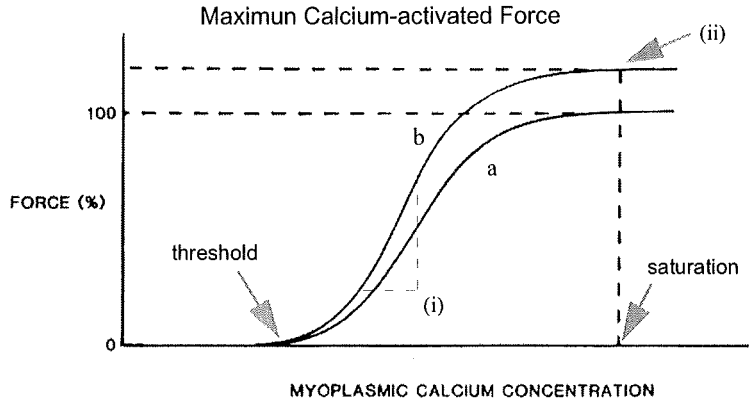


Fig 1.2.6: Schematic presentation of effects of an inotropic intervention causing an ideal increase in maximun calcium-activated force in myocytes (b). An increase in the slope of the force-pCa curve (i), and an increase in the force produced at the highest level of intracellular calcium , presented by dotted lines (ii), is seen in (b) due to the effect of the inotrope, as compared to the normal force-pCa curve (a). Force generation threshold Ca^{++} and saturation Ca^{++} levels are same in both (a) and (b). (modified from Allen, D.G. & Lee, J.A.; 1997, Modulation of Cardiac Calcium Sensitivity: A New Approach to Increasing the Strength of the Heart: Oxford University Press, NY.)

stroke". Such variables can be altered to produce greater force at a given Ca^{++} concentration by increasing the overall proficiency of myosin head–actin cross-bridge formation and interaction.

In conditions where the contractile force is altered purely by increasing the maximum Ca^{++}-activated force, the force-Ca^{++} relation curve is altered as shown in figure 1.2.6. It can be seen from the curve that the force is increased

beyond the maximum, at Ca^{++} concentration where the force response saturates under normal conditions. Thus a greater force of contraction is achieved at the same Ca^{++} concentration due to increased efficiency of cross-bridge cycling in response to a calcium transient (fig.1.2.6-(ii)). The force generation $[Ca^{++}]_i$ threshold remains unchanged, and the rate of increase in force, shown by the slope of the curve is fractionally increased. In addition, the force response is seen to saturated at the same $[Ca^{++}]_i$ as in normal conditions (fig. 1.2.6).

1.2.6.2. Calcium Sensitivity

This phenomenon is based on the concept of altering force by altering Ca^{++} occupancy of low-affinity Ca^{++}-binding sites of Troponin C. At a given Ca^{++} concentration efficient Ca^{++}-binding is a key factor in force generation, should be able to produce more force.

The occupancy of the low-affinity Troponin C sites is determined by its Ca^{++} *affinity constant* which is the ratio of the association and dissociation rate constants of calcium binding. Since the association constant is high and seems to be limited only to the availability of Ca^{++}, the dissociation rate may be retarded to increase the calcium occupancy of Troponin C. In effect, the Ca^{++} stays bound to Troponin C for a longer period of time, maintaining the force of contraction.

Prolongation of the Ca^{++}-bound state should allow formation of more cross-bridges resulting in more force generation. The outcome of increased occupancy of Troponin C manifests itself in the force-Ca^{++} curve as shown in fig 1.2.7-(b)., which shows a parallel leftward shift of the entire force-Ca^{++} relationship. More force is generated at a given myoplasmic $[Ca^{++}]$, resulting in the lowering of threshold $[Ca^{++}]$ for force generation, and also the $[Ca^{++}]$ where the force response saturates (fig. 1.2.7). Several pharmacological interventions

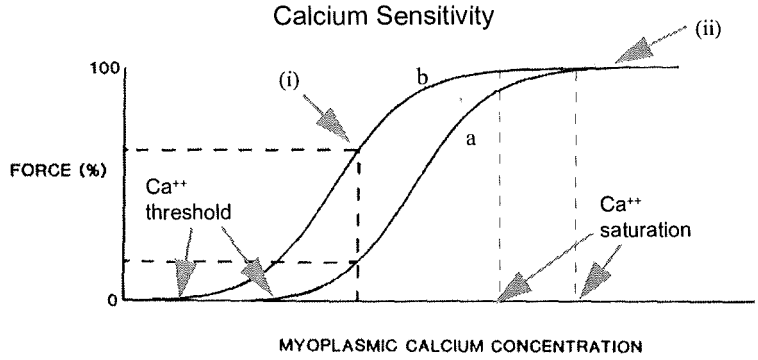


Fig 1.2.7: Schematic representation of the effects of "pure" change in calcium sensitivity of muscle fibres. A leftward shift of the force-pCa curve (b), is seen as an increase in calcium sensitivity, produced by an inotropic intervention. The arrow (i) indicates an increase in force produced in (b), compared to (a) at a given intracellular concentration of calcium. Also the maximum force (ii) is achieved at a lower calcium concentration in (a) when compared to (b). These two components form the phenomenon of calcium sensitivity. In addition, $[Ca^{++}]_i$ concentration where contraction initiates (*Ca^{++} threshold*), is lowered, and $[Ca^{++}]_i$ beyond which no further increase in force is generated (*Ca^{++} saturation*) is also decreased. (modified from Allen, D.G. & Lee, J.A.; 1997, Modulation of Cardiac Calcium Sensitivity: A New Approach to Increasing the Strength of the Heart: Oxford University Press, NY.)

are known to result in such a parallel shift of force-Ca^{++} relation and are referred as "*calcium sensitizing*" agents. The concept of increased Troponin C occupancy stands out as the most probable mechanism for calcium sensitivity alterations produced by these drugs. However, theoretically any inotropic intervention showing such a parallel shift of force-Ca^{++} relation, without any effect on maximum force produced, can be classified as an inotropic intervention affecting calcium sensitivity. This effect may involve increased calcium occupancy of Troponin C or may be due to altered molecular interactions between the various units of the troponin complex and tropomyosin.

In practice, it is difficult to categorize the response discretely as being due to modified calcium sensitivity or to maximum calcium-activated force unless a wide range of Ca^{++} concentrations are studied. It should however be clear that there are two independent ways at which force can be altered at a certain Ca^{++} concentration, which can be generally termed as myofilament calcium responsiveness. Some interventions for example Pimobendan alter force by increasing calcium sensitivity and calcium-activated force (Lee *et al.,* 1989),

while caffeine sensitizes the myofilaments but reduces the maximum Ca^{++} activated force (Wendt and Stephenson, 1983). These observations support the hypothesis of the two mechanism being independent and having the potential of being manipulated separately, provided an appropriate method is found.

1.2.7. Endogenous Calcium Sensitizing Factors of the Heart

A number of factors have been found to shift the Ca^{++}-force relation curve indicating their direct or indirect influence on myofibrillar Ca^{++} responsiveness. Change in pH, intracellular Mg^{++} or P_i concentration, ATP levels, muscle length, troponin I/myosin light chain phosphorylation, and several drugs such as caffeine are known to alter force produced in response to a given $[Ca^{++}]_i$ (Lee and Allen, 1997; Miller *et al.,* 1997).

Increased calcium sensitivity of myofilaments is known to be achieved by a number of native molecular mechanisms in the heart muscle. The Frank-Starling mechanisms stated that calcium responsiveness in cardiac muscle is dependent upon the sarcomere length and is manifested in greater force of ventricular contraction produced with increased preload (Hibberd and Jewell, 1982). On the other hand, increased P_i and H^+ during hypoxia desensitizes the myofilaments. Protein Kinase C (PK-C) and PK-A have also been implicated in influencing calcium sensitivity by phosphorylating Troponin I (TnI) and decreasing the sensitivity of the Ca^{++} by stabilizing the actin/TnI bound state. The phosphorylation of the light chain 2 of myosin (LC-2) of the thick filament is correlated with increased calcium sensitivity (Strauss *et al.,* 1997). These examples indicate the presence of a number of systems which can be modulated to increase sensitivity of the contractile apparatus to calcium. Thus therapeutic interventions can exploit these systems to ameliorate the compromised contractility in a failing heart.

1.2.8. Pharmacological Agents for Altering Mechanisms of Inotropy

Current pharmacological approaches for treatment of myocardial dysfunction mainly use inotropic agents to stimulate myocardial contractility, having a varied but limited success (Webster *et al.,* 1996). Many of these agents aim at

elevating the intracellular Ca^{++} during contraction, thereby increasing the amount of free cytosolic Ca^{++}, for the Troponin C to bind (Allen and Lee, 1997). Among the inotropic agents currently in use, are cardiac glycosides, β-adrenergic agents and phosphodiesterase (PDE) inhibitors (Lee and Allen, 1997).

Cardiac glycosides, such as digitalis, block the Na^+ pump, increasing the $[Na^+]_i$ which exchanges the high Na^+ with Ca^{++} from the extracellular space, *via* the Na^+-Ca^{++} exchanger. β-adrenergic agonists occupy the β-receptor causing an increased level of cyclic AMP through the action of G-protein and adenyl cyclase. PDE inhibitors also elevate cyclic AMP levels, and they act by reducing the rate of cAMP breakdown. Higher cAMP levels cause phosphorylation of some intracellular proteins, such as Ca^{++} channels of the myoplasmic membrane, increasing Ca^{++}-entry, and hence higher accumulation of Ca^{++}, in the sarcoplasmic reticulum (Lee and Allen, 1997).

However, these positive inotropic agents have a limited clinical value (Lee and Allen, 1991). The sarcoplasmic reticulum has a high risk of overloading if the Ca^{++} content is increased beyond a certain level. This may cause spontaneous Ca^{++} release resulting in arrhythmias(Allen and Lee, 1997). Arrhythmia is a common and serious problem with many positive inotropic drugs used to treat ischemic cardiac muscle, where the $[Ca^{++}]$ may already be at higher levels.

1.2.8.1. Calcium Sensitizing Drugs

Alternative inotropic drugs are being investigated which can increase force of contraction by mechanisms other than elevation of intracellular Ca^{++}. Increasing the sensitivity of the myofilaments towards calcium is one option whereby higher force can be achieved at a given Ca^{++} concentration. Theoretically, increased Ca^{++}-sensitivity can be brought about in at least four different ways (Lee and Allen, 1997);

i). Increasing affinity of Troponin C for Ca^{++}.

ii). Increasing the effectiveness of cross-bridge formation

iii). Increasing the rate of cross-bridge attachment or decreasing the cross-bridge detachment rate.

iv). Enhancing the myofilament cooperativity by altered feedback between attached cross-bridges and Troponin C affinity for Ca^{++}.

A newer group of inotropic agents called "Calcium Sensitizers" are in the process of development, which aim to increase the response of myofilaments to Ca^{++} and stimulate cardiac contractility in the failing heart (Lee and Allen, 1991; Edes *et al.,* 1995). These drugs have attracted interest as they are able to avoid the toxicity problems associated with Ca^{++} overload produced by agents presently used for treatment of contractile failure of the heart (Lee and Allen, 1997). Calcium sensitizers such as EMD 53998 (E. Merck-Darmstadt) have been observed to increase the sensitivity of intact ventricular muscle (Lee and Allen, 1991). Drugs have been designed to bind myofilament components such as cardiac Troponin C (Levosimendan, Orion Pharmaceuticals Inc.) which are found to increase force in skinned fiber bundles (Edes *et al.,* 1995). Several Ca^{++}-sensitizers have been found to have a phosphodiesterase inhibiting activity and may act indirectly, or have dual activity of PDE inhibition and Ca^{++}-sensitization. The molecular mechanisms involved are being investigated and agents with a more clear-cut positive inotropic effect of increasing myofibrillar sensitivity are being pursued (Lee and Allen, 1997).

1.2.9. Molecular Targets of Therapeutic Interest

Several cellular abnormalities of heart muscle are associated with contractile dysfunction. Proteins involved in these mechanisms are under investigation with a view to development of better therapeutic interventions. Most therapies are aimed at making the proteins perform better under conditions of stress and to sustain contractility, without having any adverse effect on the compromised myocardium. Several natural compensatory mechanisms triggered by the diseased myocardium, have added to the range of possible targets, which can be of therapeutic value, and may be manipulated to cure the diseased myocardium.

Most therapeutic strategies for improving ventricular systolic function have been based on inotropic agents which produce greater contractile force by raising the cytosolic Ca^{++}. Calcium is likely to already be raised in diseased muscle (Lee and Allen, 1993) and can result in adverse effects. Agents such as β-blockers and phosphodiesterase inhibitors have been found to induce fatal arrhythmias and sudden cardiac death (Lubbe *et al.,* 1992). With the discovery of the phenomenon of calcium sensitization, and with the success obtained with pharmacological calcium sensitizing drugs, the treatment of compromised contractile force without elevating the cytosolic Ca^{++} has become possible. Phosphorylation proteins such as protein kinase C (PK-C) and PK-A, thick filament myosin light chain 2 and Troponin T/Troponin I interactions, have been implicated in altering calcium sensitivity and can be targeted for modulation of the latter (Strauss *et al.,* 1997).

Troponin C is the foremost regulatory protein of the contractile system responding to inotropic stimulus of elevated calcium. Thus, this molecule was chosen for this study, as a target for therapeutic intervention for modulating calcium sensitivity of the contractile apparatus.

1.2.9.1. Troponin C

Troponin C (TnC) is a calcium-binding, 18 kilodalton (kD), acidic protein component of the regulatory Troponin complex. The protein exists in two similar but functionally distinct isoforms in the vertebrate species, namely the slow and fast TnC (Moss *et al.,* 1986). The fast TnC isoform is the exclusive isoform in adult fast-twitch skeletal muscle whereas slow TnC is expressed both in slow-twitch and cardiac muscle (Wilkinson, 1980; Gahlmann *et al.,* 1988). The fast isoform is thus referred to as *skeletal Troponin C* (*sTnC*), and the slow isoform is known commonly as the *cardiac Troponin C* (*cTnC*).

The crystal structure of Troponin C has revealed a dumbbell-shaped protein with two globular domains connected by a long central helix (Farah and Reinach, 1995), with the globular domains containing Ca^{++}-binding sites (see fig 1.2.8). The two isoforms of Troponin C have different binding affinities for

calcium. The carboxyl-terminal half of both isoforms have two high-affinity Ca^{++}-binding sites, shown as sites III and IV in fig. 1.2.8.

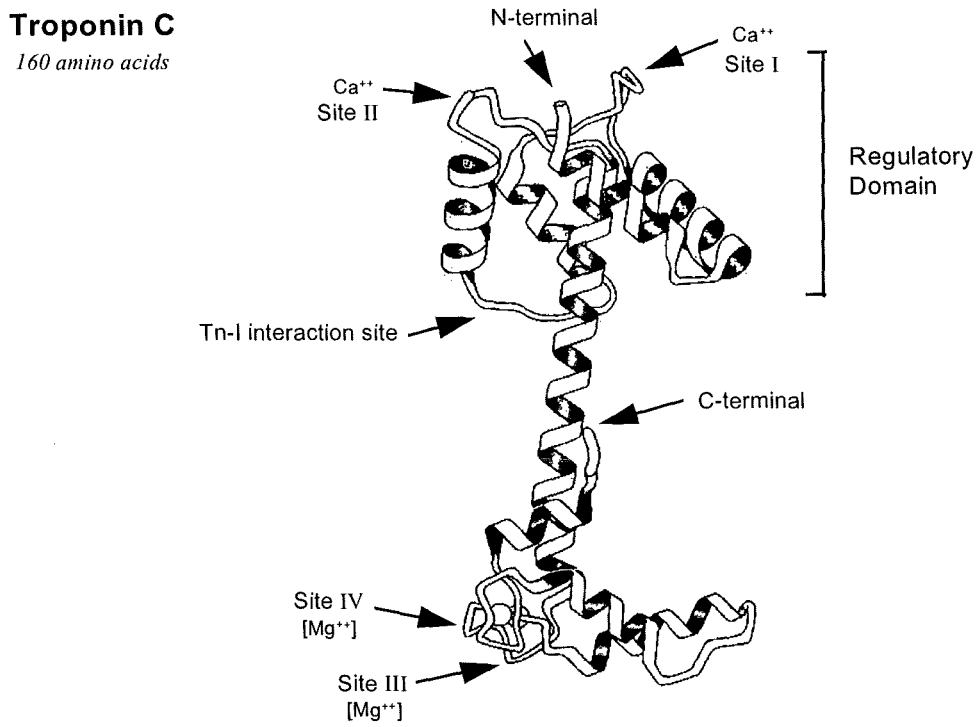


Fig 1.2.8: Structure of Troponin C protein. The protein has an Amino-end regulatory domain including the two low-affinity Ca^{++}- binding sites. The high-affinity sites at the carboxyl-end are occupied by Mg^{++} under physiological conditions. (adapted from Parmacek, M.S. and Leiden, J.M.; Structure, Function, and Regulation of Troponin C. Circulation 84(3):991-1003, 1991).

The skeletal Troponin C isoform has two additional low-affinity Ca^{++}-binding sites at the amino-terminal half of the skeletal Troponin C isoform, which are shown as sites I and II in fig. 1.2.8. The amino-terminal of the cardiac isoform has only one functional low-affinity Ca^{++}-binding site while the other site is non-functional (Gahlmann and Kedes, 1990). The four TnC Ca^{++}-binding sites are numbered I to IV according to their order in the primary protein structure. Sites I and II of the NH_2 domain bind only to Ca^{++} with a low affinity ($K=3 \times 10^5$ M^{-1}), while sites III and IV of the COOH-terminal domain bind Ca^{++} with high affinity ($K=2 \times 10^7$ M^{-1}) and a higher affinity ($K=2 \times 10^3$ M^{-1}) to Mg^{++} , and hence occupied by the latter under physiological conditions (Grabarek *et al.,* 1992; Farah and Reinach, 1995). Calcium-binding site I is non-functional in cardiac Troponin C isoform owing to some amino acid substitutions (Grabarek *et al.,* 1992).

1.2.9.2. Troponin C Isoform Substitution Studies

The functional difference between the cardiac and skeletal isoforms of Troponin C have been under investigation for some time. Selective extraction and reconstitution of protein subunits, in skinned or permeabilized muscle fibres has become an important tool for probing into the mechanism underlying the regulation of contractility (Babu *et al.,* 1988; Gulati *et al.,* 1989; Moss, 1992). This methodology has been used to exchange native protein with mutated or fluorophore-labeled proteins or myofibrillar proteins obtained in bacterial expression systems, for analyzing protein functions and interactions (Akella *et al.,* 1997; Gulati *et al.,* 1997). Most protein substitution studies have been done in skeletal muscle fibres but the technique is also workable in cardiac myocytes and myofibres. Myosin light chain 2 (MLC-2) and Troponin C have been reversibly extracted from cardiac fibres (Margossian, 1985; Gulati *et al.,* 1989; Moss, 1992). In addition, transgenic mice have been developed which express the sTnC isoform in heart and have yielded substantial information on the role of the skeletal isoform in heart (Metzger *et al.,* 1993).

The role of two Troponin C isoforms in determining the force-calcium relationship was investigated by extracting most of the native isoform of skeletal muscle fibre and reconstituting with cardiac Troponin C. Results showed an influence of the isoform of TnC present in the thin filament, on the tension-pCa relationship. The results apparently depend strongly on the number of low-affinity Ca^{++}-binding sites on TnC and their relative affinities for Ca^{++} and also affect the degree cooperativity in the thin filaments (Moss *et al.,* 1986). Similarly, a reciprocal experiment was done to study the length-dependent change in performance of myocardium, using skinned cardiac trabeculae, where the cardiac isoform of Troponin C was substituted for the skeletal isoform. The length-induced response was found to be greater in the presence of cardiac Troponin C than skeletal Troponin C indicating that cTnC may be the length sensor for the Frank-Starling mechanism (Babu *et al.,* 1988; Akella *et al.,* 1997). Such protein substitution experiments have clearly provided evidence that the fact that isoform of Troponin C present in the

myofiber, influences the form of tension-pCa^{++} relation (Moss, 1992). In another study, pH sensitivity of contraction was found to be significantly increased in skeletal muscle fibres, where the native sTnC was extracted and substituted with the cardiac isoform (Metzger and Moss, 1991). Studies on transgenic mice expressing sTnC isoform in heart, have also shown that the skeletal isoform of the protein is more resistant to pH changes (Metzger *et al.,* 1993).

1.2.10. Experimental Hypothesis: Rationale for Targeting of Troponin C

The project is designed to initiate an investigation into therapies involving gene transfer, which are aimed at sensitization of contractile filaments of a compromised myocardium, in the course of a disease such as myocardial ischemia and progressive heart failure. It is known that $[Ca^{++}]_i$ levels in ischemia are rarely altered, yet contractile response is reduced due to desensitization of myofilaments. This study forms the groundwork of a long-term project designed to investigate ways of manipulating myofibrillar proteins which are directly susceptible to desensitization during ischemia.

Troponin C is a key Ca^{++}-binding regulatory protein of the myofilament. The skeletal isoform of this protein differs from the cardiac isoform in having an extra low-affinity binding site. Both isoforms have been characterized, cloned and sequenced (Gahlmann *et al.,* 1988). It is suggested that partial or complete substitution of the cardiac (cTnC) isoform by skeletal (sTnC) isoform in cardiomyocytes, will have a positive inotropic effect on myocardium, by way of increasing the calcium sensitivity of contractions.

Troponin C was selected on the basis of results from Troponin C isoform-substitution experiments, where native sTnC isoform was substituted for cTnC in skeletal muscle fibres (Moss *et al.,* 1986) and a similar reciprocal experiment (Babu *et al.,* 1988) discussed in the previous section. In addition to possible high affinity for calcium, the sTnC isoform was shown to be less sensitive to lower pH when tested in transgenic mice expressing the skeletal isoform in the heart (Metzger *et al.,* 1993). This experiment clearly identified cTnC as an attractive target for therapies designed to treat myocardial

ischemia, where contractility is compromised due to several adverse intracellular conditions, such as acidosis.

Pharmacological calcium sensitizing agents are under investigation and are known to have a positive inotropic effect by enhancing Ca^{++}-sensitivity of myofilaments (Strauss *et al.,* 1997). Some compounds are designed to interact with myofibrillar components, such as Levosimendan, which is designed to increase the Ca^{++}-sensitivity of cardiac Troponin C by binding to N-terminal domain of the protein in a Ca^{++}-activated state (Edes *et al.,* 1995; Haikala *et al.,* 1995). In this project we use a gene transfer approach to overexpress sTnC in cardiomyocytes, using adenoviral transfer vectors. This gene transfer approach has been successful in achieving expression of the foetal isoform of Troponin I, its myofilament incorporation and subsequent enhanced contractile function in adult rat myocytes (Westfall *et al.,* 1997). Therefore our gene transfer approach may also result in partial substitution of the native cTnC by the sTnC protein and provide useful data to address the phenomenon of reduced Ca^{++}-sensitivity during ischemia.

1.3. Gene Transfer to the Myocardium

A crucial component of any gene transfer protocol is the delivery system available for transferring the desired gene. This factor becomes more important when therapeutic genes are expected to be transferred directly to a patient in a routine clinical setting. The future of gene therapy is to be determined largely by the development of effective *in vivo.* gene transfer vectors which can safely and effectively deliver genes into the human body (French, 1997).

The ideal gene transfer vector should have the ability to introduce foreign DNA into the cell, protect it from enzyme degradation while it travels through the cytoplasm to the nucleus, facilitate transport across the nuclear membrane and protect from nuclear endonucleases, eventually retaining it for a certain time period to express as required. Viruses are known to carry out these processes very efficiently and are being used widely for this purpose (Dunckley *et al.,* 1992; Lynch *et al.,* 1992; Stratford-Perricaudet *et al.,* 1992). Non-viral systems such as cationic liposomes have been effectively used (Nabel *et al.,* 1990) and new mixed systems of viral proteins and non-viral macromolecules (Aoki *et al.,* 1997) are being developed. Naked DNA has also been expressed in muscle by direct injection of plasmid DNA (Wolff *et al.,* 1990; Acsadi *et al.,* 1991; Kitsis *et al.,* 1991).

1.3.1. Non-Viral Gene Transfer Systems

Liposome-based DNA transfer systems form the major part of non-viral methods of gene transfer. These methods have been improved by coupling liposomes with viral proteins and cell surface receptors to direct the DNA to specific tissues. Direct injection of DNA has also been successful in transfer and expression of genes to muscle cells *in vivo.*

1.3.1.1. Direct DNA Injection

A super-coiled plasmid can be considered as the most elementary gene transfer vector, as it can be delivered by direct injection and subsequently expressed. Eukaryotic and reporter genes under the transcriptional control of

eukaryotic promoters have been successfully injected and expressed in skeletal muscle (Wolff *et al.,* 1990) and cardiac muscle (Acsadi *et al.,* 1991). The direct DNA injection method has been successful in myogenic tissues. The relatively efficient uptake of DNA has been attributed to the presence of the transverse tubule (T-tubule) system which is a unique characteristic of the both skeletal and cardiac myocytes (Wolff *et al.,* 1990; Kitsis *et al.,* 1991). However, this process is very inefficient as only a small fraction of the injected DNA gains entry into the cell and even less DNA survives the journey to the nucleus, where it is expressed (French, 1997). Nevertheless, plasmid DNA can be obtained in milligram amounts by amplification in *E.coli* and using routine plasmid isolation procedures (Sambrook *et al.,* 1989). Direct injection of DNA is being used widely in studying promoter function (Prentice *et al.,* 1997). This approach may have clinical applications in situations where expression of minute amounts of extremely potent growth factor is required. Isner *et al.* have successfully used plasmid DNA encoding vascular endothelial growth factor (VEGF) to stimulate collateral vessel growth in patients with peripheral vascular disease (Isner *et al.,* 1996).

1.3.1.2. Cationic Liposomes

Commercial formulations of cationic lipids and non-ionic detergents are commonly being used for transfecting plasmid DNA into cultured eukaryotic cells. Lipofectin® (Gibco BRL) was the first such reagent to be used for *in vivo.* gene transfer to vascular tissue (Nabel *et al.,* 1990). Cationic liposomes are positively charged synthetic lipid vesicles which have the ability to complex with negatively charged DNA and penetrate the cells by membrane fusion (Feldman and Steg, 1997). DNA-liposome complexes contain an excess of liposomes and hence are positively charged which facilitates their fusion with cell membrane (Felgner *et al.,* 1989). On entry into the cell, the complex is routed to the lysosomal pathway and DNA-liposome complex is degraded by the lysosomal enzymes, with only about 1% of the DNA reaching the nucleus (Feldman and Steg, 1997). The foreign DNA remains extrachromosomal and the transgene expression is therefore transient. There is no size constraint for the transgene and the DNA-liposome

complexes are prepared readily by diluting the liposomes and DNA in standard buffers and mixing in correct ratios.

Various cationic liposome preparations are available commercially. Improved formulations are being researched, and new preparations are occasionally reported to mediate more efficient *in vitro.* transfections. Transfection efficiencies are reported to be as high as 90% for *in vitro.* gene transfer but similar results have not been achieved *in vivo.* Nevertheless, efficiency of transfer is known to improve by optimization of the DNA to lipid ratio (Felgner *et al.,* 1994; Gao and Huang, 1995). Despite these deficiencies, cationic liposomes are being used extensively for *in vivo.* gene transfer and have been approved for use in gene therapy clinical trials (Nabel *et al.,* 1993; Caplen *et al.,* 1995).

1.3.1.3. Conjugated Vectors

Molecular conjugate vectors comprise a group which benefits from the natural cellular mechanism of internalization of macromolecules by receptor-mediated endocytosis (Michael and Curiel, 1994). The basic design for such vectors is attachment of plasmid DNA to macromolecule ligands which can be internalized by a certain cell type. The vector is made up of polycations, such as polylysine, which are chemically linked to a ligand. The polylysine-ligand molecule can form a complex with the negatively charged transgene DNA *via* electrostatic interactions, condensing the DNA into a compact macromolecule-like structure with the ligand presented on the surface. The ligand DNA-polylysine-ligand complex binds to a cell membrane receptor facilitating the internalization of the whole complex into the cell vesicle system.

Conjugate vectors were first developed by Wu *et al.* for gene transfer to hepatocytes using asialoglycoprotein as ligand both *in vitro.* (Wu and Wu, 1987) and *in vivo.* (Wu *et al.,* 1991). Later transferrin-polylysine vectors were also employed for hematopoietic gene transfer *in vitro.* (Wagner *et al.,* 1990; Zenke *et al.,* 1990). These vectors have virtually no DNA size limitation, and enter *via* a normal physiological pathway and hence are non-toxic to the cells (Michael and Curiel, 1994). Theoretically, *in vivo.* gene delivery can be

directed to the appropriate cell type with a varied choice of ligand domains. However, delivery of molecular conjugates, to cells with the compatible cell surface receptors, has generally resulted in poor gene expression (Zenke *et al.,* 1990; Zatloukal *et al.,* 1992). Moreover, *in vitro.* expression results have been inconsistent, possibly due to the fact that although efficient uptake of DNA is achieved with these vectors, the vector does not possess a mechanism for protection of the internalized DNA (Michael and Curiel, 1994).

1.3.1.3.i. : Conjugated Vectors Associated with Hemagglutinin Virus of Japan (HVJ)

HVJ-liposome vectors incorporate liposomes, DNA and UV-inactivated HVJ particles. The HVJ virus has a capability of fusing with the cell membrane at neutral pH by virtue of activity of its fusion protein. This property of the virus is used to introduce DNA directly into the cytoplasm avoiding lysosomal degradation. The DNA is first trapped into liposomes and then fused to the UV-inactivated HVJ to form a "fusigenic viral liposome" containing DNA, which is 500nm in diameter (Dzau *et al.,* 1996). HVJ-liposome can fuse with the plasma membrane within 30 minutes at 37°C which is a great advantage for *in vivo.* gene deliveries.

HVJ-liposomes have been used for *in vivo.* gene transfer in a number of cardiovascular disorders. Vascular proliferative disease such as restenosis have been successfully inhibited by transferring antisense (AS) oligodeoxynucleotides (ODN), specific for cell cycle regulatory genes, *via*HVJ-liposomes into balloon-injured rat carotid artery (Dzau *et al.,* 1996). Recently, Aoki *et al.* (Aoki *et al.,* 1997) established an efficient *in vivo.* reporter gene transfer into the myocardial infarction model in rats, using the HVJ-liposome.

1.3.2. Viral Vectors

Viruses have emerged as the obvious candidate for use as vectors for gene therapy because of their high efficiency of introducing and expressing their genetic material in a variety of host cells (Ali *et al.,* 1994). A number of viruses have been modified for use in gene therapy protocols. Basic recombinant modifications include deletion of portions of the genome to accommodate the

gene of interest and ensure the virus is replication defective, so as to prevent proliferation in the host cells.

1.3.2.1. Retroviruses

Most retroviral vectors are based on the murine moloney leukemia virus (MoMuLV). Retroviruses have a glycoprotein envelope with a protein core and two copies of 8.8 kb genomic RNA molecules. Upon retroviral infection into host cell, the genomic RNA is reverse transcribed by the viral reverse transcriptase into a linear double-stranded DNA. The DNA enters the nucleus and is spliced into the host genome by viral integrase and exists as a *provirus* (Nicolas and Rubenstein, 1988).

All gene therapy protocols use disabled retroviral gene transfer vectors where the viral *gag, pol* and *env* genes are replaced by the therapeutic gene of interest (Anderson, 1992). The recombinant retroviral vector is capable only of a single infectious cycle, as it lacks the indispensable viral genes. "Helper" producer cell lines are used to make and propagate these viruses, and are engineered by introducing the *essential*, (*gag, pol* and *env*) genes into immortal cell lines. The producer cell line provides all replication and assembly proteins of the virus, but have no packaging signal in the provirus, which cannot be packaged (Morgenstern and Land, 1990).

For generating a retroviral vectors, a retroviral genome, with the essential genes replaced by a therapeutic gene, but retaining the encapsidation (packaging) and replication signals, is made. This engineered genome is introduced into helper cell lines, usually as a plasmid (Miller and Rosman, 1989). Since the two retroviral genomes (provirus and plasmid) are complementary, infectious particles are produced by packaging of RNA transcribed from the engineered viral genome, carrying the therapeutic gene. Titres up to 10^8 colony forming units (cfu) of recombinant viruses can be obtained from these helper cell lines in culture (Morgan and Anderson, 1993).

Since retroviruses have the capability of integration into the host genome, persistence gene expression of the transgene is expected. Foreign genes of up to 8 kb can be accommodated in these vector and there is no inflammatory

response from the host due to absence of any viral gene products. However, retroviruses can integrate only into host cells which are capable of replication. Also persistent gene expression may not be required for many genes of therapeutic benefit. Cardiocytes are terminally differentiated cells and thus retroviruses may not be useful for these as they infect dividing cells only (Miller *et al.,* 1990). However, therapies involving other dividing cells of the heart tissue matrix may utilize retroviral vectors. For example, retrovirus may be able to express the master regulatory gene for muscle cell differentiation MyoD, in cardiac fibroblasts, converting them to myoblasts (French, 1997).

1.3.2.2. Adeno-associated Virus (AAV)

The adeno-associated viruses (AAV) are small single-stranded DNA viruses. The virion is icosahedral with a diameter of 20-30 nm, is non-enveloped and contains a DNA genome of 4.68 kb (Berns and Linden, 1995). Wild-type AAV have the ability to integrate stably into chromosome 19q13 of the human cell genome (Feldman and Steg, 1997). The virus has a broad host range and is able to replicate in any cell line provided a helper virus, usually an adenovirus, is present. AAV integrates into the host genome with high efficiency in the absence of a helper virus and is known to remain latent for prolonged periods. Latently infected cells subsequently infected with helper virus, rescue the AAV from the site of integration and trigger the complex lytic cycle (Flotte and Carter, 1995).

AAV genome includes two 145 nucleotide inverted terminal repeats (ITRs) which are the minimal essential *cis*-acting sequences necessary for replication, package and rescue. In addition to the ITRs, the genome consists of two open reading frames (ORF) encoding *Cap* (capsid) protein and the *Rep* (replication) proteins (Ali *et al.,* 1994). Three promoters are present in the genome which are activated in a cascade-type mechanism, initiating with adenovirus E1A protein activating the AAV p5 promoter.

AAV vectors used for gene transfer are constructed firstly by substituting the *Cap* and *Rep* genes by the foreign DNA, on a bacterial plasmid. This plasmid is then cotransfected with a helper plasmid which provides the *Cap* and *Rep*

functions *in trans*. Recombinant vector is produced upon infection of the cotransfected cells with adenovirus. The recombinant AAV particles are then separated from the helper virus physically by buoyant density centrifugation.

AAV has several properties which has made it a vector of choice for some gene therapy applications. AAV are not associated with any disease and has a minimal number of viral antigens to induce host immune response (Berns and Linden, 1995). AAV vectors have been shown to transduce cells stably at high efficiency and they have the ability to infect non-dividing cells (Podsakoff *et al.,* 1994). AAV integration into the host chromosome has not been encountered after an *in vivo.* infection and not shown to occur naturally in humans (Flotte and Carter, 1995).

The small size of the AAV genome allows a maximum foreign DNA insert size of 4.2 kb which appears to be the only definite limitation for this vector (Ali *et al.,* 1994). The packaging and purification of large quantities of AAV-based vectors is somewhat limited by technical factors (Flotte and Carter, 1995). In spite of the limited size insert accommodated in AAV vectors, a significant number of potentially therapeutic genes have been cloned into AAV vectors and used to infect cells *in vivo.* These vectors have been evaluated in preclinical models for cystic fibrosis (Flotte *et al.,* 1993) and Parkinson's disease (Kaplitt *et al.,* 1994).

1.3.2.3. Adenoviruses

Adenovirus-mediated gene transfer is being used widely to introduce potentially therapeutic genes into the myocardium and coronary vasculature, to treat cardiovascular disorders (Dzau *et al.,* 1993). Certain characteristics of this virus make it an excellent candidate for gene transfer to the heart cells. Adenoviruses are not associated with any severe disease and of approximately 50 serotypes identified to date, only a third are known to cause disease. Adenoviruses can infect the respiratory tract, eye and gastrointestinal tract, but in most cases the infection in asymptomatic and subclinical (Ali *et al.,* 1994; Yeh and Perricaudet, 1997). Moreover, the virus can be grown to high titres and can infect slow-replicating and nonreplicating,

terminally differentiated cells, such as neurons and cardiac myocytes. The adenovirus genome generally does not integrate into the host cells and exists episomally, therefore vectors based on these viruses provide transient expression of the transferred foreign gene (Ali *et al.,* 1994).

1.3.2.3.i. Adenovirus Biology

Adenoviruses are intermediate sized DNA viruses with a genome comprising a linear double-stranded DNA molecule of approximately 36,000 base pair. The genome is traditionally divided into 100 map units (mu) with 1 mu representing 360 base pairs (Ginsberg, 1984; Horwitz, 1990; Berkner, 1992). The virion particles lack an envelope and are 65-90 nm in diameter, with a spiked isocahedral morphology. The viral capsid is made up of protein units called *capsomers* and encloses the DNA genome at its core. The *hexons*, *pentons* and *fibre* are the major capsomers with the hexons being the major part of a viral capsid. Fibres are slender stalks with a globular head, attached

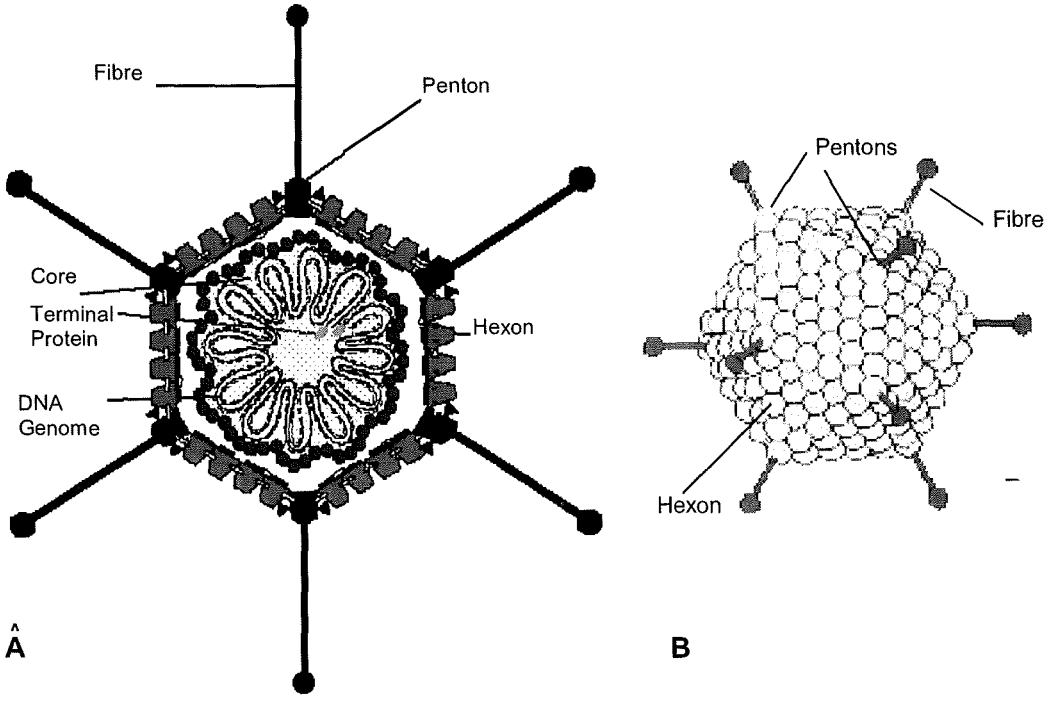


Fig 1.3.1 : Structure of adenovirus particle showing the major capsid proteins (**B**). DNA lies at the centre of the capsid, bound to protein, forming the core of the viral particle (**A**).

to the penton, which are embedded in the main body of the capsid. Fiber and penton-base proteins are involved in the process of attachment of the virus particle to the host cell receptors and subsequent internalization. There are

252 capsomers with 240 hexons and12 pentons positioned at apices of the isocahedron. Human adenoviruses have been classified serologically into 47 serotypes (Ali *et al.,* 1994).

1.3.2.3.ii. Genome Organization

A typical adenovirus genome comprises of a 35935 base pairs of double-stranded DNA with Inverted Terminal Repeats (ITR) at either end of the linear genome. The genome encodes at least 30 mRNA species, organized into several early and late transcriptional regions (Ali *et al.,* 1994). Generally, the transcriptional units are grouped into "early" and "late" expression units which transcribe mRNA before and after the viral DNA replication.

The gene expression exhibits a cascade regulation (Berkner, 1992; Ali *et al.,* 1994; Spector and Samaniego, 1995). There are four early regions (E1-E4) and one major late region with five main coding regions (L1-L5) (Berkner, 1992; Ali *et al.,* 1994; Graham and Prevec, 1997). The E1 region is active

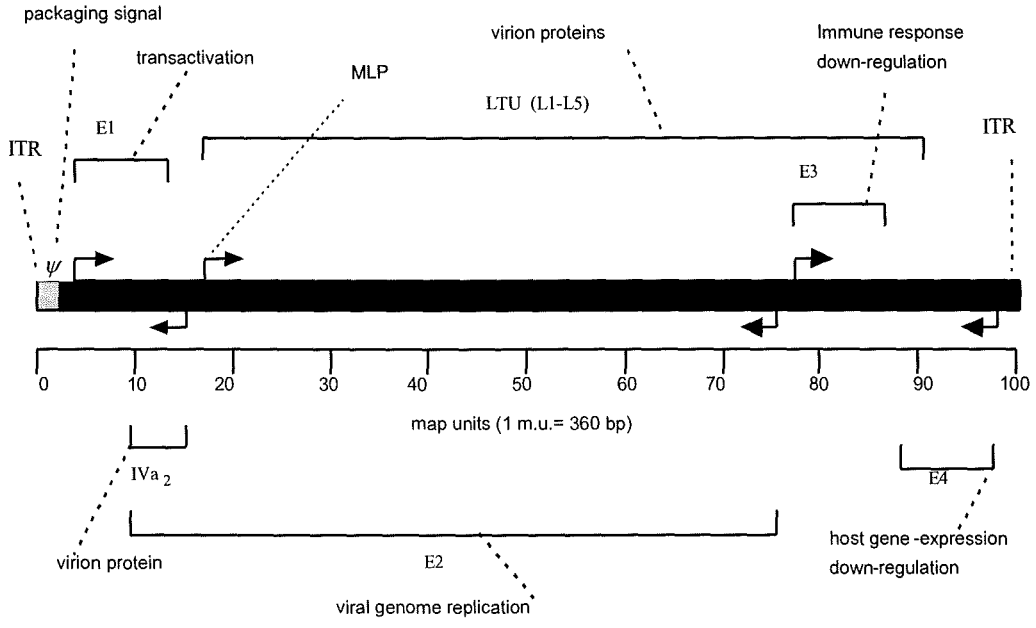


Fig 1.3.2 : Schematic presentation of the adenoviral genome showing the main transcriptional units encoding principal viral proteins, the inverted terminal repeats (ITRs) and the packaging signal (Ψ). Arrows indicate promoters and the direction of transcription. Main function of some proteins are also shown.

upon entry of the genome into the host cell nucleus, encoding proteins which activate the other early region genes. These gene transcripts in turn produce proteins which initiate DNA replication and cooperate to transcribe the late

genes from the Major Late Promoter (MLP) (Jones and Shenk, 1979; Bridge and Pettersson, 1996). E2 region encodes viral DNA replication proteins such as the DNA-binding protein and DNA polymerase (Horwitz, 1990; Berkner, 1992; Yeh and Perricaudet, 1997) while E3, which is dispensable *in vitro*, is involved in evasion of the infected host cell from immune response (Wold and Gooding, 1991; Berkner, 1992). E4 region shuts down the host gene expression in favour of the virus gene expression (Ginsberg, 1984; Halbert *et al.,* 1985; Horwitz, 1990) and also serves to upregulate transcription from E2 and late regions (Ali *et al.,* 1994), therefore effecting the early to late transition (Cutt *et al.,* 1987). The major late region codes for most of the polypeptides which make up the capsid (Ginsberg, 1984; Zenke *et al.,* 1990).

1.3.2.3.iii. Life Cycle

The adenoviral life cycle begins with receptor-mediated attachment of virus to the host cell membrane and subsequent internalization into endosomes. The virion then escapes to the cytoplasm by inducing endosomolysis and is transported to the nucleus where the DNA genome enters the nucleus through the nuclear pores and various transcription regions are expressed. On its way to the nucleus, the virion sheds its capsid proteins in a gradual manner at various steps and upon entry into the nucleus, most capsid proteins are already lost (Greber *et al.,* 1993; Ali *et al.,* 1994; Yeh and Perricaudet, 1997). The E1 region activates its own expression together with that of E2 and E3. After about 6- 8 hours, viral DNA replication ensues and then late proteins are expressed leading to assembly of 10^4-10^5 progeny virions within the infected cell nucleus. The whole host cell translational machinery is geared towards preferential processing of adenoviral transcripts and assembly of virion particles. 30 to 40 hours post infection, cell death by attrition rather than lysis occurs (Ali *et al.,* 1994) releasing the progeny viruses.

1.3.3. Adenoviral Gene Transfer Vectors

The human adenovirus type 2 and 5 (Ad2, Ad5) have been the most studied in terms of genomic organization and gene expression pattern, with their

complete nucleotide sequences available (van Ormondt and Galibert, 1984; Berkner, 1992). Consequently, these serotypes are at the forefront of development of vectors for gene transfer (Ali *et al.,* 1994). Most vectors encoding therapeutic genes been constructed using the Ad5 backbone.

In this study, Ad5-based vectors were employed for gene transfer into the myocytes. Replication-defective human type 5 adenovirus (Ad5) have emerged as highly effective vector for delivery of various genes into several types of cells, *in vitro.* (Mestril *et al.,* 1996; Hajjar *et al.,* 1997; Westfall *et al.,* 1997), *in vivo.* (Barr *et al.,* 1994; Leor *et al.,* 1996; Muhlhauser *et al.,* 1996; Rothmann *et al.,* 1996; Franz *et al.,* 1997). First generation recombinant adenoviruses have been manipulated to create capacity of up to 8.3 kb for accommodating the promoter-cDNA foreign DNA cassette in E1 and E3 regions. In most of such recombinants, The E1 region only is substituted for the foreign DNA, while the dispensable E3 region is deleted to allow accommodation of larger inserts in the E1 region (Bett *et al.,* 1994). These recombinants can be grown to high titres (Ali *et al.,* 1994) in permissive cell lines, such as HEK 293, which are able to provide the missing E1 function in *trans*. Moreover, these viruses have been modified in a way that allows propagation in permissive cell lines only, performing the function of gene delivery to target cells not undergoing a replicative cycle in the latter. It is postulated that inverted terminal repeats (ITRs) and the packaging signal sequence (ψ), are the only *cis*-acting signals required for adenovirus replication and packaging. In theory therefore, all remaining viral DNA can be substituted with foreign DNA, however for propagation of such viruses, special cell lines providing all function *in trans* will be required (Ali *et al.,* 1994; Yeh and Perricaudet, 1997).

1.3.4. HEK 293 Permissive Cell Line

For propagation of replication-defective ($E1^-$) recombinant adenoviruses, the functional gene products of the E1 are provided *in trans* by the permissive Human Embryonic Kidney (HEK) 293 cell line (Ali *et al.,* 1994; Bett *et al.,* 1994), was obtained by transformation of HEK cells with sheared Ad5 DNA

(Graham *et al.,* 1977). This cell line has the left-hand Ad5 genome sequences extending from nucleotides 1 to 4344, inserted in the pregnancy-specific β-1-glycoprotein 4 (PSG 4) gene (Louis *et al.,* 1997). The 293 cell line expresses the E1 gene products, essential for virus growth and propagation, and therefore behaves as a *permissive* cell line for growth of Ad5 virus, providing the essentially required E1 proteins *in trans* to adenoviruses defective in the E1 function. All first generation replication-defective recombinant adenoviruses are made with a foreign gene replacing the E1 region, which makes replication and propagation possible in 293 cells only. This is a preferred approach as it is a workable mode for biological containment of the recombinant virus.

1.4. Recombinant Adenovirus Construction

Ad5 recombinants are made using a two component system comprising of the viral backbone DNA and an adenoviral plasmid shuttle vector carrying the Ad5 left-end genome fragment and the foreign gene. The two DNA molecules have the ability to undergo homologous recombination when cotransfected into 293 cells, and rescue the foreign gene into the viral genome.

1.4.1. Viral Backbone DNA

The adenovirus used in this study for making recombinants is an Ad5 mutant named dl309. It is a common adenovirus backbone used for constructing the "first-generation" replication-defective adenovirus for gene transfer (Graham and Prevec, 1997). The wild-type Ad5 has four *Xba*I sites at 4, 29, 79 and 85 map units while its dl-309 mutant has a unique *Xba*I site which cuts in the E1 region at 4 map units (Graham *et al.,* 1977), inactivating the E1 region. This feature of the mutant is utilized for generating recombinants by the procedure of homologous recombination (Graham and Prevec, 1997). In addition, the Ad5-dl309 genome has a deletion-substitution in the E3 region which extends from Ad5 bp 30005-30750 and is substituted with 642-bp of heterologous DNA that shows homology to salmon DNA (Bett *et al.,* 1995).

1.4.2. Design of Adenoviral Shuttle Vectors

Adenoviral shuttle vectors are used for constructing Ad5 vectors with foreign gene inserts in the early (E1) region. Shuttle plasmids are designed to provide the essential left-end of an adenovirus type 5, but with the E1A region deleted to accommodate the foreign DNA insert.. The shuttle plasmid can undergo recombination with adenoviral genome, due to presence of a 2.4 kb region of homology, in both DNAs. The recombined product is a replication-defective Ad5, with the E1 region being replaced by the foreign DNA cassette. Two types of adenoviral shuttle vectors were used to make recombinants; the Microbix shuttle vectors and the pLES53 based vector.

1.4.3. Conventional Method of Recombinant Construction

The generally used procedure for Ad5 recombinant construction is based on the method of (Stow, 1981). In this method, the full Ad5-dl309 genome DNA is extracted from the virus, purified and digested with *Xba*I. This DNA is cotransfected into 293 cells along with a shuttle vector (see section 2.9.2) carrying the left-end of Ad5 genome, with foreign DNA insert. The replication-defective recombinant is generated by *in vivo.* homologous recombination (Berkner, 1992). The *Xba*I-cut viral DNA essentially forms the viral backbone, having all but the left-end of the genome. The pLES53-based shuttle plasmid provides the missing left-end of the virus genome, along with the foreign DNA which replaces the E1 region genes. The recombinant adenovirus is generated by recombination between regions of homology in the virus and shuttle vectors.

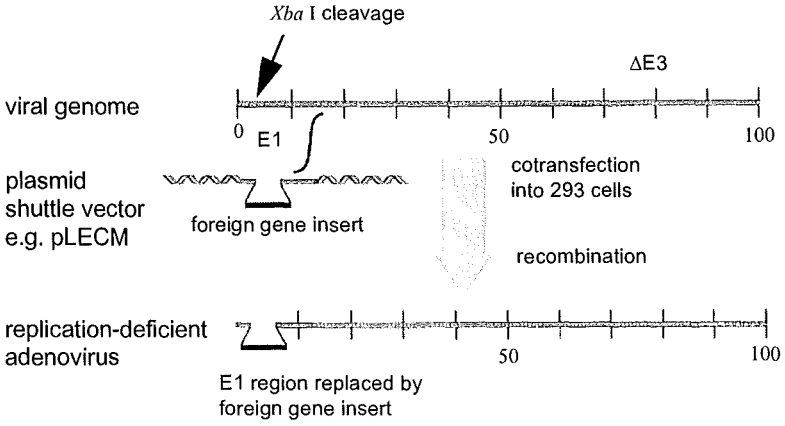


Fig 1.3.3: Strategy for rescue of foreign DNA into the Ad5 genome, by homologous recombination in 293 cells (modified from Graham, F.L. and Prevec, L.; Methods for Construction of Adenovirus Vectors. Molecular Biotechnology 3(3):207-220, 1995.)

1.4.3.1. Shuttle Vectors Based on pLES53

The pLES53 adenoviral shuttle plasmid vector was obtained from Keith Leppard, University of Warwick, UK. This vector conformed to the basic design of an adenoviral shuttle vector as described in section 1.4.2.

The shuttle plasmids have a pBR322 plasmid backbone with the ampicillin resistance selectable marker: It contains adenovirus type 5 (Ad-5) sequences from bp 1 (0 mu) to bp 5788 (16.0 mu), with a deletion of E1 sequences

(ΔE1) extending from bp 544 to bp 3328 (1.0-9.8 mu). Thus a region spanning 2460 bp (from bp 3328 to 5788) is available for recombination with a homologous region in the adenovirus genome. Cloning sites in this vector are limited with only *Xba*I and *Bgl*II restriction enzyme sites at the E1 deletion site, for the cloning of the foreign gene.

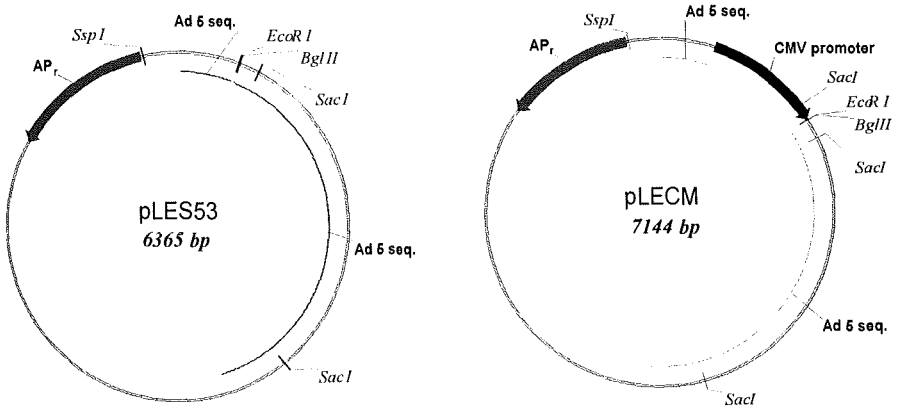


Fig 1.3.4: Plasmid maps of promoterless pLES53 and its derivative pLECM which has a Human Cytomegalovirus Immediate Early (CMV-IE) 780 bp enhancer/promoter.

The vector pLECM is a pLES53 derivative with Human Cytomegalovirus Immediate Early promoter (780 bp), following cloning sites, inserted at the E1 deletion region. cDNAs can be inserted as an *Eco*RI/*Bgl*II fragment for making a ubiquitously expressing expression vector.

1.4.4. The "Large Plasmid" Method of Recombinant Construction (Graham *et al.*)

The main system used in the study was developed by Frank Graham and colleagues which produces replication-defective adenoviruses with E1 region replacement (Graham and Prevec, 1995; Hitt *et al.,* 1995; Graham and Prevec, 1997). It encompasses use of two bacterial plasmids, one a shuttle plasmid for carrying the foreign DNA and the other an adenoviral genome-carrying plasmid with a bacterial pBR322 insert (McGrory *et al.,* 1988; Bett *et al.,* 1994).

1.4.4.1. Adenoviral Genome-carrying Plasmid pJM17

The Ad5 genome plasmid pJM17 is based on the complete 35700 bp genome of Ad5-dl309 with a 4.4 kb insert of pBR322 DNA at the *Xba*I site in the E1 region (McGrory *et al.,* 1988). This insert results in the plasmid DNA size exceeding the packaging constraints for the adenovirus (Bett *et al.,* 1993). Upon cotransfection with shuttle plasmid carrying the left-end sequences, the viral genome in pJM17 can recombine with homologous region in the shuttle vector, producing the recombinant adenovirus. The packaging constraint of pJM17 act as a selection, favouring the rescued viruses produced by recombination (Bett *et al.,* 1994). This results in a very low background of nonrecombinant, parental-type infectious progeny (McGrory *et al.,* 1988). The Ad5-dl309 backbone is able to accommodate up to 5.2 kb insert in the E1 region.

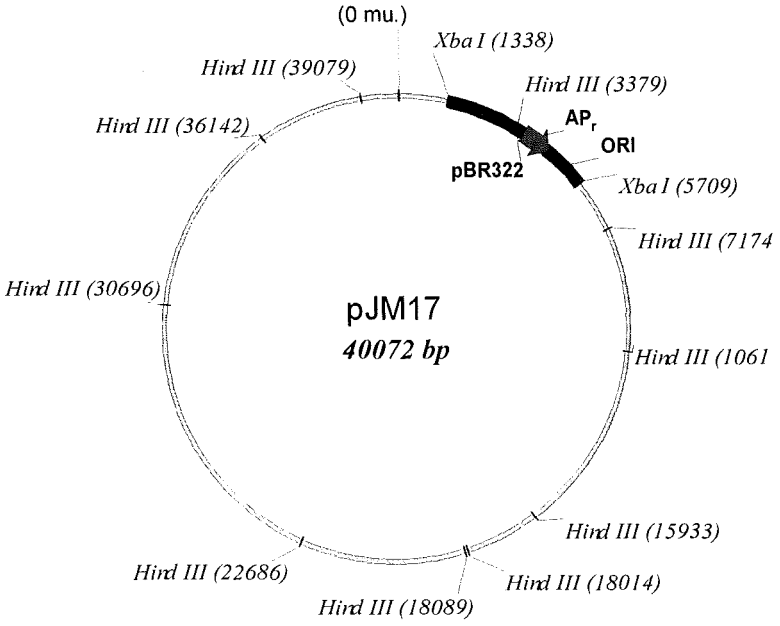


Fig 1.3.5 : Plasmid map of adenoviral large plasmid pJM17, carrying the complete Ad5-dl309 genome showing *HindIII* restriction sites. The pBR322 insert at *XbaI* site at 3.7 mu is also shown with the Ampicillin resistance marker (AP_r) and the bacterial origin of replication (ORI).

1.4.4.2. Microbix Shuttle Vectors

The shuttle plasmids from Microbix Biosystems Inc. have a bacterial plasmid backbone with the ampicillin resistance selectable marker. On a pBR322 backbone, the shuttle vector contains adenovirus type 5 (Ad-5) sequences

from bp 22 (0 mu) to bp 5790 (16.1 mu), with a deletion of E1 sequences (ΔE1) extending from bp 342 to bp 3523 (1.0-9.8 mu): Thus a region spanning 2267 bp (from bp 3523 to 5790) is available to allow recombination with a homologous region in the adenovirus genome. A multiple cloning site (MCS) with several restriction enzyme sites is inserted in the deletion region, facilitating subcloning of the promoter-gene cassette into the shuttle vector. These shuttle vectors were constructed by Frank Graham *et al.* and obtained commercially from Microbix Biosystems Inc. Toronto, Canada, and used for the generation of constructs used in this study, with the ultimate aim of making an Ad-5 replication-defective recombinant virus.

1.4.4.2.i. Basic Vectors

The pΔE1sp1A is a shuttle vector with the characteristics described in the previous section. In addition, it has a MCS with *Cla*I, *Bam*HI, *Xho*I, *Xba*I, *Eco*RV, *Eco*RI, *Hind*III, *Sal* I and *Bgl*II inserted at ΔE1 in the clockwise orientation. The pΔE1sp1B shuttle vector is similar except for the orientation of the MCS between the *Cla*I and *Bgl*II is reversed.

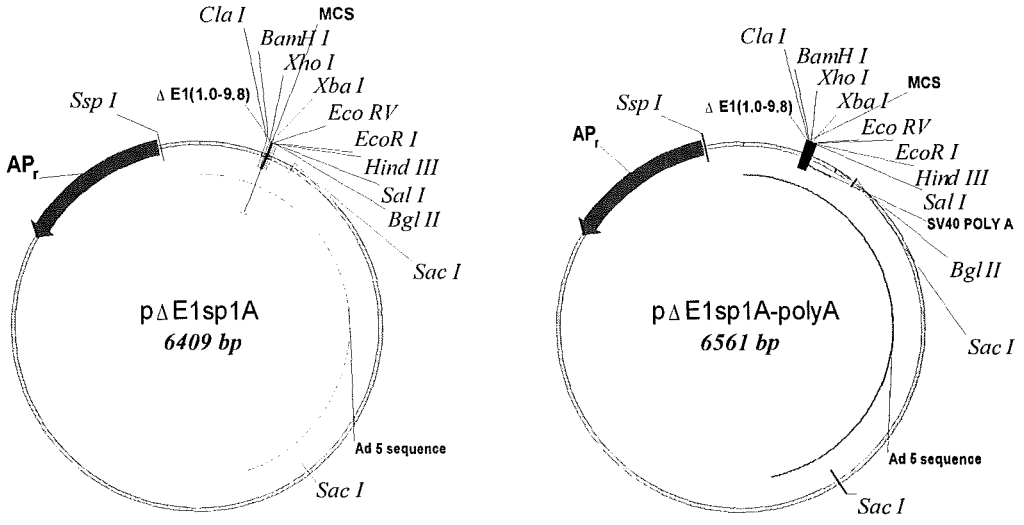


Fig 1.3.6: Plasmid maps of the basic pΔE1sp1A adenoviral shuttle vector and its derivative pΔE1sp1A polyA with the SV40-polyA sequence subcloned from pCA14.

pΔE1sp1A and pΔE1sp1B vectors do not contain a polyadenylation signal, 3' to the cDNA insertion site. A 160 bp fragment of SV40 polyadenylation signal was cloned into the vector downstream of the MCS to produce plasmids pΔE1sp1A-polyA and pΔE1sp1B-polyA (see section 3.1).

1.4.4.2.ii. Vectors with a Constitutive Promoter

The pCA13 and pCA14 shuttle vectors have the characteristics of an adenoviral shuttle vector with an addition of a Human Cytomegalovirus, Immediate Early (HCMV-IE) promoter (–299 to +72), inserted 5′ to the MCS.

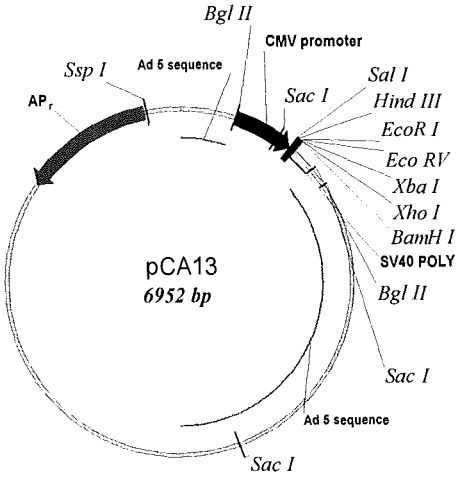


Fig 1.3.7: Plasmid map of pCA13 plasmid with the Human Cytomegalovirus Immediate Early (shorter) CMV-IE promoter used for making ubiquitously expressing adenovirus.

At the 3′ end of the MCS, the SV40 polyadenylation signal (SV40-polyA) sequence is present. pCA13 has a multiple cloning site with cutting sites for *Sal* I *Hind*III, *Eco*RI, *Eco*RV, *Xba*I, *Xho*I, *Bam*HI restriction enzymes. The orientation of the cloning sites is reversed in pCA14.

1.4.4.3. Process of Recombinant Generation

For rescuing of foreign genes into the viral genome, firstly, standard recombinant DNA manipulation techniques are used to clone the promoter-cDNA cassette into the appropriate shuttle plasmid. The pJM17 plasmid and the shuttle plasmid are propagated in *E.coli* and extracted using the CsCl-density gradient centrifugation (Sambrook *et al.,* 1989) and cotransfected into 293 cells, using the Calcium Phosphate transfection method.

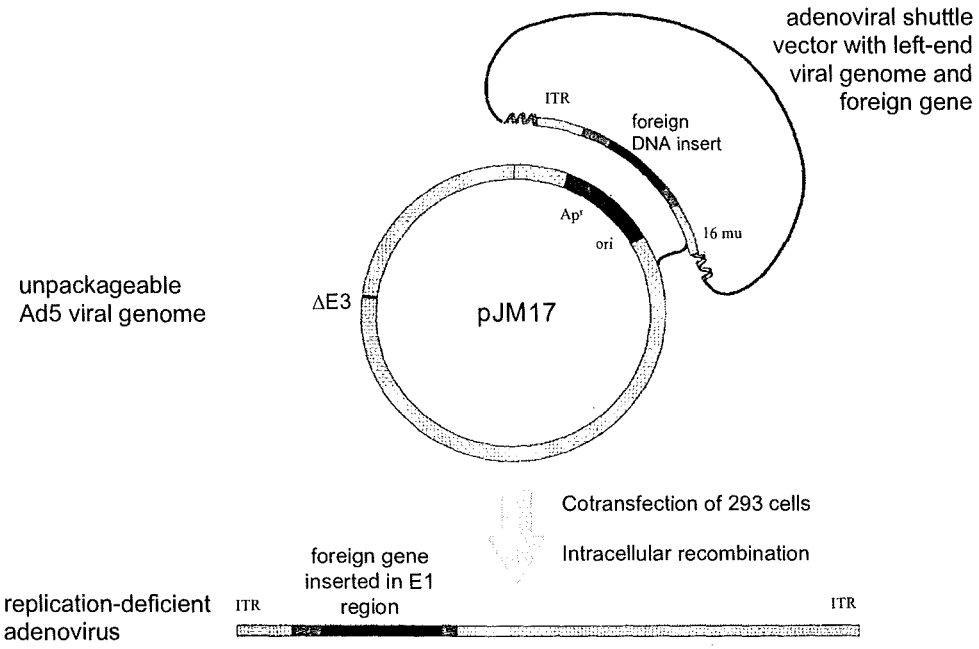


Fig 1.3.8: Strategy for rescue of foreign DNA inserts into the E1 region of Ad5 virus using pJM17. Ad5 recombinant virus are generated by overlap recombination between cotransfected bacterial plasmids carrying viral sequences. (modified from Hitt, M. *et al.*, Techniques for Human Adenovirus Vector Construction and Characterization. In: Viral Techniques, edited by Adolph, K.W. Academic Press Inc., Orlando, Florida, USA:)

A modified method based on the original protocol from Frank Graham (Graham and Van der Eb, 1973), was developed for post-transfection screening of the recombinants. This method used standard commercially available plaque overlay medium and low melting point agarose, in order to minimize the variables affecting 293 cells after cotransfection with the pJM17 and shuttle plasmids. Recombinants are generated by homologous recombination of similar regions in pJM17 and the adenoviral shuttle plasmid, in the 293 cells (see fig. 1.3.8).

1.4.5. Adenoviral Vectors for Gene Transfer to the Heart

Adenoviral vectors have been successfully used for gene transfer into heart. Both *in vitro.* gene transfer to cultured cardiomyocytes (Mestril *et al.,* 1996; Donahue *et al.,* 1997; Westfall *et al.,* 1997), and *in vivo.* transfers to hearts of mammals (Leor *et al.,* 1996; Magovern *et al.,* 1996; Rothmann *et al.,* 1996), have shown efficient gene transfer and stable expression, using replication-defective adenovirus recombinants.

Adenoviral gene transfer and expression *in vitro.* using cultured neonatal or adult cardiocytes has been a useful tool for assessing recombinant vector transduction. In addition, adenoviruses expressing genes of myofilament protein Troponin I (Westfall *et al.,* 1997), membrane proteins like SERCA and (Hajjar *et al.,* 1997), β_2-adrenergic receptor (Drazner *et al.,* 1997) and HSP-70 protein induced during stress in the myocardium (Mestril *et al.,* 1996) have been overexpressed in cardiac myocytes in culture and their effect studied.

Adenovirus vectors expressing reporter genes have been used successfully to transduce rat (Guzman *et al.,* 1993; Kass-Eisler *et al.,* 1993; Muhlhauser *et al.,* 1996) and porcine (French *et al.,* 1994) myocardial cells, by direct injection into the myocardium. Direct administration of β-galactosidase expressing adenovirus by intramuscular injection into porcine hearts *in vivo.* has shown transduction of myocytes in a well-localized area of the myocardium. The myocardial β-gal activity peaked at 3-6 days after the injection, returning to control level within one month (Muhlhauser *et al.,* 1996). On the other hand, catheter-mediated infusion of replication-defective adenovirus into coronary arterial circulation of adult rabbit *in vivo.*, has achieved efficient reporter gene transfer and expression, both in the coronary vasculature and in adjacent myocardium. Reporter gene activity was detected at 2 weeks post-infusion, while no activity was detectable at 1 month, in either myocardium or coronary vasculature (Barr *et al.,* 1994). Adenoviral gene transfer and expression has also been assessed in infarcted rat myocardium model. The transducing efficiency of a β-gal reporter gene-expressing adenovirus was lower in the infarcted, compared to normal hearts. Histological examination of transduced hearts at 7 days post-injection, showed β-galactosidase gene expression limited to the viable myocytes at the border of the infarct. (Leor *et al.,* 1996).

Adenoviral vectors are shown to be the most efficient vector system for transgene expression in myocardium *in vivo.* (Rothmann *et al.,* 1996). Improved vectors are now being developed to target the expression of therapeutic transgenes to ventricular myocardium by using cardiac muscle-specific promoters (Rothmann *et al.,* 1996; Franz *et al.,* 1997). Generally, the

timing of gene expression in adult myocytes transduced *in vivo.* in animals, ranged from 3 days to 1 month (Jennings and Reimer, 1981; Kass-Eisler *et al.,* 1993; Barr *et al.,* 1994), in many cases, it may be ample for a transgene to express and show its therapeutic benefits on the myocardium.

Chapter 2

Materials and Methods

2.1. Chemicals and Reagents

The majority of chemicals and solvents were Analar grade, and obtained from British Drug House Ltd., Poole Dorset. Other more specialized and molecular biology reagents, except where stated otherwise, were obtained from Sigma Chemical Company Ltd. Poole, Dorset. Commonly used restriction enzymes were obtained from Promega and New England Biolabs, except where stated. T4 DNA ligase was obtained from Gibco-BRL., Paisley, UK Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim, GmbH, Germany. T4 polynucleotide kinase and other DNA modifying enzymes were from Promega. DNase II, RNase I, Lysozyme and Proteinase K enzymes were obtained from Sigma Chemical Company Ltd. Poole, Dorset. DNA size marker (1kb ladder) was obtained from Gibco-BRL, Paisely, UK and Protein size markers were from BioRAD Laboratories Ltd., Hercules, Ca, USA. The random priming kit used for radiolabeling DNA was obtained from Boehringer Mannheim, GmbH, Germany. RNAzol B for RNA extraction was obtained from Biogenesis Ltd. Bournemouth, UK.

All radioisotopes, DNA sequencing kit, ^{33}P labelled dideoxy-nucleotides and Hybond membranes were supplied by Amersham International plc., Buckinghamshire. Synthetic oligonucleotides were synthesized on an Applied Biosystems automated DNA synthesizer, or obtained from Gibco-BRL or Genosys Ltd.

Plasmid with the adenovirus dl309 genome (pJM17) and compatible shuttle vector plasmid were obtained from Microbix Biosystems, Toronto, Canada. Other plasmid vectors for subcloning of DNA fragments were obtained from Promega unless otherwise stated.

Agar, Tryptone and yeast extract for bacterial cultures and serum, media and supplements for eukaryotic cell culture were supplied by Gibco, Paisley, except where otherwise stated. The standard Phosphate Buffered Saline (PBS) used in tissue culture and immunostaining was without Ca^{++} and Mg^{++} and obtained from Sigma or Gibco. Another PBS of slightly different composition used for resuspending virus infected cell was obtained from

Gibco (referred to as PBS with Ca^{++} and Mg^{++}). Plasticware for cell culture were supplied by Nunc Intermed, Denmark, Sterilin Ltd. Hounslow, Middlesex and Falcon Becton Dickinson Labware, New Jersey, USA.

2.2. Preparation of Solutions, Glassware and Plasticware

All solutions used in the isolation and manipulation of nucleic acids were prepared using molecular biology grade chemicals and distilled water and sterilized by autoclaving or by filtration through a 0.22μM filter.

All solutions used for RNA extraction were made in distilled water treated to minimize degradation of RNA by contaminating RNase. Distilled water was treated with Diethylamine pyrocarbonate (DEPC), as a 0.1% v/v solution and then autoclaved. Disposable rubber gloves were worn at all times during handling of RNA. RNA grade reagents, new, unopened packs of disposable microfuge tubes and pipette tips were used for autoclaving and these items were also handled wearing rubber gloves at all times.

2.3. Plasmid DNA Purification

Plasmid DNA was propagated in *E.coli* host and extracted when required for subcloning. Different methods of plasmid extraction were used for obtaining varying quality and amounts of plasmid DNA for transfections, use in subcloning, screening or long-term storage of plasmid DNA.

2.3.1. Small-scale Isolation of Plasmid DNA (minipreps)

Plasmid minipreps were done for screening recombinant plasmids during subcloning of promoter-cDNA constructs or for obtaining plasmid DNA for long-term storage of subcloned DNA.

2.3.1.1. Crude Alkaline Lysis Method

This method was employed mainly for analysis of potential recombinant plasmids in DNA subcloning experiments. An individual bacterial colony was picked from an agar plate and inoculated into 5 ml of L-Broth (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 100 μg/ml ampicillin, in a 25 ml universal container. Subcultures were incubated at 37°C

overnight with orbital shaking at 180-200 rpm. One day later, 1.8 ml of the overnight culture was removed and transferred to a 2 ml Eppendorf tube for centrifugation (at 14000 rpm for 3 minutes in a microfuge). The bacterial pellet was resuspended very thoroughly in 100μl of cell resuspension solution (TGE; 25mM Tris-HCl, pH 8.0, 50mM glucose, 10mM EDTA, pH 8.0, by repeated pipetting. 200μl of alkaline-SDS (0.2M NaOH, 1% sodium dodecyl sulphate) was then added to the tubes which were mixed by inverting several times. After 5 minutes of incubation on ice until the solution became clear and viscous, 150μl of neutralization solution (3M Potassium, 5M Acetate) was added, the solution was mixed by inversion until a white flocculant precipitate became visible. Tubes were incubated on ice for 5 minutes and then microfuged (14000 rpm for 5-7 minutes) to remove the white chromosomal DNA and cell debris. Approximately 430μl of clear supernatant was transferred to a fresh 1.5 ml Eppendorf tube while avoiding removal of any of the white precipitate. Cold 100% ethanol (900μl) was added, tube sealed and mixed by inversion and transferred to a −70°C freezer for 30-45 min for precipitation of DNA. Tubes were then centrifuged at 14000 rpm for 15 minutes and the DNA pellet washed in 70% ethanol, air-dried and resuspended in 50μl of TE pH 8.0 with 1μl of RNase A (10mg/ml). The solution was incubated at room temperature for 10 minutes after which 10-20μl of the DNA solution was employed for restriction analysis.

2.3.1.2. Kits

Commercially available kits were used for obtaining high quality plasmid DNA for sequencing and for long-term storage purposes.

2.3.1.2.i. Qiagen Minipreps

For obtaining microgram amounts of plasmid DNA of high purity, for using as vector or insert DNA for cloning, QIAprep Spin Plasmid kit was used. The DNA isolation protocol uses alkaline lysis of bacterial cells for releasing of plasmid DNA and adsorbing on to silica spin-columns followed by washing off the salts and finally elution of the plasmid DNA. The prescribed protocol

(QIAprep Plasmid Handbook 03/95) for the kit was followed except that the DNA was eluted in 50μl of TE pH 8.0.

2.3.1.2.ii. Hybaid Minipreps

Plasmid DNA for sequencing of new clones, was done using Hybaid Recovery™ Plasmid Miniprep Kit. The kit protocol is based on alkaline lysis of the bacteria and binding of the released DNA to a silica gel matrix and entrapment in a spin-filter followed by washing and collection of plasmid DNA by elution. The kit protocol (version 5/96) was followed.

2.3.2. Large-scale Isolation of Plasmid DNA

Generally, larger quantity of plasmid DNA was required for transfections and for obtaining vector and insert DNA in subcloning procedures. Commercially available kits and CsCl preps were used to obtain milligram amounts of plasmid DNA.

2.3.2.1. Plasmid Extraction for Caesium Chloride Preps

Caesium Chloride-Ethidium Bromide (CsCl-EtBr) density gradient centrifugation was the method of choice of obtaining large amounts of plasmid DNA for transfection. For this purpose, 10 ml of overnight cultures were initiated from an inoculum (loop) of a glycerol stock or a single colony picked from a bacterial dish, as previously described. The following day 2 ml of the subculture was inoculated into L-broth (800 ml containing 100μg/ml of ampicillin), in a 2 litre bevelled conical flask. Incubation was carried out at 37°C with orbital shaking at 180-200 rpm. For pBR322 based low copy number plasmids, a plasmid amplification step was employed. Chloramphenicol (170μg/ml final concentration) of was added after 6-8 hours of shaking (OD 600 = 0.6), after which the incubation was continued overnight. Cells were harvested by centrifugation (at 5000 rpm for 10 min, at 4°C in a Beckman Model J2-21 centrifuge with a JA-14 rotor. The bacterial pellet was resuspended in 18 ml of TGE (25mM Tris-HCl, pH 8.0, 50mM Glucose, 10mM EDTA, pH 8.0), by repeated pipetting using a 10 ml pipette. 2ml of lysozyme solution (50 mg/ml in TGE) was added and the solution mixed by swirling after which the tube was incubated for 20 minutes at room

temperature. 40 ml of alkaline-SDS (0.2M NaOH, 1% sodium dodecyl sulphate), freshly prepared from stock solutions, was added and the solution mixed by inversion. The cell lysis reaction was incubated on ice for 10 minutes and then neutralized by adding 20 ml neutralizing solution (3M Potassium, 5M Acetate). The tube was mixed well by inversion and incubated on ice for 10 minutes. 5 ml of sterile distilled water was added and tubes were centrifuged for 10 minutes at 7000 rpm as above. The clear supernatant was filtered through a sterile gauze into a fresh tube to remove cell debris. 0.6 volumes of isopropanol was added to precipitate the DNA and the mixture was incubated at room temperature for 20 minutes. DNA was pelleted by centrifuging for 25 minutes (7000 rpm at 20-22°C in a Beckman J-20 centrifuge). The supernantant was immediately discarded and the pellet gently washed with 70% ethanol. Tubes were centrifuged for 5 minutes at 7000 rpm and the ethanol wash aspirated off completely and air-dried. The DNA pellet was dissolved in 3 ml of 0.1X SSC (15mM NaCl, 1.5 mM Na citrate, pH 7.0)

2.3.2.1.i. CsCl-EtBr Density Gradient Centrifugation

For DNA obtained from a 400-800 ml culture, the gradient was prepared as follows; 8 g of caesium chloride was weighed out in a universal container and dissolved in 3 ml of 0.1X SSC. The solution was placed at 37°C in a waterbath to aid the dissolving process. DNA solution was added to the universal tube and the CsCl dissolved completely. 640µl of ethidium bromide (10 mg/ml in water) was added and mixed. Tubes were incubated on ice for 20 minutes and then spun in a IEC Centra-7R refrigerated centrifuge at 2500 rpm at 4°C for 25 minutes The supernatant was then transferred to Beckman Quick-Seal tubes (Polyallomar #342412) using a short-form Pasteur pipette as a funnel. The remainder of the tube was filled with liquid paraffin and tubes were sealed and centrifuged in a Beckman Ti70 rotor at 49000 rpm for 16 hours at 20°C in a Beckman L8 Ultracentrifuge. Plasmid DNA band was visualized using a long wavelength UV light source and the DNA band was drawn off using a 21G hypodermic needle and syringe. Ethidium bromide was removed by successive extractions with TE-saturated butanol until the

organic supernatant became colorless. The DNA solution was dialysed against distilled water or TE for 12-48h at 4°C with 3-4 changes of buffer, using dialysis tubing. After dialysis, nucleic acids were analyzed on a 1% agarose gel to check the integrity of DNA and the possible presence of any contaminating RNA. If RNA was found to be present, the DNA solution was treated with RNase (10μg/ml), and subjected to phenol-chloroform extraction and one chloroform extraction to remove any traces of phenol. DNA was precipitated using 1/10th volume of 3M Na-acetate pH 5.2 and 3 volumes cold 100% ethanol. If no RNA was visible upon electrophoretic analysis, the DNA was ethanol precipitated as described and resuspended in a desired volume of TE or 0.1X SSC.

2.3.2.2. Wizard Maxipreps

For large scale plasmid preparations where the quality of DNA obtained was not critical such as those employed for obtaining higher amount of vector or insert DNA required in subcloning of genes of interest, the Promega Wizard™ Maxipreps DNA purification systems were used. The procedures for bacterial growth and DNA amplification (when required) were as described above. The procedure is based on alkaline lysis of bacterial cells and binding of DNA to resin and separation on a column followed by washing off excess salts and finally eluting the plasmid DNA. The alkaline lysis of bacteria and DNA isolation protocol outlined in Promega Technical Bulletin No. 139, revision 10/94 was followed with minor modifications. The solutions provided in the kit were at times replaced with further solutions prepared according to the compositions supplied in the bulletin. DNA elution was carried out using an increased amount (2-2.5 ml) of preheated (70-80°C) TE buffer in circumstances where plasmid amplification was employed.

2.4. DNA Manipulations for Subcloning

Troponin C and other foreign cDNAs and promoters were subcloned into adenoviral shuttle plasmid vectors as a first step for making recombinant adenoviruses. In addition, molecular tagging of cDNA with epitope coding sequence was also carried out.

2.4.1. Plasmid Vectors Used for Subcloning

The source of the skeletal Troponin C cDNA was plasmid LK419 obtained from Larry Kedes' Lab. at the University of Southern California, CA, USA (Gahlmann *et al.,* 1988). Plasmid pSP72 used as an intermediary vector in the cloning, was obtained from Promega Corporation, Madison, WI, USA. Adenoviral shuttle plasmid vectors pCA3, pCA4, pCA13 and pCA14, plasmids pΔE1sp1A, pΔE1sp1B and plasmids pFG140 and pJM17 were purchased from Microbix Biosystems Inc., Toronto, Ontario, Canada. Adenoviral shuttle vectors pLES53 and pLECM were provided by Dr. Keith Leppard, University of Warwick, UK. All plasmids required for subcloning purposes were extracted using Wizard Maxipreps while those required for transfections were purified by CsCl-EtBr density centrifugation.

2.4.2. Oligonucleotides for PCR, Sequencing and Epitope-tagging

Oligonucleotides were obtained from Gibco-BRL or Genosys in lyophilized form, reconstituted with sterile distilled water. Stocks of oligonucleotides were made at concentrations of 200μM and maintained as frozen stocks at −20°C. Working solutions were made by diluting 1:20 for PCR and 1:100 for sequencing reactions, in fresh sterile distilled water. Oligonucleotides to be used for making epitope tag sequences were reuspended in TE to a final concentration of 2μg/μl unless otherwise stated. All primers were designed empirically or in some cases using GCG package or the Vector NTI software. Primer annealing temperature and other characteristics including possibility of hairpin loop formation were checked using the Primer Master Version 1.0 public domain software.

2.4.3. Agarose Gel Electrophoresis of DNA

A 1% (w/v) agarose solution was made up in 0.5X TBE buffer (0.0445M Tris base, 0.0445M Boric acid. 0.001M EDTA, pH 8.0) and heated in a microwave to boiling point to dissolve the agarose. The solution was cooled down to 40-45°C and EtBr was added to a final concentration of 0.1μg/ml and poured into a horizontal gel tray set with the appropriate gel comb. The gel was transferred to a electrophoresis gel tank with 0.5X TBE, the comb removed

ready to load the DNA samples. The DNA samples were mixed with one-sixth volume of 6X gel-loading buffer (50% glycerol, 1% bromophenol blue). Higher strength (1.2-2%) was used when smaller fragments (<300 bp) needed to be resolved. Lower strength gels (0.5-0.8%) gel was used to resolve larger (>4000 bp) bands or for DNA fragments which required extracting from the gel for cloning purposes.

2.4.4. Restriction Digests of DNA Fragments

Restriction endonuclease digestion were carried out using a 1.5 to 2 times excess of the 1 Unit of enzyme per μg of DNA recommendation of the supplier. Fragments were separated by agarose gel electrophoresis and purified by electroelution or gel extraction as described in section 2.4.5. Micropure EZ™ columns (Millipore Corp.) were used for removal of interfering enzymes from the DNA as per instruction of the manufacturer. Other manipulations done on the DNA are discussed later under the appropriate headings.

2.4.5. Extraction of DNA Fragments from Agarose Gels

DNA restriction fragments were isolated from agarose using the QIAprep Gel Extraction Kit. The prescribed protocol of QIAprep Plasmid Handbook revision 03/95 was followed for extracting DNA, with the exception of omitting the optional step #7 and eluting the DNA in TE. $1/10^{th}$ of the eluted DNA fragment was run on a 1% agarose gel to confirm and visually quantify the amount of DNA eluted.

In case of low yield of some small sized DNA fragments, electroelution was employed as an alternative. The gel piece carrying the fragment was cut out and placed in prepared dialysis tubing with sterile TBE secured by dialysis clips. The tubing was then placed in a gel tank and electroelution carried out at 70-80V for 1-2 hours. After the eluted DNA was carefully collected from the tubing and extracted with phenol/chloroform once and then chloroform once and ethanol precipitated. DNA pellet was suspended in the appropriate volume to obtain the required concentration of DNA for use in ligations or other manipulations.

2.4.6. Preparation of Double-stranded DNA Oligonucleotides

Double-stranded DNA oligos comprising the epitope tag coding sequence were made for incorporation into the sTnC cDNA. The appropriate oligos were synthesized, annealed and ligated into the sTnC cDNA to produce cDNA coding for skeletal Troponin C protein with a single copy of carboxyl-terminal epitope tag.

2.4.6.1. Annealing of ss DNA Oligonucleotides

Double stranded short DNA inserts were made for incorporating epitope tag sequences in the cDNA at the 3'-end. Single stranded oligonucleotides of the sense and the antisense DNA were synthesized with appropriate restriction sites and stop codon, annealed and then phosphorylated to obtain the double stranded epitope tag insert. The annealing reaction consisted of 20μl each of the sense and antisense oligonucleotides with 10μl of 10X Klenow buffer in a total volume of 100μl. The annealing reaction was heated to 95°C in a thermal cycler block for 5 minutes and then transferred to a waterbath which was preheated to 70°C. The waterbath was then switched of and the annealing mix allowed to cool over a 2 to 3 hour time period to allow annealing the oligonucleotides. The single stranded oligonucleotides and the annealed DNA was run on a 2.5% agarose gel to check for proper annealing: The annealed DNA band travelled slower than its two parent oligonucleotides.

2.4.6.2. Phosphorylation of Annealed DNA

5μl of annealed DNA was used in the phosphorylation reaction with 5μl of 10X Kinase buffer, 2.5μl of ATP (10mM) 2μl of T4 Polynucleotide Kinase (Promega cat. no. M4101) and 35.5μl of distilled water. The reaction was incubated at 37°C for 1 hour. 2μl of 0.5M EDTA pH 8.0 was added at the end if incubation and the reaction heated at 75°C for 10 minutes, and then transferred to ice. The phosphorylated DNA was precipitated with 0.1 volume (5.5μl) of Na-acetate and 200μl of cold 100% ethanol and kept at −70°C for 1 hour. The DNA was spun at 14000 rpm in a microfuge for 20 minutes, the pellet washed with 50μl of 70% ethanol, dried and resuspended in 20μl of TE

(final concentration of DNA being 100 μg/μl). 1-3μl of this DNA was used in ligation reactions.

2.4.7. End-filling of Protruding and Recessed Termini of DNA

Protruding or recessed end of DNA were filled in to produce blunt ends where required. DNA fragment was isolated from the gel as described in section 2.4.5. and added to a reaction mix containing 1X Klenow buffer, 40μM of each dNTPs and 1U of DNA Klenow polymerase (Boehringer Mannheim cat. no. 1008 404) per microgram of DNA, and incubated at 37°C for 3 hours. After the completion of reaction, the DNA was ethanol precipitated as described in section 2.5.2., and resuspended in a suitable volume. Alternatively, the enzyme and unused nucleotides were removed using Micropure EZ™ for removing the polymerase and Microcon™-30 for concentrating the DNA and removing the unused nucleotides as described in section 2.5.3.

2.4.8. Dephosphorylation of DNA

Vector DNA cut with an enzyme was prevented from religating back, favouring insert ligation, by dephosphorylation of restricted cohesive termini using Calf Intestinal Phosphatase (CIP; Boehringer Mannheim cat. no. 713023). 1 Unit of CIP enzyme was added per 50 pmol of DNA 5'-recessed, 3' recessed or blunt-ended DNA termini, to a vector restriction digest at the end of digest reaction and incubated at 37°C for 1-1.5 hours. Buffer adjustments were made using the restriction enzyme buffer rather than CIP buffer, before adding the enzyme. After the completion of the dephosphorylation reaction, the CIP was inactivated by adding 1/10th volume of 200mM EGTA and heating at 65°C for 10 minutes. The enzyme was completely removed either by using Micropure EZ™ columns or by phenol/chloroform extraction and ethanol precipitation as described in section 2.5.2.

2.4.9. Ligations

DNA ligation reactions for splicing insert DNA into vectors were carried out using 20-50ng of vector DNA and 50-200ng of insert. 1:1, 1:2 and 1:5 DNA

molar ratios were used for the vector and insert DNA. Typically, 50ng of vector was mixed with twice molar amount (1:2 ratio) with 2μl of 5X ligase buffer, 1μl of ATP (10mM) and 1μl of T4 DNA ligase (Gibco-BRL cat. no. 15224) in a 10μl reaction. For a protruding end ligation, the reaction was incubated at 4°C overnight and for blunt-end ligations, the incubation was at room temperature overnight. Control ligations were set up containing cut plasmid vector only (no foreign insert) plus ligase and used as background for vector DNA restriction quality.

2.5. Additional Nucleic Acid Procedures

The classical procedures for nucleic acid manipulation were employed for cleaning, concentrating and estimating DNA and RNA as described by Sambrook *et al* (Sambrook *et al.,* 1989). Radiolabeled probing of nucleic acids was performed according to the protocols provided with Amersham Hybond-N+ membranes and modified wherever required.

2.5.1. Organic Extraction of Nucleic Acid Solutions

This method was used to remove cellular debris or contaminating proteins from crude DNA preparations. Equal amounts of Phenol washed in Tris buffer (Fisher Scientific, Loughborough, UK) was mixed with 24:1 chloroform-isoamyl alcohol and stored in a dark bottle at 4°C. The phases were allowed to separate. An equal volume of the lower organic phase of the phenol-chloroform was added to the DNA solutions or crude DNA extracts and vortexed. The extraction mix was then centrifuged at 12000g in a microfuge at room temperature. The lower phenol phase was removed using a disposable fine pastette and an equal amount of chloroform-isoamyl alcohol was added, mixed by vortexing and centrifuged at 12000g in a microfuge for 5-7 minutes. The upper aqueous phase was aspirated off carefully into a fresh Eppendorf tube and ethanol precipitated and resuspended in the desired volume of buffer or water.

2.5.2. Concentration of DNA Samples by Ethanol Precipitation

Na-Acetate/Ethanol precipitation was used to concentrate dilute DNA solutions, or where the buffer of the DNA solution is required to be changed. To the DNA solution, $1/10^{th}$ volume of 3M sodium acetate and 2.5 volumes of cold ethanol were added, mixed thoroughly, and frozen at −70°C for 1-2 hours to precipitate the DNA. The DNA was pelleted by centrifugation at 14000 rpm in biofuge and washed with 70% ethanol, spun, air-dried and then resuspended in water or TE.

2.5.3. Concentration of Dilute DNA Samples

Other than ethanol precipitation, DNA was also concentrated using Microcon-30 (for volumes up to 500µl) or Centricon-30 (for volumes up to 2 ml) columns (Millipore Corporation). DNA solutions were concentrated following the protocol described by the supplier. The total amount of DNA spun per column was kept below 400µg to prevent clogging of the filter membrane.

2.5.4. Quantitation of Nucleic Acids

DNA, RNA solutions and oliogonucleotide stocks were diluted 1:100 to 1:500 in distilled water or preferably the buffer they were originally resuspended and the nucleic acid concentration and 260/280 ratio measured on the Pharmacia Biotech Ultrospec 2000 UV/VIS spectrophotometer, using the nucleic acid mode. The displayed results for concentration were multiplied by the dilution factor

2.5.5. Preparation of Radiolabeled DNA Probes

Radiolabeled single stranded DNA probes labelled with ^{32}P were generated using the Random Primed DNA Labeling Kit (Boehringer Mannheim, cat. no. 1004-760). 50 ng of DNA (from a 25 ng/ml solution) was denatured by heating to 100°C for 10 minutes and immediately placed on ice. 2µl of denatured DNA was taken in an Eppendorf tube and placed on ice: 1µl each of dATP, dTTP and dGTP and 2µl of reaction buffer mix was added to the reaction followed by 5µl of [α-^{32}P]dCTP (50µCi) and 7µl of distilled water.

Finally, 1μl of Klenow enzyme added, mixed and incubated at 37°C for 30 minutes to 1 hour in a waterbath covered with a lead pot. The reaction was stopped by heating. All non-incorporated dNTPs were removed using Nick® columns Sephadex® G-50 DNA-grade from Pharmacia Biotech (cat. no. 17-0855-02)

2.5.6. Hybridization of Radiolabeled Probe

DNA and RNA transfers to Hybond-N+ membrane (see sections 2.12.2. and 2.15.4.) were subjected to hybridization immediately after transfer or stored at 4°C wrapped in Saran-Wrap. The membrane was prehybridized at 65°C for 1 hour in prehybridization buffer (0.5X Denhardt's solution, 5X SSC, 0.5% SDS, 20 μg/ml of heat denatured salmon sperm DNA) using a hybridization tube. The prehybrization solution was removed and replaced with hybridization solution (0.5X Denhardt's solution, 5X SSC, 0.5% SDS, 10% Dextran sulphate, 20 μg/ml of heat denatured salmon sperm DNA) to which the denatured (single stranded) labelled probe had been added (not exceeding a 20 ng/ml concentration limit). Hybridization was carried out overnight at 65°C in hybridizing oven. The following day, the membrane was washed twice with 2X SSC/0.1% SDS at 65°C for 10 minutes and 1X SSC/0.1% SDS at 65°C for 10 minutes if required due to filter being too hot. A high stringency final wash of 0.1X SSC/0.1% SDS at 65°C for 10 minutes was done only if the probe was 100% homologous. The membrane was removed from the hybridization bottle onto a 3MM paper for drying and then the filter wrapped in Saran-Wrap and exposed to autoradiography.

2.6. DNA Transformation

The modified plasmid vector DNA were transformed into *E.coli* host following ligation of foreign inserts into vectors, for obtaining amplified amounts of plasmid DNA. This plasmid DNA was then extracted for screening and selection of the proper plasmid vectors with the desired subcloned promoters, cDNA and other markers.

2.6.1. Preparation of Competent Cells

A glycerol stock of *E.coli* DH5α cells of known single colony origin was used to inoculate 5 ml of L-broth supplemented with 20mM $MgSO_4$, 10mM NaCl, 5mM KCl using a 100X solution of the salts and incubated at 37°C for overnight. The following day, 100 ml of L-broth supplemented with 1 ml of the 100X solution of salts as before, in a sterile bevelled 250 ml conical flask was inoculated with 2 ml of overnight culture and incubated in an orbital shaker at 37°C for 4-5 hours. When the optical density at 600nm reached 0.45, the cells were split in two 50 ml sterile Falcon tubes placed on ice for 15 minutes. Cells were kept on ice and spun at 4°C from this step onwards. Cells were pelleted at 2500 rpm in IEC Centra-7R refrigerated centrifuge for 15 minutes and the supernatant was discarded. The cell pellet was resuspended in 20 ml/tube TFB-1 (30mM potassium acetate, 100mM rubidium chloride, 10mM calcium chloride, 50mM manganese chloride and 15% v/v glycerol, pH 5.8) gently and placed on ice for 15 minutes. Cells were again pelleted as before and resuspended in 4 ml/tube TFB-2 (10mM MOPS, 75mM calcium chloride, 10mM rubidium chloride and 15% v/v glycerol, pH 6.5) and placed on ice. The cells can now be used immediately or frozen. For freezing, the cells were aliquoted out into Eppendorf tubes which had been placed on ice, and snap-frozen in a ethanol-dry ice bath. Frozen aliquots were placed in the −70°C freezer and taken out and thawed just before use. Thawed out cells were not refrozen, but discarded after use.

2.6.2. Transformation of Bacterial Cells

Frozen competent cells were placed on ice to thaw and an Eppendorf tube was placed on ice. 5μl of ligation mix or 100ng of plasmid DNA was transferred to the tube and 50μl of thawed competent cells pipetted into the tube, mixed gently and incubated on ice for 30 minutes. The cells were heat-shocked for 90 seconds at 42°C without any shaking and placed back on ice for 5 minutes. 700μl of L-broth (without any antibiotic) was transferred to the tube and the cells incubated in orbital shaker for 45 minutes to allow expression of the ampicillin resistance gene. Cells were pelleted at 5000 rpm

for 2-3 minutes in a microfuge; all but 100μl of the supernatant was discarded and the cells resuspended and spread on a LB-agar plate with 100μg/ml of ampicillin. The plates were incubated at 37°C overnight.

2.6.3. Screening of Putative Recombinants

Single colonies were picked from the plates of ligation transformations and inoculated in 5 ml broth with 100 μg/ml of ampicillin and incubated at 37°C overnight. 2 ml of the culture was used for crude DNA minipreps. The DNA was digested with the appropriate enzymes to determine the successful ligation of the insert. In case of subcloning of PCR amplified fragment, after the confirmation of the presence of the insert, the inserted fragment was sequenced using the appropriate primers.

2.7. Sequencing of Plasmid DNA

All sequencing reactions were carried out using Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science cat. no. US 79750) and Redivue™ ^{33}P labeled dideoxynucleotides (Amersham Life Sciences AH 9538). This method is based on PCR amplification of the DNA to be sequenced, using ^{32}P-labelled dideoxynucleotide terminators and has the advantage of producing good results using low amount of template DNA.

For each plasmid to be sequenced, 2μl of the termination master mix (dGTP) was dispensed in four thin walled PCR tubes (termination tubes) and labelled as G, A, T and C. 0.5μl of each of the four ^{33}P labeled dideoxynucleotides (ddNTPs) was added to the respective tubes and kept on ice. 200-600μg of DNA was mixed with 2μl of reaction buffer, 2 pmol of primer made up to 18.5μl with sterile distilled water: Lastly, 1.5μl (4U/μl) of Thermo Sequenase polymerase was added. 4.5μl of this reaction mix was added to each of the four termination tubes, mixed well and layered with mineral oil. Tubes were capped, placed in a thermal cycler and cycled 30 times with each cycle consisting of heating at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. 4μl of stop solution was added to each termination mix tube. The samples were frozen at −20°C at this stage for running the next day.

Tubes were heated at 70°C for 2-5 minutes and placed on ice, immediately before the loading on a glycerol tolerant sequencing gel. 3-5μl of reaction was loaded in each well.

2.7.1. Denaturing Polyacrylamide Gel Electrophoresis for Sequencing

The gels was cast in the Biorad Sequi-Gen sequencing cell system. The glass plates were thoroughly cleaned with detergent and then wiped to dryness with 70% ethanol. The plates were assembled in the apparatus, separated by red 0.4 mm spacers. 6% polyacrylamide, 8M urea gel was made up using 10X TTE buffer (108g Tris base, 36g Taurine, 2g $Na_2EDTA.2H_2O$ per litre, pH 8.0) to a final 1X TTE concentration, 30% Acrylamide/bis-acrylamide 19:1 solution (Biorad cat. no. 161-0154) and polymerized using 0.001 volume of ammonium persulphate and TEMED (N,N,N',N'-tetramethylethylenediamine) each. Gel was poured using a 50 ml syringe and allowed to set overnight. Electrophoresis conditions were usually constant 1800 Volts and the gel was pre-run for 1-1.5 hours to bring up the temperature to 45-50°C before the samples were loaded. The voltage was altered to keep the temperature of the gel between 50-55°C. At the end of the run, the plates were separated and the gel transferred to a Whatman 3MM paper, covered in Saran-wrap and dried in a Hybaid Gel-Vac gel-drier for 1 hour. The gel was then exposed to Kodak Biomax MR film and the autoradiograph developed 16-24 hours later.

2.8. Eukaryotic Cell Culture

Animal cell culture was used routinely for maintaining and propagating established and primary cell lines required for generating adenoviruses and as gene expression systems.

2.8.1. Growth and Maintenance of Cell Lines

Generally cells were maintained in Minimal Essential Medium (MEM; Gibco-BRL cat. no. 21090) or Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL 41965), supplemented with 10% Foetal Bovine Serum (FBS or FCS; Gibco-BRL cat. no. 10108), 2mM Glutamine (using 100X (200mM)

Glutamine, Gibco-BRL cat. no. 25030) and 100 IU/ml of Penicillin and 100μg/ml of Streptomycin using 100X Penicillin/Streptomycin (10,000 IU Penicillin- 10,000 μg Streptomycin, cat. no. 15140). The usual method for propagation of cells was as monolayers to logarithmic phase in a 175 cm^2 tissue culture flask. Cells were normally subcultured upon reaching 70-80% confluency. The cell monolayer was washed twice with 8 ml PBS and detached using 1 ml of Trypsin-EDTA (Gibco-BRL cat. no. 45300). The detached cell were diluted 10-20 fold in the appropriate medium and pelleted by centrifugation (IEC Centra-7R table top refrigerated centrifuge), at 1000 rpm for 10 minutes The pellet was resuspended in 10 ml of appropriate medium and split in the required aliquots into fresh tissue culture vessels.

Cells with special growth requirements such as HEK-293, and primary cardiocytes were treated differently. Milder methods of cell detachment and varied conditions of medium were employed for these cells which are discussed later.

2.8.2. Freezing and Thawing of Cell Lines

Most cell lines were frozen down for storage by first detaching the cells by trypsinizing. Detached cells were diluted 20 fold and pelleted as described above and resuspended in appropriate medium supplemented with 20% serum except where stated. An equal amount of medium with 20% DMSO was then added dropwise over a period of 3-5 minutes to avoid shock to the cells. The cells were then aliquoted into cryovials (1.8 ml) and frozen by cooling at the rate of 1°C/min using a Nalgene Cryo 1°C freezing container in a −70°C freezer overnight after which they were transferred to the vapour phase of liquid nitrogen.

293 cell were first detached from the culture vessels using citric saline as described above. Detached cells were diluted in 5 volumes of growth medium and the cells were pelleted by centrifugation (200g for 5 minutes). The supernatant was discarded and the pellet resuspended in 100% foetal bovine serum. $1/10^{th}$ volume of sterile DMSO was added slowly to the serum/cell suspension and the mixture was aliquoted into sterile freezer vials. The vials

were placed in Nalgene Cryo 1°C freezing container for 1 hour at 4°C followed by 16-20 hours at −70°C. Vials were then moved to the vapour phase of liquid nitrogen, for long term storage.

Frozen vials of all cell lines were thawed rapidly in a 37°C water bath. Vials were washed with 70% ethanol after which the cell suspension was transferred under aseptic conditions to a sterile tissue culture petri dish. 5-6 ml of appropriate medium was added to the cells dropwise over 3-5 minutes while gently agitating the vessel. A further 10 ml of medium was added and cells were then incubated at 37°C in 5% CO_2 overnight. The medium was changed the next day to remove the cryopreservative containing medium and cells were then allowed to grow as described above.

2.8.3. Special Buffers and Solutions for Cell Culture

DNase stock was prepared as 2 mg/ml Deoxyribonuclease II (Sigma Cat. No. D-8764) in 0.15M NaCl, filter sterilized and frozen down at −20°C in aliquots. Trypsin (100 mg/ml solution in sterile water) was dissolved by stirring overnight at 4°C, filtered through a Whatman No 1 filter paper and filter sterilized using a bottle top filter. Trypsin stock was stored at −20°C in aliquots. 100X BrdU (30.74 mg 5-Bromo-2'-deoxyuridine, Sigma cat. no. B-5002) was dissolved in 10 ml of MEM, filter sterilized, aliquoted and frozen at −20°C : Once thawed, aliquots of BrdU stock were not refrozen.

Calcium and Bicarbonate Free Hanks with HEPES (CBFHH) buffer was made from stock solution of its components. The stock components were: NaCl (200g/L), 40 ml to 136.9mM; KCl (40g/L) 10 ml to 5.36mM; $MgSO_4 .7H_2O$ (20g/L) 10 ml to 0.81mM; KH_2PO_4 (6g/L) 10 ml to 0.44mM; $Na_2HPO_4.7H_2O$ (9g/L), 10 ml to 0.34mM; HEPES pH 7.4 (47.66 g/L), 100 ml to 20mM and Glucose (100g/L) 10 ml to 5.55mM: 800 ml of distilled water was added and the pH was adjusted to 7.5 with NaOH. 10 ml of Penicillin-Streptomycin (100X) was added and the volume raised to 1000 ml. The buffer was filter sterilized using a bottle top filter and stored at 4°C.

Citric Saline used for detaching 293 cells was made as 10X stock (1.350M KCl and 150mM sodium citrate), sterilized by autoclaving and stored at room temperature. Working concentration for citric saline was 1X and was made using the stock when required and sterilized by autoclaving.

2.8.4. Cell Lines

Established human and rodent cell lines were used as *in vitro.* systems for analysing foreign gene expression and for making recombinant adenoviruses. The cell lines were maintained and propagated according to the cell culture protocols provided by the suppliers. In addition, primary cells such as cardiocytes were routinely isolated and cultured when required, using specially devised methods described later in this section.

2.8.4.1. Human Embryonic Kidney 293 Cells

Low passage cells (at passage 25), were obtained from Microbix Biosystems, Toronto, Canada. A frozen vial was thawed and plated on to a 100 mm tissue culture petri dish in Minimal Essential Medium (MEM) supplemented with 10% foetal bovine serum, 2mM Glutamine and 100 IU/ml Penicillin/ 100 µg/ml Streptomycin, and incubated at 37°C in 5% CO_2. Cells were grown to 80-90% confluency and split 1:3. A fraction of this batch was frozen down at the lowest (27-29) passage possible in order to provide stocks of low passage cells.

Cells were maintained in logarithmic growth by subculturing every 3-4 days in 175 cm^2 tissue culture flasks (Nunc). Cells were split when at 80-90% confluency by aspirating off spent medium, and washing twice with 10 ml. 1X citric saline (135mM KCl and 15mM sodium citrate). After the wash, 2ml citric saline was layered on the monolayer and incubated at 37°C for 5 min to detach the cells. Complete detachment of the cells was achieved by tapping the sides of the flask. 10-12 ml. of fresh medium was added to the dislodged cells and these were dispersed by pipetting. The suspended cells were split 1:3 or 1:4 and aliquoted into fresh 175 cm^2 tissue culture flasks. Media was added to a final volume of 18-22 ml. per flask and incubated as described above.

2.8.4.2. C2-C12 Myogenic Cell Line

Cells were obtained from our lab liquid nitrogen stock. The cells were cultured in Dulbecco's Modified Eagles Medium with sodium pyruvate (Gibco cat. no. 41965) supplemented with 20% foetal bovine serum, 1% chick embryo extract and 100U/ml Penicillin/100µg/ml Streptomycin. Cells were passaged every 2-3 days and generally split 1:10 to 1:20 as required, after detaching by trypsinization using Trypsin-EDTA.

2.8.4.3. Cardiac Fibroblasts

Cardiac fibroblasts were obtained as a by-product of the primary cardiocyte preparation, during the preplating step. These cells are maintained in MEM (5% FCS, 2mM Glutamine and 100µg/ml of Streptomycin and 100U/ml of Penicillin.) At 70-80% confluency, the cells were subcultured by detaching the cells using Trypsin-EDTA (Gibco-BRL) as described earlier.

2.8.4.4. NIH-3T3 Mouse Fibroblasts

NIH-3T3 Swiss mouse fibroblast cells were obtained as a frozen stock from the European Collection of Cell Culture (ECACC) at the Centre for Applied Microbiology & Research (CAMR), Salisbury, UK. Cells were thawed out and maintained in DMEM supplemented with 10% Calf serum, 2mM Glutamine and 100U/ml of Penicillin/100µg/ml Streptomycin. Upon reaching 70-80% confluency, the cells were subcultured by detaching the cells using Trypsin-EDTA (Gibco-BRL) and diluting 1:10 to 1:20 as required.

2.8.4.5. Primary Culture of Neonatal Cardiocytes

Rat neonate cardiocyte culture procedure was modified from protocols devised by Paul Simpson (Simpson and Savion, 1982). Sprague-Dawley rats from day of birth to 4 days old, were used to obtain hearts for primary cardiocyte culture. 30 to 40 animals were sacrificed by Euthatal injection and hearts exposed and isolated using sterile curve forceps and scissors. Isolated hearts were transferred to a 100mm tissue culture dish containing 10-12 ml of PBS. From this stage onwards, all manipulations were done under sterile conditions in a class II safety cabinet.

All non-cardiac tissues were removed from the heart using fresh sterile surgical tools and the cleaned hearts were transferred to a 100 mm dish

containing 10 ml of CBFHH buffer. Hearts were cut in half and CBFHH buffer aspirated off. Hearts were rinsed twice with 10 ml of the buffer to remove blood. 4-5 ml of buffer was added and hearts were finely chopped into about 2 mm size cubes using pointed scissors over 5-8 minutes. Heart tissues were transferred into a 50 ml falcon tube using a 10 ml pipette. Remaining tissue was transferred completely by rinsing the plate twice and transferring the mixture to the falcon tube. After the tissue segments have settled, the CBFHH supernatant is removed carefully.

10 ml Trypsin solution was added to the cells and the tube shaken for 20 minutes on an automatic shaker at room temperature. Tissue pieces were allowed to settle and supernatant transferred to a collecting tube with 2 ml of foetal bovine serum. Another 10 ml of trypsin solution was added to the tissue and shaken as before for 15 minutes The supernatant was transferred to the collecting tube. 9 ml of DNase solution was added to the tube and the tissues pipetted up and down several times to shear and degrade the DNA released from the cells. The trypsinization and DNase treatment steps were repeated until the pellet became white.

Collecting tubes with cardiocytes were spun at 1000 rpm for 5 minutes. Supernatant was collected carefully and discarded. Cell pellets were pooled after addition of 2 ml of MEM per tube and gentle, thorough resuspension. Final cell volume was made up to 20 ml. 200µl DNase stock was added after which cells were pipetted up and down several times until visible clumps were dispersed. Cells were then pelleted at 1000 rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 32 ml of MEM medium.

Four 100mm tissue culture plates were taken and the lids removed in aseptic conditions under a flow hood. A sterile steel sieve was prewetted with MEM medium and 8 ml of cell suspension was poured through the sieve into each of the four plates. The plates were incubated at 37°C in a 5% CO_2 for 45 minutes. This pre-plating was done to eliminate the fibroblast content of the cell suspension.

After the pre-plating, plates were swirled to detach any partly attached cardiocytes and the cell suspension transferred to a 50 ml falcon tube. 2 ml of MEM was added to each of four plates which were tapped to dislodged incompletely detached cardiocytes and the cell suspension was then transferred to the falcon tube. 5 ml of MEM was pipetted through the four plates to completely detach the remaining non-adhering cardiocytes which were then transferred to the falcon tube. The final volume of the cardiocytes suspension was raised to 45 ml. In addition, the adherent cardiac fibroblasts were then grown by adding 10 ml of MEM medium and maintaining as described in section 2.8.4.3.

Cardiac myocytes were centrifuged at 1000 rpm for 5 minutes and the pellet resuspended in 32 ml of MEM medium with 1% BrdU. Cells were plated into 60mm or 100mm tissue culture dishes and incubated at 37°C in 5% CO_2. After 24 hours, cardiocytes were checked for contractions after 24 hours incubation and used either for transfections or viral infections.

2.9. Transfection of DNA into Eukaryotic Cells

Plasmid DNA was transfected into cells in culture for analysing gene expression and for making recombinant adenoviruses. Generally, caesium purified DNA was used for transfecting the cell lines, employing the $CaPO_4$ or the liposome transfection methods.

2.9.1. $CaPO_4$ Transfection Method

$CaPO_4$-DNA coprecipitation method was used mainly for transfections in the production of recombinant adenoviruses. A modification of the method devised by Graham F.L. *et al* (Graham and Van der Eb, 1973) was used. 250µl of 2X HBS (50mM HEPES, 274mM NaCl, 10mM KCl, 1.5mM Na_2HPO_4, and 11mM Glucose, pH 7.1) was taken in a 5 ml polystyrene tube and 0.002 volume of salmon sperm DNA (10 mg/ml) was added and the buffer-carrier DNA solution syringed through a 21G hypodermic needle a few times to shear the carrier DNA. 5-10 µg of plasmid DNA to be transfected, is made up in an Eppendorf tube to 225µl with tissue culture grade distilled

water and 25µl of 2.5M $CaCl_2$ added dropwise with slow vortexing. The DNA-$CaCl_2$ mix was added dropwise to the HBS-carrier DNA in the tube and incubated for 5 minutes at room temperature or to the point of formation of a visible milky precipitate. The calcium phosphate-DNA precipitate was then added to the medium on a dish of appropriate cells and incubated for 6-18 hours. After this incubation time the media was replaced with 5 ml of fresh medium and left for 36-48 hours to allow for expression of the desired gene. In case of production of recombinants, the media is removed after incubation and overlayed with soft agar consisting of BME Basal medium with 1.1% agarose as described in section 2.10.2., allowed to solidify at room temperature and incubated at 37°C in a 5% CO_2 incubator.

2.9.2. Liposome-mediated Transfection

Cationic liposome-mediated transfection of DNA was used mainly to study transient expression of genes using plasmid DNA. Cells were grown to a confluency of 70-80% using the appropriate medium with a reduced serum supplement (2-5% FCS) in a 60 mm tissue culture dish. Spent medium was replaced with 5 ml of fresh low-serum medium, 3 to 12 hours before the transfection. The liposome, DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) from Boehringer Mannheim (cat. no. 1202375) was used for transfection. 5µg of plasmid DNA was diluted in HBS (HEPES Buffered Saline, 20mM HEPES, 150mM NaCl, pH 7.4) to a final concentration of 0.1 µg/µl, in a total volume of 50µl using an Eppendorf tube. A separate polystyrene analyzer cup was used to dilute 30µl of DOTAP to 100µl using HBS. The DNA solution was added to the diluted DOTAP and mixed carefully by gentle pipetting several times. The transfection mixture was incubated at room temperature for 10-15 minutes and then transferred to the cell and mixed thoroughly with medium by rocking the dish. The cells were incubated at 37°C in a 5% CO_2 incubator with the DOTAP/DNA mix for 4-16 hours and then replaced with fresh medium and incubated for another 36-48 hours to allow for expression of transfected gene.

2.10. Construction of Recombinant Adenoviruses

Recombinant adenoviruses were constructed using the method developed by Frank Graham *et al* (Graham and Prevec, 1995; Graham and Prevec, 1997) which utilizes the phenomenon of homologous recombination between the large adenoviral genome-carrying plasmid and smaller shuttle plasmid carrying the foreign gene of interest in 293 cells.

2.10.1. Cotransfection of Adenoviral and Shuttle Plasmids

The adenoviral genome carrying plasmid pJM17 and the appropriate shuttle plasmid carrying the gene of interest were cotransfected into 293 cell lines using CaPO4-mediated DNA transfection described in section 2.9.1. The 293 cells were split 2 days before the transfection and grown to 60% confluency. The spent medium was replaced with fresh MEM with 5% FCS and 1mM glutamine, 12 to 24 hours before the transfection. For the transfection, the larger pJM17 and the smaller shuttle plasmids (pCA13, pCA14 or pΔE1sp1A or pΔE1sp1B, carrying the appropriate cassette of promoter and cDNA) were mixed in quantities of 5μg + 5μg, 5μg + 10μg and 10μg + 10μg. The DNA was coprecipitated and each of the three mixes transferred to the 60 mm dish of 293 cells at 70 to 80% confluency. The DNA-CaPO4 precipitate was kept over the cells to settle for at least 6 hours, allowing uptake of DNA-CaPO4 precipitate, after which it was overlayed with soft agarose medium.

2.10.2. Soft Agarose Overlay

After the transfection, the cells were overlayed with BME with soft agarose and incubated at 37°C in 5% CO_2 incubator. This overlay was prepared using equal volumes of 2X BME (Basal Medium (Eagle); cat. no. 21017), and melted and cooled 2.2% LMP agarose (2.2% w/v agarose type VII, Sigma cat. no. A-4108 in distilled water, autoclaved) supplemented with 5% CELLect GOLD foetal bovine serum (ICN cat. no. 2916849). The agarose was layered onto the transfected cells when cooled down to about 32-34°C gently and allowed to solidify at room temperature for 15-20 minutes. The dishes were observed daily for cell health. On day 3, post-cotransfection, the cells were overlayed with another 5 ml of BME/agarose overlay and incubated further.

Cells were observed daily for any signs of rounding up of cells or initiating plaques. On day 7 of transfection, cells were overlayed with 3 ml of MEM/agarose prepared by mixing equal amounts of 2X MEM (cat. no. 21935) and 2.2% LMP agarose, supplemented with 10% foetal bovine serum and 100 IU/ml of Penicillin and 100μg/ml of Streptomycin. Incubation was continued in the same conditions as before, and the dishes screened daily for plaques.

2.10.3. Plaque Selection and Recombinant-screening

Plaques were marked and picked up as soon as they were visible by the naked eye. The agarose layer was stabbed with plugged short-form pasteur pipettes with bulb attached to suck in the agar plugs. The agar plugs were transferred to an Eppendorf tube with 500μl of Dulbecco's PBS (with Ca^{++} and Mg^{++}) and rinsed thoroughly to mash and suspend the agarose. 150μl of this plaque suspension was used to infect 293 cells grown to 70-80% confluency in a 60 mm tissue culture dish. The dish was incubated at 37°C in a 5% CO_2 atmosphere for 45 minutes to 1 hour and then 3-4 ml of MEM was added to the dish and the incubation continued up to 7 days or until the appearance of a full cpe. Once the desired level of cpe was reached, the cells were harvested as described in section 2.11.1. and the cell lysate used for DNA minipreps as described in section 2.11.5. 4/5 th of the DNA isolated was digested with *Hin*dIII restriction enzyme and the banding pattern produced compared with the Ad5-dl309 bands to check for potential recombinants. All recombinants were passed through at least two rounds of plaque purifications to eliminated any wild type virus contamination.

2.11. Adenovirus Growth, Propagation and Analysis

Recombinant adenoviruses were amplified in 293 cells grown in culture and subsequently purified on a discontinuous caesium gradient if required. Recombinant purity was analysed using *Hin*dIII restriction analysis of viral DNA.

2.11.1. Adenovirus Propagation

Both replication competent Ad-5 dl309 and all the replication deficient adenoviruses were propagated in Human Embryonic Kidney (HEK) 293 cells. Cells were grown to 80-90% confluency in an appropriate tissue culture vessel and seeded with a known adenovirus stock at 10^6 pfu. Infected cells were incubated at 37°C with 5% CO_2 for up to 7 days or until the appearance of cytopathic effect (cpe), apparent by the rounding up of cells and consequently forming of bunches and detaching from the surface of the culture dish. Cells were harvested at the stage where half the cells were rounded up and formed bunches and half rounded up but still adhering to the surface. Cells were scraped off using a cell scraper, transferred with the spent medium to a universal container and spun at 1000 rpm in a IEC Centra-7R centrifuge for 10 minutes. The cells pellet was resuspended in an appropriate volume of Dulbecco's PBS (with Ca^{++} and Mg^{++}) and transferred to an Eppendorf tube. To obtain the virus, the cell suspension was freeze-thawed in dry-ice and a 37°C waterbath 3-4 times, the cell debris removed by centrifugation at 8000 rpm for 3-5 minutes in a microfuge, and supernatant transferred into a fresh tube and labelled appropriately. This crude lysate was then either purified on a CsCl gradient or used *per se*. The crude virus suspension was titred for the amount of virus present before proceeding with its application.

2.11.2. Titration of Adenovirus

Purified virus solution or crude virus preparations were titred for their potential of forming individual plaques on 293 cells. This amount reflected the actual potency of the virus solution in terms of plaque forming units (pfu). The virus suspension was diluted serially down to a dilution of 10^{-14} in Dulbecco's PBS (with Ca^{++} and Mg^{++}), and 100µl each of dilutions from 10^{-5} to 10^{-14} were used to infect 60 mm 293 cell dishes at 70-80% confluency. The dilutions were spread evenly over the monolayer by rotating and tilting the dishes and incubated at 37°C in a 5% CO_2 atmosphere for 30-45 minutes. After the adsorption, all free running liquid was aspirated off the monolayer, and the

agar overlay was prepared: MEM (supplemented with 10% FCS, 2mM Glutamine, 100U/ml of Penicillin and 100μg/ml of Streptomycin) was warmed to 37°C and 0.2 volume of 3.2% Noble agar, melted and cooled to 38-40°C, was added and mixed thoroughly. The soft-agar medium was overlayed over the infected cell monolayer and allowed to solidify at room temperature for 15-20 minutes. The dishes were observed daily for plaque formation. After 5-6 days, all dilution plates which showed countable plaques were counted. The number of plaques appearing in a plate was multiplied by the dilution factor and two or three consequent dilution counts were averaged to give the plaque forming units (pfu) per ml.

2.11.3. Plaque Purification of Adenoviruses

The phenomenon of a chance recombination between the recombinant virus genome and E1A region in the 293 cells necessitates that all recombinants are plaque purified at regular time intervals to maintain the purity of the virus stock, and avert contamination by fortuitously forming replication competent viruses. Also new recombinants were subjected to three rounds of plaque purification immediately after their production. The virus lysate/suspension was diluted serially down to dilution of 10^{-8} in Dulbecco's PBS (with Ca^{++} and Mg^{++}), and 100μl each of dilutions from 10^{-2} to 10^{-8} were used to infect 60 mm 293 cell dishes at 70-80% confluency, spread evenly over the monolayer and incubated at 37°C in a 5% CO_2 atmosphere for 30-45 minutes. After the adsorption, all liquid was aspirated off the monolayer, and the agar overlay was prepared: MEM (supplemented with 10% FCS, 2mM Glutamine, 100 IU/ml of Penicillin and 100μg/ml of Streptomycin) was warmed to 37°C and 0.2 volume of 3.2% Noble agar, melted and cooled to 38-40°C, was added and mixed thoroughly. The soft-agar medium was overlayed over the infected cell monolayer and allowed to solidify at room temperature for 15-20 minutes. The dishes were observed daily for plaque formation. After 5-6 days, several individual, well isolated plaques were picked and reinfected onto 293 cells for amplifying the virus as described in section 2.11.1. Part of the lysates from these dishes were used to extract DNA and their *Hin*dIII digest pattern

observed as described. Lysates showing the expected or non wild-type DNA *Hin*dIII digest pattern were retained. Retained virus lysates were diluted 1:4 in 5X virus storage buffer (100mM NaCl, 50mM Tris-HCl, 5mM $MgCl_2$, 0.5% bovine serum albumin (BSA), 50% glycerol), and frozen at −70°C in properly labeled tubes. The expected *Hin*dIII digest pattern of a recombinant was determined using the Vector NTI software as described in section 2.16.

2.11.4. Purification of Adenovirus Using CsCl Gradient Centrifugation

Three to four 175 cm^2 tissue culture flasks with 293 cells at 80% confluency were seeded with 10^8 titre virus stock at 1 ml per flask. The virus infected cells were taken through the steps of virus propagation to the cell lysate stage as describe previously. Cell lysate containing the high titre virus was then loaded on to a discontinuous CsCl density gradient prepared in a 14 ml transparent tube (Beckman Ultra-Clear™ Centrifuge Tube cat. no. 344060). The gradient was made by pipetting 2 ml of 1.45g/ml CsCl (20.5 g of CsCl, 2.9 ml 500mM Tris pH 7.9, distilled water 25.8 ml) into the tube first followed by layering 3 ml of 1.32g/ml CsCl (32 g CsCl, 6.8 ml 500mM Tris pH 7.9, distilled water 61.2 ml). A third layer of 2 ml 40% glycerol (40 g glycerol, 10mM Tris pH 7.9, 1mM EDTA pH 8.0) was pipetted gently onto the caesium layers and topped up with virus lysate. Any remaining space from the top of the tube was filled up with Dulbecco's PBS (with Ca^{++} and Mg^{++}). The tubes were balanced and spun in LKB Bromma 2332 Ultrospin 85 ultracentrifuge using RPS40T-859 rotor at 25000 rpm for 1.5 hours at 4°C. The virus band was drawn out by piercing the side of the tube with a 18G hypodermic needle and the opalescent band with the virus was allowed to drip into a bijou bottle. The purified virus was desalted by dialysing against 10mM Tris pH 8.0, 1mM EDTA for 3 hours to overnight with several buffer changes. The virus was titred and then stored in 1X virus storage buffer using 5X virus storage buffer (see section 2.11.3.), and stored at −70°C.

2.11.5. Small-scale Isolation of Viral DNA (minipreps)

293 cells were grown as described in 60mm culture dishes, to a confluency of 80-90% and overlayed with a plaque agar suspension, for screening of

recombinants, or with crude cell lysate of a high virus concentration (10^6-10^8pfu/ml) or with purified viral solution. 100-150µl of viral solution was layered on to the cell monolayer (per 60 mm dish) after aspiration of growth medium. After an incubation of 45 minutes with the virus suspension, MEM was added and cells incubated till the appearance if cpe and harvested as described in section 2.11.1. The cell pellet was resuspended in 600-750µl of Dulbecco's PBS (with Ca^{++} and Mg^{++}; Gibco-BRL cat. no. 140040) and transferred to a sterile 1.5 ml Eppendorf tube. The cell suspension was frozen on dry-ice and thawed in a 37°C waterbath with 3-4 repetitions to lyse the cells and release virus. Cell debris was removed by centrifugation in a microfuge at 8000 rpm for 3-5 minutes and the supernatant containing the released virus was collected.

Cell lysates containing virus were transferred to fresh Eppendorf tubes and digested with Proteinase-K (Sigma cat. no. P-2308) at 1 mg/ml in presence of 5mM EDTA pH 8.0 and 0.5% SDS at 37°C for 4 hours. The digest mixture was phenol-chloroform extracted once, chloroform extracted and then ethanol precipitated as described in section 2.5.2. DNA was resuspended in 35-40µl of TE and the solution RNase treated with 1µl of RNase (10 mg/ml). 25-30µl of the DNA was employed for restriction analysis, typically by *Hind*III digestion where the restriction pattern was compared with that of the wild type Ad5-dl309 virus or with the known digest pattern of the recombinant virus.

2.11.6. Transduction of Cells by Recombinant Adenovirus

Cells grown in culture were infected with recombinant adenoviruses to establish expression of the transgene. Generally, the appropriate cell line was grown to 60% confluency in a 100mm tissue culture dish as described in the sub-section of section 2.8.4. The spent medium was aspirated off and 250-300µl of adenovirus lysate or suspension, at a titre of 10^{-6} to 10^{-10}, was layered on to the cells and incubated for at least 45 minutes at 37°C. Fresh medium was pipetted on the infected cells and incubated at 37°C in a 5% CO_2 incubator for 24 to 48 hours to allow for expression of the transgene.

The transduced cells were analysed qualitatively for gene expression by staining with appropriate stains or immunostaining, or harvested for protein analysis and western blotting.

2.12. Gene Amplification and Analysis

DNA amplification was generally done to obtain larger amounts of DNA for subcloning. PCR was also used as a first screening step for checking plasmid and adenovirus recombinants. Southern analysis was performed to confirm recombinants with the appropriate DNA inserts.

2.12.1. Polymerase Chain Reaction (PCR)

Polymerase chain reaction was routinely used for amplification of DNA for obtaining high amount of insert DNA with specific restriction sites incorporated at either ends, facilitating directional cloning. Screening of positive recombinant plasmids with the inserts cloned and for checking for the presence of recombinant adenoviruses in a mixed population, or for contamination of wild type viruses in recombinant stocks, was also carried out using PCR amplification using the appropriate primers.

2.12.1.1. DNA Amplification

A typical PCR reaction was carried out in a total volume of 50µl using Promega PCR kit (cat. no. 18038) with 1X PCR buffer, 5mM Mg^{++}, 200µM of each dNTPs (made from a 5mM dNTPs mix prepared using 4 dNTPs, (Promega cat. no. U1240), 100 ng of template DNA, 1nmol each of the two primers.

Proof-reading polymerase were also used to amplify DNA for subcloning purposes. *Pfu* polymerase (Stratagene cat. No. 600135) was used in a *Pfu-Taq* mix of 1:6 and 1:10. A typical 50µl reaction mix consisted of 1X *pfu* buffer, 200µM each of dNTPs (made from a 5mM dNTP stock mix, 100 ng of template DNA, 1nmol each of the two primers and 0.5µl of the polymerase mix. The EXPAND™ High Fidelity PCR System (Boehringer Mannheim, cat. no. 1-681-834) was also used with similar concentrations of dNTPs, template

DNA and primers as described above for *Pfu* (for primer details, see table 2.1)

All PCR reactions was overlayed with a few drops of light mineral oil (Sigma cat. no. M-3516) and subjected to an appropriate thermal cycling program in a Hybaid Omni-gene thermal cycler. Thermal cycling programs were generally designed with an initial denaturing step at 94°C for 3-4 minutes. This was followed by 20-30 cycles of a 45 seconds denaturing at 94 C, 30 seconds primer annealing and then an extension step at 72 C. Primer annealing temperature was kept at 3 to 5 C higher than the calculated annealing temperature for the primer pair. Primer extension time was kept at 1 minute per kilobase of amplified product for Taq, and 1.5 minutes per kilobase for proof-reading polymerases.

2.12.1.2. PCR Screening of Viral Recombinants

For screening viral recombinants, the viral DNA was obtained as described in section 2.11.5. and 1/20th of the DNA used as template for PCR reactions as described in the previous section, using appropriate primers. The reactions and run on a 1% agarose gel as described in section 2.4.3. and checked for the samples amplifying the DNA bands of the expected sizes.

2.12.1.3. Screening of Recombinant Plasmids Using Colony PCR Method

For screening plasmid recombinants for subcloning, the colony PCR method was used for primary screening. Several single colonies of transformed *E.coli* DH5α were picked using a sterile tooth pick, and each suspended in 50μl of sterile distilled water in a PCR tube. The same tooth pick was then spotted on to a fresh, grid-lined bacterial culture plate. This was done to keep the colonies viable for later use, and the tubes and corresponding spotted culture labeled correctly. The suspended bacteria were then heated at 95-99 C in the heating block of a thermal cycler, or boiled in a waterbath for 5 minutes and allowed to cool down to room temperature. The cell debris was pelleted by microcentrifugation for 2 minutes at 10,000 rpm, and 10μl of the supernatant carrying the plasmid DNA transferred to a fresh PCR tube.

The bacterial culture plate was incubated at 37°C for overnight and then stored at 4°C for storage. PCR reaction was performed using appropriate primers on the template DNA in the supernatant as described in the previous section. The amplified bands were run on a 1% agarose gel as described in section 2.4.3. and the colonies amplifying the DNA bands of the expected sizes were retained for future use.

2.12.2. Southern Blot Analysis

DNA was digested with the appropriate enzymes and separated on a 1% agarose gel as described in section 2.4.3. The gel was photographed after aligning with a fluorescent ruler, and the 1kb ladder and any part not required was removed using a scalpel blade, before setting up the transfer. The transfer to Hybond-N+ membrane (Amersham International plc, cat. no. RPN 303B), was carried out using 0.4M, NaOH buffer as per instruction described in the protocol by Amersham. The pre-treatment recommended for DNA fragments greater than 10kb was not used due to the absence of any fragments of the size. The transfer process was done for 2-3 hours or overnight. After successful transfer of the DNA, the membrane was washed briefly in 2X SSC (300mM NaCl, 30mM Na citrate), with gentle agitation. Filter hybridization was carried out using the appropriate probe, labelled with ^{32}P as described in section 2.5.5. The DNA hybridization was carried out using the method described in section 2.5.6.

2.13. RNA Extraction and Purification

RNA was extracted from isolated tissues or cells grown in culture to study expression levels of endogenous and foreign genes at the level of transcription. RT-PCR or Northern Blot analysis were used to detect the specific RNA species from total RNA isolated.

2.13.1 RNA Isolation

Total cellular RNA isolation from tissue samples and cells grown in monolayers using RNAzol™B (Biogenesis Ltd, Poole, England). All tissue samples were frozen directly in liquid nitrogen and used immediately or

frozen at −70°C to later use. Frozen tissue was placed in a sterile ceramic mortar with pestle which was pre-cooled to freezing temperature by rinsing with liquid nitrogen and kept on dry ice. 2 ml of RNAzol™B was added per 100 mg of tissue and the tissue crushed to break into smaller pieces and then homogenized into a paste consistency.

Cells grown in monolayer were lysed directly in the tissue culture dish by adding 2 ml of RNAzol™B per 60 mm dish. RNA was solubilized by passing the lysate through the blue tip of pipette of a micropipette, a few times. RNA extraction for both material was similar from this point onwards. The cell lysate/homogenate and transferred to Eppendorf tube. 0.1 volume of chloroform was added to the homogenate, capped and shaken vigorously for 15 seconds and incubated on ice for 5 minutes. The suspension was centrifuged in a microfuge for 15 minutes at 4°C at 14000 rpm and the upper aqueous phase transferred to a fresh tube. An equal volume of isopropanol was added and the tube incubated on ice for 15 minutes. RNA was precipitated by centrifugation in a microfuge for 15 minutes at 14000 rpm. The supernatant was removed and the RNA pellet washed once with excess of cold 75% ethanol by vortexing and subsequent centrifugation for 8 minutes at 8000 rpm at 4°C. The pellet was dried briefly for 10-15 minutes, taking care not to over-dry, and then dissolved in appropriate amount of TE made in DEPC treated water. RNA was run on a 1% agarose gel prepared with fresh TBE buffer and run in cleaned tank with fresh running buffer to check for the quality of RNA. If no degradation was observed, the RNA was stored at −70°C until further use.

2.13.2. Agarose Gel Electrophoresis of RNA

A 1-1.5% (w/v) denaturing formaldehyde-agarose gel was made up by dissolving agarose in 10 ml of 10X MOPS (0.2M 3-[N-morpholino] propanesulphonic acid, 50mM sodium acetate, 10mM EDTA, adjusted to pH 7.0) and 73 ml DEPC treated distilled water. This was allowed to cool and 17 ml of 37% (v/v) formaldehyde added (final concentration of 0.66M formaldehyde) and EtBr was added to a final concentration of 5 μg/ml, the

solution mixed and poured into a gel mould. 15-20μg RNA was used and samples were concentrated by ethanol precipitation and redisolving in 25μl denaturation buffer (50% formamide (v/v), 1X MOPS, 6% formaldehyde) was added. This was heated at 65°C for 5 minutes, cooled on ice and 2.5μl of 6X loading buffer was added and the samples loaded into the wells and run in 1X MOPS buffer.

2.14. Protein Analysis

Analysis for the expression of recombinant proteins was carried out using SDS-PAGE and subsequent western blotting. Recombinant adenoviruses or plasmids were expressed *in vitro.* using cardiocytes or NIH-3T3 mouse fibroblasts systems, the total protein extracted and analysed for presence of the recombinant protein.

2.14.1. Extraction of Proteins for Analysis

Cells were infected with virus or transfected with expression plasmid vectors as described in section 2.11.6., and 48-72 hours later harvested for analysis. Cell monolayers were scraped off in Dulbecco's PBS and pelleted by spinning at 1000 rpm for 5-10 minutes in a IEC Centra-7R refrigerated centrifuge. The cells were subjected to either a freeze-thaw cycle or detergent lysis followed by TCA precipitation of proteins.

2.14.1.1. Cell Lysis by Freeze-thawing

The cell pellet obtained above was resuspended in 700μl of distilled water containing 1X of protease inhibitor mix made using 1000X stock (40mg/ml PMSF; Sigma cat. no. P-7626, 1mg/ml Aprotinin; Sigma cat. no. A-1153, 1mg/ml Pepstatin A; Sigma cat. no. P-4265). The cells were subjected to 3 to 4 cycles of freezing on dry-ice and thawing at 37°C. The cell lysate was microfuged briefly to pellet the cell debris and the supernatant collected. Protein concentration assay was done on these lysates and the appropriate amount used for loading on the protein gels.

2.14.1.2. Detergent-lysis and TCA Precipitation

The cell pellet was resuspended in 750µl of distilled water and 6.25µl of 2% (w/v) sodium deoxycholate added to lyse the cells followed by 250µl of 24% (w/v) Trichloroacetic acid (TCA) for precipitating the proteins. The reaction was kept on ice for 15 minutes with shaking every few minutes. The TCA precipitated protein was pelleted by centrifugation in a microfuge for 5 minutes at 14000g. The supernatant was discarded and the 75µl of 1M Tris base (high alkalinity) was added and the Eppendorf tube left at room temperature for 30 minutes. The pellet was then resuspended by repeated vigorous pipetting and 75µl of SDS-sample buffer (40mM Tris, pH 6.8, 4mM 2-mercaptoethanol, 2% SDS, 10% glycerol, 0.2% bromophenol blue) added and the protein mix and boiled for 10 minutes. The protein samples were loaded onto PAGE gels for resolving the proteins for western blotting or stored at −20°C for later use.

2.14.2. Quantification of Proteins

Protein concentrations were determined using the Bio-Rad Protein Assay Kit II (cat. no. 500-0002), following the Microassay procedure described in the kit protocol. Known standard dilutions of 3.5, 7.0, 14.0, 21.0 and 28 µg/ml were made and 0.8 ml of these standard dilutions were mixed with 0.2 ml of Dye Reagent Concentrate in an Eppendorf tube and mixed thoroughly. 0.8 ml of sample buffer with .2 ml of Dye reagent was used as a blank. The test sample were diluted 1:4, 1:10 and 1:100 and 0.8 ml of these dilutions mixed with 0.2 ml of Dye reagent. Absorbance of the test samples and standard were measured at 595 nm wavelength against the sample buffer/reagent blank after a period of 10 minutes to 1 hour. A graph for absorbance versus concentration of standards was plotted. The test sample concentrations were read off from this standard curve, and final concentrations determined, taking the dilution factor into account.

2.14.3. SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out in vertical slab gels for separating the proteins based on their molecular weights, mainly as a first step for carrying out immunoblotting. The gels were cast and run in a Hoeffer SE600 Electrophoresis Unit. The resolving gel was 15% Acrylamide/bisacrylamide 29:1 made up using 4X resolving gel buffer (1.5M Tris-HCl, pH 8.8, 0.4% SDS) polymerized with .01 volume of 10% ammonium persulphate and .001 volume TEMED. The gel solution was mixed and poured into an assembled gel sandwich till about 1.5 cm from the top of the plate. A few ml of 0.1% SDS was gently layered up to 1-3 mm above the gel, and left at room temperature for 1-2 hours, to allow for the gel to polymerize. The stacking gel was made as 5% Acrylamide/bisacrylamide 29:1, made using 4X stacking gel buffer (0.5M Tris-HCl, pH 6.8, 0.4% SDS), polymerized with .01 volume of 10% ammonium persulphate and .001 volume TEMED. The comb is placed in position and the stacking gel poured above the resolving gel after it has polymerized. After polymerization of the stacking gel, the gel unit was transferred to a Hoeffer SE600 Electrophoresis Unit, with electrophoresis buffer (25mM Tris, 192mM Glycine, 0.1% SDS, pH 8.3). 20-40μg of protein mixture, prepared as described in section 2.14.1., was loaded per well and run along with Kaleidoscope Prestained Standard molecular weight markers (Biorad cat. no. 161-0324), at 80-100V for 3-4 hours or at 40V overnight, for up to 16-20 hours. If required for western blot analysis, the separated protein bands in the resolving gel were transferred to a nitrocellulose membrane as described in section 2.14.5.1 and stained, if required, as described in section 2.14.3.1.

2.14.3.1. Staining of SDS-PAGE Protein Gels

The resolving gel was stained for visualizing protein bands, after the blotting process to check for evidence of transfer of proteins and to have a profile of the gel. The gel was soaked in Coomassie gel stain (1% w/v Coomassie Blue R-250, 10% acetic acid, 45% methanol)and agitated gently for 10-15 minutes, after which the stain was decanted gently and the gel rinsed with a

few changes of distilled water. The gel was then destained by submerging in Coomassie gel destain (10% methanol, 10% acetic acid) for up to 1 hour. For complete destaining, the destain solution was changed and left overnight with shaking.

2.15. Analysis of Gene Expression in Transiently Transfected Cells

Cardiocytes and mouse fibroblast cells were infected with recombinant viruses and the expression of the transgene analysed. Initially, β–galactosidase encoding adenovirus was used to transfect fibroblasts and later the adenovirus encoding skeletal troponin C, tagged with HA antigen, was used. Northern blot analysis was also carried out on some specific constructs. Western blot analysis was carried out for studying expression of the tagged protein.

2.15.1. β-galactocidase Expression and Staining

Cells infected with β-galactosidase-expressing adenovirus or transfected with a β-galactosidase-expressing plasmid were qualitatively checked for expression of β-galactosidase by staining the cells with X-gal. Cells were infected as described in section 2.11.6. and given 36-48 hours for expression of the reporter gene. Cells were then treated with Fixing solution (2% formaldehyde, 0.2% Gluteraldehyde in PBS) at 4 ml per 60 mm dish for 10 minutes at room temperature. The fixed cells were washed twice with PBS and then stained with X-gal staining solution (1 to 5mM X-gal, 5mM Potassium Ferricyanide, 5mM Potassium Ferrocyanide, 2mM Magnesium Chloride). The dishes were incubated in a 5% at 37°C for at least two hours to overnight and observed for blue stained cells under the microscope.

2.15.3. Reverse Transcribed - PCR

Total RNA was isolated from transfected/infected cells grown in culture using RNAzol™B as described in section 2.13.1. The RNA was reverse transcribed using MMLV Reverse Transcriptase using the Advantage™ RT-for-PCR Kit (Clonetech cat. no. K1402-1). The total RNA was reverse transcribed converting the population of mRNA molecules to cDNA by priming the RT

reaction with oligo$(dT)_{18}$ primer. The cDNA thus generated was used for PCR amplification using the appropriate specific primers using the method described in section 2.12.1.

2.15.4. Northern Blot Analysis

Total RNA was fractionated on a denaturing formamide/agarose gel as described in section 2.13.2., and transferred to Hybond-N+ membrane. Before proceeding with the transfer process, the integrity of the RNA separated was checked. This was done by visualizing the RNA under UV-light in the presence of EtBr and looking for undegraded 28S and 18S ribosomal RNA bands. The gel was photographed with a fluorescent ruler aligned along the side of the gel. The marker ladder, if present, and any excess gel, was removed with a scalpel blade, before setting up the transfer.

Transfer to the Hybond-N+ membrane was performed using the alkali transfer protocol outlined by protocols provided by Amersham. Transfer buffer was 0.5M NaOH and the process was continued for 2-3 hours, followed by rinsing the blot with 2X SSC (300mM NaCl, 30mM Na citrate) gently. The filter was hybridized with the appropriate probe labelled with ^{32}P as described in section 2.5.6.

2.15.5. Western Blot Analysis

The hemagglutinin (HA) epitope-tagged skeletal Troponin C expression was detected through western blotting by probing with the HA tag specific high-affinity rat monoclonal antibody.

2.15.5.1. Western Transfer

Protein samples were resolved on a SDS-PAGE gel as described in section 2.14.3. The electrophoretically resolved protein bands on the gel were electroblotted on to a ProBind 45 nitrocellulose membrane (Pharmacia Biotech, cat. no. 80-1247-86), and the expression of the protein of interest detected using immunochemical techniques. The resolving gel, the ProBind membrane and the foam were soaked in the transfer buffer (24mM Tris base, 194mM glycine, 20% v/v methanol) and the gel and membrane were sandwiched between blotting paper and foam. The transfer was carried out at

room temperature in a Hoeffer FE Series Transphor Electrophoresis Unit at 100V (constant voltage) for 1-1.5 hours or at 30V for overnight transfers.

2.15.5.2. Immunodetection of Tagged Foreign Protein

After the transfer of protein, the nitrocellulose membrane was washed a couple of times with Tris-buffered saline (TBS; 50mM Tris-HCl, 150mM NaCl, pH 7.5) and non-specific binding sites blocked by covering the membrane with 10 ml of Blocking solution (5% skimmed milk in TBS), with shaking for 2 hours to overnight at room temperature. The membrane was washed 3 times with TBS and covered with the appropriate dilution of the protein or epitope-tag specific antibody and incubated overnight with shaking at 4°C. The membrane was washed 3 times with Wash buffer (TBS with 0.1% Tween®20). and incubated with the compatible Horseradish peroxidase, (HRP)-conjugated secondary antibody, diluted in blocking solution, for 1-2 hours with shaking. Membrane was washed once with washing buffer to remove any excess secondary antibody and then three times with TBS to remove traces of Tween®20. Excess wash buffer was dripped off for 30-45 seconds and the membrane transferred to a shallow container with the protein side up. 1ml each of solution A and B of the ECL Western Blot detection reagent kit (Amersham Life Sciences RPN-2109) were pipetted into one side of the container. The solutions were mixed thoroughly and the container tilted so that the surface of the membrane is evenly coated with the developing reagents. The membrane was then lifted up using a forcep, dripping off the excess liquid, and put in between two pieces of Saran-Wrap. The membrane was then immediately exposed to X-ray film for exposure times ranging from 5 seconds up to 10 minutes.

2.15.6. Immunocytochemistry

This method was specially used to detect the expression of the HA epitope-tagged sTnC protein, following the appropriate recombinant adenovirus infection on cells grown in tissue culture. For expression studies involving immunostaining, the cells were grown in Slide Flask (Nunc cat. no. 170920) and grown overnight to 48 hours and then infected with the virus at a

titre of approximately 10^5 to 10^6 as described in section 2.11.6. After allowing 48-72 hours for expression, the media was aspirated off and the cells were fixed with 1-2 ml of freshly prepared 4% paraformaldehyde (Sigma P-6148; w/v in PBS), for 10 minutes. The fixed cells were then washed three times with PBS allowing 3-5 minutes per wash, and the cells permeabilized by incubating with 0.1% Triton X-100 in PBS for 3-5 minutes at room temperature. Slides were washed once quickly with 0.1% Triton X-100, made up in distilled water. Non-specific binding sites were blocked by incubating the permeabilized cells with the Blocking solution (0.5% Bovine Serum Albumin (BSA) Sigma A-4503, in PBS) for 15 minutes.

Three procedures of immunocytochemistry, employing varying primary antibodies were tried to obtain optimal results. The (1) direct method used a primary antibody conjugated to a fluorochrome and (2) indirect method used unconjugated primary antibody with (a) secondary fluorochrome-conjugated antibody or (b) secondary antibody conjugated to biotin.

2.15.6.1. The Direct Method

The direct methodology for immunocytochemistry is time saving as it does not require the secondary antibody. The cells were grown and treated in a similar way as described above. After the permeabilization of cells and blocking for non-specific binding, an appropriate concentration of the Anti-HA-rhodamine (Boehringer Mannheim, cat. no. 1666959), diluted in blocking solution, was used to cover the cells and incubated for 30-45 minutes. Rhodamine-labeled Phalloidin (Sigma P-1951) was also added along with the primary antibody at a concentration of 2μg/ml to detect the cells by staining the actin cytofilaments. The cells were washed 3 times with PBS allowing 5 minutes per wash. The flaskette part of the slide flask was removed and a small drop of Aquamount (BDH 36226-2H) mounting medium was dropped on the cells and a clean coverslip placed gently on it avoiding any air bubbles being trapped. Any excess mounting medium was blotted off and allowed to dry in the dark, at room temperature. After drying, the slides

were stored at 4°C or at -20°C until screening under a fluorescent microscope using the appropriate filters.

2.15.6.2. The Indirect Methods

This method uses unconjugated primary antibody, with a choice of using a fluorochrome-conjugated secondary antibody or a biotin-conjugated secondary antibody. The biotin-conjugated secondary requires a third streptavidin-enzyme or a streptavidin-fluorochrome conjugate. The streptavidin-fluorochrome conjugate can be visualized directly, whereas the streptavidin-enzyme conjugate is detected using a color development staining system.

2.15.6.2.i. Using Fluorochrome-conjugated Secondary Antibody

The appropriate cells were grown, infected, and then permeabilized and blocked as described above. Following the blocking step, cells were incubated with the Anti-protein or Anti-tag mouse monoclonal primary antibody in the appropriate dilution made in the blocking solution, for 30 minutes at room temperature. Phalloidin TRITC (Sigma P-1951) was also added as described earlier. The cells were washed three times with PBS and incubated with a suitable dilution of the Anti-mouse IgG (whole molecule) TRITC-conjugate (Sigma T-2402) secondary antibody for 30 minutes at room temperature. Cells were washed three times and the immunostained cells mounted by covering with a drop of Aquamount mounting solution and placing a coverslip on it. The cells were examined under a fluorescence microscope fitted with the appropriate filters or the slides stored at -20°C in the dark for later observations.

2.15.6.2.ii. Using Biotin-conjugated Secondary Antibody

This method uses a three-step immunostaining protocol followed by detection by Vector® Red staining which can be visualized under an ordinary microscope. Cells were grown, infected and permeabilized and blocked as described in section 2.15.6. Following the blocking step, cells were incubated with the High-Affinity Anti-HA primary antibody (Boehringer Mannheim cat. no. 1867423), in the appropriate dilution made in the blocking solution, for 30

minutes at room temperature. The cells were washed three times with PBS and incubated with appropriate dilution of the Anti-rat Ig-biotin $F(ab')_2$ fragments secondary antibody (Boehringer Mannheim cat. no. 1348779), made in blocking solution, for 30 minutes at room temperature. Cells were again washed three times and then incubated with a dilution of Streptavidin-Alkaline Phosphate conjugate (Boehringer Mannheim cat. no. 1089161), made in blocking solution, for 10 Minutes. Cells were again washed three times, with PBS and stained as described in section following.

2.15.6.2.ii.a. Vector® Red Staining and Counterstaining

The alkaline phosphatase immunolabeled cells were then stained using the Vector® Red Alkaline Phosphatase Substrate Kit (Vector Laboratories cat. no. SK-5100). The Vector® Red substrate working solution was made by adding 2 drops each of the Reagents 1,2 and 3 of the kit to 5 ml of assay buffer (100mM Tris-HCl, pH 8.2 treated with levamisole, Vector® Red, cat. no. SP-5000, to inhibit endogenous alkaline phosphatase activity). The substrate working solution was made immediately before use and the cells incubated with the solution at room temperature, in the dark for 20-30 minutes. After the required staining intensity was reached, the cells were washed with assay buffer for 5 minutes and then rinsed with distilled water. The cells were then counterstained with hematoxylin to stain the nuclei. A few drops of Haemlum (Mayer's) solution (BDH cat. no. 35060-4T) was dropped onto the slides and incubated for 15-20 seconds and washed of with distilled water. Slides were then rinsed with 100% ethanol for 2-5 minutes to increase the intensity of the Vector® Red fluorescence if required. Excess liquid was tapped off from the slide and the cells mounted using Aquamount (BDH 36226-2H) and examined under a light microscope or kept at 4°C for later observations.

2.16. Vector NTI Software

The Vector NTI Molecular Biology Software (InforMax Inc. Maryland, USA) was used for maintaining the information database and performing electronic manipulations on all DNA molecules. Data for all plasmids, adenoviruses and Ad5-recombinants was maintained in the Vector NTI software's Molecule

manager whereas, sequencing oligonucleotide and PCR primer database was maintained in the Primer Manager.

All the adenoviral plasmid shuttle vectors, additional intermediate plasmids, commercially obtained plasmids and cDNA sequences employed in the construction of these, were obtained from various sources. The source of the Troponin C cDNA sequences, the commercial vectors, the wild-type adenovirus 5 genome was Genbank EMBL., which was accessed using a GCG package version 9 available on the Glasgow University UNIX mainframe. The sequences for adenovirus shuttle vectors obtained from Microbix were purchased from the same company on disks and were entered into the Vector NTI database. Most plasmid entries were modified to present the plasmids diagramatically, clearly showing restriction enzyme sites, genetic markers and functional maps.

Typically, adenoviral shuttle vector for making Ad5 recombinants, were made following the cloning steps carried out in actual benchwork, using the simple cutting and pasting functions of the Microsoft® Windows® environment. Recombinant adenoviruses were then constructed by manipulation of these vectors and Ad5-dl309 genome sequences in a similar manner.

As the sequence for dl309 mutant genome of adenovirus 5 was not available from any accessible databank, Ad5-dl309 sequence was extracted out from the sequence of the Ad5-dl309 genome-carrying plasmid vector pJM17 (sequence obtained from Microbix Biosystems Inc.). The pBR322 bacterial plasmid sequence of pJM17 was removed by cutting out the *Xba*I fragment of the plasmid and rejoining cohesive *Xba*I ends to circularize the adenoviral DNA. The circular Ad5-dl309 genome was then linearized beginning at 0 mu of the adenoviral genome.

Representation of molecular structures of recombinant adenoviruses were made by joining the appropriate shuttle vector and the Ad5-dl309 genome, simulating the outcome of a single recombination event between homologous regions of shuttle vector and the adenovirus genome. For this purpose, the fragment inclusive of bp 1 to the first *Sac*I site downstream of the SV40-polyA

sequence, of the appropriate shuttle vector which carries the foreign gene, was copied to the clipboard. This fragment was then pasted immediately 5′ to the *Sac*I site at bp 3644 of the Ad5-dl309 genome. The resulting genome is a representation of the actual recombinant carrying the gene of interest. A *Hind*III digest pattern for this recombinant was obtained by using the Vector NTI's Analyse/Restriction Site function. This pattern was checked against the *Hind*III cut fragments of the actual recombinants.

Vector NTI was also employed for the design of some PCR primers used in the study. Primer duplex analysis was also carried out on some previously made, empirically designed primers, using the software's PCR primer analyse function.

Table 2.1.A: DNA Oligonucleotides Used as PCR Amplification Primers

•Troponin C Primers

Primer name [pair]	**size** in bases	**Description** *Hybridization Location and Orientation*	**Sequence (5' → 3')** *degenerate bases are shown in italics: Restriction sites are underlined*
G153583 [C88303 T43429 and C166762]	34	5' primer incorporating an *EcoRI* site, used for amplifying full length and some truncated sTnC cDNAs. *bp 27-44 forward*	*GGTGGAGTGCGA* *ATTC*GGCGACCT GCAACAGAGG
C88303 [G153583]	34	3' primer for G153583; incorporates a *BglII* site *bp 558-575, reverse*	*CACCCTAGGGAG* *ATCT*GATCTTGG TAGAGGCGAC
T43429 [G153583]	36	3' primer for modifying the sTnC cDNA to delete the last 12 amino acid codons and contains a *BglII* site. The oligo was designed for substituting the last 12 codons with HA epitope tag sequence *bp 491-508, reverse*	*CTTCAGGAAAGA* *GATCT*CGTCAAT GCGGCCGTCGTT
C166762 [G153583]	40	3' primer for modifying the sTnC cDNA, by deleting the last 9 codons and substituting a HA epitope tag sequence: Adds a *BglII* site. *bp 479-517, reverse*	*CCATCATCTTAGA* *GATCT*CCTCGTC GAAGTCAATGCG GCC
A1901D04 (sTnC:XhoI/EcoRI)	42	5' primer with characteristics similar to G153583 with an extra *XhoI* site, upstream of the *EcoRI* site *bp 27-44 forward*	*AACAAGACTACTC* *GAG GAATTC*GGC GACCTGCAACAG AGGAG

Table 2.1.A: DNA Oligonucleotides Used as PCR Amplification Primers

•Troponin C Primers (continued)

Primer name [pair]	**size** in bases	**Description** *Hybridization Location and Orientation*	**Sequence (5' → 3')** *degenerate bases are shown in italics: Restriction sites are underlined*
1669-077 (sTnC/EcoRI:T-tag) [sTnC:d8-T/NheI, sTnC full/NheI]	32	5' primer for amplifying full length and truncated sTnC cDNAs, incorporating an *EcoRI* site. This primer was designed to produce SV40 large T-antigen epitope tagged sTnC cDNA. *bp 13-30 forward*	*TCATAGTAGAATTC* TGGAGTGCAAAG GAGGCG
1669-073 (sTnC:d8-T/NheI) [sTnC/EcoRI:T-tag]	34	3' primer for modifying the sTnC cDNA designed to delete the last 8 codons, adding a *NheI* site and substituting with T-antigen epitope tag sequence *bp 500-520, reverse*	*TCATAGTAGCTAG* CGAACTCGTCGA AGTCAATGC
1669-074 (sTnC-full/NheI) [sTnC/EcoRI:T-tag]	33	3' primer for modifying the sTnC cDNA by deletion of the last 9 codons for substituting with HA epitope tag sequence: Adds a *NheI* site. *bp 526-544, reverse*	*TCATAGTAGCTAG* CCTGCACGCCCTC CATCATC

Table 2.1.B: DNA Oligonucleotides Used as PCR Amplification Primers

•Adenovirus Primers

Primer name [pair]	**size** in bases	**Description** *Hybridization Location and Orientation*	**Sequence (5' → 3')**
Ad5 280-99 [Ad5 3580-98;rev]	20	5' primer hybridizing to adenoviral sequences upstream of the foreign insert; designed for screening Ad5 recombinants and inserts in adenoviral shuttle vectors *bp 280 to 298 forward*	*CGGGAAAACT GAATAAGAGG*
Ad5 3580-98;rev [Ad5 280-99]	19	3' primer hybridizing to adenoviral sequences downstream of the foreign insert/ poly A sequence, for screening Ad5 recombinants and inserts in adenoviral shuttle vectors *bp 3580 to 3598 reverse*	*GCTGCTGCAA AACAGATAC*
Ad5-E1A:629/for [Ad5-E1:1045/rev]	20	5' primer hybridizing to adenoviral sequences within the E1A region: Used to screen presence of E1A region in recombinants *bp 629 to 648 forward*	*ATCGAAGAGG TACTGGCTGA*
Ad5-E1:1045/rev [Ad5-E1A:629/for]	20	3' primer hybridizing to adenoviral sequences within the E1A region: Used to screen presence of E1A region in recombinants *bp 1026 to 1045 reverse*	*CCTCCGGTGA TAATGACAAG*
Ad5-E1;for [Ad5-E1;rev]	18	5' primer hybridizing to adenoviral sequences within the E1A region: Used to screen presence of E1A region in recombinants *bp 1469 to 1486 forward*	*CTGTGGAATG TATCGAGG*
Ad5-E1;rev [Ad5-E1;for]	18	3' primer hybridizing to adenoviral sequences within the E1A region: Used to screen presence of E1A region in recombinants *bp 2951 to 2968 reverse*	*TCAGACAGGA TACCCAAG*

Table 2.2: DNA Oligonucleotides Used as Sequencing Primers

Primer name	size in bases	Gene	Location and Orientation	Sequence (5' → 3')
G182861	18	skeletal Troponin C	bp 94-76 reverse	*GGACCTGGCCTCAGC CTG*
G182927	18	skeletal Troponin C	bp 514-529 forward	*GACGAGTTCCTGAAG ATG*
C929111	19	skeletal Troponin C	bp 411-429 forward	*TGGCTGAGATTTTCA GGGC*
sTnC (200-214)	15	skeletal Troponin C	bp 200-214 forward	*ATGCTGGGCCAGACA*
sTnC (262-245/rev)	18	skeletal Troponin C	bp 262-245 reverse	*CTGCTCATCCACCTC CTC*
sTnC (233-247)	14	skeletal Troponin C	bp 233-247 forward	*GACGCCATCATCGA*
sTnC (368-382/for)	15	skeletal Troponin C	bp 368-382 forward	*ATCTTCGACAGGAAT*
sTnC (382-368/rev)	15	skeletal Troponin C	bp 382-368 reverse	*ATTCCTGTCGAAGAT*

Table 2.3: DNA Oligonucleotides Used for Epitope Tagging

Primer name	size in bases	Description	Sequence (5' → 3') *
A80698	39	sense oligo for 3 codon spacer and 9 codon influenza virus hemagglutinin antigen epitope tag sequence terminating in a stop codon	GATC*TCTTACCCTTAT GATGTCCCTGACTAC GCCTAAGA*
A80716	39	complementary antisense oligo for A80698: The resulting ds DNA has protruding termini for ligation to *BglII* cut vector/cDNA ends	GATC*TCTTAGGCGTAG TCAGGGACATCATAAG GGTAAGA*
1669-075 T-tag: XbaI/BamHI/TOP	27	sense oligo for 2 codon spacer and 6 codon SV40 large T-antigen epitope tag sequence terminating in a stop codon	CTAG*ACCCCCCGAGC CCGAGACCTAAG*
1669-076 T-tag: XbaI/BamHI/ BOTTOM	27	complementary antisense oligo for T-tag:XbaI/BamHI/ TOP: The resulting ds DNA has protruding termini for ligation to *XbaI* at the 5' end and *BamHI* at the 3' end	GATC*CTTAGGTCTCGG GCTCGGGGGGT*
A1901D05 HA-tag:/BamHI	44	3' primer complements to the last 8 codons of the HA epitope tag sequence, designed to modify the 3' *BglII* site to *BamHI* site, facilitating the subcloning of HA tagged cDNA with different cDNAs	*TCATTCGATGG*GATC*C* CTTAGGCGTAGTCAG GGACATCATAAGG *raised, italicised letters indicate the mismatched bases*

* ***sequence underlined present the 5' protruding termini; double underlined sequence shows the stop codon. Bold letters show the coding sequence of epitope tags.***

Chapter 3

Results

Subcloning

3.0 Subcloning of Promoters and cDNA into Adenoviral Shuttle Plasmids

Skeletal Troponin C cDNA was subcloned into a range of shuttle plasmids with ubiquitous and tissue specific promoters. A typical foreign DNA insert in a shuttle vector comprised of (5′ to 3′) a promoter and a sTnC cDNA followed by SV40-polyA sequence. sTnC cDNA was first cloned into intermediary vectors, then removed by cutting with restriction enzyme sites available in the intermediary vector, and then ligated into shuttle vectors. Tissue-specific promoters were cloned directly into the shuttle vectors from their source plasmids, followed by inserting the sTnC cDNA from the intermediate vector. The promoter-cDNA cassette was then cut out and inserted into a basic promoter vector with the SV40 polyadenylation signal completing the entirety of the insert. Some clones were made using PCR amplification of the cDNA, incorporating specific restriction enzyme sites at either ends of the product, thereby facilitating cloning.

3.1. Subcloning of SV40-polyA Signal into Basic Adenoviral Shuttle Vectors

Vectors pΔE1sp1A and pΔE1sp1B do not have the SV40-polyA sequences necessary for mRNA stability. These sequences were cloned in from pCA13 and pCA14. pΔE1sp1A was cut with *Xho*I and *Bgl*II (see section 2.4.4.), and the large gel fragment separated and gel purified: The *Xho*I/*Bgl*II fragment, containing the SV40-polyA sequences and part of the MCS was cut out from pCA14, gel purified and ligated (see section 2.4.9.) to the pΔE1sp1A backbone. This ligation restores the MCS and in addition adds the SV40-polyA 3′ to the MCS (See figure 3.1). Similarly, pΔE1sp1B was cut with *Hind*III and *Bgl*II and the backbone ligated to a *Hind*III/*Bgl*II fragment from pCA13 carrying the SV40-polyA sequence. In both cases, positive clones were screened by cutting with *Cla*I and *Bgl*II. The new clones were designated as pΔE1sp1A-polyA and pΔE1sp1B-polyA. Positive clones were sequenced for confirmation of the correct MCS insert and the ligation

junctions, using primer Ad5 280-99, before proceeding with further cloning. Vectors pΔE1sp1A-polyA #5 and pΔE1sp1B-polyA #8 were retained for further subcloning purpose.

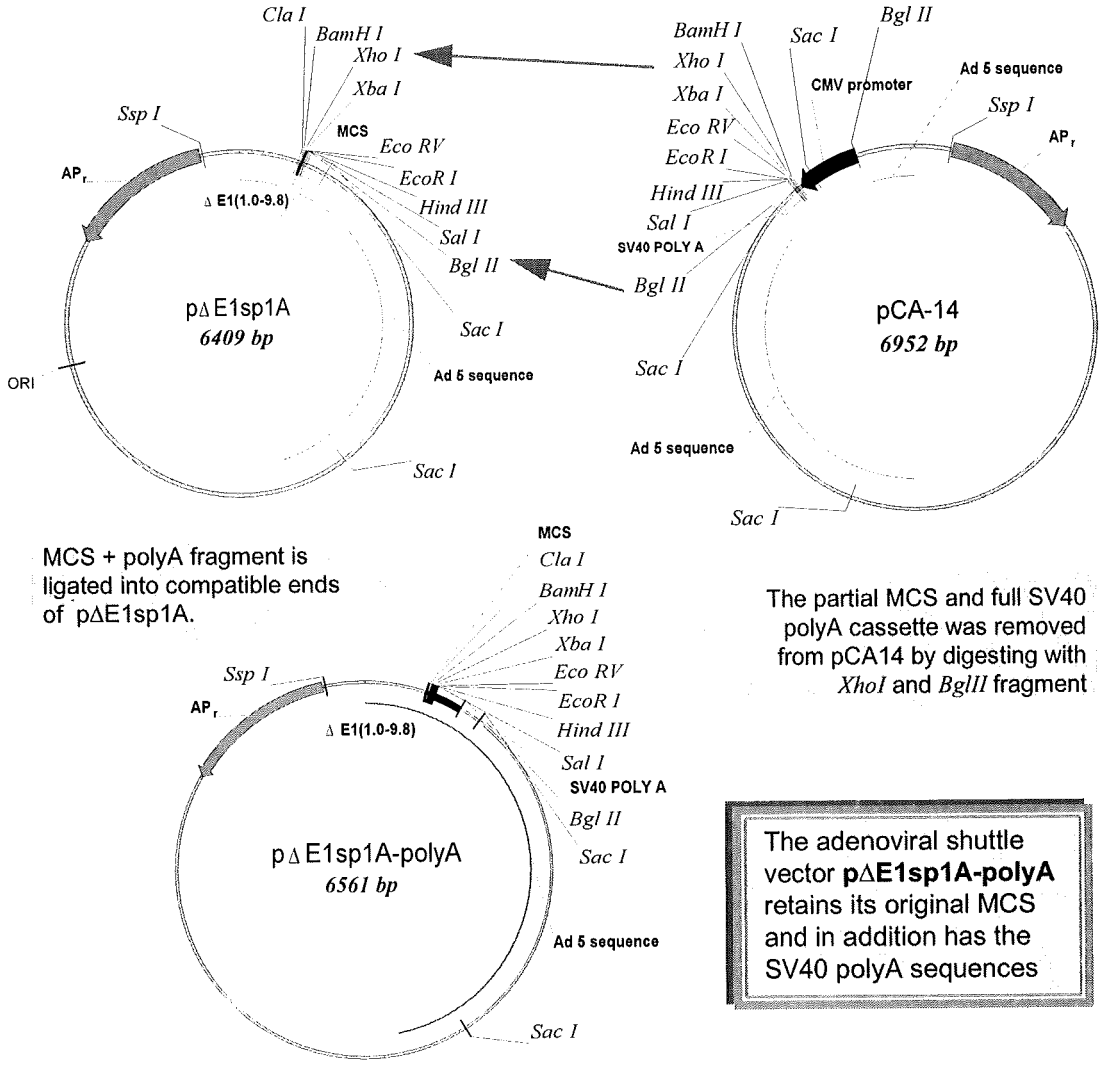


Fig 3.1: Schematic presentation of steps involved in subcloning of SV40-polyA sequence from pCA14, into pΔE1sp1A. Similar strategy was also carried out for pΔE1sp1B (see section 3.1.1.).

3.2. Subcloning of Skeletal Troponin C cDNA

The source of the skeletal Troponin C cDNA was pLK419, obtained from Larry Kedes' Lab., University of Southern California, CA, USA. This cDNA was isolated from a human muscle cDNA library by R. Gahlmann *et al* and completely sequenced (Gahlmann *et al.,* 1988). The entire sequence of skeletal Troponin C cDNA has been published and available from Genbank

(accession no. X07898). The cDNA comprised of 677 bp sequence with a 64 bp 5′ untranslated region (UTR) from bp 1-64, a 483 bp coding region from bp 65 to bp 547 and a 130 bp 3′ UTR ranging from bp 548 to 677. The sTnC cDNA was cloned into the shuttle vectors with ubiquitously expressing CMV-IE promoter and with different tissue-specific promoters.

3.2.1. Subcloning of cDNA into Vector with Ubiquitous Promoter

The sTnC cDNA was subcloned into adenoviral shuttle vectors with CMV-IE promoter, using PCR amplification and direct subcloning of a fragment of the cDNA. Both pCA13 and pLECM vectors were used for this purpose.

3.2.1.1. Using PCR-amplified DNA

The skeletal Troponin C cDNA was PCR-amplified on plasmid LK419 template, using 5' primer G153583 which incorporates an *Eco*RI site, and the 3′ primer C88083 adding a *Bgl*II site (see table 2.1.A.) using conditions described in section 2.12.1. The PCR product was separated on an agarose gel, purified and cut with *Eco*RI and *Bgl*II. pLECM vector was cut with the same pair of restriction enzymes and gel purified to lose the smaller fragment and the *Eco*RI/*Bgl*II-cut PCR product ligated to the vector. The positive clones were checked out using *Eco*RI/*Bgl*II which cuts out the whole 500kb cDNA fragment. Six positive clones were obtained and were designated pLECM/sTnC and screened further by sequencing (see section 2.7.), for the integrity of the inserted PCR product. One clone was retained for further use as it was found to contain no mutations.

Another similar clone of skeletal Troponin C was made using the cloned and sequenced PCR product from pLECM/sTnC. This was done to obtain a clone having SV40-polyA sequence at the 3′ end of the sTnC gene. The *Eco*RI/*Bgl*II insert fragment was cut from pLECM/sTnC and ligated into pCA13 vector, which was cut with *Eco*RI and *Bam*HI and gel purified to lose the small MCS oligonucleotide. The new plasmid was named pCA13/sTnC and two clones were retained for further use.

3.2.1.2. Using cDNA Fragment *via* an Intermediary Vectors

The fortuitous occurrence of a *Bst*EII site just upstream of the translational start site in the sTnC cDNA, and of an *Avr*II site downstream of the stop codon, was exploited for cloning of the cDNA into various vectors *via* an intermediary vector pSP72 (Promega cat. no. P2191; Genbank Accession no. X65332)

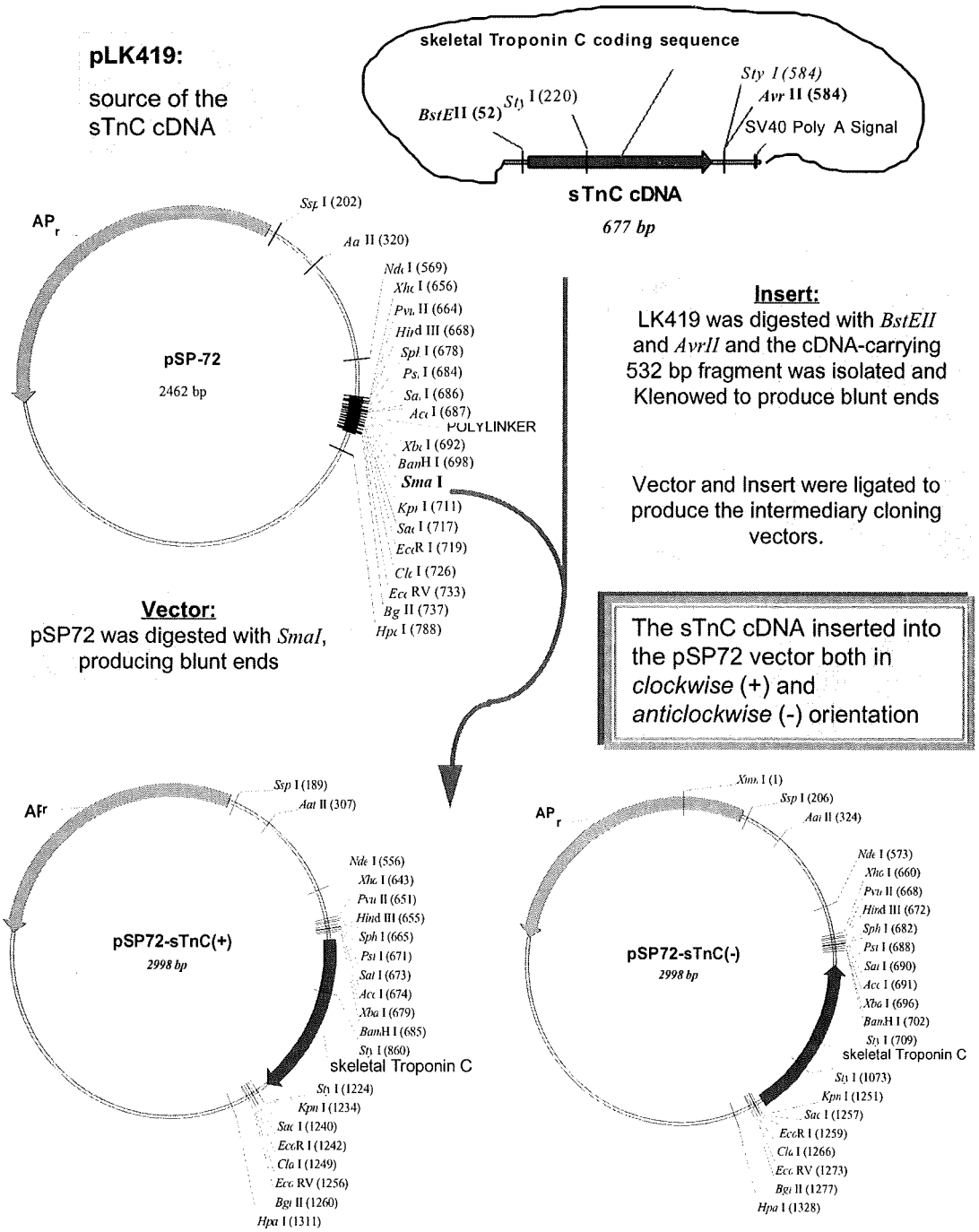


Fig. 3.2: Flowchart highlighting the main steps involved in subcloning of skeletal Troponin C cDNA, into intermediary vector pSP72.

The sTnC cDNA containing fragment was cut using *Bst*EII and *Avr*II and gel purified by electroelution, as described in section 2.4.5., and then blunt-ended using Klenow as described in section 2.4.7. The pSP72 vector was cut with *Sma*I and the blunt ends dephosphorylated (see section 2.4.8.), to prevent self-ligation. The vector was cleaned using the Micropure EZ™ columns and the sTnC carrying insert ligated into the blunt ends produced by the *Sma*I cut (see fig. 3.2). Positive clones with sTnC cDNA were checked out by cutting with the flanking restriction enzyme sites *Eco*RI and *Xba*I. The positive cloned were further screened for the orientation of the insert by cutting with *Xba*I and *Sty*I: The cDNA inserted in a clockwise orientation produced a 364 bp and a 181 bp fragment, while the anticlockwise orientated insert showed a 364 bp fragment only. A clockwise orientation clone pSP72/sTnC(+) and an anticlockwise-inserted clone pSP72/sTnC(–) were selected for further cloning purposes.

3.2.1.2.i. Subcloning cDNA Fragment from Intermediary Vector

Skeletal Troponin C cDNA was cut from the intermediary vector pSP72/sTnC(–) with *Eco*RI and *Bam*HI and ligated to pCA13 vector cut with the same enzyme pair. Positive clones were screened by cutting with *Bam*HI and *Sst*I (*Sac*I). Positive clones showed a 2 kb and a 953 bp fragment. Five positive clones were retained for sequencing the ligation junctions and were retained for generating recombinant adenovirus.

3.3. Subcloning of Tissue-specific Promoters into Shuttle Vectors

Tissue-specific promoters were inserted into adenoviral shuttle plasmids as first step to obtain promoter-cDNA cassettes which express specifically in cardiocytes and myocytes.

3.3.1. Human Cardiac Actin Promoter Subcloning

The upstream region of the human cardiac actin (HCA) gene has been characterized and cloned (Minty and Kedes, 1986). The cloned DNAs were obtained in plasmids LK339 (human cardiac actin; -177 to +70) and LK359 (human cardiac actin; -485 to +70). Promoters were flanked by *Eco*RI and

*Hind*III at the 5′ and 3′ end of the promoters respectively, in both the plasmids. The complete promoter sequence has been published and available from the Genbank database (accession no. M13483).

The human cardiac actin (HCA) promoters, both –177 (–177 to +72) and -485 (–485 to +72) were cloned into the vector pΔE1sp1A by removing the promoters from LK339 and LK357 by cutting with *Hind*III and *Eco*RI and ligating to the compatible sites in pΔE1sp1A. The positive clones were checked by cutting with *Hind*III/*Eco*RI and with *Sst*I to confirm the presence of the promoters in an adenovirus shuttle plasmid. *Hind*III/*Eco*RI digest showed a 259 bp fragment in HCA (–177) and a 549 bp fragment with HCA (–485): The *Sst*I digest cut out a 2 kb fragment, characteristic of the adenoviral shuttle vector. The new HCA promoter vectors were designated p1A/HCA (–177) and p1A/HCA (–485) and were used for cloning sTnC cDNA.

3.3.2. Human Skeletal Actin Promoter Subcloning

The regulatory upstream region of the human skeletal α-actin gene have been characterized, cloned and partially sequenced by Taylor, A. *et al* (Taylor *et al.,* 1988). Plasmid clones with two versions of human skeletal α-actin promoter were obtained from Larry Kedes' Lab., University of Southern California, CA, USA. Plasmid LK496 carried the -1282 to +239 bp of the human skeletal α-actin promoter as a 5′ *Xba*I - 3′ *Hind*III fragment. LK480 carried the -2000 to +239 region of the gene flanked by *Hind*III on either side of the promoter. The sequence for the promoter up to -708 has been published (Taylor *et al.,* 1988) and available from the Genbank database (accession no. M20543). Sequence from –1282 to –709 was obtained from Larry Kedes' Lab., University of Southern California, CA, USA.

The skeletal actin –1282 promoter (–1282 to +239) was cut out as a *Xba*I/*Hind*III fragment from plasmid LK496. This fragment was ligated into the compatible ends of pΔE1sp1A and positive clones checked by cutting out the same 1520 bp fragment. The plasmid was named p1A/skact(–1282) .

The skeletal actin –2000 promoter (–2000 to +239) was cut out as a 2.3 kb *Hind*III fragment from plasmid LK480 obtained from Larry Kedes' lab. This fragment was ligated into pΔE1sp1B *Hind*III cut and dephosphorylated and positive clones screened by cutting with *Xba*I: The clones with the insert in the right orientation showed a 1520 bp fragment. This plasmid vector was named p1B/skact(–2000).

3.4. Subcloning cDNA with Tissue-specific Promoters

Troponin C and β-galactosidase cDNA were subcloned with tissue specific promoters in a two-step cloning procedure to ensure the presence of SV40-polyA sequence with the cDNA. was first subcloned into vectors with the relevant promoter and then the whole promoter-cDNA was removed and inserted into a pΔE1sp1A-polyA or pΔE1sp1B-polyA adenoviral shuttle plasmid.

3.4.1. Cardiac actin promoters with sTnC cDNA

Skeletal Troponin C cDNA was cloned into the HCA promoter carrying adenoviral shuttle vectors, by first cutting out the sTnC cDNA from pSP72/sTnC(+) as a *Sal* I /*Bgl*II fragment and ligating into compatible ends of both p1A/HCA(–177) and p1A/HCA(–485). The new clones were named p1A/HCA(–177)/sTnC and p1A/HCA(–485)/sTnC. Positive clones were analyzed by cutting with *Eco*RI; the p1A/HCA(–177)/sTnC cut out a 785 bp fragment and the p1A/HCA(–485)/sTnC gave a 1115 bp band. Seven clones of p1A/HCA(–177)/sTnC and seven of p1A/HCA(–485)/sTnC were positive and were kept for later use.

Finally, the promoter-cDNA cassette was cloned into a vector with the SV40-polyA sequence. The pΔE1sp1A-polyA was cut with *Eco*RI and dephosphorylated to prevent recircularization of the plasmid. From p1A/HCA(–177)/sTnC and p1A/HCA(–485)/sTnC the promoter-cDNA cassette was cut out with *Eco*RI cutting at the 5′ end of the promoter and downstream of the sTnC cDNA, and ligated to the *Eco*RI-linearized pΔE1sp1A-polyA. Positive clones were initially screened by cutting out the insert with *Eco*RI.

New plasmids were named p1A/HCA(–177)/sTnC/pA and p1A/HCA(-485)/sTnC/pA. Those showing inserts of the right size were further screened for the orientation of the insert by cutting with *Bam*HI and *Sst*I. Inserts with the right orientation, in case of p1A/HCA(–177)/sTnC/pA showed fragments of 297 bp and 560 bp and for p1A/HCA(–485)/sTnC/pA, 580 and 560 bp, along with the characteristic 2 kb *Sst*I fragment of the parent shuttle vector. Seven clones of p1A/HCA(–177)/sTnC/pA and three of p1A/HCA(–485)/sTnC/pA were retained for making adenovirus recombinants.

3.4.2. Skeletal Actin Promoters with sTnC cDNA

pSP72/sTnC(+) was used to cut out a *Bam*HI and *Bgl*II fragment carrying the sTnC cDNA and this was ligated to a p1A/skact(–1282) vector, linearized with *Bgl*II and dephosphorylated to prevent self-ligation. The presence and orientation of the insert was determined by cutting with *Sst*I: Insert with the right orientation produced fragments of 1420 bp and 150 bp. The new plasmid was named p1A/skact(–1282)/sTnC and four clones were retained for future use.

As a final step, the 2112 bp *Xba*I/*Eco*RI fragment which comprised of the promoter-cDNA cassette, was cut out from a randomly chosen clone of p1A/p1A/Skact(–1282)/sTnC and ligated to compatible sites in pΔE1sp1A-polyA. (See figure 3.3) Clones were screened by cutting with *Sst*I which cut out a 1420 and 290 bp fragment from the positive clones: Five clones were obtained and one randomly chosen to generate an adenovirus recombinant.

For cloning of sTnC with larger human skeletal α-actin promoter, the pSP72/sTnC(–) was used to cut out a *Eco*RI and *Bam*HI fragment carrying the sTnC cDNA. This fragment was ligated to a p1B/skact(–2000) vector, which had been cut with the same enzyme pair. Positive clones were screened for the presence of insert by cutting with *Sst*I which produced fragments of 2 kb, and a doublet of 888/820 bp. The new plasmid was named p1A/skact(–2000)/sTnC, for which four clones were retained.

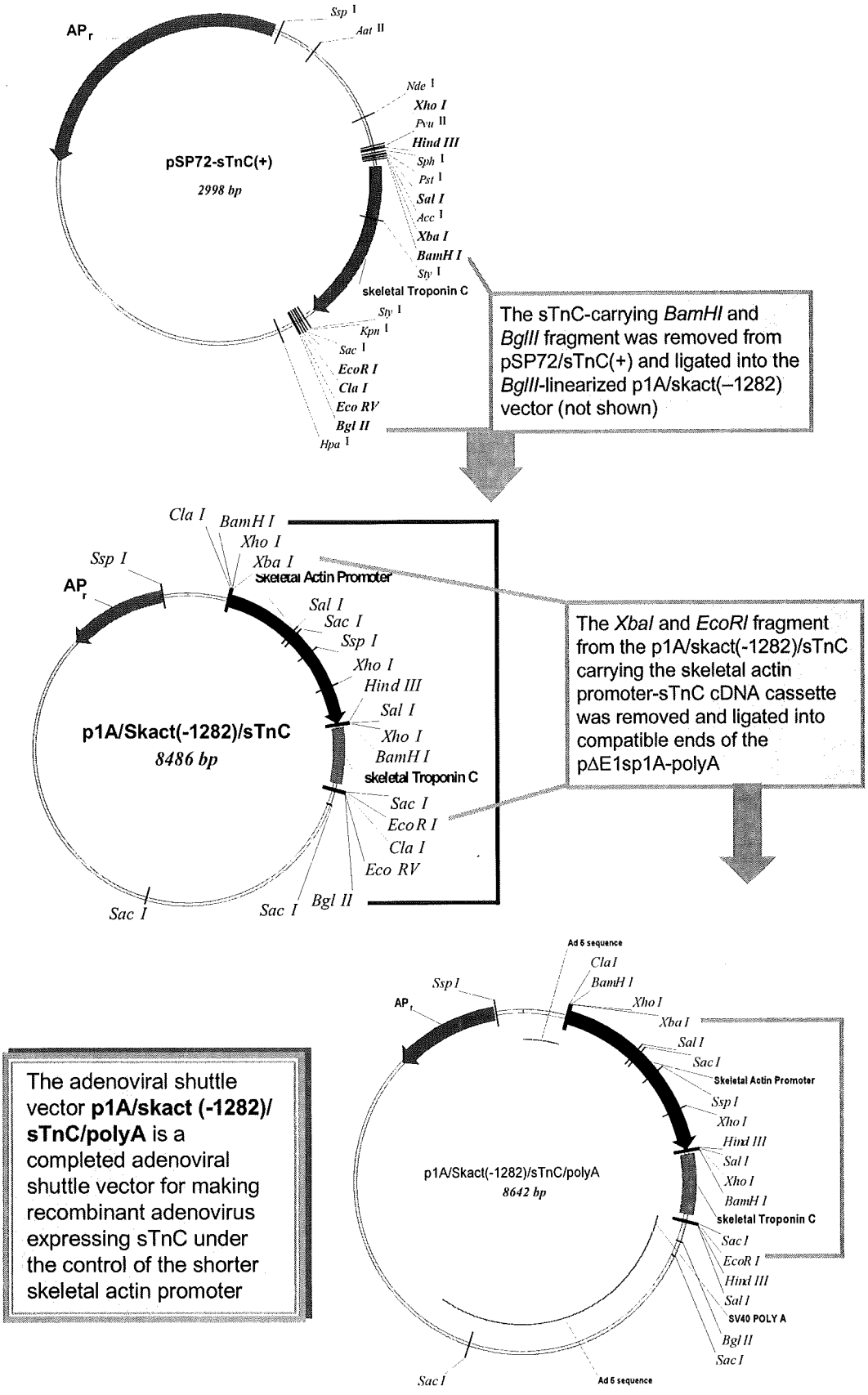


Fig 3.3: A typical strategy adopted for subcloning of sTnC fragment from pSP72/sTnC intermediary vector into adenoviral shuttle vectors with tissue-specific promoters.

Finally, the complete promoter-cDNA cassette was cut out from a randomly chosen clone of p1B/skact(–2000)/sTnC as a *Cla*I/*Bgl*II fragment and ligated to compatible sites in pΔE1sp1B-polyA. Clones were screened by cutting with *Sty*I, which cuts out a 1276 bp and a 330 bp fragment in positive clones in addition to 3 fragments of the parent vector sized 685, 998 and 1100 bp: Five clones were obtained and one randomly chosen to generate an adenovirus recombinant.

3.5. Subcloning of Epitope-tagged Troponin C cDNA

Epitope tags were incorporated at the carboxyl end of the Troponin C protein by altering the 3′ end of the sTnC cDNA. Double-stranded oligonucleotides encoding the amino acids of the epitopes were generated and ligated to a truncated PCR amplified sTnC cDNA: The template plasmid was LK419 and specially designed primers having the desired restriction enzyme sites were used for the amplification. Two kinds of epitope tags were added to the Troponin C protein; the influenza virus haemagglutinin epitope tag (HA-tag) and the SV40 large T antigen epitope tag (T-tag).

3.5.1. Subcloning of HA-epitope-tagged sTnC cDNA (sTnC:HA)

The influenza virus epitope tag sequences (Wozniak *et al.,* 1994) were added to the 3' end of the skeletal Troponin C cDNA, by substituting the last 12 codons of the sTnC cDNA with the HA-epitope coding sequence. The HA tag sequences were synthesized and added to a truncated PCR product of the sTnC.

3.5.1.1. Generating Double-stranded HA-tag Adaptor

Two single stranded complementary 39-mer oligonucleotides were synthesized for making a double stranded oligonucleotide having coding sequences for the influenza virus haemagglutinin antigen epitope terminating into a stop codon having *Bgl*II sites at either ends. The sense oligonucleotide (A80698) and the antisense oligonucleotide (A80716) were annealed by heating and slow cooling as described in section 2.4.6.1. and then

phosphorylated using T4 polynucleotide kinase as described in section 2.4.6.2. and resuspended in TE at a final concentration of 1μg/ml.

3.5.1.2. Amplification of sTnC Truncated (Δ12) cDNA

A truncated cDNA was amplified by PCR using Taq polymerase as described in section 2.12.1.using plasmid LK419 as the template. Amplification was carried out using a top (sense strand) primer (G153583), which anneals along bp 27 to 44 and incorporates an *Eco*RI restriction enzyme site 5′ to the amplified DNA. The bottom (antisense strand) primer (T43429) annealed (complementary) along bp 491 to 508, adding a *Bgl*II site at the 3′ end of the final PCR product (see table 2.1.A.). The amplified DNA produced a sTnC cDNA with 36 bp of coding sequences (corresponding to 12 amino acids at the carboxyl end in the protein) deleted at the 3′ end. This PCR product was digested with *Eco*RI and *Bgl*II to produce cohesive termini and was ligated into compatible ends in the vector pLECM (see fig. 3.4). Positive clones were checked by cutting the insert back out. Six clones were identified as positive and each was sequenced till one without a mutation was found. This plasmid was called pLECM/sTnC-Δ12; the Δ12 denoting the deletion of 12 amino acids at the carboxyl terminal. The sequenced clone with no mutations was used to insert the epitope tag encoding oligonucleotide to complete the cDNA.

3.5.1.3. Insertion of HA Epitope-tag Adaptor Sequence

Vector pLECM/sTnCΔ12 was cut with *Bgl*II and dephosphorylated to prevent self-ligation. The annealed and phosphorylated HA-tag oligonucleotide was ligated with a 1:1 and 1:2 end-mole ratio of vector to oligonucleotide. Potential positive clones were cut with *Eco*RI/*Bgl*II and the digested miniprep DNA was separated on a 2% agarose gel: The digest removed approximately 500 bp fragment with a visible faint band of very small size in all positive clones.

To establish if the insert was present and in the correct orientation, the potentially positive clones were sequenced using primer C929111 (see table 2.2) which reads into the tag. The first clone reading into the tag with the right

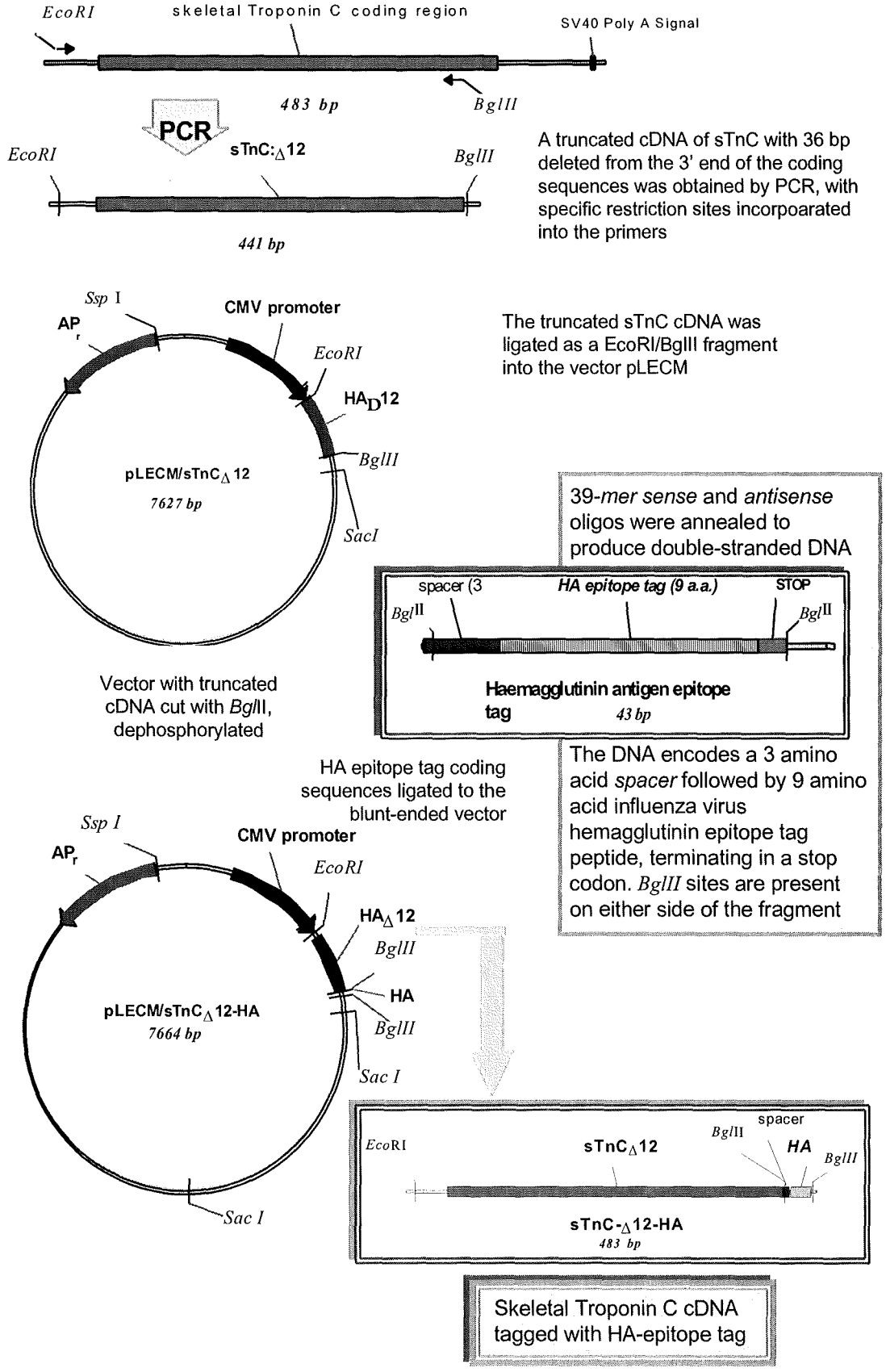


Fig 3.4: Flow diagram for subcloning strategy of influenza virus hemagglutinin (HA) antigen epitope-tagged coding sequence into skeletal Troponin C.

frame, and having no mutations was retained for making recombinants and further modifications. This clone was named pLECM/sTnCΔ12-HA.

3.5.1.4. Subcloning sTnC:HA into pCA13 and Intermediary Vector

The skeletal Troponin C cDNA with HA epitope tag (sTnCΔ12-HA) was further subcloned into pCA13 to ensure the presence of a SV40-polyA sequence at the 3′ end of the sTnCΔ12HA cDNA. For this purpose, a PCR cloning approach was adopted, using pLECM/sTnCΔ12-HA plasmid as template to amplify the whole sTnC cDNA with the 3′ *Bgl*II site modified to a *Bam*HI enzyme recognized sequence. In this case, a simple *Eco*RI/*Bgl*II fragment cutting and ligating approach was not possible due to the presence of two *Bgl*II sites on either side of the tag, with the possibility of losing the tag even in a partial digest. The sense-strand (top) PCR primer was A1901D04 (sTnC:XhoI/EcoRI), while the antisense (bottom) primer was A1901D05 (HA-tag/BamHI; see tables 2.1.A. and 2.3 for primer details). The top primer added

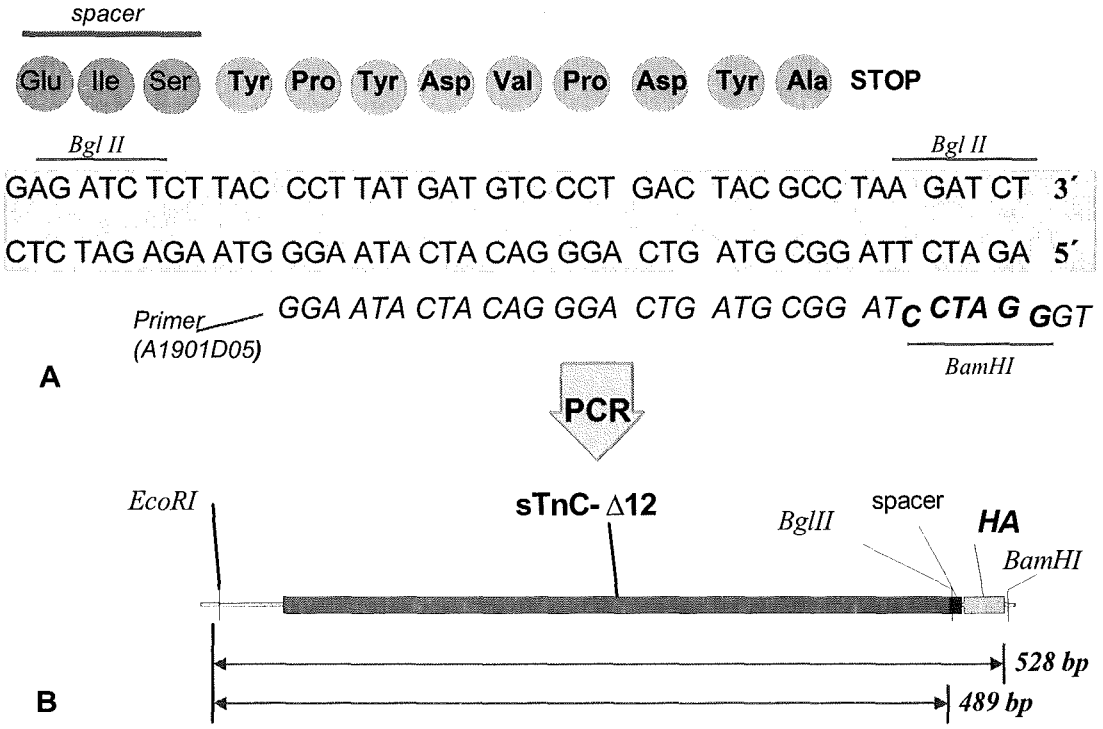


Fig 3.5: PCR mutagenesis of *BglII* site to *BamHI* site. The alignment of the 3′ primer with the two mismatched bases (underlined) is shown in (**A**). The transformed restriction enzyme site on sTnC cDNA facilitates removal and replacement of truncated cDNA, without disturbing the epitope tag coding sequence (**B**).

a *Xho*I and a *Eco*RI site 5′ to, the sTnC cDNA. Whereas the bottom primer was complementary to sequences on the HA tag coding region and the stop codon but the 3′ *Bgl*II site was altered to a *Bam*HI site (see fig. 3.5). A 1:10 mix of *Pfu* proof-reading polymerase and *Taq* polymerase, in a Pfu buffer, was used for PCR amplification as described in section 2.12.1. The PCR product was cut with *EcoRI* and *Bam*HI and cleaned by phenol/chloroform extraction. The digested PCR product was ligated to pCA13, *EcoRI/Bam*HI-digested and gel purified vector DNA (see fig. 3.6).

In addition, the same PCR product was also cloned into compatible sites of the vector pGEM-7Zf(+), named as pGEM/sTnCΔ12:HA (see fig. 3.6), and used to make further tagged cDNA, described later. The pCA13 clones were screened by colony PCR method (using primers Ad5 280-99 and Ad5 3580-98-rev; see table 2.1.B.), and clones showing an amplified product of 1.2 kb were retained. Eleven clones were further screened by digesting with *Eco*RI/*Bam*HI and *Eco*RI/*Bgl*II separately and run on a 2% agarose gel.

The *Eco*RI/*Bam*HI digest on cuts out the whole sTnC:HA cDNA of 528 bp while a *Eco*RI/*Bgl*II digest cuts out only the sTnC, leaving out the tag (489 bp fragment) and other vector sequences. The difference between the tagged and the non-tag fragments was evident on a 2% gel (see fig. 3.9-A). The positive clones were kept and sequenced to check the integrity of the PCR product. The new clone was named pCA13/sTnCΔ12:HA and one sequenced clone with no mutations and correct reading frame was retained to make recombinant adenovirus.

3.5.1.5. Subcloning sTnC Δ9 HA Tag

Due to the close proximity of the HA tag to one of the Ca^{++} binding sites at the carboxyl terminal, another tagged skeletal Troponin C cDNA was constructed. This cDNA was amplified such that 27 bp at the 3′ end of the original coding region were deleted and replaced by the HA-tag coding sequences. The final protein was designed to have 9 amino acids replaced by HA-tag thereby making the tagged sTnC protein longer by 3 amino acids.

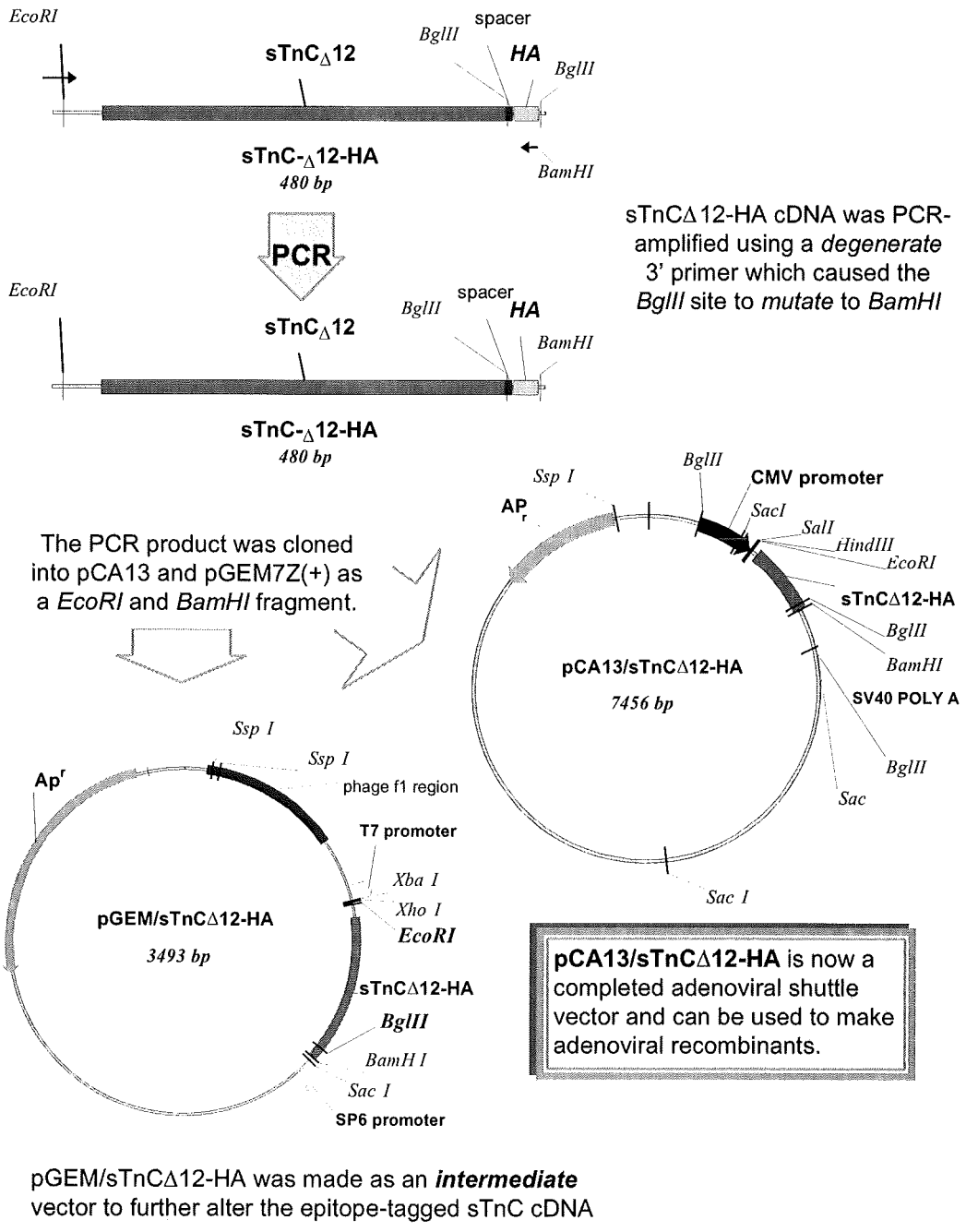


Fig 3.6: Subcloning of HA epitope-tagged sTnC cDNA into the pCA13 shuttle vector and the pGEM7Z+, intermediate vector. The tagged sTnC from pGEM/sTnCΔ12-HA was used to produce a tagged sTnC cDNA with lesser deletion of amino acids.

For this purpose the pGEM/sTnCΔ12:HA was the starting clone which was constructed to avoid the cumbersome cloning and screening procedures required for cloning in oligonucleotides of the tag. The sTnCΔ12 cDNA was removed by cutting with *Eco*RI/*Bgl*II, leaving the HA-tag sequence behind in

the pGEM vector. The vector backbone fragment was gel-purified and cleaned for later use.

PCR amplification of a truncated sTnC cDNA from the pLK419 template was done using custom-designed primers, using a 1:10 *Pfu*/Taq polymerase mix in *Pfu* buffer (see section 2.12.1.). The sense (top) PCR primer was G153583 which added an *Eco*RI site 5′ and an antisense (bottom) primer was C166762, adding a *Bgl*II site at the 3′ end of the final PCR product (see table 2.1.A.). The amplified DNA produced a sTnC cDNA which had 27 bp of coding sequences (9 amino acids in the protein) deleted at the 3′ end (sTnCΔ9:HA). This amplified DNA was cut with *Eco*RI/*Bgl*II, cleaned and ligated to the pGEM vector with the HA-tag (see fig. 3.7). The clone was named pGEM/sTnCΔ9:HA and the new insert was sequenced. pGEM/sTnCΔ9:HA #19 was found to contain no mutations and a correct reading frame, was used for the next cloning step.

The *Eco*RI/*Bgl*II fragment of pGEM/sTnCΔ9:HA #19 was cut out and ligated into the compatible ends of vector pCA13. Clones were screened by digesting with *Sst*I. All positive clones showed the 2 kb vector fragment and a 936 bp fragment coming from the inserted DNA. These were named pCA13/sTnCΔ9:HA for which 16 clones were obtained and one was selected at random to generate recombinant adenovirus.

3.5.1.6. Subcloning sTnCΔ9:HA cDNA with HCA(–485) Promoter

The HA-tagged skeletal Troponin C cDNA was cloned into p1A/HCA(–485) plasmid to produce a tissue-specific sTnCΔ9:HA expressing clone (see fig. 3.8). pCA13/sTnCΔ9:HA was cut by performing a complete digest with *Hind*III followed by a partial digest with *Bgl*II and the fragments separated on a 1.2% agarose gel. The 750 bp fragment carrying the sTnCΔ9:HA cDNA and the SV40-polyA sequence was cut out and gel-extracted as described. This fragment was ligated into a p1A/HCA(–485) vector cut with *Hind*III/*Bgl*II and cleaned by gel extraction.

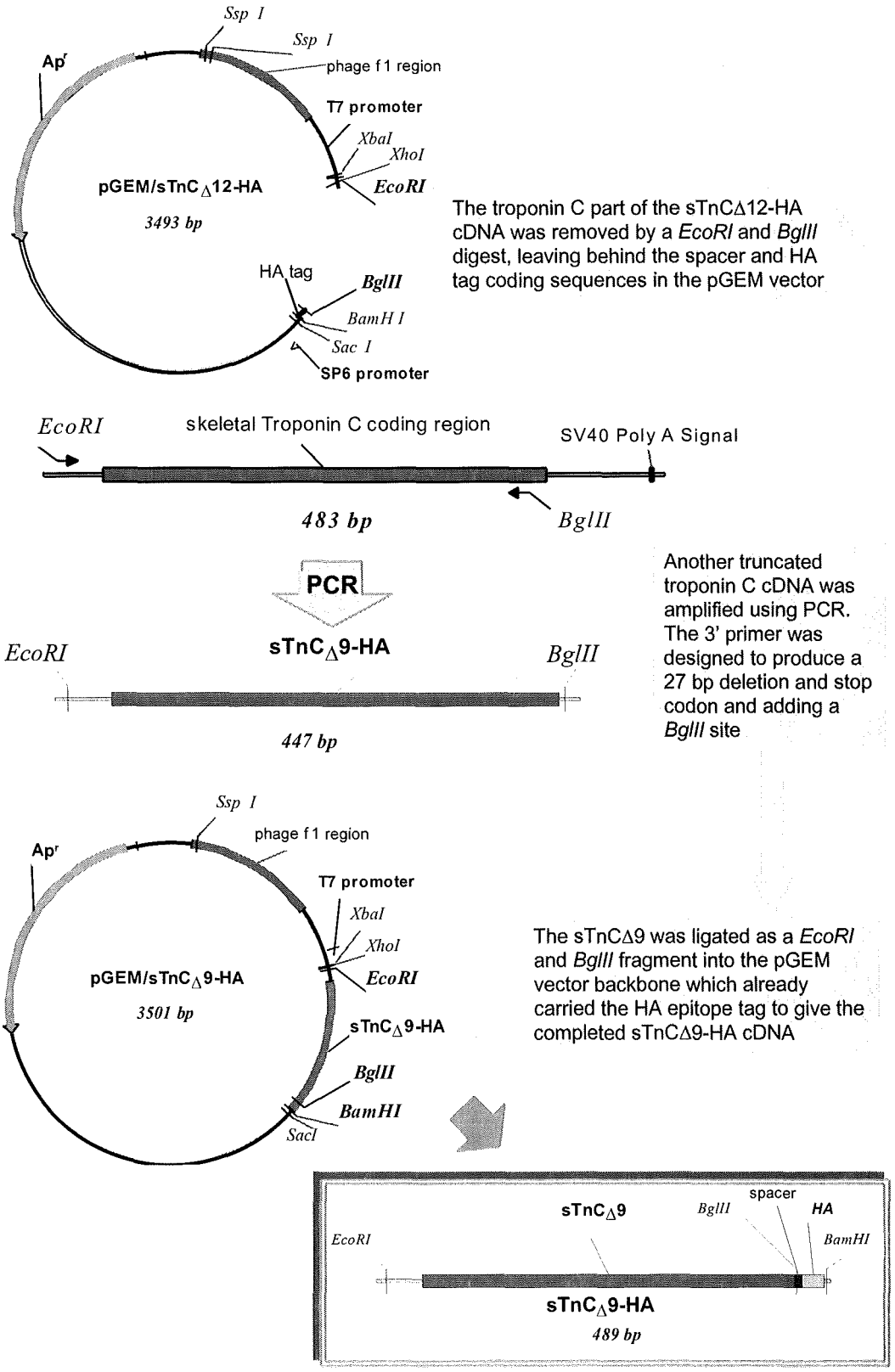


Fig 3.7: Schematic presentation of strategy for obtaining an HA epitope-tagged sTnC cDNA having a tagged sTnC protein with only the last 9 amino acids deleted and substituted by the HA tag peptide sequence. The subcloning was carried out via an intermiediary pGEM vector.

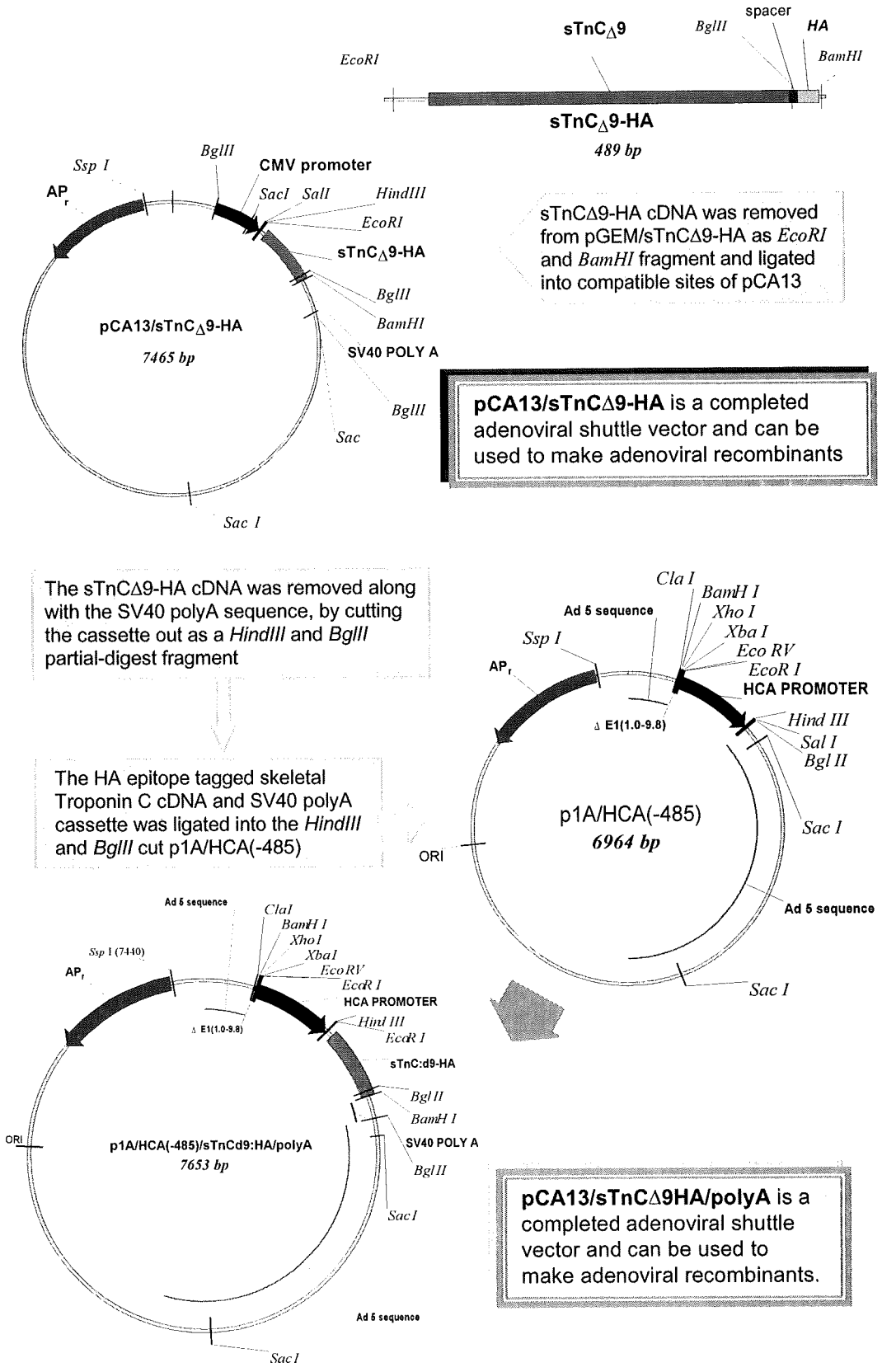
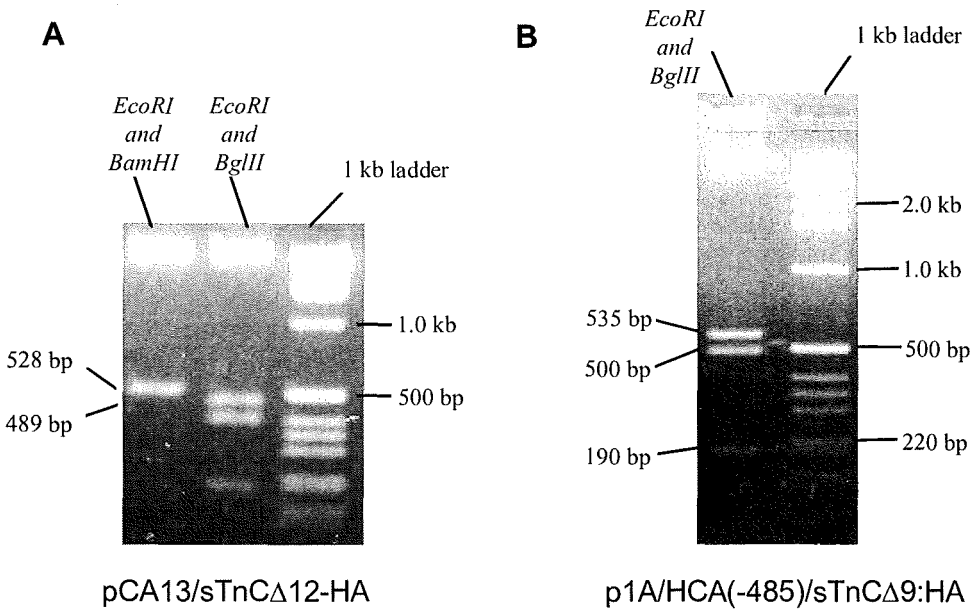


Fig 3.8: Subcloning of HA epitope-tagged sTnC cDNA (sTnCΔ9-HA) into the pCA13 shuttle vector and subsequently into the tissue-specific Human Cardiac Actin; HCA(-485) promoter adenoviral shuttle plasmid.

Putative clones were screened by cutting with *Eco*RI and *Bgl*II and separating on a 1.5% agarose gel. Positive clones showed a 535 bp sTnCΔ9 fragment, a 500 bp HCA(–485) fragment and a HAtag/SV40-polyA fragment of 190 bp (see fig. 3.9-B). 14 clones were obtained and named as p1A/HCA(–485)/sTnCΔ9:HA one was chosen randomly to generate recombinant adenovirus.



pCA13/sTnCΔ12-HA p1A/HCA(-485)/sTnCΔ9:HA

Fig 3.9: Restriction digests of adenoviral shuttle vectors pCA13/sTnCΔ12-HA and p1A/HCA(-485)/sTnCΔ9-HA. **A**. pCA13/sTnCΔ12-HA DNA digested with *EcoRI* and *BamHI* removes the full, HA-tagged sTnC cDNA. *EcoRI* and *BglII* digest cuts out a tagless, truncated sTnC cDNA fragment. The DNA fragments were separated on a 2% agarose gel. **B**. p1A/HCA(-485)/sTnCΔ9-HA. DNA digested with *EcoRI* and *BglII* removes a 535 bp fragment with the tagged sTnC cDNA, a 500 bp frgament of HCA (-485) promoter and a 190 bp fragment comprising of the HA tag and the SV40-polyA sequence. DNA fragments were separated on a 1.5% agarose gel.

3.5.2. Subcloning of SV40 large T Antigen Epitope Tag with sTnC

First, a double stranded oligonucleotide with sequences encoding the T-antigen epitope tag (Yoshihara and Hall, 1993) was made and ligated into the pCA13 vector. This adenovirus shuttle plasmid vector could be used to clone cDNA for expressing proteins which required an SV40 large T antigen epitope tag at the carboxyl terminal. DNA amplified by PCR using the original cDNA template was ligated 3′ to the epitope tag, such that the reading frame was not disturbed. This amplified DNA was modified such that it eliminated a part

of the 3′ sequences to accommodate the tag sequences, keeping the final protein to the same length. Also the stop codon was eliminated and appropriate restriction sites incorporated using custom-made primers

3.5.2.1. Generating Double-stranded SV40 Large T Antigen Epitope Tag Oligonucleotide

An adaptor-duplex was designed for adding SV40 T antigen epitope tag at the carboxyl terminal of the sTnC protein. Two single-stranded 27-mer oligonucleotides were designed such that one strand contained the complete

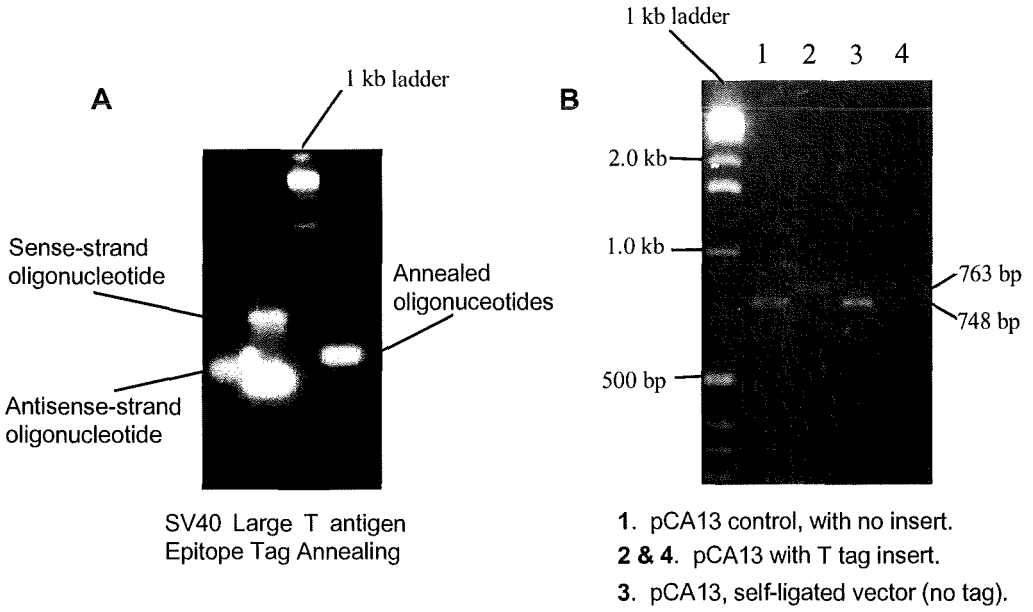


Fig 3.10: Subcloning of SV40 large T antigen epitope tag sequence into pCA13. **A.** Single-stranded oligonucleotides and ds annealed DNA were run on a 2% agarose gel to confirm annealing. The Annealed ds-DNA migrates to a postion between the two parent oligos The bottom oligo probably has a secondary structure and shows more staining and faster migration. **B.** The T-tag subclones were screened by colony PCR using adenoviral primers. The PCR products were run on a 2% agarose gel and a 15 bp difference could be detected between clones with and without a T-tag insert.

coding sequence for epitope tag, while the other oligonucleotide was complementary to the first. These two oligomers hybridized such that the core sequence coded for the epitope tag, with 5′ protruding ends compatible for ligating to restriction enzyme sites. The sense (coding) oligonucleotide was 1669-075 (T-tag:XbaI/BamHI TOP) and its complementary oligonucleotide was 1669-076 (T-tag:XbaI/BamHI BOTTOM; see table 2.3), which hybridized to form an epitope tag-encoding double-stranded (ds) adaptor-duplex, having

a *Xba*I compatible protruding end at the 5′ and a *Bam*HI protruding end at the 3′ terminal of the adapter-duplex. The *Xba*I end was able to ligate to DNA cut with *Nhe*I, *Avr*II and *Spe*I, in addition to *Xba*I itself. The two oligomers were annealed by heating and cooling down slowly (also see fig. 3.10-A), and then phosphorylated using T4 polynucleotide kinase enzyme as described in section 2.4.6.

3.5.2.2. Subcloning of Tag Adaptor into pCA13

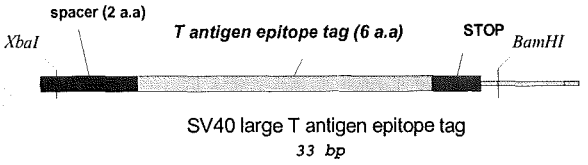
The vector pCA13 was cut with *Xba*I and *Bam*HI and the large fragment gel-purified. The SV40 large T antigen epitope tag adaptor-duplex was ligated into the pCA13 vector with a 1:1 and 1:2 end-mole ratio of vector to adaptor-duplex. Putative positive clones were screened by colony PCR using primers Ad5:280-99 and Ad5-3580-98;rev (see fig. 3.10-B).

These primers amplified a 763 bp fragment on the positive clone DNA templates and a 748 bp product on a negative (pCA13 only) clone template. The PCR products were run on a 2% agarose gel: The positive clones were selected on the basis of comparison of the putative positive clone amplified DNA with a pCA13-only, negative template. The positive clones showed a slower migration and the 15 bp difference in the fragments was evident (see fig. 3.10-B). These clones were named pCA13:T and were sequenced using properly and were kept for later use. 3 clones were found to carry the tag sequence inserted.

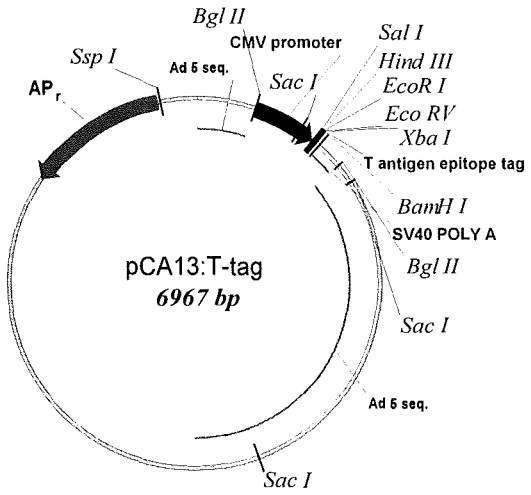
3.5.2.3. Subcloning of sTnCΔ8 and sTnC-full cDNA into pCA13:T

Two truncated cDNAs named sTnCΔ8 and sTnC-full were generated by amplification of LK419 cDNA template using EXPAND™ High Fidelity PCR System (Boehringer Mannheim, cat. no. 1681834). sTnCΔ8 was skeletal Troponin C cDNA which was amplified with specially designed primers,

29 mer sense and antisense oligonucleotides were annealed to produce a double stranded DNA encoding the 6 amino acid SV40 virus large T antigen epitope tag peptide and a 2 amino acid spacer junction

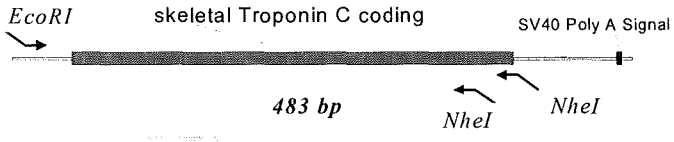


This adaptor-duplex DNA was designed to produce a 5' *XbaI* and a 3' *BamHI* site to facilitate cloning into the pCA13 adenoviral shuttle vector

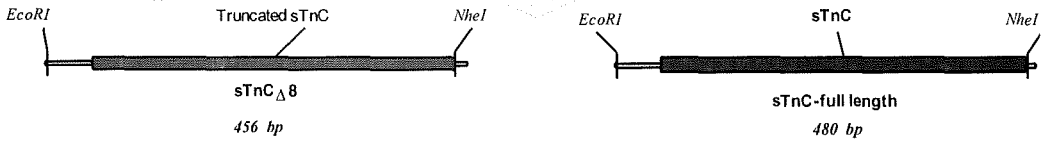


The vector **pCA13:T-tag** can be used for inserting a T antigen tag at the COOH-terminal of any protein.

Two truncated cDNAs were amplified by PCR from the sTnC cDNA template (pLK419) with specially designed primers



PCR



sTnCΔ8 cDNA has the last 8 codons deleted, the sTnC native stop codon and a *NheI* site added at the 3' end.

sTnC full length cDNA has the sTnC native stop codon omitted and a *NheI* site added at the 3' end.

Both truncated sTnC cDNAs were ligated into the *EcoRI* and *XbaI* sites (*NheI* compatible) of pCA13:T, to obtain the tagged sTnCΔ8:T and sTnC:T cDNA

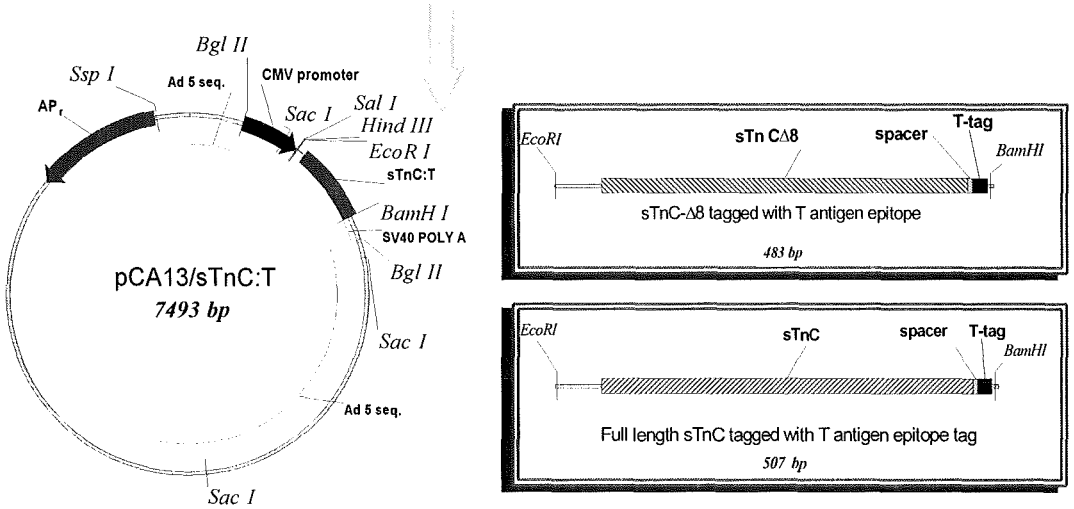
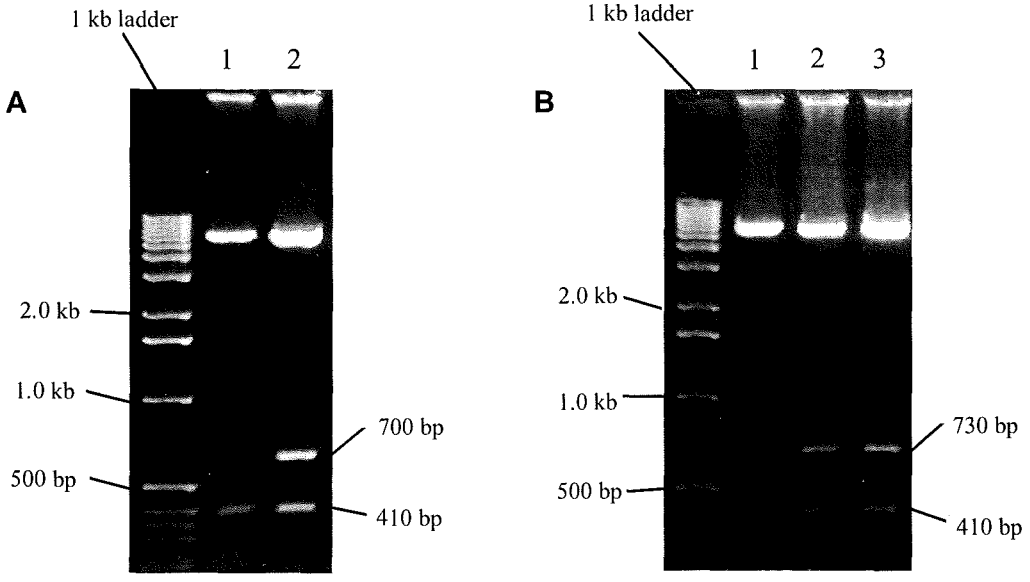


Fig 3.11: Flow diagram for subcloning of SV40 large T antigen epitope tag sequence with skeletal Troponin C. Two types of tagged cDNAs were made by modifying the 3' end of sTnC cDNA.

producing a cDNA with the last 24 bases of wild type sTnC cDNA eliminated. This was done by using the 5′ primer 1669-077 (sTnC/EcoRI:T-tag) which added an *Eco*RI restriction enzyme site immediately 5′ to its hybridization site (from sequence 13 bp to 30 bp on the sTnC cDNA). The 3′ (antisense) primer was 1669-073 (sTnC-d8-T/NheI) which hybridized from bp 500 to 520 and added a *Nhe*I site 3′ to the cDNA. This cDNA had coding sequence for all but the last 8 amino acids of the protein and after cloning into pCA13:T, the sTnC protein with the last eight amino acids replaced by a T-tag epitope should be expressed.

Similarly, the sTnC-full was amplified using the LK419 template with the same 5′ primer as sTnCΔ8 but with a different 3′ (antisense) primer. Antisense primer was 1669-074 (sTnC-full/NheI) which was complementary to bp 526 to 544 of the sTnC cDNA eliminating only the stop codon. This primer also incorporated a *Nhe*I site at the 3′ end of the cDNA facilitating cloning into the pCA13:T. Thus the new amplified cDNA had the complete coding sequence



1. pCA13--tag without insert.
2. pCA13-T-tag with sTnCΔ8 insert.

1. pCA13--tag without insert.
2 & 3. pCA13-T-tag with sTnC-full insert.

Fig 3.12: Restriction digests of SV40 large T antigen epitope tagged sTnC cDNA inserts. *EcoRI* and *BglII* digests of DNA minipreps of subclones of **A.** pCA13/sTnCΔ8:T and **B.** pCA13/sTnC:T. The 700 bp (approx.). fragment cuts out the sTnC with the tag sequence and the poly A sequence..

for the protein and after cloning into pCA13:T, a full sTnC protein with additional T-tag sequence should be expressed (see fig. 3.11).

Both PCR amplified truncated cDNAs were cut with *Eco*RI and *Nhe*I and then ligated to the pCA13:T plasmid vector which was cut with *Eco*RI and *Xba*I. Potential clones were screened by digesting with *Eco*RI and *Bgl*II. Positive clones cut out an approximately 700 bp and a 410 bp fragment with the sTnCΔ8 insert (fig. 3.12-A), and approximately 730 bp and 410 bp fragments with the sTnC-full insert (fig. 3.12-B). The new clones were named pCA13/sTnCΔ8:T, with the Δ8 denoting the deletion of the last eight amino acids of the wild type protein and pCA13/sTnC:T, for non deleted T tagged Troponin C.

Positive clones were further screened by sequencing the whole span of the PCR product and the tag sequences. Clones with insert having the correct reading frame and no base mutations were retained for making recombinants.

The SV40 T antigen tagged skeletal Troponin C clones were made as an alternative for the HA tagged sTnC. These plasmids were not analyzed for expression in this project. Recombinant adenoviruses were not generated using these plasmids, as these developments were beyond the scope of the current project.

3.6. Adenoviral Shuttle Vectors and Other Plasmids Constructed During the Project

The range plasmids constructed during the project comprise of adenoviral shuttle vectors, intermediary vectors and modified basic vectors. The completed adenoviral shuttle vectors (Tables 3.3 -3.7) are ready for using to make recombinant adenoviruses. Modified basic vectors (Table 3.1), comprised of basic shuttle vectors which had promoter or SV40 poly A signal inserted, while intermediary vectors (Table 3.2) were commercially available vectors, used to subclone sTnC gene, and both sets of plasmids were employed for further subcloning to obtain completed shuttle vectors.

Table 3.1. Modified Basic Adenoviral Shuttle Vectors

Plasmid	Promoter	MCS available	Description
pΔE1sp1A-poly A	none	ClaI, BamHI, XhoI, XbaI, EcoRV, EcoRI, HindIII, SalI,	pΔE1sp1A basic vector with SV40 poly-A sequence inserted 3´ of the MCS
pΔE1sp1B-poly A	none	ClaI, SalI, HindIII, EcoRI, EcoRV, XbaI, XhoI, BamHI,	pΔE1sp1B basic vector with SV40 poly-A sequence inserted 3´ of the MCS
p1A/HCA(-177)	Human Cardiac Actin (-177)	HindIII, SalI, BglII	pΔE1sp1A basic vector with the shorter cardiac actin promoter inserted as a EcoRI/BglII fragment
p1A/HCA(-485)	Human Cardiac Actin (-485)	HindIII, SalI, BglII	pΔE1sp1A basic vector with the full-length cardiac actin promoter inserted as a EcoRI/BglII fragment
p1A/skact(-1282)	Human Skeletal α-actin (-1282)	HindIII, SalI, BglII	pΔE1sp1A basic vector with the shorter skeletal actin promoter inserted as a XbaI/HindIII fragment
p1A-skact(-2000)	Human Skeletal α-actin (-2000)	SalI, BglII	pΔE1sp1A basic vector with the full-length skeletal actin promoter inserted as a HindIII fragment
p1A/skact(-2000) [reverse]	Human Skeletal α-actin (-2000)	EcoRI, EcoRV, XbaI, XhoI, BamHI, ClaI.	pΔE1sp1A basic vector with the full-length skeletal actin promoter inserted as a HindIII fragment, in reverse orientation
p1B/skact(-2000)	Human Skeletal α-actin (-2000)	EcoRI, EcoRV, XbaI, XhoI, BamHI, BglII.	pΔE1sp1B basic vector with the full-length skeletal actin promoter inserted as a HindIII fragment
p1B/skact(-2000) [reverse]	Human Skeletal α-actin (-2000)	SalI, ClaI,	pΔE1sp1B basic vector with full-length skeletal actin promoter, inserted as a HindIII fragment, in reverse order

Table 3.2. Intermediary Vectors with Skeletal Troponin cDNA and Promoter DNA

Plasmid	Promoter	Gene	Description	Restriction enzyme sites available[a] to remove promoter-cDNA/ cDNA
pSP72/sTnC (+)	none	sTnC	sTnC coding region removed using BstEII and AvrII from pLK419, and inserted as a blunt-end fragment into the SmaI site of pSP72, in forward orientation	SalI, XbaI, BamHI, [sTnC], EcoRI, ClaI, EcoRV, BglII [b]
pSP72/sTnC (-)	none	sTnC	sTnC coding region removed using BstEII and AvrII from pLK419, and inserted as a blunt-end fragment into the SmaI site of pSP72, in an reverse orientation	EcoRI, [sTnC], BamHI, XbaI, Sal I, HindIII, XhoI [c]
p1A/HCA(-177)/sTnC	HCA(-177)	sTnC	sTnC coding sequence removed from pSP72 (+), using SalI /BglII restriction digest, is ligated into compatible sites in p1A/HCA(-177) plasmid.	ClaI, BamHI, XhoI, XbaI, EcoRV,[promoter-cDNA], EcoRI, ClaI, EcoRV, BglII
p1A/HCA(-485) /sTnC	HCA(-485)	sTnC	sTnC coding sequence removed from pSP72 (+), using Sal I /BglII restriction digest, is ligated into compatible sites in p1A/HCA(-485) plasmid	ClaI, BamHI, XhoI, XbaI, EcoRV,[promoter-cDNA], EcoRI, ClaI, EcoRV, BglII
p1A/skact(-1282)/sTnC	skact(-1282)	sTnC	sTnC coding sequence removed from pSP72 (+), using BamHI/BglII restriction digest, is ligated into BglII site in p1A/(skact-1282) plasmid.	ClaI, BamHI, XhoI, XbaI, EcoRV, EcoRI, HindIII, SalI,[promoter-cDNA], EcoRI, ClaI, EcoRV, BglII
p1B/skact(-2000)/sTnC	skact(-2000)	sTnC	sTnC coding sequence removed from pSP72 (-), using EcoRI/BamHI restriction digest, is ligated into compatible sites in p1A/(skact-2000) plasmid.	ClaI, SalI, EcoRI, [promoter-cDNA], BamHI, BglII.

a- only restriction enzyme sites available in adenoviral shuttle vectors also are shown here.

b- more sites compatible to adenoviral shuttle vectors are available 5′ of SalI, but are not used due to presence of a potential ATG start codon in the SphI site.

c- more sites compatible to adenoviral shuttle vectors are available 5′ of EcoRI, but are not used due to presence of a potential ATG start codon at the junction of ClaI and EcoRI.

Table 3.3. Completed Vectors with Constitutive Promoter and Skeletal Troponin cDNA

Plasmid	Promoter	Gene	Description	Poly A
pLECM/sTnC	CMV-IE	sTnC	sTnC coding sequence DNA is PCR-amplified with 5´ EcoRI and 3´ BglII restriction enzyme sites, and ligated into compatible sites in pLECM plasmid.	E1b
pLECM/ sTnCΔ12:HA	CMV-IE	sTnCΔ12	Truncated sTnC coding sequence DNA is PCR-amplified with 5´ EcoRI and 3´ BglII restriction enzyme sites, and ligated into compatible sites in pLECM plasmid. The HA epitope tag double-stranded oligonucleotide is then inserted as a BglII fragment	E1b
pCA13/sTnC-(PCR)	CMV-IE (-299-+72)	sTnC	sTnC coding sequence DNA is PCR-amplified with 5´ EcoRI and 3´ BglII restriction enzyme sites, and ligated into EcoRI/BamHI sites in pCA13.	E1b
pCA13/sTnC	CMV-IE (-299-+72)	sTnC	sTnC coding sequence removed from pSP72 (-), using BamHI/BglII restriction digest, is ligated into compatible sites in pCA13 plasmid.	SV40

Table 3.4. Completed Vectors with Tissue-specific Promoters and sTnC cDNA

Plasmid	Promoter	Gene	Description	Poly A
p1A/HCA(-177)/sTnC/polyA	HCA(-177)	sTnC	Promoter-sTnC cDNA cassette is removed from p1A/HCA(-177)/sTnC, as EcoRI fragment and ligated into pΔE1sp1A-polyA.	SV40
p1A/HCA(-485) /sTnC/polyA	HCA(-485)	sTnC	Promoter-sTnC cDNA cassette is removed from p1A/HCA(-485)/sTnC, as EcoRI fragment and ligated into pΔE1sp1A-polyA.	SV40
p1A/skact(-1282)/sTnC/polyA	skact(-1282)	sTnC	Promoter-sTnC cDNA cassette is removed from p1A/skact(-1282)/sTnC, as XbaI/EcoRI fragment and ligated into pΔE1sp1A-polyA.	SV40
p1B/skact(-2000)/sTnC/polyA	skact(-2000)	sTnC	Promoter-sTnC cDNA cassette is removed from p1B/skact(-2000)/sTnC, as XbaI/BamHI fragment and ligated into pΔE1sp1B-polyA.	SV40
p1A/HCA(-485)/sTnCΔ9:HA/ polyA	HCA(-485)	sTnCΔ9:HA	sTnCΔ9:HA cDNA and SV40 poly A sequence are removed from pGEM/sTnCΔ9:HA, using *Eco*RI/*Bgl*II digest and ligated into p1A/HCA(-485)	SV40

Table 3.5: Adenoviral Shuttle Vectors with Influenza Virus Hemagglutin Antigen (HA) Epitope Tag

Plasmid	Promoter	Gene	Description	Poly-A
pLECM/ sTnCΔ12:HA	CMV-IE	sTnCΔ12	Truncated sTnC coding sequence DNA is PCR-amplified with 5´ EcoRI and 3´ BglII restriction enzyme sites, and ligated into compatible sites in pLECM plasmid. The HA epitope tag double-stranded oligonucleotide is then inserted as a BglII fragment	E1b
pCA13/sTnCΔ12:HA	CMV-IE	sTnCΔ12	sTnCΔ12 cDNA is PCR-amplified from the pLECM/ sTnCΔ12:HA plasmid. Degenerate 3´ primer is used to mutate the BglII restriction enzyme site to BamHI site, and the fragment ligated into EcoRI/BamHI sites in pCA13.	SV40
pCA13/sTnCΔ9:HA	CMV-IE	sTnCΔ9:HA	sTnCΔ9 cDNA is removed from the intermediary vector pGEM/ sTnCΔ9-HA, and the fragment ligated into EcoRI/BamHI sites in pCA13.	SV40
pGEM/ sTnCΔ12:HA	none	sTnCΔ12:HA	sTnCΔ12:HA cDNA was removed from pCA13/sTnCΔ12:HA as a EcoRI/BamHI fragment and ligated into compatible ends of pGEM-7Z(+).	none
pGEM/ sTnCΔ9:HA	none	sTnCΔ9:HA	The sTnCΔ12 part of the sTnCΔ12:HA cDNA was removed from pGEM/sTnCΔ12:HA, leaving the HA tag behind. New truncated sTnC coding sequence, (sTnCΔ9) was PCR-amplified with EcoRI and BglII ends, and used to replace the removed fragment producing a sTnCΔ12:HA tag cDNA.	none

Table 3.6: Adenoviral Shuttle Vectors with SV40 Large T- antigen (T) Epitope Tag

Plasmid	Promoter	Gene	Description	Poly-A
pCA13-T	CMV-IE	none	SV40 large T-antigen epitope tag sequence inserted as a XbaI/BamHI oligo, into pCA13. This construct can be used to incorporate the T-antigen epitope tag at the 3´ end of cDNA for any protein, in order to tag the protein with a COOH-terminal.	SV40
pCA13/sTnCΔ8-T	CMV-IE	sTnCΔ8:T	Truncated sTnC coding sequence is PCR-amplified with 5´ EcoRI and 3´ NheI restriction enzyme sites, and ligated into compatible sites in pCA13-T, to produce a sTnCΔ8:T cDNA.	SV40
pCA13/sTnC-T	CMV-IE	sTnC:T	sTnC coding sequence is PCR-amplified with 5´ EcoRI and 3´ NheI restriction enzyme sites, eliminating the stop codon only. The modified cDNA is ligated into compatible sites in pCA13-T, to produce a sTnC:T cDNA, coding for full-length sTnC with T-antigen epitope tag.	SV40

Table 3.7. Completed Vectors with Tissue-specific Promoters and β-galactosidase Reporter Gene

Plasmid	Promoter	Gene	Description	Poly-A
p1A/HCA(-177)/β-gal	HCA(-177)	β-galactosidase	β-galactosidase encoding sequence is removed along with the SV40 polyA, from pCA17, as a BamHI/ BglII fragment, and ligated into the BglII site of the intermediary vector p1A/HCA(-177)	SV40
p1A/HCA(-485) / β-gal	HCA(-485)	β-galactosidase	β-galactosidase encoding sequence is removed along with the SV40 polyA, from pCA17, as a BamHI/ BglII fragment, and ligated into the BglII site of the intermediary vector p1A/HCA(-485)	SV40
p1A/skact(-1282)/ β-gal	skact(-1282)	β-galactosidase	β-galactosidase encoding sequence is removed along with the SV40 polyA, from pCA17, as a BamHI/ BglII fragment, and ligated into the BglII site of the intermediary vector p1A/skact(-1282)	SV40
p1B/skact(-2000)/ β-gal	skact(-2000)	β-galactosidase	β-galactosidase encoding sequence is removed along with the SV40 polyA, from pCA17, as a BamHI/ BglII fragment, and ligated into the BglII site of the intermediary vector p1A/skact(-2000)	SV40

Chapter 4

Results

Recombinant Adenovirus Construction

4.0 Recombinant Adenovirus Construction

For all adenoviral construction work, a mutant of adenovirus type 5 (Ad5) named dl309 (described in section 1.3.5.1) was used as the backbone for generating replication-defective recombinants. Recombinant adenoviruses were constructed using two methods employing varying sources of the viral backbone DNA.

4.1. Recombinant Construction and Improvement of Methods

Initially, the method developed by Thomas Shenk, was employed to generate recombinant adenoviruses. Due to some shortcomings of this method, another method using an altered source of the viral backbone, developed by Frank Graham *et al.* was used. Both methods are based on recombinant adenovirus generation by homologous recombination.

4.1.1. Conventional Method of Recombinant Adenovirus Production

This method of adenovirus construction is based on homologous recombination between Ad5 viral genome DNA and compatible adenoviral shuttle plasmid vector DNA, carrying the gene of interest. These DNA are cotransfected into Human Embryonic Kidney (HEK) 293 cells, where they recombine to generate replication-defective recombinant adenovirus (see section 1.3.6).

The Ad5-dl309 genomic DNA is obtained in large amounts by first obtaining a high titre, CsCl-purified Ad5-dl309 virus preparation as described in section 2.11.4, and digesting the protein coat by Proteinase-K (see section 2.11.5.) to release the viral DNA. The DNA is then phenol-chloroform extracted and ethanol precipitated and quantified as described in section 2.5. A known amount of DNA is then digested with *Xba*I, which cuts within the E1 region making it non-functional. This *Xba*I-cut Ad5-dl309 genome is then cotransfected with a pLES53-based adenoviral shuttle vector (see fig 1.3.4), with the expression cassette of interest.

4.1.1.1. Lipofection and Recombinant Screening

Transfections were performed by lipofection using DOTAP as described in section 2.9.2. 5μg of Ad5-dl309 DNA and 5μg of pLES53-based vector plasmid was used for the transfection into 293 cells. Cells were grown in liquid medium for four to five days and then harvested and freeze-thawed to release any virus generated. The cell lysate was then plated onto fresh 293 cells using a range of 10-fold serial dilutions for isolation of single plaques of newly made virus. Cells infected with viral dilutions were overlaid with MEM containing 0.6% noble agar after which they were cultured for several days. Plaques which began appearing after 4-5 days were picked and reinfected onto fresh 293 cells until completion of a full cpe. Amplified plaques were then harvested for extraction of viral DNA as described in section 2.11.5. Comparison of HindIII digests for recombinants and Ad5-dl309 virus were then performed.

The screening of the plaques by this method was time-consuming, as a considerable number of the plaques screened showed an Ad5-dl309-like pattern for the *Hind*III digest. This may have been due to religation of the *Xba*I cut Ad5-dl309, resulting in an intact Ad5-dl309 genome. This resulted in a bias towards production of the parental Ad5-dl309 virus rather than homologous recombination taking place to generate a recombinant.

4.1.2. Frank Graham's Method of Recombinant Production

The large plasmid method uses the same Ad5-dl309 mutant adenovirus backbone as the Tom Shenk's method, but with the virus genome in a plasmid form such that large scale production of viral DNA, is not required. Frank Graham and colleagues have modified the viral genome such that it has the ability to replicate in a bacterial *E.coli* DH5 host and can be purified by CsCl density gradient centrifugation (see section 2.3.2.1.1.). The large adenoviral plasmid pJM17 (Microbix Biosystems Inc., Toronto, Canada; described in section 1.3.7.1) was used in the generation of majority of the recombinants in the present study. pJM17 can yield progeny that comprise 90-95% recombinants. In the event of parental type virus arising, this may

either be due to chance elimination of the bacterial pBR322 fragment after transfection or a rare recombination event occurring between the plasmid and the E1 region of the 293 cell genome.

4.1.2.1. Transfections and Recombinant Screening

5 to 15μg each of pJM17 and the appropriate adenoviral shuttle vector was typically transfected into 293 cells by the $CaPO_4$ transfection method. After exposure to the DNA/$CaPO_4$ containing transfection mix for 6-8 hours, cells were overlaid with a modified soft agarose medium and dishes were cultured for several days (see section 2.10.2.). Dishes were examined daily for plaques, which typically began appearing from day 5 of culturing, but could arise up to 13 days after transfection. Plaques appearing as holes on the cell sheet with rounded up cells in the center, were picked as they are detected, and reinfected on to fresh 293 cells to amplify the virus. The reinfected cells were harvested when they reached full cpe, and freeze-thawed to release the virus. At this stage, half of this lysate was used for extraction of viral DNA as described in section 2.11.5.

Viral DNA was digested with *Hind*III, separated on a 1% agarose gel and compared with the restriction pattern of the parental type (Ad5-dl309) viral DNA. The restriction pattern of a variety of different viruses was compared to a simulated digest carried out on Vector NTI software (see section 2.16.). All potential recombinants whose restriction digest pattern corresponded with that identified by the Vector NTI program were retained and subjected to plaque purification to eliminate potential parental virus contamination.

After plaque purification, some viruses were subjected to screening by PCR using E1 region specific primers (for details see section 2.1.B.). Verified recombinant adenovirus isolates without any parental virus contamination were amplified on a large scale and subjected to CsCl density gradient centrifugation and stored at –70°C in storage buffer as described in section 2.11.3.

4.2. Optimization of Recombinant Construction Methods

The pLECM-based construction method was used at the early stages of the current project, but proved to be inefficient in our hands. A high background of the parental Ad5-dl309 virus was obtained which may have resulted from religation of the *Xba*I-cut cohesive termini. Such religation would result in a functional genome with an intact E1A region, which is likely to have an adaptive advantage over the recombinant virus. Thus, under these circumstances, a larger number of parental type virus than recombinants would be generated. Presence of parental-type virus makes the recombinant isolation process lengthy and necessitates the screening of 50 or more plaques for a recombinant to be found.

The transfection protocols used in the T. Shenk's methods proved inefficient when the Microbix plasmids were used. Use of pJM17 plasmid should result predominantly in production of recombinant viruses. When Shenk's method was employed, cells were grown in liquid medium and harvested after 4 days or left to show signs of cpe and then reinfected to screen for any recombinants generated. This method resulted once more in a frequency of recombinant of about 1%, making the isolation procedure cumbersome. Two recombinant adenoviruses were generated using this method, only after screening up to 100 plaques for each.

4.2.1. Optimized Method for Recombinant Isolation

A third protocol which represented a modification of the method of Frank Graham *et al.* (Graham and Van der Eb, 1973; Graham and Prevec, 1995) was then successfully employed to generate recombinants reproducibly. The media and other reagents used were all standard commercially available products, which were chosen to assist in minimizing the inter-experiment variability.

4.2.1.1. Transfections

Low passage 293 cells were split at low density and grown to a confluency of 70-80%, which normally takes about 48 hours. One day before the transfection, the original medium on the cells was replaced by fresh MEM with

5% FCS and 2.5mM Glutamine, to induce slow growth of cells: Slow growing 293 cells were found to be an essential requirement for allowing uptake and consequent homologous recombination of the plasmids.

Production of the transfection mix remained the most intrinsically variable step of the protocol. This transfection stage involved mixing the HBS-Phosphate and DNA/ $CaCl_2$ mix, forming a fine $CaPO_4$/DNA precipitate which is endocytosed by the 293 cells. The 1X HEPES-buffered-saline-phosphate (HBS-PO_4) was obtained by making a 2X HBS-phosphate buffer and diluting with equal volume of the DNA/$CaCl_2$ solution as described in section 2.10.1. Different mixing methods were tried and it was found that mixing by adding the DNA/$CaCl_2$ solution to an equal volume of 2X HBS-PO_4 (with 10μg/ml of salmon sperm carrier DNA) immediately was the most efficient for obtaining a fine precipitate and avoiding a flocculant precipitate. The gradual, drop-wise addition method generally gave flocculant precipitate which could result in cellular toxicity, but on shorter incubation did result in successful recombinant plaques.

4.2.1.1.i. Role of Carrier DNA

The use of carrier DNA in the transfection procedure was also a critical factor in obtaining recombinant adenoviruses. This maybe due to better transfection efficiencies obtained by using additional DNA. It has been postulated that this carrier DNA may act as a decoy, engaging most of the DNA-degrading enzymes encountered at a stage after the DNA/$CaPO_4$ uptake and during its transport to the nucleus of the cell. This would make it possible for the pJM17 and the smaller shuttle vector plasmids to enter the nucleus un-degraded and undergo homologous recombination.

4.2.1.2. Agarose Overlay

Following the cotransfection, the 293 cells were overlayed with soft agar medium composed of 2X Basal Minimal Eagle's (BME) medium and 2.2% Low Melting Point (LMP) agarose with 5% Foetal calf serum. 2X BME and 2X MEM, also used in the overlay, are commercially available from Gibco in batches of reproducible quality. BME was chosen over 2X MEM as the media

in the first overlay for its similarity of constituents to the regular MEM particularly glucose at 1 g/litre: Amino acid and vitamin composition of this medium was marginally lower, however, this factor is seen to contribute to the essential requirement of slow growth of cells post transfection. The overlay-purpose MEM had 4.5 times more glucose, even though all other components were the same as the regular MEM. Highly purified FCS (Gold CELLect foetal bovine serum from ICN-Flow) was incorporated into the agarose overlay to avoid the toxicity associated with regular serum.

All subsequent overlays were carried out using 2X MEM. The overlay contained 10% regular FCS. This FCS was not deleterious for the cells in the second overlay onwards, but sometimes induced breaking in the cell monolayer, giving a false effect of plaques.

Genuine viral plaques were seen to appear between 9 to 13 days after transfection. Generally 3-4 plaques were obtained in a transfection using 10μg of pJM17 and 10μg of the appropriate shuttle vector, which represented a suboptimal efficiency for recombinant generation. In general, 60% of plaques represented true viral plaques containing correct, while 40% of the plaques did not give rise to a cpe upon reinfection: These latter plaques may represent product of defective adenoviral particles arising due to anomalous homologous recombination. The highest number of plaques obtained was 9 in one case, but on a number of occasions, only a single plaque was obtained which was found to represent a recombinant with the correct DNA digest pattern.

4.4. Recombinants Made and Studied

Attempts to generate recombinant adenoviruses were made using a range of shuttle plasmids with various foreign gene inserts. The plasmids used and the success achieved is presented in table 4.1. All putative recombinants were initially screened by comparison with Vector NTI simulated *Hind*III digest patterns for each, as described in section 2.16. The parental Ad5-dl309 virus digested with *Hind*III produces fragments of sizes 8010, 5446, 5322, 4597, 3437, 2937, 2802, 2081, 994 and 75 base pairs. The 75 bp fragment was not taken into consideration because it does not differ between recombinants and wild type virus, and cannot be visualized on the 1% agarose gels used in our analysis. The 3437 bp and the 2802 bp fragments of the parental type virus pattern differ in all recombinants and were absent or altered in size. Additional bands are seen in the digest pattern depending upon which *Hind*III sites are removed or added along with the inserted foreign DNA fragment from the shuttle vector. The 3.4 kb and 2.8 kb fragments may thus increase or decrease in size or alternatively give rise to smaller fragments.

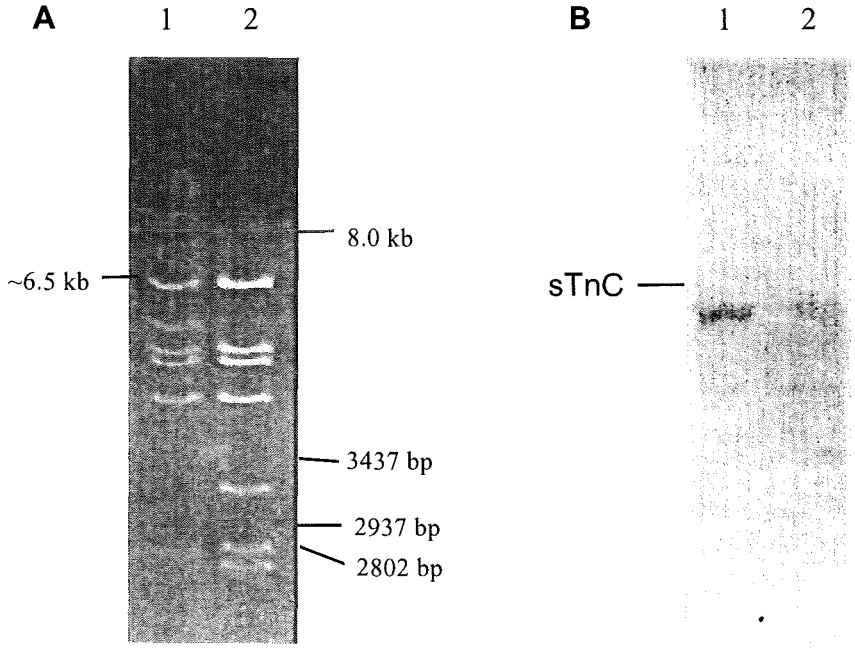
4.4.1. Recombinant Adenoviruses Constructed Using Shenk's Method

One recombinant adenovirus was constructed using the conventional (T. Shenk's) protocols for recombinant generation. 5μg each of Ad5-dl309 *Xba*I cut DNA and the shuttle plasmids pLECM/sTnC were cotransfected using DOTAP and putative recombinants screened as described in section 2.10.3. Numerous plaques were screened of which one plaque displayed a recombinant type pattern *i.e.* the 2.8 kb fragment missing and additional bands appearing. This recombinant was retained for further screening by southern blot analysis and named to as Ad5/CMV/sTnC.

This recombinant was analysed by southern blotting as described in section 2.12.2. The membrane was probed with a skeletal Troponin C specific probe described by Gahlman *et al.*, 1988. The probe DNA was obtained as a 460 nucleotide, *Hind*III and *Eco*RI fragment from plasmid LK975. The DNA was separated using a 1% agarose gel and extracting the DNA out as described in sections 2.4.3. and 2.4.5 and labeling with [α-^{32}P]dCTP as described in

section 2.5.6. Ad5/CMV/sTnC recombinant was made and analyzed as a collaborative work with Dr. Yvonne Alexander (H.P. Labs).

The recombinant showed hybridization of skeletal Troponin C cDNA specific probe to an approximately 6.5 kb *Hind*III fragment, confirming the presence of the foreign gene insert (see fig. 4.1.). The reason for the different *Hind*III digested fragment size for these recombinants may represent an anomalous



1. Ad5/CMV/sTnC recombinant adenovirus
2. Ad5-dl309 parental adenovirus (control)

Fig 4.1: Southern blot analysis of skeletal Troponin C-expressing recombinant adenovirus.
A. Ad5/CMV/sTnC recombinant and Ad5-dl309 parental type virus DNA isolated, digested with *HindIII* and resolved on a 1% agarose gel. DNA from the gel was blotted on to Hybond N+ membrane and probed as described in section 2.12.2.
B. Hybridization of radiolabeled sTnC-specific probe to the *HindIII* fragment confirming the presence of sTnC gene in the recombinant virus.

recombination event. Nonetheless, the latter did not interfere with the incorporation of the promoter-cDNA cassette or subsequent foreign gene transcription.

4.4.2. Recombinant Adenoviruses Constructed Using Frank Graham's Method

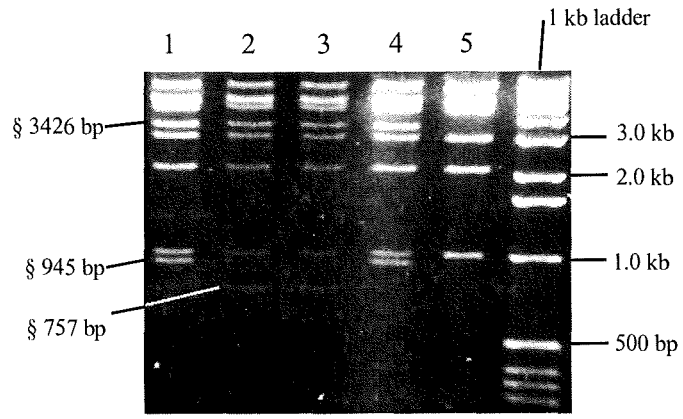
A number of recombinants were generated using this protocol and were confirmed by matching the restriction digest patterns with those generated by the Vector NTI software.

10μg each of the large adenoviral genome carrying pJM17 plasmid and the small adenoviral shuttle plasmids was used. Plaques obtained from transfected and agarose-overlaid plate, were amplified as described in section 2.10.3. The DNA was subjected to *Hind*III digest to determine whether the recombinants were correct. The presence of a correct cDNA insert was also confirmed by PCR analysis (see section 2.12.1).

4.4.2.1. Ad5 Epitope Tagged Troponin C Recombinants

Adenovirus recombinants expressing Troponin C protein tagged with a HA antigen epitope tag were made, both with the ubiquitous CMV-IE promoter and the tissue-specific HCA(–485) promoter. The shuttle plasmid pCA13/sTnCΔ9:HA was used to make the CMV-IE driven tagged troponin C recombinant. *Hind*III digest pattern for this recombinant exhibited the parental background pattern with the 3437 bp fragment altered to 3426 bp which was not noticeable on a 1% agarose gel. However, the 2.8 kb fragment was replaced by approximately 750 bp and revealed the recombinant nature of virus, designated Ad5/CMV-IE/sTnCΔ9:HA (see fig. 4.2). The tissue specific Ad5 recombinant was made using the shuttle plasmid p1A/HCA(–485)/sTnCΔ9:HA: The *Hind*III digest pattern was similar for the 3437 bp fragment as explained above but the 2.8 kb fragment was replaced by one approximately 950 bp (see fig. 4.2). This recombinant was designated as Ad5/HCA(–485)/sTnCΔ9:HA.

A



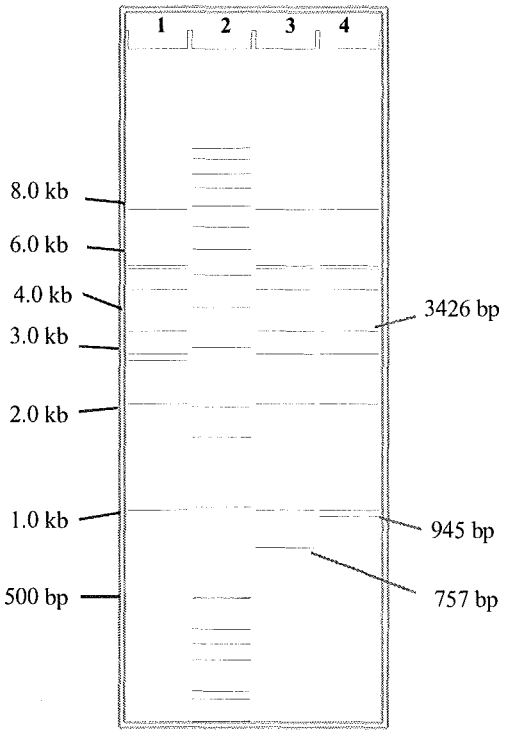
§ new or altered fragments appearing in recombinants only

1& 4: Ad5/HCA(-485)/sTnCΔ9-HA recombinant

2 & 3: Ad5/CMV-IE/sTnCΔ9-HA recombinant

5: Ad5 recombinant showing an unexpected pattern

B



1	2	3	4
8010	1 kb	8010	8010
5446		5446	5446
5322		5322	5322
4597		4597	4597
3437		***3426***	***3426***
2937		2937	2937
2802		2081	2081
2081		994	994
994		***757***	***945***
75		75	75

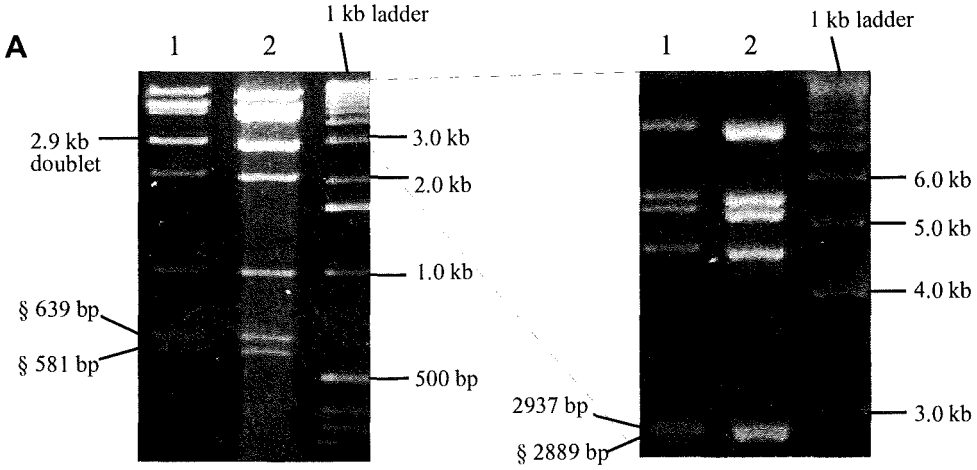
1. Ad5-dl309
2. 1 kb marker DNA
3. Ad5/CMV-IE/sTnCΔ9-HA recombinant
4. Ad5/HCA(-485)/sTnCΔ9-HA recombinant

Fig 4.2: *Hin*dIII digest patterns of HA epitope-tagged skeletal Troponin C-expressing recombinant adenoviruses. **A.** Recombinant DNA digested with *Hin*dIII and resolved on a 1% agarose gel. **B.** Simulated *Hin*dIII digest fragment pattern produced by the Vector NTI software, for recombinant viruses shown in A and the parental Ad5-dl309 for comparison. Fragment lengths of the three viruses shown in the table were also generated by Vector NTI: Lengths shown in Bold/italics are characteristic of the recombinant. Lane 3 & column 3 of B corresponds to lanes 2 and 3 of A, while lane 4/column 4 of B shows expected results of virus shown in lane 1 and 4 of A.

4.4.2.2. Ad5 Troponin Recombinants with Tissue-specific Promoters

Recombinants with the sTnC coding driven by Human cardiac actin (HCA) tissue-specific promoter were made using the shuttle plasmid p1A/HCA(-485)/sTnC/pA and p1A/HCA(–177)/sTnC/pA, containing the full length and truncated HCA promoters respectively. The *Hind*III digest pattern for the full length HCA(–485)/sTnC recombinant was similar to the parental virus pattern but with the 3437 bp fragment replaced by approximately 2.9 kb while the 2802 bp fragment was replaced by smaller 950 and 580 bp fragments (see fig. 4.4). The recombinant was designated Ad5/HCA(–485)/sTnC. The shorter HCA promoter recombinant displayed a 2889 fragment in place of 3437 bp fragment, while the 2802 bp fragment replaced by 640 and a 580 bp fragments when digested with *Hind*III (see fig. 4.3). This recombinant was designated Ad5/HCA(–177)/sTnC.

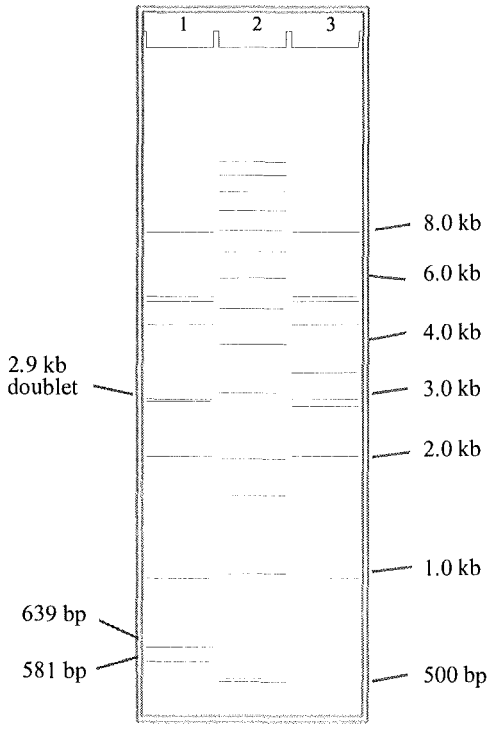
The Adenovirus encoding skeletal Troponin C cDNA under human skeletal α-actin promoter were made similar to procedures described above. The shuttle plasmid p1B/skact(–2000)/sTnC/pA was used to generate a recombinant adenovirus with skeletal Troponin C coding sequence transcribed using the full length human skeletal α-actin promoter. The *Hind*III digest of the recombinant virus was based on the parental background pattern with the 3437 bp fragment replaced by one slightly increased to 3446 which displayed a difference which was undetectable on a 1% agarose gel. The 2.8 kb fragment was replaced by a 2.2 kb and a 360 bp fragment (see fig. 4.4) This recombinant was designated Ad5/skact(–2000)/sTnC. To make adenovirus coding sTnC under the control of the Skeletal actin (–1282), the p1A/skact(–1282)/sTnC/pA plasmid was employed. The *Hind*III digest pattern displayed a replacement of 3437 fragment with one of 2.9 kb, and the 2.8 kb fragment replaced by a 1.9 kb and a 580 bp fragment, by comparison to the parental type *Hind*III digest pattern (see fig. 4.5). The recombinant virus was designated Ad5/skact(–1282)/sTnC.



§ new or altered fragments appearing in recombinants only

Lanes 1 & 2: Ad5/HCA(-177)/sTnC

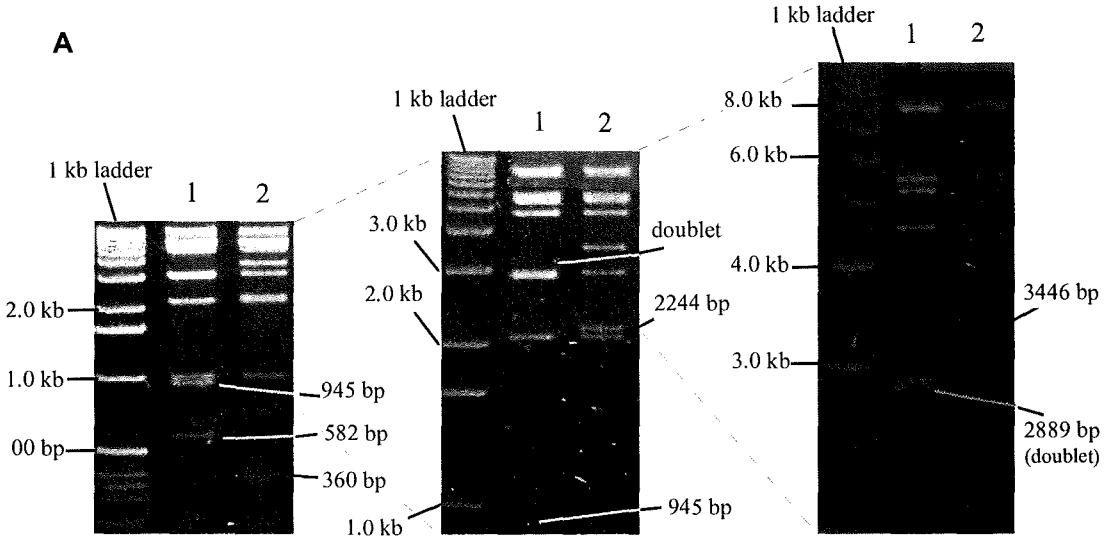
B



1	2	3
8010	1 kb	8010
5446		5446
5322		5322
4597		4597
2937		3437
2889		2937
2081		2802
994		2081
639		994
581		75
75		

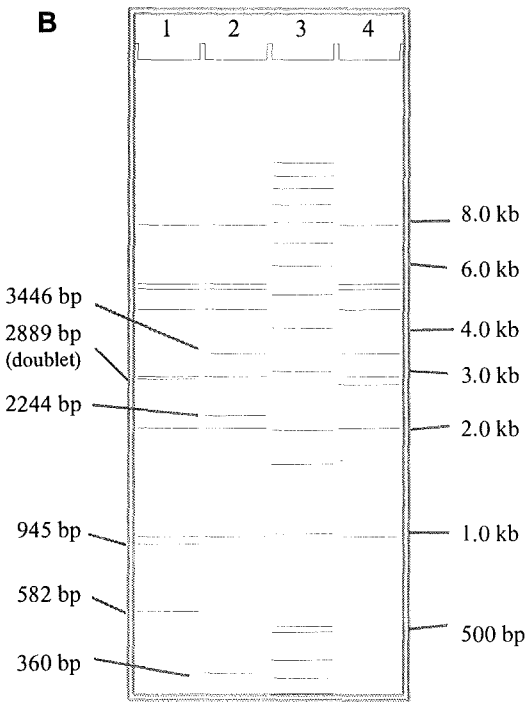
1. Ad5/HCA(-177)/sTnC recombinant
2. 1 kb ladder marker
3. Ad5-dl309

Fig. 4.3: *Hind*III digest pattern of cardiocyte-specific skeletal Troponin C-expressing recombinant adenoviruses transcribed by the shorter Human Cardiac Actin promoter. **A.** Recombinant virus DNA digested with *Hind*III and resolved on a 1% agarose gel. (The gel on the left is the same as the one on right but was run longer to resolve the fragment doublet at 2.9 kb.) **B.** Simulated *Hind*III digest fragment pattern produced by the Vector NTI software for recombinant viruses and the parental Ad5-dl309 for comparison. Fragment lengths of the two viruses shown in the table were also generated by Vector NTI: Lengths shown in Bold/italics are characteristic of the recombinant. Lane & column 3 of B corresponds to the two lanes shown in A.



* fragments of lengths shown on the right side of the gels are observed only in recombinants

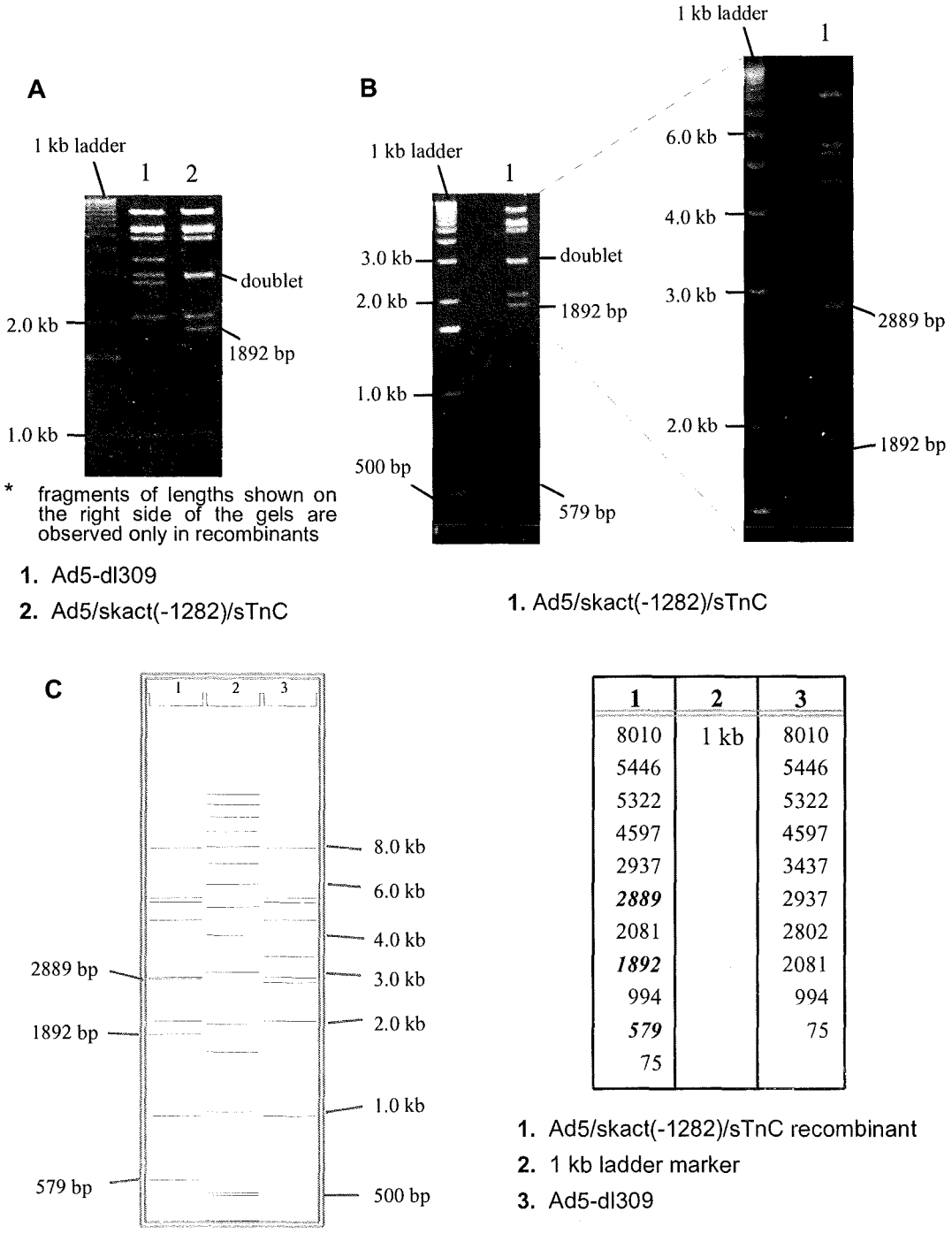
1. Ad5/HCA(-485)/sTnC
2. Ad5/skact(-2000)/sTnC



1	2	3	4
8010	8010	1 kb	8010
5446	5446		5446
5322	5322		5322
4597	4597		4597
2937	***3446***		3437
2889	2937		2937
2081	***2244***		2802
994	2081		2081
945	994		994
582	***361***		75
75	75		

1. Ad5/HCA(-485)/sTnC recombinant
2. Ad5/skact(-2000)/sTnC recombinant
3. 1 kb ladder marker
4. Ad5-dl309

Fig. 4.4: *Hin*dIII digest pattern of cardiocyte-specific (human cardiac actin full-length promoter driven), and myocyte-specific (skeletal α-actin full-length promoter driven) skeletal Troponin C-expressing recombinant adenoviruses. **A.** Recombinant virus DNA digested with *Hin*dIII and resolved on a 1% agarose gel. (The three pictures are of the same gels run for increasing length of time, in order to resolve and confirm the fragment doublets appearing at approximately 3 kb.) **B.** Simulated *Hin*dIII digest fragment pattern produced by the Vector NTI software for recombinant viruses and the parental Ad5-dl309 for comparison. Fragment lengths of the three viruses shown in the table were also generated by Vector NTI; lengths shown in Bold/italics are characteristic of the recombinant. Lane & column 1 of B corresponds to lane 1 of A, while lane/column 2 of B shows expected results of virus shown in lane 2 of A.



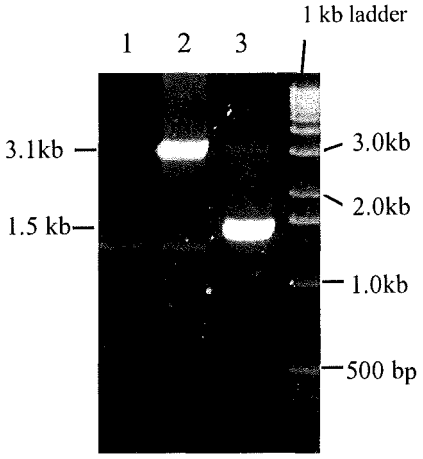
1	2	3
8010	1 kb	8010
5446		5446
5322		5322
4597		4597
2937		3437
2889		2937
2081		2802
1892		2081
994		994
579		75
75		

Fig. 4.5: *Hin*dIII digest patterns of parental Ad5-dl309 virus and Ad5/skact(-1282/sTnC recombinant virus with shorter skeletal α-actin promoter for tissue specific expression in myocytes. **A.** Recombinant virus and parental virus DNA digested with *Hin*dIII and resolved on a 1% agarose gel for comparison. **B.** recombinant adenovirus DNA digested and separated on a 1% agarose gel. (both pictures are of the same gels run for increasing length of time, in order to resolve and confirm the fragment doublets appearing at approximately 3 kb.) **C.** Simulated *Hin*dIII digest fragment pattern produced by the Vector NTI software for recombinant viruses and the parental Ad5-dl309 for comparison. Fragment lengths of the two viruses shown in the table were also generated by Vector NTI; lengths shown in Bold/italics are characteristic of the recombinant. Lane & column1of C corresponds to lane 2 of A and 1 of B. Lane/column 3 of C shows expected DNA digest pattern of of virus shown in lane 1 of A. Fragment lengths of table shown in B

4.5. Additional Recombinants Screening Procedures

The majority of recombinants were initially screened by PCR using Ad5 primers, before a *Hind*III digest or southern analysis was carried out. Recombinants were also checked for purity by screening for contamination by parental-type virus, using E1-specific primers (see table 2.1-B).

PCR analysis was carried out using the Ad5 primers which hybridized to bp 280-289 (sense primer) and bp 3580-3598 (antisense primer) on the wild type adenovirus genome. These primers were designed to flank any foreign DNA, promoter-cDNA cassette insert and amplify the same in a PCR reaction (also see table 2.1-B.).



1. pJM17 (control)
2. Ad5/skact(-2000)/sTnC
3. Ad5/HCA(-485)/sTnC

Fig 4.6: Screening of Ad5 recombinants by PCR analysis. PCR amplification was done using viral DNA isolated from recombinants as template DNA. The Ad5 280-99 and Ad5 3580-99 primers amplified a 3.1 kb (approximately) fragment on the Ad5/skact(-2000)/sTnC recombinant. The Ad5/HCA(-485)/sTnC DNA template amplified a 1500 bp fragment using the same primer pair. The control pJM17 cannot amplify the DNA under conditions used and shows non-specific bands.

On the Ad5-dl309 virus genome template, the Ad5 primers amplify a 3319 bp fragment using these primers whereas on an E1 region-deleted adenovirus genome, a fragment of 138 bp is amplified. The pJM17 plasmid template which contains a pBR322 bacterial plasmid insert at 1339 bp, would amplify a fragment of size 7690 under altered PCR conditions, but no product is obtained under the PCR conditions used for screening the recombinants. The

Ad5 primer pair will amplify different size fragments depending upon the length of the promoter-cDNA cassette contained in the recombinant adenovirus (see fig. 4.6).

Table 4.1: Recombinant Adenoviruses Constructed and Studied for Expression of the Transgene

Shuttle Vectors used to make recombinants	Success	Adenovirus Name	Recombinants Confirmed By	Expression Studies Done (+/-)	E1-region Screening
pLECM/sTnC	Yes	Ad5/CMV/sTnC	Southern Blot	Northern Blot (+)	Negative
pLECM/sTnCΔ12-HA	Yes	Ad5/CMV/sTnCΔ12-HA	not done	not done	Negative
pCA13/CMV-IE/sTnC	No				
p1A/HCA(–177)/sTnC/pA	Yes	Ad5/ HCA(–177)/sTnC	Vector NTI simulated *HindIII* digest.	not done	Negative
p1A/HCA(–485)/sTnC/pA	Yes	Ad5/HCA(–485)/sTnC	Vector NTI simulated *HindIII* digest	not done	Negative
p1A/HCA(–485)/β-gal	No				
p1A/skact(-1282)/sTnC/pA	Yes	Ad5/skact(-1282)/sTnC	Vector NTI simulated *HindIII* digest	not done	Negative
p1A/Skact(–2000)/sTnC/pA	Yes	Ad5/Skact(–2000)/sTnC	Vector NTI simulated *HindIII* digest	not done	Negative
p1A/Skact(–1282)/β-gal	No				
p1A/Skact(–2000)/β-gal	No				
pCA17/CMV-IE/β-gal	No				
pCA18 /CMV-IE/Luciferase	No				
pCMV-IE/sTnCΔ9:HA	Yes	Ad5/CMV-IE/sTnCΔ9:HA	Vector NTI simulated *HindIII* digest	Western Blotting and Immunocytochemistry (+)	Negative
p1A/HCA(–485)/sTnCΔ9:HA	Yes	Ad5/ HCA(–485)/sTnCΔ9:HA	Vector NTI simulated *HindIII* digest	Immunocytochemistry (+)	Negative

Chapter 5

Results

Expression Studies

5.0 Expression Studies

A number of recombinant adenoviruses produced in this project were analyzed for expression under culture conditions. Expression studies were either carried out at the RNA level using Northern analysis or at the protein level using Western blot analysis and Immunocytochemistry. Reporter gene-expressing Ad5/β-gal recombinant virus was used to establish transduction protocols *in vitro.* and *in vivo.* NIH3T3 mouse fibroblasts, primary neonatal rat cardiocyte and cardiac fibroblasts were the standard *in vitro.* systems used for studying expression.

The β-galactosidase-expressing adenovirus (obtained from Prof. Ron Hayes, University of St. Andrews), was used to standardize the *in vitro.* virus transduction protocols at the initial stages of the project. Typically, Ad5/β-gal adenovirus was transduced at a titre of 10^7-10^8 pfu/ml into cells in culture as described in section 2.11.6. Infected cells were given 36-48 hours for expression of the reporter gene. The cells were then fixed and stained with X-gal as described in section 2.15.2.

5.1. Analysis of Skeletal Troponin C Expression

Due to non-availability of skeletal Troponin C-specific antibody, the protein was tagged with the human influenza virus hemagglutinin (HA) epitope tag at the DNA level. The protein expression was analysed using the HA epitope tag-specific antibody using western blots and immunocytochemistry. Wild type (non-tagged) sTnC-expressing recombinant adenoviruses could only be studied at the transcriptional level for reasons described above and were studied using Northern blot analysis.

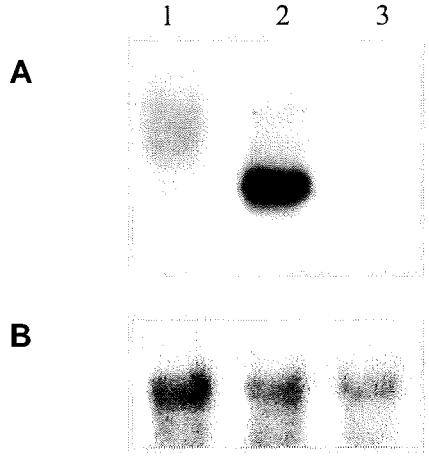
5.1.1. Northern Blot Analysis

The expression of the wild-type skeletal Troponin C-expressing recombinant adenovirus, Ad5/CMV-IE/sTnC, was analysed by Northern Blot analysis. Neonatal rat cardiocytes were isolated and cultured in 60mm dishes as described in section 2.8.4, and infected with the Ad5/CMV-IE/sTnC virus

solution at a titre of 10^5-10^6 pfu/ml, following the procedure described in section 2.11.6. The cells were allowed 48-72 hours for expression of the transgene and the total RNA isolated using RNAzol™B as described in section 2.13. The quality of RNA was checked on a 1% agarose gel and then quantified as described in section 2.5.4.

10μg of each of the test and control RNA samples were fractioned on a 1% denaturing formaldehyde-agarose gel made up in MOPS buffer as described in section 2.13.2. The separated RNA was transferred to Hybond N+ membrane using the alkali transfer protocol as described in section 2.15.4. The membrane was probed with a skeletal Troponin C specific probe described by Gahlman *et al*, 1988. Probe DNA was obtained by removing a 460 nucleotide, *Hind*III and *Eco*RI fragment from plasmid LK975, separating on a 1% agarose gel and extracting the DNA out as described in sections

Northern Blot Analysis of sTnC-expressing Recombinant Adenovirus



1. Control; uninfected cardiocytes only
2. Ad5/CMV/sTnC recombinant adenovirus
3. Control; cardiocytes infected with Ad5/β-gal

Fig 5.1: Northern blot analysis of Ad5/CMV/sTnC recombinant adenovirus. Ad5/CMV/sTnC recombinant was analyzed for expression at the level of transcription by infecting cultured cell with viruses and extracting total RNA. 20μg of each sample of RNA was resolved on a 1% formamide-agarose gel and analysis was carried out as described in section 5.1.1. **A.** Resolved total RNA was probed with sTnC-specific radiolabeled DNA. Lane 2 shows hybridization of sTnC-specific probe to a transcript indicating expression of the gene of interest. **B.** Total RNA was probed with 28S ribosomal RNA to show equal loading of samples, as described in section 5.1.1.

2.4.3. and 2.4.5. DNA was labeled with [α-^{32}P]dCTP as described in section 2.5.6. The membrane was reprobed with human ribosomal 28S RNA oligonucleotide (Clonetech Ltd. cat. no. 9034-1). This analysis was carried out in collaboration with Dr. Yvonne Alexander, Dept. of Medicine and Therapeutics.

5.1.2. Western Blot Analysis

The recombinant adenoviruses generated were infected into cells and subsequently analysed for expression of the skeletal Troponin C protein *in vitro*. The protein was tagged with the human influenza virus hemagglutinin (HA) epitope, at the carboxyl terminal as described in section 3.1.5. The tagged protein expression was analysed by western blot analysis and immunocytochemistry of cultured fibroblasts and cardiocytes infected with the HA epitope tagged sTnC expressing recombinant adenoviruses. The HA epitope tag was inserted at the DNA level, by deleting bases encoding the 9 amino acids from the carboxyl terminal end of the sTnC protein cDNA. The deletion was substituted by a 12 amino acid HA tag encoding sequence thereby making the protein longer by 3 amino acids.

5.1.2.1. Virus Infection and Sample Preparation for Western Blot Analysis

Cells were split into 100mm tissue culture dishes and grown to approximately 50 or 60% confluency as described in section 2.8.4. Cardiocytes were isolated and cultured as described in section 2.8.4.5., and cultured in 60mm dishes for 72 hours before they were infected with the viruses.

Four 100mm dishes with NIH3T3 were infected with 150µl of (1), PBS only for cell line control; (2), virus solution of Ad5-dl309 for adenovirus control; (3), virus solution of Ad5/CMV-IE/sTnCΔ9-HA; and (4), virus solution of Ad5/HCA(-485)/sTnCΔ9-HA, at an estimated titre of 10^6-10^7 pfu/ml. The cells were incubated at 37°C in 5% CO_2 for 48-60 hours to allow the virus to express the foreign protein.

Cardiocytes were infected in the same manner as described above for 3T3 cells, but the medium was supplemented with 1X BrdU to prevent the growth

of cardiac fibroblasts. Infections were done with 100μl of (1) PBS only, for cardiocyte-only control and with virus extracts at an estimated titre of 10^6-10^7 pfu/ml of (2) Ad5/CMV/sTnCΔ9-HA and (3) Ad5/HCA(-485)/sTnCΔ9-HA. Cardiocytes were then incubated at 37°C in 5% CO_2 for 48-60 hours to allow the virus to express the foreign protein.

Infected NIH3T3 cells and cardiocytes were harvested as described in section 2.14.1 and freeze-thawed to extract the proteins as described in section 2.14.1.1. The lysates were transferred to a fresh tube and frozen at -20°C until being subjected to electrophoretic separation on a SDS-PAGE gel.

The protein concentration of the samples was determined using the Biorad Protein Assay Kit as described in section 2.14.2. Cell lysate containing approximately 100μg of total protein from each sample was taken in a fresh tube and an equal volume of SDS-sample buffer (40mM Tris, pH 6.8, 4mM 2-mercaptoethanol, 2% SDS, 10% glycerol, 0.2% bromophenol blue) added, mixed and boiled for 10 minutes in a boiling waterbath. Samples were allowed to cool down to room temperature and one fifth (20μg) of each protein sample was loaded on SDS-PAGE gels to resolve the proteins for western blotting, or stored at −20°C for later use.

5.1.2.2. SDS-Polyacrylamide Gel Electrophoresis and Electroblotting

SDS PAGE gels were made and run as described in section 2.14.3. The resolving gel strength was 12% Acrylamide/bis 29:1 with 2% stacking gel. Test samples with appropriate controls were loaded. 10μl prestained protein standards (Kaleidoscope Prestained Standards, Biorad cat. no. 161-0324) were also loaded as markers for checking the protein migration in gels, and run at 30V for overnight (about 16 hours) at room temperature or until the blue dye had migrated to the bottom of the gel. After the desired separation was reached, the protein bands were transferred from the gel to the membrane by electroblotting at 100V for 1 to 1.5 hours as described in section 2.15.5.1 in a pre-chilled buffer at room temperature. The protein transfer was confirmed by the successful transfer of prestained protein markers.

5.1.2.3. Immunoblotting

After the transfer of protein, the blot was probed with HA tag-specific antibody using the protocol described in section 2.15.5.2. The primary and secondary antibodies were reconstituted and stored as stock solutions according to the instructions of the manufacturer, and diluted as required with 1% skimmed milk in TBS. Briefly, the membrane was washed once with Tris-buffered saline (TBS) and non-specific binding sites blocked with 10 ml of Blocking solution (5% skimmed milk in TBS), with shaking for at least 2 hours at room temperature. The membrane was washed with TBS and incubated with the primary anti-HA high-affinity rat monoclonal (clone 3F10) antibody (Boehringer Mannheim cat. No. 1-867-431) diluted 1:1000 (100ng/ml) in 1% skimmed milk/TBS) and incubated overnight with shaking at 4°C. The membrane was washed 3 times with TBS T Wash buffer (TBS with 0.1% Tween®20) allowing approximately 5 minutes for each wash.

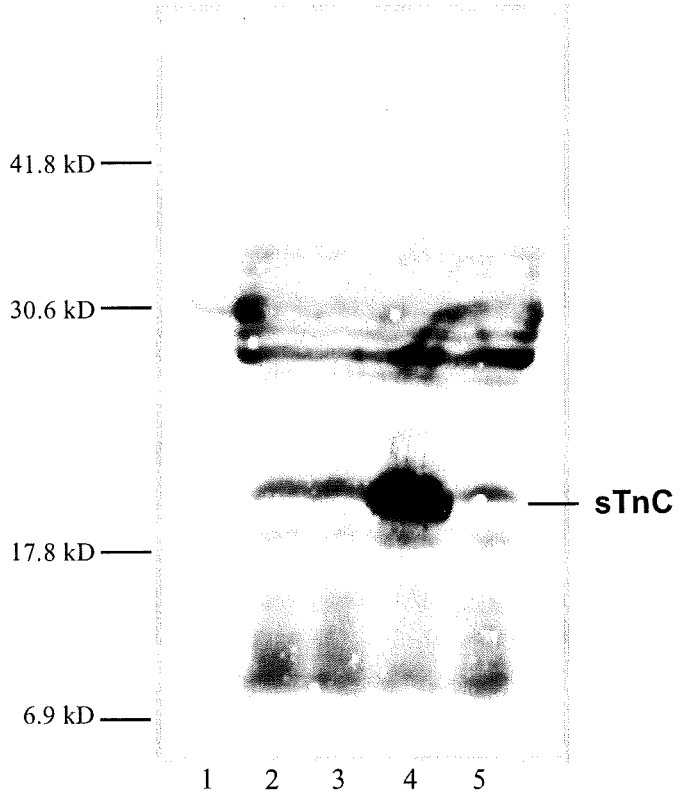
The membrane was then incubated with the anti-rat peroxidase-conjugated secondary antibody (anti-rat Ig-POD, Fab fragments; Boehringer Mannheim cat. No. 1-348-752). The secondary antibody was diluted 1:3000 in 1% skimmed milk/TBS with 0.05% Tween®20, and incubated for 1-2 hours at room temperature, with shaking. Membrane was washed three times with TBS T wash buffer to remove any excess secondary antibody and then three times with TBS to remove traces of Tween®20. Excess fluid was dripped off and the membrane transferred to a shallow container with the protein side up, and 2ml each of premixed solution A and B of the ECL Western Kit (Amersham Life Sciences RPN-2109) were pipetted into one side of the container. The two solutions were mixed thoroughly, and tilted to coat the surface of the membrane completely. The membrane was then drip-dried and layered between two pieces of Saran-Wrap and exposed to X-ray film for exposure times ranging from 5 seconds up to 10 minutes. Fig. 5.2 shows a 15 seconds exposure of the blot.

5.1.2.4. Western Blot Analysis Results

The Ad5/CMV-IE/sTnCΔ9-HA recombinant adenovirus showed expression of the HA-tagged sTnC protein in NIH3T3 mouse fibroblast cells (see fig. 5.2.,

Lane 4). The immunostained protein was estimated as a protein with molecular weight of approximately 21 kD although sTnC wild type protein has

Western Blot Analysis of Cells Infected with Recombinant Adenoviruses



1. Control; Uninfected Cardiocytes
2. Control; uninfected 3T3 mouse fibroblast cells
3. Ad5-dl309 infected 3T3 mouse fibroblast cells
4. Ad5-CMV-IE/sTnCΔ9-HA infected 3T3 mouse fibroblast cells
5. Ad5-HCA(–485)/sTnCΔ9-HA infected 3T3 mouse fibroblast cells

Fig 5.2: Western Blot analysis of cardiocytes and cultured 3T3 fibroblasts infected with recombinant skeletal Troponin C (HA-tagged) expressing adenoviruses. 20μg of total protein from infected cells was loaded on the gel, and the resolved protein bands blotted onto a membrane. The HA epitope-tagged protein was probed with the high-affinity HA primary rat monoclonal antibody and detected using anti-rat Ig-Peroxidase secondary antibody and ECL chemiluminescence method (see text for details). Molecular weights were estimated from the prestained markers used and are represented in kilodaltons (kD). Lane 4 shows the expression of the HA epitope tagged sTnC at approximately 21 kD.

a

calculated molecular weight of 18 kD. It is known that the gel pH affects the migration of proteins (Yates and Greaser, 1983) which could have brought about incorrect co-migration of the markers used and the sample. Also the

Troponin C molecule may have been contaminated by other cellular proteins which failed to dissociate from it during denaturation in sample preparation or during migration in the SDS-PAGE gel.

The Ad5/HCA(-485)/sTnCΔ9-HA recombinant (fig. 5.2, lane 5) did not show any expression in the 3T3 cells as was expected due to the tissue-specific nature of the Human Cardiac Actin promoter. Ad5-dl309 (fig. 5.2, lane 3) was used as a parental virus background control. This control was included to assess if any viral protein may react with the anti-HA primary antibody, producing a false positive result. This virus showed a background similar to the no-virus control (uninfected 3T3 cells) which was the standard background control for the expression system (fig. 5.2, lane 2).

The overall background of the blot was high due to higher amount of protein loading and the use of a directly conjugated secondary antibody, contrary to the recommendation of the protocols outlined in the primary antibody manufacturers guidelines. This problem can be overcome by use of a biotin-conjugated secondary antibody followed by a streptavidin-peroxidase labeling. Also, using less than 10μg of sample can also minimize the background on the blot, as highlighted in the manufacturer's protocols.

After successful detection of expression by ubiquitously-expressing Ad5/CMV-IE/sTnCΔ9-HA, by immunoblotting, the equivalent virus recombinant, but under the control of the cardiac actin promoter, was analyzed for expression. This was carried out using immunofluorescence microscopy of Ad5/HCA(-485)/sTnCΔ9-HA infected primary neonatal rat cardiocyte culture. The Ad5/CMV-IE/sTnCΔ9-HA was also analyzed in the latter system and in NIH3T3 fibroblasts, by immunocytochemical analysis.

5.1.3. Immunocytochemistry

The HA epitope-tagged skeletal Troponin C protein expression by the recombinant adenovirus was further confirmed by immunocytochemical analysis of virus infected cells in culture. Three procedures of immunocytochemistry, employing varying primary/secondary antibodies and

immunodetection methodology were employed. The first was a direct method using primary anti-HA antibody conjugated to Rhodamine. The second was an indirect method using primary anti HA antibody with a secondary Rhodamine-conjugated antibody. The third method was similar to the second but with a secondary antibody conjugated to biotin. Detection of the immunolabeled cells was done using a Streptavidin-Alkaline Phosphatase conjugate followed by staining with Vector® Red staining procedure.

5.1.3.1. Cell Culture and Viral Transduction

NIH3T3 mouse embryo fibroblast cells were grown in tissue culture slide chambers (Nunc cat. no. 170920) and were seeded at a very low density and the fibroblasts were grown until they reached a 50 to 60% confluency. Primary neonatal rat cardiocytes were cultured in 60 mm dishes. The cells were then infected with adenovirus lysate at a titre of approximately 10^6 to 10^7 pfu/ml as described in section 2.11.6.

5.1.3.2. Optimization of Immunostaining Procedures Employing HA-tag Antibody

Three Immunochemical methods were performed as described in section 2.15.6. Briefly, the media was aspirated off and cells fixed with 2 ml of freshly prepared 4% paraformaldehyde for 10 minutes. Fixed cells were then washed three times with PBS allowing 3 minutes per wash, and cells permeabilized by incubating with 0.1% Triton X-100 in PBS, for 5 minutes at room temperature. Cells were washed once quickly in 0.1% Triton X-100 made with distilled water. Permeabilized cells were incubated with Blocking solution (0.5% Bovine Serum Albumin, fraction V, in PBS) for 15 minutes to block non-specific binding sites. The flaskette chambers were removed at this stage and the immunostaining was carried out for the different methods. From the three methods tried, the indirect method using the biotin-conjugated secondary antibody was successful. The other two methods failed to give as discrete results as the one obtained with the former method.

The Direct immunostaining method employed HA-tag specific primary, mouse monoclonal antibody, conjugated to rhodamine, eliminating the use of a secondary antibody. After fixing the cells and blocking non-specific sites (as

outlined above), the Anti HA rhodamine (Boehringer Mannheim, cat. no. 1 666 959) stock was diluted in blocking solution to 1mg/ml and cells incubated with it for 30-45 minutes at room temperature. Phalloidin TRITC at 2mg/ml was added along with the primary antibody to detect cells by staining the actin cytofilaments. Cells were washed 3 times with PBS allowing 5 minutes per wash. The flaskette cover was removed and a drop of Aquamount mounting medium (BDH 36226-2H) was dropped on the cells and a clean coverslip placed gently on it avoiding any air bubbles being trapped. Any excess mounting medium was blotted off and allowed to dry in the dark at room temperature. Once dried, the slides were stored at 4°C or at -20°C until screening under a fluorescent microscope using the appropriate filters.

The Indirect immunostaining methods involving different primary and secondary antibodies were used. For these methods, the two-step immunostaining procedure involved a secondary antibody conjugated to rhodamine. Following the blocking step, cells were incubated with the primary Anti-HA mouse monoclonal antibody (Boehringer Mannheim cat. no. 1 583 816) at 2mg/ml dilution made in the blocking solution, for 30 minutes at room temperature. Phalloidin TRITC was also added as described earlier. Cells were washed three times with PBS and incubated with a 1:2000 dilution of the Anti-mouse IgG-TRITC secondary antibody (sigma T-2402), for 30 minutes at room temperature. Cells were washed three times and the immunostained cells mounted by placing a drop of mounting solution and placing a coverslip on it. The cells were examined under a fluorescence microscope fitted with the appropriate filters or the slides stored at −20°C in dark for later observations.

These two methods were unable to detect the expression of the HA tagged sTnC. A possible explanation could be the primary antibody used which was a common factor in both the methods.

5.1.3.3. Immunostaining Using Biotin-conjugated Secondary Antibody

This method used a three-step immunostaining protocol which immunolabeled the protein of interest with alkaline phosphatase (AP) enzyme. AP is then

detected by a substrate conversion/ color production reaction using the Vector® Red staining Kit (Vector Laboratories cat. no. SK-5100), which can be visualized under an ordinary light microscope. In addition, primary antibody used was a new high-affinity anti-HA rat monoclonal antibody which had proved successful in western blot analysis.

For the purpose of immunostaining, NIH3T3 mouse fibroblasts were infected as described in section 2.11.6. with (1), PBS only, as a control; and (2), Ad5/CMV-IE/sTnCΔ9-HA recombinant virus. The Cardiocytes were infected with (1), Ad5/CMV/sTnC; (2), Ad5/HCA(-485)/sTnCΔ9-HA; and (3) Ad5/CMV/sTnCΔ9-HA. Following infection, both 3T3 cells and cardiocytes were incubated for 48-72, hours, prior to analysis for expression of the protein. The cells were then fixed and blocked as described in section 2.15.6.

Following blocking, the cells were treated as described in section 2.15.6.2.ii. Briefly, the blocked cells were incubated with a 1:1000 (100ng/ml) working concentration of Anti-HA High-Affinity primary rat monoclonal (clone 3F10) antibody (Boehringer Mannheim cat. no. 1 867 431) diluted in blocking solution, for 30 minutes at room temperature. Cells were washed three times with PBS and incubated with a 1:100 dilution (2mg/ml) of Anti-rat Ig-biotin $F(ab')_2$, fragments, secondary antibody (Boehringer Mannheim cat. no. 1 348 779), for 30 minutes at room temperature. Cells were washed three times with PBS and then incubated with 1:3000 dilution of Streptavidin-AP conjugate (Boehringer Mannheim cat. no. 1 089 161) for 30 minutes. Cells were again washed three times with PBS.

5.1.3.3.i. Vector® Red Staining

The alkaline phosphatase-immunolabeled cells were stained using the Vector® Red Alkaline Phosphatase Substrate Kit (Vector Laboratories cat. no. SK-5100) as described in section 2.15.6.2.ii.a. 2.5ml of assay buffer (100mM Tris HCl, pH 8.2) was taken and 25ml of levamisole stock (100X) added to inhibit endogenous AP activity of the cells. The substrate working solution was made immediately before use, by adding a drop each of reagents 1, 2 and 3 of the kit to the 2.5ml of assay buffer and mixing thoroughly.

Cells were incubated with the freshly made substrate solution at room temperature, in the dark for 20-30 minutes, or until the required staining intensity was reached. Cells were washed with assay buffer for 5 minutes and then rinsed in distilled water. A drop of hematoxylin solution (Haemlum (Mayer's) solution; BDH cat. no. 35060-4T) was added to each slide and incubated briefly for approximately 15 seconds, and then rinsed in distilled water. Slides were then further rinsed with 100% ethanol for 2-3 minutes to increase the intensity of the Vector® Red fluorescence. Excess liquid was tapped off from the slide and the cells and mounted, using Aquamount as described in section 2.15.6.2.ii.a, and examined under a light microscope or kept at 4°C for later observations.

5.1.3.4. Immunocytochemistry Results

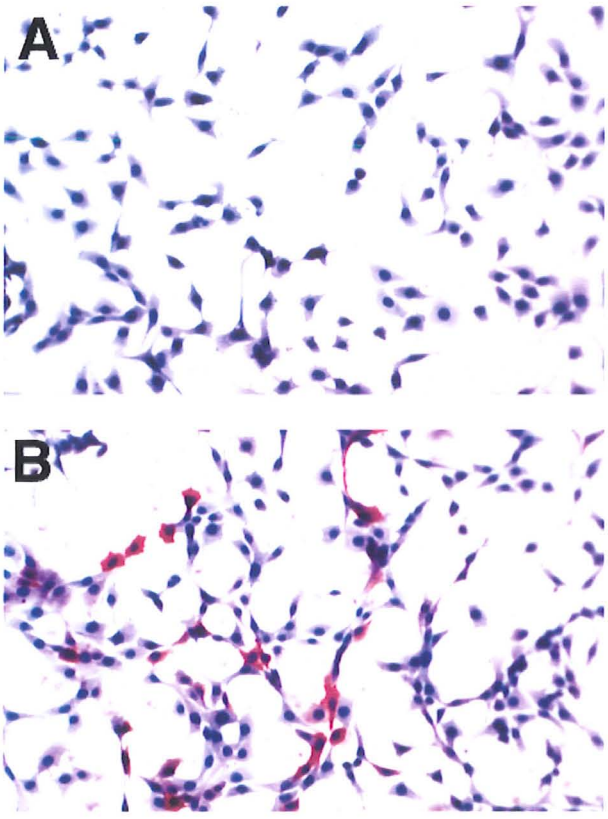
Immunocytochemical analysis of infected mouse fibroblasts and primary cardiocytes proved useful in confirming the expression of HA tagged sTnC by the recombinant adenoviruses. The NIH3T3 cells showed expression of the Ad5/CMV-IE/sTnCΔ9-HA, which was anticipated from infections of mouse fibroblasts with a CMV promoter (fig. 5.3.B). The Ad5/CMV-IE/sTnCΔ9-HA recombinant also expressed in primary cardiocyte cultures, where immunostaining of both cardiac fibroblasts and cardiac myocytes was observed (fig. 5.3.C).

Ad5/HCA(-485)/sTnCΔ9-HA recombinant virus did not show expression in NIH3T3 cells when tested by western blot analysis (see fig. 5.2) and therefore it was not subjected to further analysis by immunocytochemistry, in the same expression system. However, cultured cardiocytes were used and this virus showed visible expression in a few cells (fig. 5.4.B). No staining was observed in the cardiac fibroblasts, whereas somewhat inconspicuous, but visible red staining could be observed in groups of cardiocytes. Some intracellular staining was observed without any distinct morphological indication of cellular structures. This pattern of staining indicated the possibility of localization of the tagged Troponin molecule in the sarcomeres of the cardiocytes resulting in decreased visibility of the staining due to intricacies of the myofilament

structure. The brighter staining observed may be attributed to the tagged molecules stained in ruptured cardiocytes. Nevertheless, the absence of staining in the cardiac fibroblasts and some staining in cardiocytes reflected strong evidence of tissue-specific expression of the recombinant adenovirus.

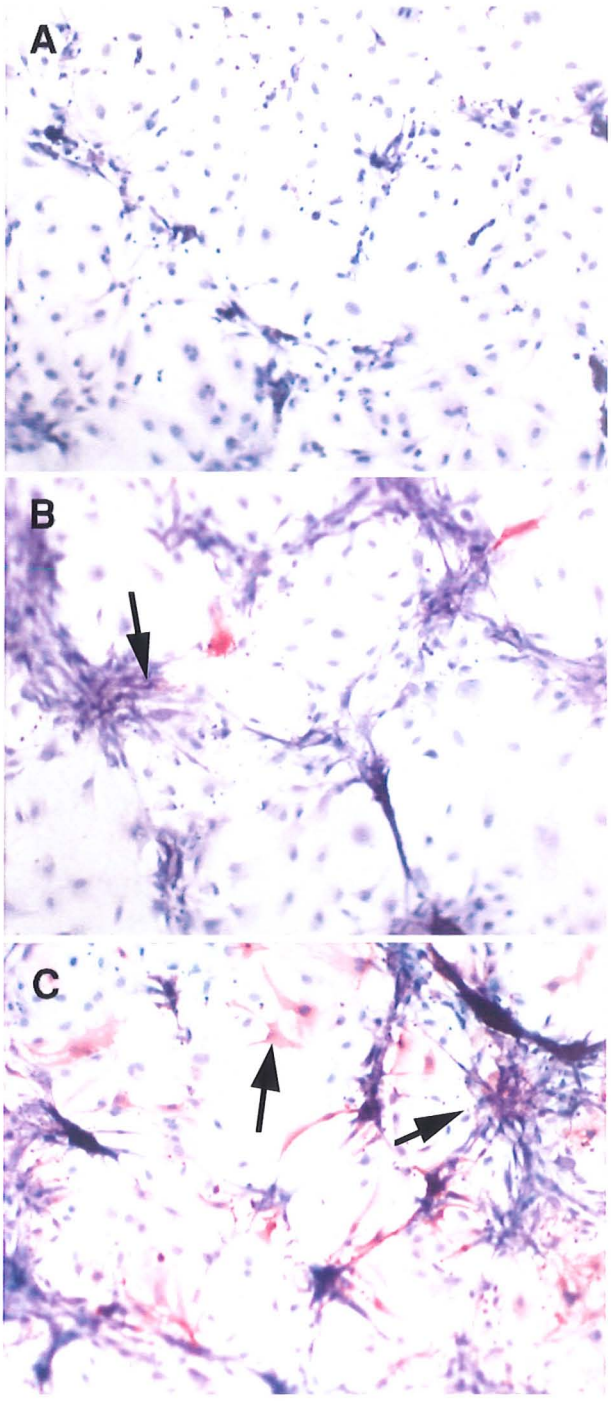
The non-infected controls used in the immunocytochemical staining showed no background staining in NIH3T3 cells (fig. 5.3.A). The non-tagged virus control used in cardiocyte culture also did not show any background staining eliminating the possibility of false positive results being produced by the sTnC protein (fig. 5.4.A).

Immunocytochemistry of mouse fibroblast cells infected with recombinant adenoviruses



A. NIH3T3 (no-virus control); cells treated with PBS only
B. NIH3T3 cells infected with Ad5/CMV-IE/sTnCΔ9-HA recombinant

Fig. 5.3: Immunocytochemical analysis of NIH3T3 mouse fibroblast cells in culture, infected with recombinant virus expressing the skeletal Troponin C tagged with HA epitope tag. Cells were immunolabeled with alkaline phosphatase and stained with Vector Red stain; (see section 5.1.3.3.i for details).



Primary Cardiocytes in culture, infected with;

A. Ad5/CMV-IE/sTnC recombinant (non-tagged)

B. Ad5/HCA(-485)/sTnCΔ9-HA recombinant

C. Ad5/CMV-IE/sTnCΔ9-HA recombinant

Fig. 5.4: Immunocytochemical analysis of primary cardiocytes in culture, infected with recombinant adenoviruses expressing skeletal Troponin C tagged with HA epitope. Arrow in B points to cardiocytes showing some visible staining indicating tissue-specific expression of the virus used. Arrows in C point to stained cardiac fibroblasts and groups of stained cardiocytes indicating non-specificity of the recombinant adenovirus used.

Appendix

to

Chapter 5

A-5.1. Western Blot Analysis

In order to probe into the anomaly seen in migration of the epitope tagged protein described in section 5.1.2.4, further western blot analysis was carried out on the recombinant adenoviruses using the cultured neonatal cardiocyte system. The premise of the experiment was to compare the native cardiac troponin C protein in the cardiocytes with the HA epitope-tagged sTnC transgene protein.

A-5.1.1. Cell Culture and Viral Transduction

Neonatal rat cardiocytes were isolated and cultured as described in section 2.8.4.5., and maintained in 60 mm dishes for 72 hours before they were infected with the viruses. The cells were then infected with adenovirus lysate at a titre of approximately 10^5 to 10^6 pfu/ml as described in section 2.11.6.

Seven 60mm dishes of neonatal cultured cardiocytes were treated as follows; (1), was untreated cardiocytes, while the rest of dishes were infected with 100μl of (2), PBS only, (3), virus suspension of Ad5-dl309 for adenovirus control, (4), virus suspension of Ad5/CMV/sTnC, (5), Ad5/CMV-IE/sTnCΔ9-HA, (6) virus suspension of Ad5/HCA(-485)/sTnC, (7), virus suspension of Ad5HCA(-485)/sTnCΔ9-HA. The cells were incubated at 37°C with 5% CO_2 for 72 hours to allow the virus to express the foreign protein.

A-5.1.2. Sample Preparation and SDS-PAGE

Treated cardiocytes were harvested as described in section 2.14.1 and freeze-thawed to extract the proteins as described in section 2.14.1.1. The lysates were transferred to a fresh tube and estimated for protein content as described in section 2.14.2. Cell lysate containing approximately 40μg of total protein from each sample was taken in a fresh tube and an equal volume of SDS-sample buffer (40mM Tris, pH 6.8, 4mM 2-mercaptoethanol, 2% SDS, 10% glycerol, 0.2% bromophenol blue) added, mixed and boiled for 10 minutes in a boiling waterbath. Samples were allowed to cool down to room temperature and one fifth (5-8μg) of each protein sample was loaded on SDS-PAGE gels to resolve the proteins for western blotting, along with 20μl of prestained protein standards as described in section 5.1.2.2. In addition, to

the samples and markers, 500ng of purified Human cTnC protein (cat. no. TrTd2, Advanced Immunochemical Inc. CA, USA) was also loaded as a positive control for anti-cTnC antibody. This protein was diluted into a total volume of 20μl and mixed with an equal volume of 6X SDS loading buffer and boiled for 10 minutes in a waterbath before loading on the gel.

A-5.1.3. Immunoblotting

The immunoblotting for the influenza virus hemagglutinin epitope tag was carried out as described in section 5.1.2.3. Similar methods were used for the cardiac Troponin C protein screening, with some modifications. The membranes were first probed with the cardiac Troponin C antibody and then reprobed after stripping, with the HA-epitope-specific antibody.

A-5.1.3.1. Probing with Cardiac Troponin C Antibody

For probing the native cardiac Troponin C protein, anti-Troponin C mouse monoclonal antibody (clone 1A2, cat. no. HG2AC3-1A2, Advanced Immunochemical Inc., CA, USA), was used. The antibody was diluted 1:1000 (2.2μg/ml.) in 1% skimmed milk/TBS and incubated at RT for 4 hours. The secondary antibody was HRP anti-mouse IgG (sheep) (cat. no. S081-201, SAPU, Lanarkshire, Scotland), diluted 1:1000 with 1% skimmed milk/TBS.

A-5.1.3.2. Stripping of Membrane and Reprobing

After probing with the cardiac Troponin C antibody, the membrane was stripped by incubating at 50°C with 100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH 6.7, for 45 minutes, with occasional agitation. The membrane was then washed with large volumes of TBS-T, twice, 10 minutes each time at RT.

The membrane was then blocked using 5% skimmed milk/TBS for 2 hours and reprobed with the HA-specific antibody as described in section 5.1.2.3.

Western Blot Analysis of Neonatal Rat Cardiocytes Infected with Recombinant Adenoviruses

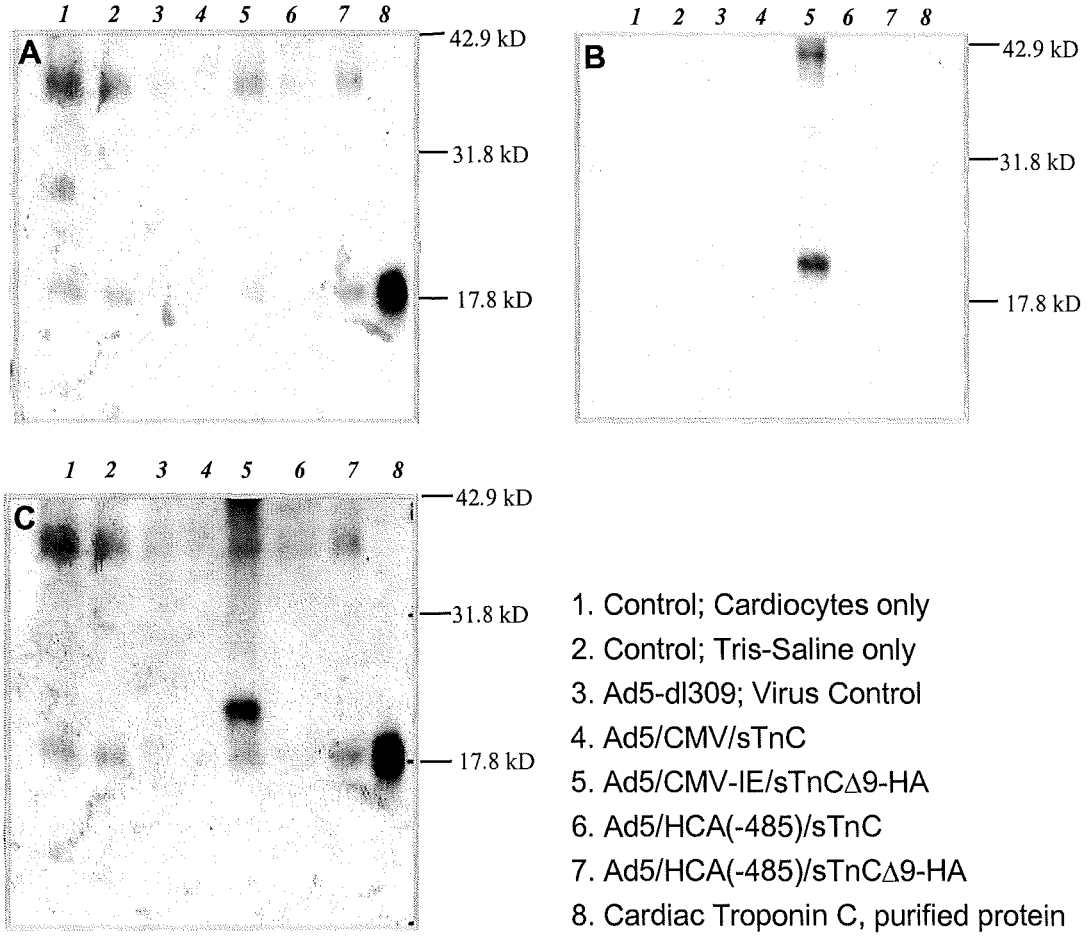


Fig 5.5: Western Blot analysis of cultured neonatal cardiocytes infected with recombinant skeletal Troponin C (HA-tagged) expressing adenoviruses. 5-8μg of total protein from infected cells and purified cTnC protein were resolved on the gel, and the protein bands blotted onto a membrane. **A.** The membrane was first probed with anti-cardiac Troponin C, mouse monoclonal primary antibody: Purified cTnC protein (Lane 8) was used as a positive control for the anti-cTnC antibody. All lanes show the presence of the native cTnC protein. **B.** The membrane was stripped and reprobed with the high-affinity HA primary rat monoclonal antibody. Lane 5 shows the expression of the HA epitope tagged sTnC at approximately 21 kD. **C.** The two autoradiographs were then superimposed for comparing the bands obtained in the two experiments. The HA-tagged sTnC protein from the transgene has slower mobility (~21kD) compared to the native cTnC protein (18kD). No cross-reaction of the cTnC antibody was obtained with the sTnC-HA tagged protein. Molecular weights were estimated from the prestained markers used and are represented in kilodaltons (kD).

A-5.1.4. Results and Discussion

The native cardiac Troponin C (cTnC) of the cardiocytes was detected by the anti-cTnC antibody. However, the expression detected was very low and only

detected after a 2 hour exposure of the ECL-treated membrane to the film as shown in Fig. 5.5-A. This may be due to lower affinity of the primary antibody and possibly due to less sample loaded.

The sTnC-HA expression was detected in cardiocytes infected with the ubiquitously-expressing recombinant adenovirus Ad5/CMV-IE/sTnCΔ9-HA, as shown in Fig. 5.5 B. No expression was detected in the tissue-specific Ad5/HCA(-485)/sTnCΔ9-HA, possibly due to very low activity of this promoter.

The two autoradiographs were superimposed, revealing a clear difference in the migration of the native cTnC and the HA-tagged sTnC expressed by the transgene (Fig 5.5-C). The HA-tagged sTnC migrated slower than the native cTnC from the cardiocytes, with an estimated molecular weight of approximately 21 kD, as observed previously (see section 5.1.2.4.).

Furthermore, the sTnC protein did not cross-react with the anti-cTnC antibody as was expected. This could be due to the conditions used for anti-cTnC probing, which were not optimal, as seen by the low level of detection of the native cardiocyte cTnC.

Chapter 6

Discussion

6.0 Discussion

The current project has resulted in the development of a range of replication-defective adenoviruses. Made to express skeletal Troponin C gene. Some of these adenoviral recombinants have been analyzed for expression and successfully express the sTnC gene in fibroblasts and cardiocytes. The adenoviral vectors obtained were designed to express the sTnC gene constitutively, or in a tissue-specific manner, by placing the gene under control of ubiquitously-expressing and muscle-specific promoters.

6.1. Subcloning of sTnC Gene in Adenoviral Shuttle Vectors

Development of adenoviral shuttle vector constructs with promoter-sTnC cDNA cassette, was the first step for making recombinant adenoviruses. Different strategies of subcloning were adopted to ensure integrity of the cDNA, and stability of transcription from the foreign DNA cassette. During the course of subcloning of sTnC, a range of useful plasmids were constructed (see Tables 3.1-3.7) which have wider and more general applications for future use in Adenovirus recombinant construction.

6.1.1. Insertion of SV40 poly A Sequence into Basic Adenoviral Shuttle Vectors

Achievements, of the work carried out include the improvement of basic adenoviral shuttle vectors, by adding a SV40 poly A sequence, at the 3′ end of the multiple cloning site of plasmid vectors pΔE1sp1A and pΔE1sp1B. A strong polyadenylation (poly A) signal is important for heterologous gene expression. Both the adenoviral E1b and SV40 poly A, are known to confer stability on the transcribed transgene RNA (Berkner, 1992), with more efficient termination of transcript being observed with cDNA having the SV40 pA signal sequence (Berkner *et al.,* 1987).

The pLES53-based plasmid pLECM is expected to utilize the indigenous poly A signal of E1b gene of the adenovirus. This sequence is located at bp 4038 (van Ormondt and Galibert, 1984), which is 3′ to the site of foreign DNA

insertion, and is known to perform well in obtaining efficient foreign gene expression (Berkner, 1992).

6.1.2. Subcloning Promoter-sTnC gene Cassette in Adenoviral Shuttle Vectors

Skeletal Troponin C cDNA was subcloned into the adenoviral shuttle plasmids, with the constitutive, Cytomegalovirus Immediate Early (CMV-IE) promoter, and tissue-specific promoters such as Human Cardiac α-actin (HCA) and Human Skeletal α-actin (SkAct). In the course of subcloning the sTnC with tissue-specific promoters, several basic vectors containing promoters were also obtained. These vectors are useful for subcloning any gene desired, and were used to subclone the β-galactosidase reporter gene and phosphodiesterase (PDE) gene (J. Queen, personal communication), with HCA and SkAct promoters.

Cloning of sTnC coding region DNA into the center of the multiple cloning site (MCS) of the pSP72 vector, has provided the gene with several commonly used restriction enzyme sites, on either side of sTnC gene, thereby facilitating any further subcloning of this gene. The sTnC cDNA is pSP72 is inserted in either orientation, which further increases the choice of restriction enzyme sites which can be used to remove the insert.

6.1.3. Development of Epitope-tagged sTnC and Subcloning into Adenoviral Shuttle Vectors

As the skeletal Troponin C protein is not recognized by a specific antibody, the direct assessment of expression of the wild-type skeletal Troponin C transgene at the translational level, was not possible. In order to carry out protein expression studies of the sTnC transgene, the protein was tagged with influenza virus hemagglutinin (HA) and the simian virus 40 (SV40) T-antigen epitope tags, enabling detection of expression using immunochemical techniques. The tags were inserted at the DNA level, by deletion of 3′sequences of the sTnC cDNA by PCR amplification and inserting a synthesized oligonucleotide coding for the epitope tag sequences (see figs.

3.4 -3.8 and fig. 3.11). The 3′ insertion of the epitope tag sequence corresponded to the tag appearing at the carboxyl-terminal of the translated protein. Both the HA and T-antigen tagged sTnC cDNAs were made and subcloned into the adenoviral shuttle plasmid pCA13, which has a CMV-IE constitutive expressing promoter.

Two different forms of the deletion/substitution tagged construct were made for sTnC with HA epitope tag. Initially, sTnCΔ12-HA was designed by deleting the last 12 codons of the sTnC coding sequence and the stop codon. This deletion was replaced by a 3 codon spacer and a 9 codon HA tag sequence, terminating in a stop codon.

The 12 amino acid (36 base pair) deletion from the carboxyl-terminal end (3' end in the cDNA) of the protein, encroaches on the high-affinity Ca^{++}-binding site IV, which extends from amino acids 140 to 151 of the 160 amino acids sTnC protein (Parmacek and Leiden, 1991). In order to eliminate this imperfection of the sTnCΔ12-HA construct, another tagged skeletal Troponin C molecule was designed with a lesser 3′deletion (sTnCΔ9-HA.). This protein is designed with a deletion of 9 codons (27 base pairs) from the coding region and the stop codon, and addition of 36 base pairs (3 codon spacer and a 9 codon HA tag) sequence, terminating in a stop codon. This modification increases the size of the coded protein molecule by 3 amino acids. The sTnCΔ9-HA construct was also cloned with the HCA promoter in the adenoviral shuttle vector pΔE1sp1A.

The T-antigen tagged sTnC (sTnCΔ8-T) was designed with a deletion of 8 codon from the coding region of sTnC gene, and the stop codon. A sequence coding for 2 codon spacer and 6 codon T-antigen epitope sequences, and a stop codon, were added to the truncated cDNA to produce 8 amino acid-deleted sTnC, with a carboxyl-terminal T-antigen epitope tag. Another tagged protein cDNA, sTnC-T was made, which did not have any deletion in the sTnC coding region. In this modified sTnC, only the stop codon was eliminated and replaced by sequence coding for 2 codon spacer and 6 codon T-antigen epitope sequences and a stop codon. This addition made the sTnC protein

longer by 8 amino acids. The two sTnC-T tag constructs were subcloned into the pCA13 adenoviral shuttle plasmids.

The HA and T-antigen tagged sTnC cDNA were made as two alternate epitope tag-sTnC constructs for establishing protocols for detection of expression of skeletal Troponin C in heart cells *in vitro.* and *in vivo.* With the success of the HA tagged sTnC in *in vitro.* expression studies, the T-antigen tagged sTnC constructs were not pursued further. However, the strategy of subcloning of T-antigen tag constructs in pCA13, was performed in such a way that an intermediary vector pCA13-T was also obtained. This construct is in effect the pCA13 plasmid with a T-antigen tag inserted 3′ to the multiple cloning site (MCS) of the vector. PCA13-T can be used to make carboxyl-terminal, T-antigen tag constructs of any gene, using methods outlined in section 3..5.2.2. (also see fig. 3.11).

6.2. Recombinant Adenovirus Construction

Several adenoviral shuttle vector constructs with skeletal Troponin C and reporter genes were made during the project (see Tables 3.3 -3.7), with the ultimate aim of obtaining a recombinant adenovirus expressing the genes. Two methods of recombinant generation were tried, of which the T. Shenk's method did not yield good results in our hands. Frank Graham's method gave better results, but only after some modifications on the protocols. The $CaPO_4$ transfection method, based on the protocols outlined before (Hitt *et al.,* 1995; Spector and Samaniego, 1995; Graham and Prevec, 1997), was used, after further optimization of protocols. Precipitate formation and efficiency of transfection was highly dependent on variables such as the quality of DNA used, presence of carrier DNA, mixing method and duration of exposure of cells to the $CaPO_4$-DNA precipitate. The physiological status of the 293 cells such as confllluency, doubling time and passage number (Spector and Samaniego, 1995), were factors which affected final outcome of the transfection i.e. generation of recombinant virus. However, despite the difficulties, 8 recombinants were successfully generated.

The recombinants obtained were initially checked against the simulated digest patterns carried out using the Vector NTI software. Some of the recombinants selected for the correct *Hind*III digest pattern, were screened by PCR using Ad5 specific primers which amplified a specific length of DNA, if the foreign DNA insert was present. All recombinants were screened for presence of E1 region by PCR using E1 specific primers (Dion *et al.,* 1996), and were found to be negative, indicating the purity of the replication-defective adenoviruses.

6.3. Analyzing Foreign Gene Expression by the Adenoviral Vectors

Recombinant adenoviruses generated during this project were analysed for expression by northern blotting, western blotting and immunocytochemistry of cells infected *in vitro.*

6.3.1. Transcriptional Analysis of the Constitutively-expressing sTnC Adenovirus

The NIH3T3 mouse fibroblast cell line was used, for *in vitro.* analyses of expression of the sTnC transgene, under the control of the CMV promoter, which shows a ubiquitous expression in all cells. The NIH3T3 cell line was chosen for its acceptance as a good *in vitro.* mammalian expression system. Moreover, as a non-muscle cell line, NIH3T3 cells do not have endogenous skeletal Troponin C gene activity. Northern blot analysis was carried out on total RNA isolated from NIH3T3 cells, infected with Ad5/CMV/sTnC recombinant adenovirus. RNA extracted from cells infected with control and sTnC adenovirus and probed with the 460 base sTnC-specific probe (Gahlmann and Kedes, 1990). Successful hybridization of the labeled probe to the RNA, showed presence of sTnC RNA transcripts in the infected cells, while no such hybridization was present in the control cells.

6.3.2. Translational analysis of the HA Epitope-Tagged sTnC Adenovirus Recombinant

The HA tagged sTnC-expressing adenoviral constructs were also analysed at the protein level, by transducing NIH3T3 cells *in vitro.* and performing western blot analysis and immunocytochemistry. A few different combinations of

primary and secondary antibodies were tried, both with western analysis and immunocytochemistry, before success was achieved. The primary antibody that performed well in both systems was a high-affinity Anti-HA, rat monoclonal antibody. The secondary antibody for westerns was an anti-rat HRP-conjugated antibody, whereas, a biotin-conjugated anti-rat Ig, secondary antibody, detectable by a streptavidin-Alkaline Phosphatase conjugate produced good results in immunocytochemistry.

Western blot analysis of NIH3T3 cells infected with control and recombinant adenoviruses, showed overexpression by the Ad5/CMV-IE/sTnCΔ9-HA transferred gene, with no similar background observed in the mock-infected or Ad5-dl309-infected controls. No tagged protein expression was detected in the cells infected with Ad5/HCA(-485)/sTnCΔ9-HA, which was anticipated. This recombinant adenovirus is expected have a cardiac-specific expression, due to the presence of a human cardiac actin full-length promoter.

6.3.4. *In situ* analysis of the HA Epitope-Tagged sTnC Adenovirus Recombinant Expression Using Immunocytochemistry

The positive results of the western blot analysis showing overexpression of the sTnCΔ9-HA protein by adenovirus was an successful achievement of this project. To further assess the possibility of detecting the HA tagged sTnC protein expression *in situ*, immunocytochemical analyses were carried out on infected NIH3T3 cells. Ad5/CMV-IE/sTnCΔ9-HA-infected and mock-infected NIH3T3 cells were immunostained using a variety of methods described in section 2.15.6. Positive results from the western analysis suggested the greater efficacy of the high-affinity anti-HA primary antibody in epitope detection, which was used, and gave comparable results in immunocytochemistry.

Ad5/CMV-IE/sTnCΔ9-HA-infected and mock-infected NIH3T3 cells, showed *in situ* expression of the sTnCΔ9-HA protein in the virus infected cells, with no background staining in the mock-infected samples. In this analysis, the high-affinity anti-HA primary antibody was used and the secondary antibody was an anti-Rat Ig-biotin conjugate, which was labeled using a streptavidin-

alkaline phosphatase conjugate. Color development of labeled cells was achieved with Vector® Red staining. The three-step immunolabeling procedure was adopted to eliminate background staining and was highly successful in achieving the desired goal.

6.3.5. *In situ* Analysis of Tissue-specific Expression of the HA Epitope-Tagged sTnC Adenovirus

Neonatal rat cardiomyocytes were used for assessing expression of Ad5/HCA(-485)/sTnCΔ9-HA recombinant, which expresses the HA tagged sTnC protein (sTnCΔ9-HA) under the control of the full-length, cardiac muscle-specific, human cardiac actin promoter. Immunocytochemical staining showed no detectable expression in the cardiac fibroblasts, which are non-myogenic cells of the heart, and some visible staining in the cardiomyocytes. This result was in agreement with the anticipated behaviour of a cardiac promoter in heart cells. The adenovirus constitutively expressing the tagged protein, (Ad5/CMV-IE/sTnCΔ9-HA), showed expression in both cardiomyocytes and cardiac fibroblasts, while no staining was observed in cells infected with ubiquitously-expressing non-tagged wild-type skeletal Troponin C adenovirus, indicating the specific staining of the HA tag antibody.

6.3.6. Additional Expression Analyses Required

Three of the eight skeletal Troponin C adenoviral vectors, developed in this project have been analyzed and express the sTnC gene *in vitro*. Other adenoviral constructs which were designed with the wild-type sTnC gene under the transcriptional control of muscle-specific promoters, were not analyzed for expression, due to limitations of time for the project. However, the Ad5/HCA(-485)/sTnCΔ9-HA has shown the utility of using such promoters in gene transfer protocols.

Functional analysis of the Adenovirus recombinants expressing sTnC was outwith the scope of this project. However, functional studies have been initiated, and are being carried out with our collaborators. Ad5/CMV/sTnC virus is being studied by Dr K. Webster's group at the University of Miami, Florida, who are carrying out edge detection analysis on cardiocytes in

culture, with normal and simulated conditions of ischemia. Similarly, methods for measurement of contractility of rat neonatal cardiomyocytes, in culture are being optimized, by our collaborators Dr. G. Smith at the Department of Physiology, University of Glasgow. Contractility and other physiological studies will further characterize the functional efficacy of these gene transfer vectors.

6.4. Future Experiments Following from Our Vector Development

Studies relating to *in vivo.* sTnC gene transfer and expression and protein isoform substitution can be immediately initiated using the recombinant viruses made in this project. Contractility studies using the virus can also be carried out both *in vitro.* and *in vivo.*, or using *ex vivo* models such as isolated muscle fibres.

6.4.1. *In vivo.* Expression Studies using sTnC-HA tag-expressing Adenovirus

Establishment of working immunocytochemistry protocols for HA-tagged sTnC expression *in vitro,* was another milestone of the project. Therefore, an immediate follow-up of this project could encompass the use of the sTnCΔ9-HA protein-expressing viruses for *in vivo.* studies. This development will be useful for evaluating the extent of adenovirus-mediated gene transfer and expression, by transducing hearts *in vivo.*, and using immunohistochemical analysis. Furthermore, the Ad5/HCA(-485)/sTnCΔ9-HA recombinant can be used for studying targeted gene delivery to the heart *in vivo.* In addition, subcellular localization studies can be carried out *in vitro.* or *in vivo.*, using gold-immunolabeling, using electron microscopy, to establish the incorporation of the transgene protein into the myofilaments.

Gene expression analysis of sTnC recombinant adenovirus with muscle-specific promoters will add to the completeness of the adenovirus vectors, and mandatory before use of these viruses in any further functional studies. Due to non-availability of a sTnC-specific antibody (J. Potter; M. Greaser, personal communications), expression analysis at the translational level is

impractical. Thus northern analysis using sTnC-specific probe (Gahlmann and Kedes, 1990) should be able to indicate the presence of transcriptional activity of sTnC gene in cells infected with the virus. Myogenic cell lines such as C2C12 can be an acceptable *in vitro.* expression system for these viruses. The C2C12 cells can differentiate into myotubes, and express active transcriptional factors which can activate transcription from muscle-specific promoters (Bains *et al.,* 1984). Though some skeletal Troponin C mRNA activity will be present in the cell line, inclusion of appropriate mock-infected and non-sTnC viral controls can aid in establishing sTnC transcription from the transgene. The level of sTnC mRNA in C2C12 myogenic cells infected with sTnC-expressing adenovirus, is anticipated to be higher by several orders of magnitude, than the endogenous levels of the C2C12 cells.

6.4.2. Protein Substitution by Ectopic Overexpression of Alternate Isoforms of Contractile Protein Components

Protein isoform substitution by overexpression of alternate isoforms in myogenic cells, are known to influence the regulation of the native contractile protein isoform of the cell (Palermo *et al.,* 1995). Foreign gene expression has been found to downregulate expression of the endogenous isoform of the transgene by a feedback mechanism, either at the mRNA or protein level.

This phenomenon has been observed in myosin light chain isoform overexpression studies in transgenic mice. The transgenics expressed the ventricular myosin light chain-2 transgene (MLC-2c) gene, both in the atria and ventricles of the adult animal heart, under the control of alpha-cardiac myosin heavy chain gene (α-MHC) promoter. Ectopic expression of transgene in the atria resulted in a complete replacement of the atrial myosin light chain-2 protein isoform, although the endogenous isoform mRNA levels remain unchanged. In contrast, ventricular expression of the transgene had no effect at the protein level, despite an eight fold increase in transgene MLC-2v transcript levels. This demonstrated that sarcomeric protein stoichiometry is maintained rigorously *via* posttranscriptional regulation and that protein replacement can be achieved through a single transgenic manipulation.

Overexpression of skeletal Troponin C in cardiomyocytes using adenovirus and replacement of the endogenous cTnC isoform by the transgene, should be of interest in studying isoform regulation in myocytes. Similar studies with Troponin I (TnI) isoform substitution, have shown nearly complete replacement of endogenous cardiac Troponin I with slow skeletal TnI (Westfall *et al.,* 1997). Such a study may yield information on contractile protein gene regulation during heart failure and hypertrophy.

6.4.3. Functional Analysis of sTnC-Expressing Adenoviruses

The long-term goal of designing sTnC-expressing adenovirus was to study the effect of partial substitution of the endogenous cardiac isoform of Troponin C in cardiomyocytes, with the skeletal isoform, using a gene transfer approach. To assess the functional alterations produced by such substitution, studies can be performed on neonatal rat cardiocytes, transduced with skeletal Troponin C-expressing adenoviruses, under normal conditions and in conditions of simulated ischemia. Contractility and motion characteristics of the transduced and control cardiomyocytes can be determined using computerized motion edge analysis of single cardiomyocytes (Webster *et al.,* 1996). Single transduced cardiomyocyte preparation can be used to study the effects of the transgene expression on Ca^{++}-activated force, under conditions of reduced pH (Metzger *et al.,* 1993). Similarly, contractile behaviour of the transduced cell can be studied in hypoxia and other correlates of ischemia, induced by altering the environment of the culture of cardiomyocytes. Ca^{++}-activated force measurements can also be carried out on isolated cardiac muscle fibres obtained from an *in vivo.* transduced myocardium, using modifications of methods already described (Palmer and Kentish, 1994; Parsons *et al.,* 1997). Parameters such as Ca^{++}-sensitivity and Maximum Ca^{++}-activated force can also be measured in fibres with the substituted isoform of protein.

6.5. Properties of Our Viral Mediated Strategies for Manipulating Ca^{++}-Sensitivity

The adenovirus constructs made in this project have certain characteristics which may be of interest in elucidating the mechanism involved in the phenomenon of Ca^{++}-sensitivity. Some adenoviral constructs can be also useful in studying promoter responses to ischemia.

6.5.1. Tagged Troponin C as Mutants

The carboxyl-terminal hemagglutinin (HA) epitope tagged skeletal Troponin C cDNA made during this project were principally designed to enable protein expression and sub-cellular localization studies. However, these sTnC molecules may also be of special interest as a deletion-substitution mutant of skeletal Troponin C. The 12 codon deletion from the carboxyl-terminal end of protein, present in sTnCΔ12:HA, alters three amino acids of the high-affinity metal binding site IV (Parmacek and Leiden, 1991). Thus the tagged protein my be deficient in Ca^{++} binding, at this high-affinity site. Similarly, sTnCΔ9-HA-expressing adenovirus will also be of interest as an altered protein. Although the site IV is intact, this protein contains the tag which increases the length of the protein by 3 amino acids.

The carboxyl-terminal domain of Troponin C is postulated to have a structural role of maintaining the stability of the Troponin complex, presumably through TnC-TnI interactions (Zot and Potter, 1987), with the metal-binding sites playing a critical role. Site III has been found to be more critical for TnC association with the fibres, while site IV appears not to be essential for the structural stability of TnC within the thin filament. Moreover, alteration of sites I and II lower the binding affinity of TnC to the thin filament while mutations in sites III and IV affect the Ca^{++}-sensitivity of force development (Szczesna *et al.,* 1996).

The observations that mutations of carboxyl-terminal metal-binding sites causing alteration in Ca^{++}-sensitivity, are of great interest and relevance to the tagged sTnC molecules designed in this project. Future, physiological studies using the adenoviruses expressing the tagged sTnCΔ12-HA and sTnCΔ9-HA

protein (Ad5/CMV-IE/ sTnCΔ12-HA and Ad5/CMV-IE/ sTnCΔ9-HA), are expected to yield interesting data reflecting the effect of overexpression of the protein on the Ca^{++}-sensitivity of contraction in transduced cardiomyocytes.

6.5.2. Myogenic Promoter Studies using Adenovirus

The human cardiac α-actin (HCA) and human skeletal α-actin (SkAct) promoters used for making sTnC adenovirus recombinants have been characterized in detail for their muscle-specific activation of transcription (Minty and Kedes, 1986; Parker *et al.,* 1992). The SkAct promoter has been studied in more detail for its activation during myocardial cell hypertrophy (Bishopric and Kedes, 1991). The response of the SkAct promoter to a variety of growth factors, hormones and proto-oncogenes have also been extensively characterised and a number of specific regulatory domains subsequently identified (Bishopric *et al.,* 1992; Parker *et al.,* 1992; Karns *et al.,* 1995). Transcription of the SkAct gene is known to respond to certain components of ischemia, specifically hypoxia/reoxygenation cycles *in vitro.* (Webster *et al.,* 1993).

The available information on SkAct promoter, makes the availability of a sTnC adenovirus vector controlled by this promoter of potential interest. This virus can be used in *in vitro.* models of ischemia, to assess the response of this promoter to components of the ischemic process. As both SkAct(-2000) and SkAct(-1282) promoter-driven adenoviruses are available, further characterization of response elements of the promoter can be made. In addition to ischemia, SkAct promoters responsive to hypertrophy can be studied on single cardiomyocytes, using modifications of methods already described (Sadoshima *et al.,* 1992; Shyu *et al.,* 1995).

6.6. An Overview of Adenovirus-Mediated Strategies for Gene Therapy in the Heart

With the success of construction and subsequent demonstration of expression of the sTnC-expressing viruses, the groundwork has been laid for applying these viruses to animal heart gene transfer experiments. However, with

respect to the development of a potentially therapeutic adenoviral vector, and its transition to *in vivo.* studies and subsequent clinical trials, several issues, must be considered. Factors pertaining to the safety and efficacy of adenoviral vectors and the therapeutic gene itself, need to be addressed before our, or other related vector could be used for gene therapy protocols.

Many gene transfer approaches have been outlined for myocardial ischemia and heart failure (Leor *et al.,* 1996; Leor *et al.,* 1997). Any gene transfer method for improving contractile function of the failing myocardium must comply with certain criteria, which are fundamental to all gene therapy protocols. First, an appropriate gene or genes are chosen for manipulation and secondly, an efficient system should exist for delivering the therapeutic nucleic acids (DNA, RNA or oligonucleotides). There should be appropriate control of regulated expression of the transferred gene, to obtain expression, when required. In this project, Troponin C is the molecule targeted for alteration of Ca^{++}-sensitivity of cardiomyofilaments, by overexpression of the skeletal isoform, using an adenoviral gene transfer vector. Use of myogenic promoters is expected to provide the requisite control over expression of the transgene in cardiomyocytes.

6.6.1. Justification for Targeting Troponin C

The Troponin C protein of the thin filament has been under study for several years, for its role in eliciting Ca^{++}-induced contractile response in myocytes. Recently, drugs called calcium sensitizers, have been formulated, to influence the Ca^{++} responsiveness of muscle, mainly by modulation of the Troponin C protein. These drugs are important due to findings that $[Ca^{++}]_i$ is rarely altered in heart failure (Liao *et al.,* 1994).

6.6.1.1. Contractile Dysfunction of Heart Following an Ischemic Episode

Following ischemia, normal contractile function requires several days of reperfusion to fully recover to its normal level (Jennings and Reimer, 1991). On reperfusion, the reversibly injured myocardium undergoes a hypofunctional state of depressed mechanical function that has been termed "*Myocardial Stunning*" (Braunwald and Kloner, 1982; Katz, 1992). The cause

of stunning has not been established (Jennings and Reimer, 1991) but ATP depletion and the delay in replenishing levels of ATP have been postulated to account for this phenomenon (Katz, 1992). Ca^{++} transients are known to be sustained (Marban, 1997) or even increased (Kusuoka *et al.,* 1990) in stunned myocardium, which implies that myofilament responsiveness to Ca^{++} is abnormally low. It is now widely accepted that the decreased Ca^{++}-sensitivity of the contractile proteins presents a likely mechanism to underlie the altered mechanical function of the "stunned" myocardium (Kusuoka *et al.,* 1990; Marban, 1997). The intracellular pH and P_i are normal in stunned myocardium as reperfusion is complete (Kusuoka *et al.,* 1990). Thus Ca^{++}-desensitization is unlikely to be due to these two metabolites.

6.6.1.2. Decline in Ca^{++} Sensitivity of Myofilaments in Ischemia

The decline in Ca^{++}-sensitivity of myofilaments is an intrinsic effect of ischemia causing contractile dysfunction, which is more pronounced in stunned myocardium. Components of ischemia, such as hypoxia are known to decrease calcium responsiveness of the myofilaments (Hajjar and Gwathmey, 1990), which leads to contractile dysfunction and heart failure. Amelioration of the desensitization is therefore a viable option for recovering the compromised contractility of a compromised heart. Contractile recovery of stunned myocardium is imminent due to restored blood flow. However, if large parts of the left ventricle are affected, causing impaired global left ventricle function, the extent of stunning can be reduced by inotropic stimulation without causing further damage to the myocardium (Heusch and Schulz, 1996).

6.6.1.3. Possible Role of Ca^{++} Sensitizers in Restoration of Contractility

Pharmacological modulation of Ca^{++}-sensitivity is seen as a promising strategy for treatment of acute or chronic heart failure. Calcium sensitizers comprise a group of drugs which increase the Ca^{++} responsiveness of myofilaments by exerting a positive inotropic effect, without increasing Ca^{++} levels (Korbmacher *et al.,* 1997). The overall Ca^{++} responsiveness is seen as a composite of two fundamental components; Ca^{++}-sensitivity and maximal Ca^{++}-activated force (see sections 1.2.6).

Several pharmacological calcium sensitizer are being studied for their positive inotropic effects and the molecular mechanism underlying their characteristic behaviour. Most calcium sensitizer compounds are found to have a twin activity of PDE inhibition which maybe due to different enantiomers of the compound (Lues *et al.,* 1993; Ravens *et al.,* 1996). Calcium sensitization is achieved in a variety of ways by this group of compounds. Pimobendan increases the affinity of Troponin C for Ca^{++} (Lee *et al.,* 1989). Levosimendan stabilizes the Ca^{++}-induced conformational change of Troponin C (Haikala *et al.,* 1995), whereas MCI-154, EMD 53998 and EMD 57033 increase calcium sensitivity by direct interference with the myosin-actin interaction (Lee and Allen, 1991; Gross *et al.,* 1993; Haikala *et al.,* 1995; Pan and Johnson, 1996).

6.6.1.4. Troponin C as a Therapeutic Target

The strategy of skeletal Troponin C gene transfer and subsequent overexpression in cardiomyocytes can be seen as having certain parallel features with the pharmacological calcium sensitizing agents. Studies with these will provide information on effects produced by increased Ca^{++} affinity and the effect it has on the overall myofilament. For example, levosimendan a calcium sensitizing agent was designed to bind to cardiac Troponin C. This compound binds the N-terminal domain of cTnC, stabilizing its conformation when it is calcium activated (Pollesello *et al.,* 1994). The inotropic effect of this drug was due to conformational stability of the Troponin C rather than increased affinity for Ca^{++}, and is found to be pH independent (Haikala *et al.,* 1995). This finding corroborates the postulate of pH induced alteration in the tertiary structure of Troponin C, and its role in desensitization of the myofilament in acidosis (Wang *et al.,* 1987). In addition, the Troponin C isoform substitution experiments (Moss *et al.,* 1986) and transgenic study (Metzger *et al.,* 1993) have provided convincing information on the role of this molecule in responding to intracellular Ca^{++}. The Troponin C molecule is under extensive study and several mutated forms of this protein have been made to dissect the function of this molecule in contraction (Gusev *et al.,* 1991; Szczesna *et al.,* 1996; Akella *et al.,* 1997). In light of the above observations, overexpression of the skeletal isoform in cardiomyocytes was

postulated to confer resistance to desensitization of myofilaments, during ischemia.

6.6.2. Critical Review of Adenoviral Vectors *in vivo.*

Several attributes of the adenovirus have made this virus a vector of choice for gene transfer in general, and especially as gene transfer vector for the myocardium. Notable properties advantageous for cardiac gene transfer include the ability of adenoviral vectors to infect non-dividing cells, such as cardiac myocytes (Guzman *et al.,* 1993; Kass-Eisler *et al.,* 1993; Barr *et al.,* 1994) and their capacity to accommodate foreign DNA inserts of up to 7.8 kb (Bett *et al.,* 1994). In addition high titers of up to 1×10^{10}, are obtained, which can be improved by concentrating further, following purification, to 1×10^{12} plaque forming units (pfu) per ml, (French, 1997), are an added advantage for achieving widespread gene transduction in the ventricular walls.

Nevertheless, the adenoviral vectors are not without faults, and *in vivo.* gene transfer experiments have experienced problems. These problems have arisen with use of the first-generation adenoviruses, which have reached the stages of clinical trial protocols. Different strategies are being evolved to overcome the deficiencies of this otherwise efficient vector.

6.6.2.1. Shortcomings of Adenoviral Vectors

First-generation adenovirus-mediated gene transfer experiments have demonstrated an exceptional efficiency of virus uptake and gene expression in a variety of *in vivo.* models. Unfortunately, high-efficiency gene delivery is often not accompanied by long-term gene expression of the transferred gene, mainly due to induction of host immune response toward the adenovirus. Persistence of transgene expression by an adenoviral vector in an *in vivo.* system may not be essential in all cases of myocardial gene therapy, but can be a factor of interest in some applications. Moreover, factors influencing expression are generally of interest, for modulating the overall efficiency of the virus.

Generally, maximal gene expression peaks during the first week of infection and rapidly declines to basic undetectable levels, 80 days after infection

(Kass-Eisler *et al.,* 1993). Experience in adenovirus-mediated cystic fibrosis (CFTR) gene transfer in mice and non-human primates, indicate the transient nature of the transgene, lasting less than 21 days, and is also associated with development of inflammation. Immuno-modulation in trial animals has been found to maintain the viral genome stably in the transduced host cells for over 12 months (Kass-Eisler *et al.,* 1996). C3H-scid and Balb/c-scid immunodeficient mice, infected with human α1-antitrypsin-expressing adenovirus show indefinite gene expression from transduced hepatocytes (Barr *et al.,* 1995). Also, poor transduction efficiencies are often observed when the same virus is re-administered due to anti-adenovirus antibodies induced during the first administration (Christ *et al.,* 1997; DeMatteo *et al.,* 1997).

6.6.2.1.a. Immune Response to Adenoviral Vectors

Earlier attempts of adenoviral gene transfer into heart indicated no evidence of viral toxicity *in vivo.* (Barr *et al.,* 1994; Muhlhauser *et al.,* 1996). However, some studies found the adenoviral vectors to induce an inflammatory response when used in high titers (Leor *et al.,* 1996). The ability to use high titers of virus is the most attractive feature for use of adenoviral vectors, especially in myocardial applications. Any associated toxicity with such a feature in *in vivo.* gene transfer, is liable to cancel out the advantageous effects.

Adenovirus vectors retain about 80% of the viral genetic information, which are presumed to show a "leaky" expression, if induced by an E1-like transactivator activity of the host cell (Yeh and Perricaudet, 1997). This leads to low levels of viral protein expression, causing morphological alterations of host cell, known as a *cytopathic effect* (Zhang and Schneider, 1994; Wang and Finer, 1996). Such basal expression of viral antigens often contributes to a cellular immune response leading to the destruction of the transduced cells by T-lymphocytes (Wang and Finer, 1996; Christ *et al.,* 1997). The T-lymphocyte response results in viral elimination after a primary treatment of recombinant adenovirus, and is often followed by a potent humoral response,

which obstructs an effective subsequent adenoviral gene transfer (DeMatteo *et al.,* 1997; Gahery-Segard *et al.,* 1997).

Production of anti-adenovirus antibodies and vector clearance by the cellular immune response have emerged as the major impediments to successful gene therapy (DeMatteo *et al.,* 1996; Christ *et al.,* 1997; DeMatteo *et al.,* 1997). In order to deal with the immunogenicity of the adenoviral vectors, new vectors are being developed, with additional deletions (Kochanek *et al.,* 1996; Parks *et al.,* 1996; Wang and Finer, 1996). However, with the current first generation vectors, certain strategies of *in vivo.* gene transfer have been suggested to circumvent the inevitable immune response (DeMatteo *et al.,* 1996; Kass-Eisler *et al.,* 1996). Such procedures include the induction of tolerance to adenovirus in animals at the neonatal stage, and using viruses of varying serotypes where repeated administration of the therapeutic virus may be required (Kass-Eisler *et al.,* 1996).

Immunosuppression provides an alternative tool with potential for clinical application of adenovirus-mediated gene therapy (DeMatteo *et al.,* 1996). This has been supported by sustained gene expression observed in immunodeficient or immunologically naive rodents (Stratford-Perricaudet *et al.,* 1992). Treatment regimens with immunosuppressive drugs such as cyclophosphamide, FK506 (Ilan *et al.,* 1997) or with monoclonal antibodies that block either the T cell receptor or co-stimulation pathways, also allow prolonged transgene expression and/or readministration of adenoviral vectors (DeMatteo *et al.,* 1996; Christ *et al.,* 1997). Antigen presentation in the thymus of neonates is an important way of T-cell clone deletion which can eliminate the adenovirus-specific cellular response (Ilan *et al.,* 1996). This approach can potentially be employed to inhibit any immune response to reporter gene or therapeutic proteins which the virus has been engineered to encode (Kass-Eisler *et al.,* 1996).

Viral backbone modification has also been employed to tackle the problem of immune response against adenovirus-infected cells. Several recombinant generation systems use deletion derivatives of the viral genome that are

made by eliminating dispensable viral genes (Kochanek *et al.,* 1996; Parks *et al.,* 1996; Wang and Finer, 1996). Adenovirus itself, like other viruses has evolved strategies for escape from immune surveillance of the hosts. The gp19k gene found in the adenovirus E3 region is known to down-regulate major histocompatibility complex class-1 expression on the cell surface, enabling the infected cell to escape recognition by cytotoxic T cells (Wold and Gooding, 1991; Lee *et al.,* 1995). Vectors including the immuno-modulatory gp19k gene, fail to stimulate T-cell proliferation and thus elicit a cellular immune response against the cells infected by the adenovirus vector (Lee *et al.,* 1995). Most first-generation vectors used, have a viral backbone (e.g. Ad5-dl309), where the E3 region is deleted to accommodate more DNA in the E1 region.

6.6.2.1.b. Replication-Competent Adenovirus (RCA) Generation

Another noteworthy predicament with first-generation adenovirus-mediated gene transfer, is the generation of replication-competent adenovirus (RCA) during the propagation of the replication-defective adenovirus (RDA) in the permissive 293 cells. This cell line provides the E1 gene function *in trans* to the E1-deleted adenovirus recombinants and is extensively used as a system to generate and propagate replication-defective recombinant adenoviruses (Ali *et al.,* 1994; Graham and Prevec, 1995; Hitt *et al.,* 1995; Graham and Prevec, 1997). 293 cells have a large fragment of the left-end part of the Ad5 genome, that includes the left ITR, the packaging sequence, the complete E1 region with the protein IX coding sequence, and the distal part of gene IVa2, integrated into human chromosome 19 (19q13.2) (Louis *et al.,* 1997; Yeh and Perricaudet, 1997).

Recently, purity of the RDAs has come under question, in light of the fact that homologous recombination between the E1$^-$viral genome and E1 sequences integrated in the 293 cells, can produce adenovirus which are replication competent. This phenomenon may occur during the process of viral propagation and contaminate the recombinant with parental-type adenovirus (Louis *et al.,* 1997; Yeh and Perricaudet, 1997). These RCAs are similar to the wild-type adenovirus and can propagate in non-permissive cells. The

cytolytic nature of these adenoviruses, makes them unsuitable for *in vivo.* and clinical applications (Yeh and Perricaudet, 1997).

Strategies to circumvent unwanted RCA generation, deal with designing new permissive cell lines and remodelling the viral backbone genome (Gorziglia *et al.,* 1996; Hehir *et al.,* 1996; Imler *et al.,* 1996). The other option of modifying the vector backbone, is being used to develop adenovirus genomes which produce lethal RCAs when propagated in 293 cells.

6.6.3. Delivery Systems Available for Administration of Adenoviral Vectors to Myocardium

Gene therapy is unlikely to become a clinically important treatment option, if it cannot be administered to patients in routine clinical surroundings. This issue is being addressed for adenoviral gene transfer into myocardium to obtain targeted delivery and expression of the therapeutic gene, using non-invasive methods. Following delivery, a homogenous infection and expression in significant regions of the left ventricle, by the adenoviral vector, is also essential for the transferred gene to manifest therapeutic effects (French, 1997). A number of delivery methods have been used for transferring the adenoviral vectors into the myocardium successfully with varying degrees of expression patterns. These include direct intramuscular injection, intracoronary infusion or catheter-mediated infusion of adenovirus vector.

Intracoronary infusion of adenovirus vector is considered a less invasive method compared to Intramyocardial injection which involves thorotocomy to expose the heart for injecting the virus. With the advances in catheterization technology, intracoronary infusions also have direct clinical relevance. However, direct intramuscular injection of virus resulted in a localized and more efficient expression of virus in the myocardium (Magovern *et al.,* 1996; Muhlhauser *et al.,* 1996). The infusion method on the other hand results in transduction of coronary vasculature and non-myocyte connective tissue, scattered throughout the region supplied by the injected coronary artery (Barr *et al.,* 1994; Muhlhauser *et al.,* 1996). Transgene expression has also been observed in non-heart tissue such as lung, thymus and liver, following direct

injection of adenoviral vector in the heart (Guzman *et al.,* 1993; Kass-Eisler *et al.,* 1993). This issue of non-specific transduction by adenoviral vector needs to be addressed for safety concerns.

6.6.4. Targeted Delivery and Expression of Transgene

Therapeutic genes targeted to myocardium are required to express widely within the target organ only. This important safety issue is being addressed by using cardiac-specific promoters to obtain ectopic expression of reporter and therapeutic genes. Muscle gene promoters such as Human Cardiac Actin (HCA), (Minty and Kedes, 1986), Skeletal α-actin (Sk-Act) (Karns *et al.,* 1995), alpha-Myosin Heavy Chain (α-MHC) (Katz and Steinhelper, 1992), and Myosin Light Chain-2 (MLC-2v) (Lee, 1992; Franz, 1993), have been characterized and cloned. Specific consensus sequences within these promoters regulate associated genes, in response to binding by muscle specific transcriptional factors.

Human cardiac (alpha) actin gene is regulated developmentally, encoding the major actin species in the adult heart, and high level expression in foetal and embryonic skeletal muscle (Mayer *et al.,* 1984; Mohun *et al.,* 1984). A 485 bp, upstream region is known to modulate expression of this gene in muscle cells, but not in mouse fibroblast cells (Minty and Kedes, 1986). The skeletal α-actin gene is expressed in adult skeletal muscle under the transcriptional control of a 2000 bp promoter region. The skeletal actin promoter is found to be positively regulated by the c-fos and c-jun proto-oncogene transcriptional regulators which are induced as a hypertrophic response in the myocardium (Bishopric *et al.,* 1992). The α-MHC promoter is 685 bp upstream region of the alpha-Myosin heavy chain gene which is expressed in adult cardiac muscle and is known to be positively regulated by thyroid hormone (Kitsis *et al.,* 1991).

Recently, promoters have been subjected to extensive analysis for use in cardiac-specific gene expression. Direct DNA injection of reporter genes under the transcriptional control of α-MHC promoter have shown expression in heart but not in muscle (Buttrick *et al.,* 1992; Li *et al.,* 1997). Transgene

expression driven by Myosin Light Chain-2 (MLC-2v) promoter, has been shown to be ventricle-specific in transgenic animals (Lee, 1992; Franz, 1993). Injection of adenoviral vector expressing luciferase reporter gene transcribed by MLC-2v promoter, into neonatal rat cardiac cavity, showed expression in heart but not in skeletal muscle tissue or other tissues tested (Rothmann *et al.,* 1996).

New methods of gene regulation are being devised to regulate the expression of putative therapeutic/cardioprotective genes directly in response to ischemia-associated signals. Recently, a combination of gene regulatory components that can be used to target a product to the myocardium and limit the expression of the gene to periods of ischemia activity, has been created (Prentice *et al.,* 1997). A single set of four tandemly repeated hypoxia response element (HRE) from human erythropoietin gene, placed immediately upstream (-86 bp), of a minimal muscle-specific α-MHC promoter, conferred potent positive regulation of this promoter by hypoxia *in vitro*, and by ischemia *in vivo.* The induction by ischemia persisted for at least 4 hours, returning to baseline level within 8 hours. Such an assembly of enhancer and promoter elements allows controlled expression of a therapeutic gene in ischemic myocardium.

Use of tissue-specific promoters has been shown as a promising tool for modulating the promiscuous tissue tropism of the otherwise efficient adenoviral vectors, for gene therapy targeted to the myocardium. With development of promoters and identification of enhancer elements like the HRE, a regulatory internal "sensor" can be assembled to trigger expression of therapeutic genes in heart at the onset of ischemia.

6.7. Current Status and Future Directions of Gene Therapy for the Myocardium

Current clinical practice for treatment of heart disease normally involves drug therapy, and often surgery in severe cases. Both these interventions have limited efficacy and associated adverse effects. Moreover, these therapies are aimed at treating the consequences of a heart condition (such as ischemia),

rather than the cause (e.g. atherosclerosis). Novel therapeutic approaches for treatment of disease have become available due to recent advances in gene transfer technologies. Gene therapy is emerging as an acceptable clinical practice for treating acquired and genetic diseases.

6.7.1. Improved Adenoviral Vectors

Adenoviruses are continuously being developed for high efficiency gene transfer and long-term expression (Wang and Finer, 1996). New vectors have emerged which address the problems of cytotoxicity and immune response, by making additional mutations and deletions, exhausting the ability of the virus to replicate in the non-permissive host.

6.7.1.1. Second Generation Adenoviruses

An approach adopted for second-generation recombinant adenoviruses comprises development of temperature sensitive lethal mutants. Engelhardt *et al.* designed a vector harboring a β-galactosidase-expressing transgene, in which a temperature-sensitive mutation is introduced into the E2A gene, of an E1-deleted recombinant. At nonpermissive temperature, this virus fails to express late gene products, even when E1 is expressed *in trans*. *In vivo.* studies show that animals receiving the second-generation virus expressed β-gal for up to 70 days, compared to 14 days with first-generation viruses (Engelhardt *et al.,* 1994). Stable expression of human CF transmembrane conductance regulator gene has also been achieved in lungs of CF mice instilled with a second generation virus (Yang *et al.,* 1994).

Viral vectors having E1/E4 double deletions have also been constructed (Wang *et al.,* 1997), with parallel development of cell lines to transcomplement the functions absent in the viral backbone (Wang *et al.,* 1995). A cell line that transcomplements the E1, as well as the E4 gene functions of human adenovirus 5, has been established by introduction of the full-length Ad5 E4 region into 293 cells. The viral genome is designed with the E1 region deleted and replaced by a foreign gene, and an additional lethal E4 region deletion. This system has been used to generate several E1/E4-deleted adenovirus vectors containing a reporter gene in the E1 region (Wang

et al., 1995; Gao *et al.,* 1996; Dedieu *et al.,* 1997; Wang *et al.,* 1997). The titer and the lacZ gene expression of E1/E4-deleted adenovirus vector were comparable to those of E1-deleted vectors. The doubly-deleted vector was substantially more stable in mouse liver than was the E1-deleted construct (Gao *et al.,* 1996).

6.7.1.2. "Gutless" Adenoviral Vectors

Induction of viral genes in non-permissive host cells and immune response problems have necessitated the development of adenoviral vectors which are devoid of all viral transcriptional units (Kochanek *et al.,* 1996; Parks *et al.,* 1996; Yeh and Perricaudet, 1997). Such viral vectors are also known as "*gut-less vectors*" and have all the adenoviral genome removed from the vector, with the exception of the indispensable *cis*-acting elements required for packaging of the genome into the capsids. These vectors can accommodate foreign DNA inserts of a much larger size, limited only by the packaging constraints of the adenovirus capsid (Ali *et al.,* 1994; Yeh and Perricaudet, 1997). The major impediment for producing these vectors is providing high enough levels of Late Transcriptional Unit (LTU) function *in trans* to synthesize the viral structural proteins. Ideally, this could be done by designing a cell line with the viral LTR integrated into its genome, but can be achieved with a "helper" adenovirus (Kochanek *et al.,* 1996; Parks *et al.,* 1996).

Caskey's group have developed a new adenoviral vector with no viral coding sequences and having a capacity for an insert up to 28 kb. Successful gene transfer and expression has been demonstrated using this vector which carried a 13.8 kb full-length dystrophin cDNA under transcriptional control of a 6.5 kb muscle creatine kinase promoter, in addition to the *E.coli* lacZ gene reporter gene (Kochanek *et al.,* 1996). The vector was designed with the 28 kb insert flanked on either side by the Ad5 left terminal repeat and the complete packaging signal (Ψ).

Interest has also been generated in exploiting the *cre-lox* mechanism adopted from bacteriophage P1 for making gutless vectors. The E1-deleted

recombinant virus backbone has been modified by inserting direct repeats of loxP sites "framing" a 25 kb viral region comprising most of the LTR, protein IX gene and E2. A precise excision of the 25 kb fragment occurs when this modified virus is infected into 293 cells expressing the cre recombinase *in trans*,. This event leaves behind a mini virus with only the L5 virion protein gene, the E4 region and the essential cis-acting elements required for packaging (Lieber *et al.,* 1996). However, this method is useful only for removing the viral genome from already available E1-deleted recombinants. Similar mechanisms were involved in generating "gut-less" vectors where the helper virus was modified to expel the packaging signal (Parks *et al.,* 1996). The helper viruses were constructed with packaging signals flanked by loxP sites so that in 293 cells that stably express the Cre recombinase (293Cre), the packaging signal (Ψ) was efficiently excised, rendering the helper virus genome unpackageable. The mini virus was separated from any traces of helper virus by CsCl density gradient purification. A titer of approximately $9x10^9$ pfu/ml was obtained with less than 0.01% helper virus contamination.

6.7.2. New Therapeutic Strategies for the Heart Failure

A variety of methods are under development for therapies involving gene transfer to myocardium. Although adenovirus-mediated gene transfer is the most widely used, other viral and non-viral vectors are also being investigated to make gene transfer more clinically feasible.

Adeno-associated virus (AAV) is a gene transfer vehicle which has gained recognition for its ability to transfer genes *in vivo.*, without causing any apparent toxicity or inflammation in the host. Intramuscular injection of AAV virus encoding a reporter gene expressing into rat heart yielded no apparent inflammation, with the expression lasting at least two months. Similarly, infusion of a recombinant AAV vector into porcine coronary artery showed expression in cardiac myocytes, without any evidence of immune response or other toxicity, with the expression detectable for up to six months (Kaplitt *et al.,* 1996).

Due to the absence of regenerative capacity in cardiocytes, the myocytes lost through myocardial necrosis are irreplaceable. Cell grafting of stem cells or genetically modified cells has been attempted in normal and ischemia-injured myocardium. These methods have parallels with the surgical technique of *cardiomyoplasty*, where skeletal muscle is grafted into the infarcted myocardium to compensate for the reduced contractility. Skeletal myogenic cells transplanted into an infarcted canine myocardium showed development of new striated muscle showing a cardiac phenotype and were able to express the reporter gene (Chiu *et al.,* 1995). In a separate study, infarcted rat heart grafted with labeled and genetically modified cells displayed survival of implanted cells in the non-infarcted region only (Aoki *et al.,* 1996). Foreign expression of the myogenic determination factor MyoD in cardiac fibroblast of healing heart lesions, has elicited differentiation into muscle cells (Murry *et al.,* 1996), indicating a possible method of contractile tissue repair in myocardial infarcts.

6.8. Novel Target Therapeutic Genes

Investigations into gene expression during ischemia, progressively contribute to the understanding of molecular processes involved in pathophysiology of ischemia and heart failure. Moreover, such studies have shown changes in gene activity of proteins critical for physiology of the myocyte. Several gene expression analyses carried out in failing heart and ischemia models, have also revealed the presence of proteins expressed during ischemia, which exhibit a cardioprotective effect on the injured myocardium.

6.8.1. Variations in Gene Expression During Ischemia

Ischemia and reperfusion lead to induction of proto-oncogenes in heart and subsequent induction of genes with cardioprotective function (Knight and Buxton, 1996). Expression of stress proteins such as heat shock proteins and genes for antioxidants like Manganese superoxide dismutase (Mn-SOD) and catalase are induced in ischemia following reperfusion (Das *et al.,* 1993).

6.8.1.1. Adaptive Response

Expression of Glucose transporters GLUT1, is found to be increased in ischemia, as a mode of adapting to increased requirements of glucose uptake in persisting ischemia (Brosius, FC *et al.,* 1997). Proto-oncogenes and growth factors are operative in cellular repair processes and angiogenesis (Brand *et al.,* 1992). Fibroblast Growth Factor (FGF) and Transforming Growth Factor (TGF), are polypeptides involved in cardiac morphogenesis, which are in induced by myocardial ischemia and can re-express the fetal program of cardiac specific genes (Parker *et al.,* 1990). Such expression leads to hypertrophy and remodelling of the ventricle to adapt to the diseased condition (Parker and Schneider, 1991). These factors can supply a range of possible molecular targets for ameliorating heart injury.

Myocyte cell death in ischemia is also postulated to be due to an activation of an apoptotic program, induced in the cardiocytes by oxidative-stress. The final result of apoptosis is fragmentation of nuclear DNA, but without change in functionality of cell membrane and other subcellular organelles. In ischemic myocardium, this process is seen as an adaptive response by the heart, to sacrifice some injured myocytes to regulated apoptosis, which may contribute less to arrhythmogenesis, than formation of a necrotic scar with cells devoid of membrane function (Bromme and Holtz, 1996). Identification of apoptotic signals in heart can be helpful in designing a strategy to modulate cell death during ischemia

6.8.1.2. Transcriptional Alterations in Ischemia

Gene expression of some myogenic proteins such as ion channels, may be altered in failing hearts, having direct impact on the overall physiology of the myocyte. The sarcoplasmic reticulum channel responsible for re-uptake of cytosolic Ca^{++}, the SERCA Ca^{++}-ATPase is shows lower levels of mRNA (Arai *et al.,* 1993) and reduced protein levels (Hasenfuss *et al.,* 1995; Hasenfuss *et al.,* 1997), in failing myocardium. The Na^{+}-Ca^{++} exchanger transmembrane protein, which is responsible for calcium extrusion across the sarcolemma, is increased in failing hearts (Hasenfuss *et al.,* 1997). Overexpression of SERCA Ca^{++} ATPase in transgenic mice *in vivo.* has shown enhanced

contractility and relaxation in normal hearts (He *et al.,* 1997), implying a role for this protein in failing heart, where it is reduced. Other, proteins involved in Ca^{++}-handling such as phospholamban (Hajjar *et al.,* 1997) have also gained attention. Ca^{++}-handling in progressive heart failure is known to be altered and thus the associated proteins are considered as possible targets for gene therapy.

6.8.1.3. Cardioprotective Genes

Potentially cardioprotective genes and genes showing altered regulation during ischemia/reperfusion indicate the possible molecular processes underlying ischemic events. Dissection of these molecular process can provide insight into using these as therapeutic agents for treatment of myocardial ischemia. For example, heat shock protein HSP-70 overexpression in transgenic mice (Marber *et al.,* 1995) and using adenoviral gene transfer vectors (Mestril *et al.,* 1996), render ischemic myocytes more tolerant to ischemic injury by providing a protective effect. Genes such as antioxidants are induced to nullify the effects of toxic products of ischemia/reperfusion. HSP-70 and SOD are now being seen as possible cardioprotective molecules which can be overexpressed during ischemia to protect the heart from acute injury.

6.9. Summary

In summary the project has involved subcloning of skeletal Troponin C coding DNA into the adenoviral shuttle vectors, the generation of adenoviral recombinants using plasmids constructed in the process of the project and analysis of the adenoviral recombinants for gene expression.

The plasmid subcloning has resulted in construction of a range of plasmids containing the sTnC cDNA in combination with constitutive and tissue-specific promoters. The sTnC coding sequence has been altered to incorporate an epitope tag for production of a skeletal Troponin C protein, with carboxyl-terminal HA and T-antigen epitope tags. In the course of subcloning of the sTnC cDNA, a range of useful adenoviral shuttle vectors with tissue-specific promoters for wider applications, have also been generated.

Adenoviral recombinants with wild-type skeletal Troponin C gene under control of constitutive and tissue-specific promoters have been generated. Adenoviral recombinants expressing the HA tagged skeletal Troponin C gene with constitutive and tissue-specific promoters, have also been constructed for intracellular localization studies. Modifications of the adenovirus construction protocols, were carried out in order to obtain higher yield of recombinants.

Some of the adenoviral recombinants made have been analyzed for expression at the mRNA and protein level and have shown the desired pattern of constitutive and tissue-specific transgene expression, *in vitro*. Western blot and Immunocytochemistry protocols have been optimized for the detection of HA tag epitope expression *in vitro*. The Ad5/CMV-IE/sTnCΔ9-HA construct has demonstrated ubiquitous expression in mouse fibroblasts, cardiac fibroblast and cardiomyocytes, whereas the Ad5/HCA(-485)/sTnCΔ9-HA recombinant shows a myocyte-specific pattern, showing expression in cardiomyocytes but not in cardiac fibroblasts. The tagged constructs are currently being applied for *in vivo.* studies by other members of the group.

Future analyses employing these viruses include functional studies on infected cells, using *in vitro.* systems, such as single cardiocyte measurements for contractility, and force measurement analysis using ventricular muscle fibres isolated from *in vivo.* infected hearts of animals. Ultimate application of these sTnC-encoding adenoviruses will incorporate clinically relevant gene transfer protocols, including catheter-mediated delivery through the coronary artery, in animal models of heart disease.

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