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Embryonal Carcinoma Derived Cardiac Muscle:  
Studies in Gene Expression and Differentiation

By

Michael Anthony Rudnicki

A thesis presented to the  
University of Ottawa in  
partial fulfillment of the  
requirements for the degree  
of Doctor of Philosophy



Michael A. Rudnicki, Ottawa, Canada, 1988.

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"Everyman ought to be inquisitive through every hour of his great adventure down to the day when he shall no longer cast a shadow in the sun. For if he dies without a question in his heart, what excuse is there for his continuance?"

Frank Moore Colby

"Experience is the name everyone gives to his mistakes."

Oscar Wilde

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Abbreviations

A-MHC	- atrial-myosin heavy chain
A-MLC	- atrial-myosin light chain
BSA	- bovine serum albumin
cAMP	- cyclic adenosine monophosphate
cdNA	- complementary DNA
cpm	- counts per minute
CH-actin	- human cardiac actin
CM-actin	- mouse cardiac actin
C/S-MHC	- cardiac/skeletal-myosin heavy chain
DEPC	- diethyl pyrocarbonate
DMSO	- dimethyl sulfoxide
DNA	- deoxyribonucleic acid
EC	- embryonal carcinoma
ECM	- extracellular matrix
EDTA	- ethylene diamine tetra acetic acid
h	- hour
HBS	- HEPES buffered saline
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
M	- molar
MHC	- myosin heavy chain
min	- minute
MLC	- myosin light chain
mM	- millimolar
MOPS	- 3-[N-morpholino] propane-sulfonic acid
mRNA	- messenger RNA
MW	- molecular weight
uM	- micromolar
um	- micrometer
PBS	- phosphate buffered saline
PIPES	- piperazine-N,N'-bis[2-ethane sulfonic acid]
RA	- retinoic acid
RNA	- ribonucleic acid
rpm	- revolutions per minute
rRNA	- ribosomal RNA
SDS	- sodium dodecyl sulfate
sec	- second
TE	- Tris-EDTA
TNT	- troponin T
UT	- untranslated
V-MHC	- ventricular-myosin heavy chain
V-MLC	- ventricular-myosin light chain
VRC	- vanidyl ribonucleoside complex
LTR	- Long Terminal Repeat
TK	- thymidine kinase

Abstract

P19 embryonal carcinoma cells are multipotential stem cells that differentiate into striated muscle as well as some other cell types when aggregated and exposed to dimethyl sulfoxide (DMSO). Immunofluorescence experiments using mono-specific antibodies indicated that the majority of muscle cells were mononucleate and contained four cardiac-specific myosin isoforms; atrial- and ventricular- myosin heavy chain proteins, and ventricular-myosin light chain 1 and atrial-myosin light chain 2 proteins. Northern blot analysis of RNA isolated from differentiating cultures indicated that cardiac-actin and skeletal-actin mRNAs were co-expressed at similar levels and with identical kinetics during the differentiation of P19-derived myocytes. These results demonstrate that these myocytes are of the cardiac type and suggests that they closely resemble the cells of the early embryonic myocardium.

A cloned human cardiac actin (CH-actin) gene was transfected into P19 cells, and stable transformants were isolated. Low levels of CH-actin mRNA were present in transformed EC cells, but a marked increase in the level of CH-actin mRNA was found as these cells differentiated into cardiac muscle. The accumulation of CH-actin mRNA paralleled that of the endogenous mouse cardiac actin mRNA. A chimeric gene, which consisted of the CH-actin promoter linked to the herpes simplex virus thymidine kinase coding region, was constructed and transfected into P19 cells. In these

transformants, the thymidine kinase protein was located almost exclusively in muscle cells. These results suggest that the CH-actin promoter functions in the appropriate developmental and tissue-specific manner during the differentiation of multipotential EC cells in culture.

The CH-actin gene was transfected into rat L6 skeletal myoblasts and stable transformants were isolated. The level of the CH-actin transcript varied between clones but changed little during the differentiation of myoblasts into multinucleate myotubes. The chimeric actin-tk gene was also introduced into L6 cells, and clones of L6 transformants contained variable levels of actin-tk mRNA that did not change during differentiation. Thus, the activity of the CH-actin promoter appeared not to be up-regulated during differentiation of myoblasts into myotubes. In clones of cells expressing the actin-tk mRNA, the TK protein was not detected in myoblasts but appeared in differentiating multinucleate myotubes. We interpret these results as suggesting developmentally regulated translation of the actin-tk mRNA.

We have isolated P19 cells stably transformed with either the activated human H-ras oncogene or with a chimeric gene in which the H-ras oncogene was placed under control of the human cardiac-actin gene regulatory sequences. These P19 lines exhibited ubiquitous and muscle-specific expression of the activated H-ras protein respectively. In both kinds of P19 lines, we observed normal cardiac and skeletal muscle differentiation. Since the activated H-ras prevents

differentiation of myoblast cell lines (Olson et al., 1987; Payne et al., 1987), our results suggest a fundamental difference between the EC-derived muscle progenitor cell and continuous myoblast cell lines.

P19 cells after DMSO-treatment are induced to differentiate into cell types normally derived from the embryonic mesoderm. From DMSO-induced cultures we have isolated differentiated cell lines capable of anchorage-independent growth. Several of these cell lines derived in this fashion exhibited myogenic potential. After becoming confluent, a subset of cells would fuse and form multinucleate myotubes which occasionally showed contractile activity. Two of these cell lines, MR16 and MR322, differentiated into cultures that contained skeletal myocytes and an ECM secreting mesenchyme-like cell. These cell lines are novel in that they appear to give rise to more than one cell type and thus may represent a stem cell with limited mesodermal developmental potential.

Within differentiated P19 cultures, we have examined the distribution of striated muscle and smooth muscle actins with specific antibodies. By indirect immunofluorescence, we found smooth muscle vascular alpha-actin containing cells after both RA and DMSO induced differentiation. Our results suggest that in addition to neurons and glia formed after RA-treatment, and striated muscle after DMSO-treatment, differentiated P19 cultures may include smooth muscle cells. Thus, the P19 cell system may represent a useful culture model to study smooth muscle differentiation and gene expression.

The P19 cell culture system allows the investigation of early commitment and differentiation events that only occur in relatively small numbers of cells in the embryo. Thus, the P19 cell line appears to be unique for the study of early cardiac muscle differentiation. In addition, we suggest that the P19 cell culture system is suitable for investigations into the activation and regulation of cardiac-specific gene expression.

Résumé

Les cellules embryonnaires carcinomateuses P19 sont des cellules souches de souris, multipotentielles, capables de se différencier en cellules musculaires striées, entre autres types cellulaires, lorsqu'une masse d'entre elles, sous forme d'agrégat, est exposée au sulfoxyde de diméthyle (DMSO). L'utilisation d'anticorps mono-spécifiques, en immunofluorescence, met en évidence que la majorité des cellules est mono-nucléée et contient quatre isoformes de la myosine cardiaque: la myosine à chaîne lourde, spécifique à l'oreillette et au ventricule; la myosine à chaîne légère de type 1, spécifique au ventricule; la myosine à chaîne légère de type 2, spécifique à l'oreillette. Une analyse "Northern" de l'ARN isolé de cultures différenciées met en évidence que les ARNm des actines cardiaque et squelettique sont co-exprimés à des niveaux comparables et selon des cinétiques identiques durant la différenciation de cellules P19 en myocytes. Ces résultats démontrent que les myocytes dérivés de cellules P19 sont de type cardiaque et suggèrent que ces myocytes ressemblent aux cellules retrouvées dans le myocarde embryonnaire précoce.

Le gène humain de l'actine cardiaque (actine-CH) a été cloné et transfecté dans des cellules P19; des cellules transformées stables furent isolées. Dans ces dernières, de faibles niveaux d'ARNm de l'actine-CH étaient détectés, alors qu'une forte augmentation dans les niveaux de ces mêmes

messagers était évidente lorsque l'on forçait la différenciation de ces cellules en muscle cardiaque. La cinétique d'accumulation de l'ARNm de l'actine-CH (exogène) était très similaire à celle de l'ARNm de l'actine cardiaque de souris (endogène). Un gène chimérique, incorporant le promoteur de l'actine-CH lié en amont de la phase codante du gène de la thymidine kinase du virus Herpès simplex, fut construit et transfecté dans des cellules P19. Dans ces cellules transformées, la protéine thymidine kinase fut localisée presque exclusivement dans les cellules musculaires. Ces résultats suggèrent que le promoteur de l'actine-CH est spécifique aux cellules musculaires et que son activité est régie par les règles du développement, lors de la différenciation en culture des cellules embryonnaires carcinomateuses (EC) multipotentielle.

Le gène de l'actine-CH fut transfecté dans des cellules myoblastiques squelettiques de rat (L6); des cellules transformées stables furent isolées. Les niveaux de messagers de l'actine-CH variaient d'un clone à l'autre mais peu de changement à ces niveaux était détectable durant la différenciation des myoblastes en myotubes mono-nucléés. Le gène chimérique actine-tk fut également introduit dans des cellules L6; des clones de cellules transformées contenaient des niveaux variables d'ARNm de l'actine-tk, ces niveaux demeurant in affectés pendant la différenciation. Donc, l'activité du promoteur de l'actine-CH ne semble pas être augmentée pendant la différenciation des myoblastes en myotubes. Dans des clones de cellules exprimant l'ARNm de l'actine-tk, la protéine TK ne fut

pas détectée dans les myoblastes mais est apparue lors de la différenciation de ces derniers en myotubes multinucléés. Nous croyons que ces résultats, suggèrent que la traduction de l'ARNm de l'actine-tk est contrôlée par le développement.

Nous avons produit des cellules P19 transformées stables par transfection avec l'oncogène humain H-ras activé et avec un gène chimérique contenant les séquences régulatrices du gène de l'actine-CH placées en amont de l'oncogène humain H-ras. Dans ces lignées cellulaires de P19 qui exhibaient ou non une expression spécifique aux cellules musculaires de la protéine H-ras, nous avons observé une différenciation normale des cellules musculaires cardiaques et squelettiques. Donc, contrairement aux lignées cellulaires permanentes de myoblastes squelettiques chez lesquelles l'expression du H-ras activé bloque la différenciation, les cellules P19 semblent représenter un modèle approprié pour l'étude de l'effet de l'expression d'un oncogène sur la différenciation.

Pour arriver à trier des cellules qui ne sont pas des cellules souches EC, lors de la différenciation de cellules P19 en culture, nous avons appliqué une pression de sélection pour obtenir des cellules capables de pousser sans ancrage à un support. Plusieurs de ces lignées cellulaires, dérivées de colonies différenciées poussant sur la cellulose de méthyle, démontraient un potentiel myogénique. A confluence, une partie des cellules se fusionnaient pour former des myotubes multinucléés qui démontraient, à l'occasion, une activité contractile. Deux de ces lignées cellulaires, MR16 et MR322, se

sont différenciées en cultures contenant des myocytes squelettiques, des cellules d'apparence mésenchymateuse. Donc, ces lignées cellulaires sont innovatrices en ce qu'elles sont capables de générer plus d'un seul type cellulaire et peuvent représenter des lignées de cellules souches à potentiel de différenciation multiple mais limité.

Chez des cultures de cellules P19 en cours de différenciation, nous avons examiné la distribution des actines de muscle strié et de muscle lisse à l'aide d'anticorps spécifiques. Après induction de la différenciation par traitement avec l'acide rétinoïque (AR) ou le DMSO, nous avons observé, par immunofluorescence indirecte, des cellules contenant de l'alpha-actine de muscle lisse. Nos résultats suggèrent que les cultures de cellules P19 en cours de différenciation contiennent des cellules myofibroblastiques, en plus des neurones et cellules gliales observées après traitement avec AR et des cellules musculaires striées observées après traitement avec DMSO. Donc, le système P19 représente un modèle utile de cellules en culture pour étudier la différenciation des cellules musculaires lisses et le contrôle de leur expression génique.

Le système de cellules P19 en culture permet l'étude du phénomène d'engagement cellulaire précoce dans une voie de différenciation et d'autres phénomènes de différenciation se produisant dans un nombre relativement faible de cellules faisant partie d'un embryon. La lignée cellulaire P19 apparaît unique en son genre pour l'étude de la différenciation du muscle

cardiaque précoce. De plus, nous suggérons que le système de cellules P19 en culture est approprié pour les études portant sur l'activation et le contrôle de l'expression de gènes exprimés dans les cellules cardiaques.

## Chapter 1, General Introduction

### 1.1. Introduction:

How cell proliferation and cell differentiation is regulated during embryogenesis remains a central problem in the study of embryonic development. To approach an understanding of embryonic development, we have investigated different aspects in the differentiation of a specialized cell lineage. In particular, we have examined gene expression, gene regulation and cell differentiation during cardiac myogenesis.

In the mammalian embryo, the myocardium is amongst the earliest organs to develop and in the mouse, cardiac myocytes first appear on embryonic day 8 (Jockusch et al., 1984). Thus, the commitment of the cardiac myocyte progenitor cells and the differentiation of the first cardiac myocytes occurs in a relatively small population of cells. The small amount of material available from embryos can impose limitations on the biochemical and molecular biological analysis of the developmental processes under investigation. In the study of mammalian development, the use of cell culture systems has facilitated more experimental and analytical approaches. In this work we have made use of a cultured murine embryonal carcinoma (EC) cell line, which can be induced to differentiate in vitro into cultures that include cardiac muscle.

We have used three strategies to investigate the molecular genetics of cardiac muscle cell differentiation. We have examined the types of muscle-specific isoforms of actin and myosin expressed during the differentiation of EC-derived cardiac muscle in cell culture. We have investigated the regulation of expression of a transfected human cardiac-actin gene during this differentiation. Finally we have attempted to interfere with striated-muscle differentiation by expressing a transfected activated oncogene during muscle differentiation. With these approaches, we hope to elucidate mechanisms involved in the regulation of tissue-specific gene expression, and thus improve our understanding of development.

## 1.2. Embryonal Carcinoma Stem Cells:

### 1.2.1. Teratocarcinomas:

Teratomas are tumours which are composed of a wide variety of disorganized cell types and tissues at various levels of maturation (Stevens and Hummel, 1957). Benign teratomas are well differentiated and have a limited ability for growth. Malignant teratomas or teratocarcinomas grow continuously and have a tendency to metastasize. In mammals, these tumours are more common in females than in males. In males, testicular teratomas tend to be malignant whereas in females the tumours are usually benign dermoid cysts of the ovary (Stevens, 1967b; Damjanov and Solter, 1974; Solter and Damjanov, 1979; Martin,

1980, Stevens, 1983).

Teratocarcinomas contain both differentiated cells and undifferentiated stem cells (Stevens, 1967a). Subcutaneous injection of teratoma differentiated cells does not produce tumours. However, injection of a single undifferentiated stem cell can produce tumours with a wide range of differentiated cell types and tissues (Pierce and Dixon, 1959; Pierce et al., 1960; Kleinsmith and Pierce, 1964). Thus the stem cells of teratocarcinomas have the potential to differentiate into many cell types and tissues. These stem cells are called embryonal carcinoma (EC) cells.

Teratocarcinomas can arise either spontaneously or can be experimentally produced. Testicular teratomas can be produced by grafting male genital ridges from 12.5 day mouse embryos into the testis of an adult mouse (Stevens, 1964, 1967b, 1970; Pierce 1967). Early mouse embryos (8 days old or less) when transplanted into extra-uterine sites in a syngenic host also produce teratomas consisting of disorganized differentiated cells and tissues and the undifferentiated EC cell (Stevens, 1968, 1970; Solter et al., 1975; Iles, 1977). Therefore, teratomas can arise from either germ cells or from normal embryonic cells developing in an extra-uterine environment.

The stem cells of mouse embryo derived teratocarcinomas can be isolated from tumours and grown as a pure population of EC cells in cell culture. EC cells closely resemble the pluripotential cells of the early embryo by both morphological

(Damjanov et al., 1971) and biochemical criteria (Damjanov and Solter, 1974; Martin 1975). Furthermore, EC cells can respond to developmental signals and behave like normal embryonic cells and participate in the formation of an embryo. EC cells can be injected into a blastocyst, the blastocyst containing the EC cells can be implanted into the uteri of pregnant mice, and the embryo derived from the injected blastocyst can develop into a normal tumour-free mouse (Brinster, 1974; Mintz and Illmensee, 1975; Illmensee, 1978; Papaioannou et al., 1975; 1979; Papaioannou, 1979). The mice resulting from these experiments are chimeras in that some tissues are derived from the embryo cells and some tissues derived from the injected EC cells. In some of these mice, the germ cells are descended from the EC cell. The extent of chimerism appears to be dependent on the EC cell line used. Thus, EC cells are analogous to the undifferentiated pluripotential stem cells of the early embryo.

#### 1.2.2. Embryonal Carcinoma Differentiation in vitro:

EC cells can be adapted to cell culture and can be maintained indefinitely without loss of potential as long as they remain in exponential growth (Martin, 1975). EC cells can be induced to differentiate in vitro by either manipulating the culture conditions, by exposure to drugs, or both. In addition, different EC cell lines differ in their predisposition to spontaneous differentiation, the range of cell types capable of being formed and their response to chemical inducers (reviewed

in Rudnicki and McBurney, 1987).

Some EC cell lines tend to differentiate in cell culture under conditions of dense multilayered culture. If not subcultured for a few weeks, these EC cell lines become almost entirely differentiated (Nicolas et al., 1975). Similarly, EC colonies that grow up under sparse conditions will pile up and differentiated cells will become more and more abundant (McBurney, 1976). In addition, if the EC cells are allowed to form aggregates by culturing under conditions where they are unable to adhere to the culture dish, they will differentiate into non-EC cell types (Martin and Evans, 1975a). The EC cell lines that differentiate under these conditions often form a Reichert's membrane-like or extra-embryonic endoderm-like layer around the exterior of the cell mass. More complex differentiation can occur if the aggregates are transferred onto a surface where they can adhere. Under these conditions, many cell types such as epithelium, cartilage, neurons and muscle, can be observed (Martin and Evans, 1975b).

Drugs can be used to induce the differentiation of EC cells. The two classes of drugs most commonly used to induce differentiation of EC cells are the retinoids, such as all-trans retinoic acid (RA), and bipolar compounds, such as dimethyl sulfoxide (DMSO) and hexamethylene bis-acetamide (HMBA) (see Rudnicki and McBurney, 1987). The F9 line of EC cells, when exposed to RA while growing on plastic surfaces, differentiate into cells which resemble extra-embryonic endoderm

(Strickland and Mahdavi, 1978). These endoderm cells can be further induced by exposure to dibutyryl cAMP, to differentiate into cells that more closely resemble parietal extra-embryonic endoderm (Strickland et al., 1980). If F9 cells are simultaneously aggregated and exposed to low doses of RA, visceral endoderm-like cells form around the outside of the aggregates (Hogan et al., 1981). The EC cell lines PCCA/1 and PCC4azal, when exposed to HMBA differentiate into epithelial-like and fibroblast-like cells (Jakob et al., 1978; Speers et al., 1979). Other EC cell lines also differentiate when exposed to chemical inducers. However, for most cell lines the types of cells formed has not been well characterized (see Appendix in Silver et al., 1983).

### 1.2.3. The P19 Cell Culture System:

The P19 line of EC cells was used for the majority of the studies described in this thesis. P19 cells were derived directly from a solid primary teratocarcinoma obtained by grafting a 7.5 day C3H embryo into the testis of an adult mouse. P19 cells have a normal male karyotype and do not require the use of feeder cells to be maintained in cell culture (McBurney and Rogers, 1982). In addition, P19 cells will participate in the formation of a large variety of cell types when injected into blastocysts (Rossant and McBurney, 1982).

Little differentiation is observed when P19 cells are aggregated and not exposed to drugs. The small number of

differentiated cells that are formed resemble extra-embryonic endoderm since they contain cytoKeratin intermediate filaments (Jones-villeneuve et al., 1982) and bind Dolichos biflorus agglutinin on their cell surfaces (Smith et al., 1987).

Aggregates of P19 cells will differentiate when exposed to certain drugs (reviewed in McBurney et al., 1983). After aggregation and exposure to concentrations of RA over  $10^{-7}$  M for 48 h, P19 cells differentiate into neurons, glia and fibroblast-like cells. No striated muscle cells are present in these cultures. The neurons, as identified by their characteristic morphology and the presence of neurofilament protein, appear by 6 days. The glia cells, as identified by the presence of glial fibrillar acidic protein, appear later at around 9-10 days (Jones-Villeneuve et al., 1982). The neurons express the neuron-specific enzymes acetylcholine esterase and choline acetyl-transferase and contain tetanus toxin binding sites on their surfaces (Jones-Villeneuve et al., 1983). Furthermore, in 7 day old cultures the neurons synthesize the neurotransmitter acetylcholine, have high affinity uptake sites for gamma-aminobutyric acid but not for serotonin, and form morphologically differentiated synapses (McBurney et al., 1988). In 12 day old cultures, the neurons contain a neuron-specific enolase as well as express three enzymes involved in catecholamine synthesis, tyrosine hydroxylase, dopamine beta-hydroxylase and phenylethanolamine N-methyl-transferase. These older neurons also exhibit an exogenous catecholamine

uptake system (Sharma and Notter, 1988). Therefore, neurons derived from RA-treated P19 cells appear to mature with time in culture and acquire the ability to synthesize different neurotransmitters.

P19 cells, when aggregated and exposed to 1% DMSO for at least 96 h, differentiate into cultures which contain striated muscle and other uncharacterized cell types. No neurons or glial cells are present in these cultures (McBurney et al., 1982). The flat mononucleate muscle cells, which contain the striated-muscle myosin heavy chain (C/S-MHC) protein and often exhibit spontaneous contractile activity, first appear on the 6th day. Bipolar multinucleate myocytes begin to appear about the 8th day. The proportion of muscle cells reaches 10-30% by the 10th day. The non-muscle cells in DMSO-treated cultures are not well characterized, although some may be epithelial in origin since they express cytokeratins (McBurney et al., 1982; Edwards et al., 1983). Electron microscopic examination of the myocytes within DMSO-treated cultures has revealed abundant immature myofibrils with occasional Z-bands and the hexagonal arrangement of thick and thin filaments. Where two muscle cells abut one another, junctional complexes were often observed which resembled the intercalated disks of cardiac muscle (Smith et al., 1987). On the basis of these observations we concluded that the mononucleate myocytes within DMSO-treated cultures were most probably cardiac muscle.

The mechanisms whereby RA and DMSO induce P19 cells to differentiate remain unclear. However, the effects of both drugs appear to require cell aggregation. Retinoids are naturally occurring compounds and have been implicated in pattern formation in developing and regenerating limbs (Maden, 1982; Tickle et al., 1982; Thaler and Eichele, 1987). In contrast, DMSO is an organic compound of sulfur not synthesized by eukaryotes. DMSO may induce differentiation of P19 cells via a mechanism involving the cooperative response or cell-cell interaction of adjacent cells within an aggregate (Campione-Piccardo et al., 1985). Exposure of aggregates to DMSO promotes the formation of primitive endoderm on the periphery of the aggregates followed by development 2 to 3 days later of cardiac myocytes within the aggregate. Thus, the endodermal layer may be involved causally in the formation of the myocytes (Smith et al., 1987).

### 1.3. Thick and Thin Filaments:

The contractile apparatus of sarcomeric muscle (skeletal and cardiac muscle) consists of a complex organization of thick and thin filaments capable of generating force. The thin filaments contain the proteins actin, troponin and tropomyosin whereas thick filaments contain myosin heavy and light chain proteins. The interactions of actin and myosin produce contractile force by converting chemical energy into mechanical work (reviewed in Lehman 1976).

### 1.3.1. Myosin:

The myosin molecule is a major constituent of the contractile apparatus and is made up of two heavy chains (200,000 MW), two phosphorylatable or regulatory light chains (17,000 to 20,000 MW), and two non-phosphorylatable or alkali light chains (16,000 to 22,000 MW). These different isoforms are encoded by multigene families that exhibit complex patterns of tissue-expression characteristic of the developmental stage and muscle-type (reviewed in Buckingham et al., 1985, 1986).

The cardiac isoforms of myosin heavy chain (MHC) and myosin light chain (MLC) proteins found in the mammalian heart are expressed almost exclusively in the heart. The proportion of atrial-MHC (A-MHC) to ventricular-MHC (V-MHC) depends on the developmental stage, hormonal influences, and cardiac muscle-cell type. In small mammals, A-MHC is the major myosin isoform in both the atria and ventricle of the adult heart. In the fetal heart, A-MHC and V-MHC are co-expressed in the atria and V-MHC predominates in the ventricle (Lompre et al., 1981, 1984; Izumo et al., 1986; Pritzl and Zak, 1987). The ventricular-myosin light chain 1 (V-MLC1) and atrial-myosin light chain 1 (A-MLC1) isoforms are localized to the adult ventricle and atria respectively, whereas, the A-MLC1 and V-MLC1 isoforms are co-expressed in fetal ventricles (reviewed in Barton and Buckingham, 1985). Thus, co-expression of different cardiac MHC and MLC isoforms is normally only observed in the

fetal heart (reviewed in Buckingham et al., 1985, 1986).

### 1.3.2. Actin:

Actin filaments consist of two strands of globular molecules twisted into a helix with a diameter of about 4 nm and with 13.5 molecules per turn. The actin protein (42,000 MW) is encoded by a highly conserved multigene family. In vertebrates, there are at least six different actin genes: the striated-muscle actins, alpha-skeletal and alpha-cardiac; the smooth-muscle actins, alpha-vascular and gamma-enteric; and the non-muscle cytoskeletal, beta-actin and gamma-actin (Vanderkerckhove and Weber, 1978).

The cardiac-actin and skeletal-actin genes are expressed only in striated muscle. The relative proportions of cardiac-actin to skeletal-actin in heart and skeletal muscle is dependent on developmental stage and muscle-type. In the vertebrate fetal heart, cardiac-actin and skeletal-actin are expressed at similar levels while in the adult, virtually all of the actin is of the cardiac type (Mayer et al., 1984; Nudel et al., 1984; Minty et al., 1982; Buckingham et al., 1986). In contrast, in fetal skeletal muscle, cardiac-actin is the major actin-gene product whereas in the adult, skeletal-actin becomes the predominant isoform (Bains et al., 1984; Gunning et al., 1987, and references therein).

The cardiac actin gene is present as a single copy per haploid genome and is located on human chromosome 1 (Gunning et

al., 1984), and mouse chromosome 2 (Czosnek et al., 1983). The human cardiac actin (CH-actin) gene was cloned and sequenced by Hamada et al. (1982), and is contained entirely within a 13 kb EcoRI restriction endonuclease fragment. The transcribed region spans 5.3 kb and consists of 7 exons and 6 introns (Figure 1.1). The processed CH-actin transcript encoding the 352 amino acid cardiac-actin protein, is about 1.7 kb in length and contains a 64 nt 5'-untranslated (UT) region and a 190 nt 3'-UT region (Gunning et al., 1983). The first intron (about 700 bp in length) of the CH-actin gene disrupts the 5'-UT leader and thus the initiation of transcription site is located about 740 bp upstream of the translation start site (Figure 1.1). The 44 bp first intron contains the first part of the 5'-UT region of the CH-actin gene (Minty and Kedes, 1986).

The promotion of CH-actin transcription has been investigated by transient expression experiments where modified CH-actin genes have been assayed for activity after transfection into the mouse skeletal myoblast C2 cell line. Using this approach, a number of cis-acting sequences have been identified that appear to be required for transcription in C2 cells; a distal regulatory region located between -443 and -395 bp upstream of the transcription start site, and a proximal regulatory region located between -177 and -47 bp upstream of the transcription start site (Figure 1.1) (Minty and Kedes, 1986). Detailed deletion analysis of these regions revealed the presence of a CC(A+T rich)<sub>6</sub>GG motif [designated as a CARG box]

Figure 1.1. Nucleotide sequence of the human cardiac-actin gene. The negative numbers indicate distance upstream of the transcription start site. The CARG motifs are located at positions -100, -141 and -192, and the TATAA box is located at position -30. Slashes (/) indicate intron-exon borders. The nucleotide sequence of the promoter is from Minty and Kedes (1986), and the coding region from Hamada et al. (1982).

TGGAGATGA GAAGCGCGTG TTGCTCCCT GCGCGCGTG CCCAGCTGC AGGACCCCTC  
AGCAGAGGGC AGGGGGCCAA GCCTTCCAC TGCATGGGG <sup>-380</sup>TTATGTCCC CAAGCGTGGC  
AGGGCTCGGG AGGACCGAAT CCACAGACCA TCCAGGGAGC ACCCACACCC CAGAAAGGGG  
GAGCGGTGGG CTGGCGTCAC TTAGTCTCC CCGCGCCCT ACCCTTCCG GCGTCCCCCT  
CCCAGCTCC CTATTGGCC ATCCCGCTGA CTGCGCCCT CCGTCTCTA <sup>-192</sup>CACTGGCTGG  
GGCTCCCTG GGTGATCTC TCCCTGCCG TTGGCTCCAT <sup>-141</sup>CAATGGCGTC GGCAGTCTA  
GGGGTGGCA AGGGGACAA ATAAGCCAA GTGCGAGACC GGGCGCCCGA CCGCTGCCG  
CGGCTGCTC AACTGACCT GTCCATCAGC GTCTATATA <sup>-30</sup>GGGCGCCCTC TGGAGCCAGC  
CACCGAGGC CGGCTGCCG CGGAGCCGAG CCGACCCCGC CGGCGCGG/G TGAGTCAGCG

ACEGGCCCT CCGCG... about 670 bp ...  
CTGAG/AAA CCGCGTGAAG CTGCGCCAA ATG TGT GAC GAC GAG GAG ACC ACC GGC  
Met Cys Asp Asp Glu Glu Thr Thr Ala  
CTG GTG TGG GAC AAC GGC TCT GGG CTG GTG AAG GCG GGC TTT GGG GGC GAT GAC  
Leu Val Cys Asp Asn Gly Ser Gly Leu Val Lys Ala Gly Phe Ala Gly Asp Asp  
GGC CCC GGC GCT GTC TTC CCG TCG ATC GTG GGC GGC CGG GGC CAC GAG/G  
Ala Pro Arg Ala Val Phe Pro Ser Ile Val Gly Arg Pro Arg His Gln  
TAAACTGCC GCGAGCGCC CGTCCCACT CGCTTCTC TCAGTCCAGC GATCTAGGAA  
ATGGCTCCA CCT ... about 850 bp ...

TATATTCTGG CATGGTGAGA GCATGATTTT CACATTTTTT CTCTCATAG /GGA GTT ATG  
Gly Val Met  
GTG GGT ATG GGT CAG AAG GAC TCC TAC GTA GGT GAT GAA GGC CAG ACC AAG AGA  
Val Gly Met Gly Gln Lys Asp Ser Tyr Val Gly Asp Glu Ala Gln Ser Lys Arg  
GGC ATC CTG ACC CTG AAG TAT CCC ATC GAG CAT GGT ATC AIC ACC AAC TGG GAC  
Gly Ile Leu Thr Leu Lys Tyr Pro Ile Glu His Gly Ile Ile Thr Asn Trp Asp  
GAC ATG GAG AAG ATC TGG CAC CAC ACC TTC TAC AAT GAG CTC CGT GTT GGT CCC  
Asp Met Glu Lys Ile Trp His His Thr Phe Tyr Asn Glu Leu Arg Val Ala Pro  
GAG GAG CAC CCC ACC CTG CTC ACA GAG GGC CGG CTG AAC CCC AAG GGC AAG GGG  
Glu Glu His Pro Thr Leu Leu Thr Glu Ala Pro Leu Asn Pro Lys Ala Asn Arg  
GAG AAG ATG ACT CAG ATC ATG TTT GAG ACC TTC AAT GTC CCT GGC ATG TAC GTG  
Glu Lys Met Thr Gln Ile Met Phe Glu Thr Phe Asn Val Pro Ala Met Tyr Val

GGC ATC CAG GCA GTG CTA TCC CTG TAT GCT TGT GGC CGT ACC ACA G/  
Ala Ile Gln Ala Val Leu Ser Leu Tyr Ala Ser Gly Arg Thr Thr  
GTATGCTGG CTCTGGGAC AGTACTGAT GAATCACCAT TCCCAAGTCA CCGACCTTGC  
TGTGAATCAG ATCCCGCAGT TGAAMAAGGG ATAATCCCTT TCTCCCAT CCCTAGCAAG  
GTCTGTGCTA AGAGAAAGAG TTAACGGTAG TCCCTGAGG TTAGTTCCG AGCACAATTA  
TTATTGTCCA GCTGATAGCT TGTGGAGGTG GGCCTTCCCT CATTTAARG TCAGCGGAGT  
GTAGCAGGTT GGAGTGCAGC AGTCATTTGT TGTTTAACC ATCACAACAC CTGGGCAAGC  
ATCCCAAGG AGAATACATT CCATACAGGG ... 240 bp ...

TAGACCACTG GTGTGTCTT CAGCAATTA CCTTGTCTT GGTACTTCC CCGGGA  
G/GC ATT GTT CTG GAC TCT GGG GAT GGT GTA ACT CAC AAT GTC CCC ATC TAT GAG  
Gly Ile Val Leu Asp Ser Gly Asp Gly Val Thr His Asn Val Pro Ile Tyr Glu  
GGC TAC GCT TTT CCC CAT GGC ATC ATG CGT CTG GAT CTG GCT GGT GGC GAC CTC  
Gly Tyr Ala Leu Pro His Ala Ile Met Arg Leu Asp Leu Ala Gly Arg Asp Leu  
ACT GAC TAC CTC ATG AAG ATC CTC ACT GAG GGT GGC TAC TCC TTT GTC ACC ACT  
Thr Asp Tyr Leu Met Lys Ile Leu Thr Glu Arg Gly Tyr Ser Phe Val Thr Thr

G/GTGAGTG TGTG/TCTC ATCTGCCACA GTGGGGTCT GCTTTCCTC TCCTCTCCA  
CTGAATCCCG CTACCTCCCT ATAATTGACT TCTTGGTCCA GAGCATGACT GTGATACTCT  
TTATTTCTGT A

G/CT GAA CGT GAA ATT GTC CGT GAC ATT AAA GAG AAG CTG TGC TAT GTC GGC CTG  
Ala Glu Arg Glu Ile Val Arg Asp Ile Lys Glu Lys Leu Cys Tyr Val Ala Leu  
GAT TTT GAG AAT CAG ATG GGC ACA GCT GGC TCT TCC TCC TCC TTG GAG AAG AGC  
Asp Phe Glu Asn Glu Met Ala Thr Ala Ala Ser Ser Ser Ser Leu Glu Lys Ser  
TAT GAA CTG CCT GAT GGC CAA GTC ATC ACT AIC GGC AAT GAG GGC TTC GGC TGT  
Tyr Glu Leu Pro Asp Gly Gln Val Ile Thr Ile Gly Asn Glu Arg Phe Arg Cys  
CCT GAG ACA CTC TTC CAG CCC TCC TTC AIT G/GTGAAT GTAGGGTCTC  
Pro Glu Thr Leu Phe Gln Pro Ser Phe Ile

GTGTAGGGC ACCAATTTCC TGGAAATCTT AGGGTCTCC AGAGTAAAT CTAGAATCT  
CAAGAAGCCC TTGAGTTAAA AGAAGTCATT GTTTGGATC CCACAGCTCA ACCTCAAGTC  
CTGATGGTCC GATTAAAGGG GAAGTCTAIG TTTGCTCTA GTGATATAAT AAGGATGAC  
ACACAGGATT CATCTCAAAA TAAAGGATGA CACATAGAG TAACATGAATA CCACTAICTG  
CAG ... about 300 bp ...

CTCCAGTGTG TCTTATAGG GAACATATGT TTAGAGACA AATGGTACAG  
CTCCGCCACA CAAGAAGTT CIGTCTCCT CCCCTACCT TGACCTGAT CCACTGTGAT  
TGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT  
G/GT ATG GAA TCT GGT GGC ATC CAT GAA ACA ACT TAC AAT AGC ATC ATG AAG TGT  
Gly Met Glu Ser Ala Gly Ile His Glu Thr Thr Tyr Asn Ser Ile Met Lys Cys

GAC ATT GAT ATC CCG AAG GAC CTG TAT GGC AAC AAT GIC TTA TCT GGA GGC ACC  
Asp Ile Asp Ile Arg Lys Asp Leu Tyr Ala Asn Asn Val Leu Ser Gly Gly Thr  
AGI ATG TAC CCT GGT ATT GCT GAT GGT ATG CAG AAG GAA ATC ACT GCT CTG GCT  
Thr Met Tyr Pro Gly Ile Ala Asp Arg Met Gln Lys Glu Ile Thr Ala Leu Ala  
CCT ACC ACC ATG AAG ATT AAG/GTAAAGAA CTTTGTGAG TGGAGATGG  
Pro Ser Thr Met Lys Ile Lys

AGGCAGGCTT TGGTATTCTA ACGGAAGTAC GTTCAAATTT CTTTTCCCT CATACTCAGT  
... about 300 bp ...

TAGGTAAGTGG AGAGGTTCG\* TTTTAATAAT ACAATATATC TTTAATAATA  
TAAATGATGA AATAGAGGTC AGTTCATATA ACTTGATGG CCATATTATT TCGTGGTATG  
ACATATCCCA CATTATAGCG AATTAATATC TAAIGGTITT TGTGTGATC CTCCCAATGT  
GTATTGTCT CCGTGTCTTG GAACCTCAGA GTTCACTGGA AGTTTGTITT TCTTCTCAG/  
ATT ATT GCT CCC CCT GAG GGT AAA TAC TCT GTC TGG ATT GGG GGC TCC ATC TTG  
Ile Ile Ala Pro Pro Glu Arg Lys Tyr Ser Val Trp Ile Gly Gly Ser Ile Leu  
GCC TCT CTG TCC ACC TTC CAG CAA ATG TGG ATT AGC AAG CAA GAG TAC CAT GAG  
Ala Ser Leu Ser Thr Phe Gln Gln Met Trp Ile Ser Lys Gln Glu Tyr Asp Glu

GCA GGC CCA TCC ATT GTC CAC CGG AAA TGC TTC TAAGATGC TTCTCTCTC  
Ala Gly Pro Ser Ile Val His Arg Lys Cys Phe  
ATCTACCTTC CAGTCAGGAT GACGGTATTA TGCTTCTGG AGTCGCCAA ACCACCTTCC  
CTCACTTTC ATCAATCATT GTACAGTTTG TTTACACAGC TCGAATTTGT TGTGCTTCT  
AATATTTAT CTTTTATAA TAACCAGAC TAGGACTTGC AACCTATAA ACCCTTCTGT  
TGTTTTTGG GGTAGCGCTG GGGTGGGCA GG

Intron IV

Exon 5

Exon 1

Intron I

Exon 2

Intron 5

Intron II

Exon 3

Intron VI

Exon 7

Intron III

Exon 4

repeated several times in the upstream regions of the CH-actin gene (Miwa and Kedes, 1987). In band-shift experiments, these CARG motifs have also been shown to interact with a specific DNA-binding protein (Miwa et al., 1987). However, in these experiments, the investigators failed to demonstrate more than a modest increase in CH-actin promoted expression following skeletal myotube differentiation (Hickey et al., 1986; Minty et al., 1986).

#### 1.4. Regulation of Gene Transcription:

In eukaryotes, genes transcribed by RNA polymerase II are discrete entities in DNA which encode the information required to synthesize a specific protein. The exact mechanisms by which gene expression is regulated during development, in a tissue-specific manner, or in response to extra-cellular signals, remains unclear. Gene expression can potentially be regulated at many levels. (i) Chromatin structure and DNA-methylation may affect expression. (ii) The gene can be modified via rearrangement or the number increased by amplification. (iii) The rates of initiation and/or elongation of transcription may be regulated. (iv) The mRNA may be spliced along alternative pathways. (v) The mRNA may be stored or translated. (vi) The mRNA stability may be regulated. (vii) The rate of translation may be regulated. (viii) The level or activity of protein may be regulated by controlling stability, by controlling the level of co-factors and/or by modification.

Although most genes are probably not regulated at all of these levels, many genes appear to be subject to multi-level and complex control. In this section we will discuss the most well understood of these controls, the regulation of gene transcription.

In higher eukaryotes, genes which encode proteins are transcribed by a protein complex which includes RNA polymerase II. The transcription of protein-encoding genes is regulated in cis by two classes of DNA sequence elements, promoters and enhancers.

The promoter elements are located just upstream of the transcription start site and are typically contained within 100 bp. Genetic and functional analysis of several promoters has revealed the presence of a number of common conserved elements. The most proximal element is an AT-rich region (designated the TATA box) whereas more distally are one or more elements of 8 to 12 bp in length (designated upstream promoter elements or UPEs). There are many types of UPEs; however some of these, for example the CCAAT and GC boxes are found in certain types of promoters. In general, TATA and CCAAT boxes are found in promoters associated with enhancers whereas GC boxes tend to be found in constitutive promoters. The TATA box acts to direct the accurate initiation of transcription and the UPEs function to increase the rate of transcription (Dyran and Tjian, 1985; McKnight and Tjian, 1986; reviewed in Reeves 1986; Maniatis et al., 1987).

Mutagenesis of promoters has revealed that promoter strength is related to the number and type of UPEs, and that UPEs act independent of their orientation. However, transcription can be affected by changing the spacing between UPEs and the TATA box by adding or deleting nucleotides. Usually, the effect is less detrimental if the alteration results in a full turn rather than a half turn of the DNA helix. This suggests interaction between the proteins bound to the UPEs and TATA box is required for optimal transcription. This also suggests that this interaction requires stereospecific alignment of the proteins on the DNA helix (McKnight, 1982; Takahashi et al, 1986).

The proteins that bind promoter elements in a sequence-specific manner have been identified by DNA footprinting (Galas and Schmitz, 1978), band-shift assays (Fried and Crothers, 1981; Garner and Rezvin, 1981), and exo-III analysis (Wu, 1984). Several of these DNA-binding proteins have been purified using recognition-site affinity chromatography (Briggs et al., 1986; Jones et al., 1986; Kadonaga and Tjian, 1986; Rosenfeld and Kelly, 1986; Chodosh et al., 1986). In addition, the cDNA encoding one such transcription factor (Sp1) has been cloned. Functional analysis of Sp1 has suggested that the DNA-binding activity is localized to the C-terminal region which contains three Zn(II) finger motifs (Kadonaga et al., 1987).

Enhancers are discrete elements, that are short (50-100 bp) and often present in tandem repeats, which can act over great distances from either direction from the transcription start site, and in an orientation-independent manner. Enhancers appear to be involved in regulating the rate of initiation of transcription (reviewed in Serfling et al., 1985; Maniatis et al., 1987). Enhancers can be functionally defined as either responsive to environmental signals (inducible), or responsive to tissue-specific and developmental signals.

Like promoters, enhancers also contain discrete sequence motifs that specifically interact with DNA-binding proteins (reviewed in Serfling et al., 1985). Recently the cDNA that encodes a DNA-binding protein that binds both the MHC class I and Ig kappa gene enhancers has been cloned (Singh et al., 1988).

A model has been proposed to explain how the seemingly modular regulatory elements act in a mutually-dependent way to regulate transcription (Ptashne, 1986). In this model, the proteins bound to the different elements in DNA would interact with each other via looping out of the intervening DNA. The bound proteins would then form a complex recognized and bound by the pre-transcription complex which would become the activated transcription complex.

### 1.5. Thesis Project:

In this thesis we have pursued a number of approaches to investigate differentiation along the striated-muscle lineage. In Chapter 3, we characterized the types of isoforms of actin and myosin expressed during the differentiation of P19-derived cardiac and skeletal muscle. Our results confirmed the cardiac nature of the mononucleate myocytes and suggested that they may be analogous to the muscle cells found in the embryonic myocardium. In Chapters 4 and 5, we investigated the regulation of human cardiac-actin (CH-actin) gene expression during striated-muscle differentiation. We concluded that the transfected CH-actin promoter was subject to appropriate developmental and tissue-specific regulation during the differentiation of transfected P19 cells but not during the differentiation of rat skeletal myoblasts. In Chapter 6, we examined the effect of expression of a transfected activated H-ras oncogene on the differentiation of P19-derived striated-muscle. We found differentiation along the striated-muscle lineage was unaffected. In Chapter 7, we describe the isolation of non-EC mesodermal stem cells from differentiating cultures after DMSO-treatment of P19 cells. These cell lines appear to be multipotential since they give rise to skeletal myotube and mesenchyme-like cells. In Chapter 8, we further characterized actin expression in differentiating P19 cultures. Our results demonstrated that some of these P19-derived cells expressed smooth muscle actins.

## Chapter 2, Materials and Methods

### 2.1. Chemicals, Supplies, Biologicals and Equipment:

All chemicals were obtained from either BDH Chemicals Limited (Toronto, Ont., Canada), Sigma Chemical Company (St. Louis, Mo, USA) or Fischer Scientific Company (Fair Lawn, NJ, USA) unless otherwise stated. Biologicals were obtained from either Boehringer Mannheim Canada (Dorval, Que., Canada), Bethesda Research Laboratories (Gaithersburg, MD, USA) or New England Biolabs (Beverly, MA, USA) unless otherwise stated. Radiochemicals were obtained from Amersham Canada Limited (Oakville, Ont., Canada).

Centrifugation was performed in a Beckman J2-21 and ultracentrifugation was performed in a Beckman L8-70. Radioactivity was measured in a Beckman LS7800 Scintillation Counter. Absorbance was measured in a Beckman DU-7 Spectrophotometer. Densitometry was performed with an LKB Ultrosan XL.

### 2.2. Molecular Biological Methods:

Unless otherwise indicated, the majority of the procedures described in this section can be found in Maniatis et al., (1982).

### 2.2.1. Small Scale Plasmid Preparation:

Plasmid DNA was isolated essentially as described by Birnboim and Doly (1979). Bacteria were inoculated into 5 ml of medium (Luria broth) containing the appropriate antibiotic and incubated overnight at 37°C with vigorous shaking. Bacteria from 1.5 ml of the overnight inoculum were pelleted by centrifugation in an Eppendorf centrifuge. The pellet was resuspended in 100 ul of an ice-cold solution of lysis buffer containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl (pH 8.0) and 5 mg/ml lysozyme and stored for 5 min at room temperature. Next, 200 ul of denaturation solution containing 0.2 M NaOH and 1% SDS was added and mixed gently with the lysed bacteria. After 5 min, high salt solution containing 60% 5 M potassium acetate and 11.5% glacial acetic acid was added to neutralize the bacterial lysate. The lysate was centrifuged for 5 min in an Eppendorf centrifuge. The supernatant was extracted with an equal volume of a 1:1 mixture of phenol/chloroform. Two volumes of 95% ethanol containing 2% potassium acetate were added to precipitate the plasmid DNA. The plasmid DNA was collected by centrifugation and resuspended in 50 ul of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA) containing 10 ug/ml RNase. The plasmid preparation was stored at -20°C.

### 2.2.2. Large Scale Plasmid Preparation:

Bacteria were inoculated into 250 to 500 ml of medium (Luria broth) containing the appropriate antibiotic. Large

scale plasmid preparations were performed by scaling up the procedure described in 2.2.1. However if the plasmid was destined for transfection into eucaryotic cells, the plasmid preparation was extracted twice with phenol/chloroform (1:1) and twice with chloroform/isoamyl alcohol (24:1) and was twice precipitated after the addition of 1 volume of 7.5 M ammonium acetate and 3 volumes of 95% ethanol.

#### 2.2.3. Restriction Endonuclease Digestion of DNA:

Plasmid DNA was mixed with 2-3 u/ug of restriction endonuclease in a solution containing the appropriate digestion buffer in a 1.5 ml Eppendorf tube. The mixture was incubated at the optimum temperature (usually 37°C) for 2-3 h.

Eucaryotic genomic DNA was usually digested with 5-10 u/ug restriction endonuclease for at least 8 h.

#### 2.2.4. Isolation of Restriction Fragments of Plasmid DNA:

Fragments less than 1 kb were isolated after size fractionation by electrophoresis through low-melting temperature agarose. The DNA band of interest was excised from the agarose gel, melted at 65°C and diluted in 5 volumes of 10 mM Tris.Cl (pH 8.0) and 1 mM EDTA. After the diluted agarose had cooled to room temperature, it was extracted with phenol, phenol/chloroform (1:1) then chloroform. The DNA fragment was recovered by ethanol precipitation.

Alternatively, DNA fragments of any size were isolated by electrophoresis into troughs cut into the agarose gel. The diluted DNA fragments would be recovered by concentrating the sample by binding to a NACS (BRL) column.

#### 2.2.5. Dephosphorylation of DNA:

The 5'-terminal phosphates of DNA were removed by suspending the DNA in 0.05 mM Tris-Cl (pH 9.0), 1 mM MgCl<sub>2</sub>, 0.01 mM ZnCl<sub>2</sub>, 1 mM spermidine and 0.01 u, calf intestinal phosphatase (CIP) per ug of DNA. The reaction mixture was incubated at 37°C for 30 min then at 65°C for 15 min. The mixture was phenol/chloroform extracted and ethanol precipitated.

#### 2.2.6. Ligation of DNA Fragments:

DNA fragments were ligated together by resuspending fragments in ligase buffer containing 66 mM Tris-Cl (pH 7.5), 5 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM ATP and 1 u of T4 DNA ligase per 1 ug of DNA. The ligation mixture was incubated overnight at room temperature.

#### 2.2.7. Preparation of Transformation Competent Escherichia coli:

The Escherichia coli host we used most frequently in transformation experiments was HB101. The procedure used to transform plasmid DNA into E. coli is the calcium chloride

method described by Mandel and Higa (1970). A 100 ml culture of medium was inoculated with 1 ml of an overnight culture of JA221 and incubated at 37°C with vigorous shaking for 2.5 h. The culture was chilled on ice for 10 min before centrifuging at 4,000 g for 5 min. The bacterial pellet was resuspended in 50 ml of ice-cold 50 mM CaCl<sub>2</sub> and 10 mM Tris-Cl (pH 8.0). After incubating for 15 min on ice, the cell suspension was centrifuged for 5 min at 4,000 g. The bacterial pellet was resuspended in 6.6 ml of 50 mM CaCl<sub>2</sub>, Tris-Cl (pH 8.0) and stored at 4°C for 12-24 h.

Plasmid DNA was mixed with 200 ul of the competent bacteria and stored on ice for 30 min. The DNA-bacteria mixture was heated at 42°C for 2 min then on ice for 5 min. Next 1 ml of medium was added and the mixture incubated for 1 h at 37°C before applying to the surface of agar plates containing the appropriate antibiotic.

#### 2.2.8. Isolation of Eucaryotic Genomic DNA:

High molecular weight genomic DNA was isolated from cells grown in tissue culture by a modification of the method described by Blin and Stafford (1976). Cell monolayers were washed twice in PBS and the cells lysed in 5 ml per 100 mm dish of Tris-buffered saline solution containing 0.5% SDS, 10 mM NaCl, 10 mM EDTA and 10 mM Tris-Cl (pH 8.0) containing 50 ug/ml proteinase K. The lysate was incubated at 37°C overnight. After extracting with phenol/chloroform (1:1) the lysate was

made 50 ug/ml in RNase and incubated overnight at 37°C. The lysate was extracted again with phenol/chloroform and the DNA precipitated by the addition of 2 volumes of 95% ethanol containing 2% potassium acetate. The high molecular weight DNA was spooled out with a pasteur pipette and re-dissolved in 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA.

#### 2.2.9. Isolation of Cytoplasmic RNA:

Cytoplasmic RNA was isolated from cells grown in tissue culture as described by Skup et al. (1982). Cell pellets were washed twice in ice cold PBS and resuspended in 2 volumes of ice-cold lysis buffer containing 50 mM HEPES (pH 7.6), 0.24 M KCl, 3 mM DTT, 0.25 M sucrose, 10 mM MgSO<sub>4</sub> and 10 mM vanadyl ribonucleoside complex. The mixture was vortexed vigorously for 3 min before centrifuging for 15 min at 23,000 g to pellet the nuclei. Then a 1/10 volume of 0.2 M streptomycin sulfate was added to the supernatant and the homogenate incubated on ice for 4 h. The polysomal RNA was collected by centrifugation at 5,000 g for 15 min. The pellet was dissolved in 10 mM Tris-Cl (pH 7.6), 10 mM EDTA, 1% SDS and 50 ug/ml proteinase K and incubated for 1 h at 37°C. After extracting with phenol/chloroform (1:1) the RNA was precipitated by the addition of 2 volumes of ethanol containing 2% potassium acetate. RNA preparations were stored under ethanol at -70°C.

#### 2.2.10. Isolation of Total Cellular RNA:

RNA was isolated from whole cells grown in tissue culture after the method of Auffray and Rougeon (1980). Dispersed cells were washed twice in ice-cold PBS before being lysed in 2.5 ml per 100 mm dish of ice-cold 3 M LiCl and 6 M urea. The lysate was homogenized for 2 min on a Polytron tissue homogenizer (setting number 7) and stored at  $-20^{\circ}\text{C}$  overnight. The precipitated RNA was collected by centrifuging at 12,000 g. The RNA pellet was washed in 3 M LiCl, repelleted and subsequently resuspended in diethyl pyrocarbonate (DEPC) treated H<sub>2</sub>O. RNA was stored under ethanol at  $-70^{\circ}\text{C}$ .

#### 2.2.11. Native Agarose Gel Electrophoresis:

Double stranded DNA fragments were size fractionated by electrophoresis through native horizontal agarose slab gels. Different concentrations of agarose were used depending on the size range to be resolved; for fragments less than 1 kb, 1.5-2% agarose; for fragments between 1 and 10 kb, 1% agarose and for fragments over 10 kb, .6-.8% agarose. Both the agarose gel and the electrophoresis buffer contained 0.08 M Tris-phosphate (pH 7.8), 0.002 M EDTA and 0.5 ug/ml ethidium bromide. Samples were mixed with 1/10 volume of loading buffer containing 0.1% bromophenol blue, 0.1% xylene cyanol and 30% glycerol before loading the sample into a well cast into the agarose gel. Electrophoresis was usually at 8 V/cm for 2-8 h.

### 2.2.12. Formaldehyde Agarose Gel Electrophoresis of RNA:

RNA was size fractionated by electrophoresis through denaturing horizontal agarose slab gels in the presence of formaldehyde. Both the agarose gel and the electrophoresis buffer contained 200 mM MOPS (pH 7.0), 50 mM sodium acetate, 10 mM EDTA and 9% formalin. The RNA samples were concentrated by ethanol precipitation and then resuspended in loading buffer containing 50% formamide, 10% formalin, 200 mM MOPS (pH 7.0), 50 mM sodium acetate and 10 mM EDTA. Samples were incubated at 65°C for 15 min, cooled on ice for 5 min before adding a 1/10 volume of loading buffer containing 0.1% bromophenol blue, 0.1% xylene cyanol, 30% glycerol and 0.05 ug/ml ethidium bromide and loading the RNA into sample wells in the agarose gel. Electrophoresis was usually at 8 V/cm for 2-8 h.

### 2.2.13. Denaturing Polyacrylamide Gel Electrophoresis:

Polyacrylamide gels were cast between two glass plates with spacers such that the gel was 0.5 mm thick. The gels contained 5% acrylamide mix (consisting of a 29:1 mixture of acrylamide and N,N'-methylene bisacrylamide), 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA and 8 M urea. Polymerization was catalysed by the addition of 400 ul 10% ammonium persulfate and 20 ul TEMED (N,N,N',N'-tetramethylethylene diamine) to 75 ml of the gel mixture. The electrode buffers contained 0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA. Samples were heated at 95°C for 2 min.

in 95% formamide, 0.02% bromophenol blue, 0.02% xylene cyanol and 0.02 mM EDTA before quick cooling on ice and loading into wells cast into the gel. Electrophoresis was at 45 mA for 2-3 h after a 1 h prerun.

#### 2.2.14. Southern and Northern Transfer:

After electrophoresis of the DNA fragments was completed, the gel was immersed in several volumes of 1.5 M NaCl and 0.5 M NaOH for 1 h at room temperature with constant shaking to denature the DNA. The gel was neutralized by soaking in several volumes of 1 M Tris-Cl (pH 8.0) and 1.5 M NaCl for 1 h at room temperature. The gel was placed over 3 sheets of Whatman 3MM paper which had been presoaked and was in contact with a reservoir containing 10X SSC. Over the gel was placed Hybond-N nylon membrane (Amersham), several sheets of 3MM paper presoaked in 2X SSC and a layer of paper towels 10 cm thick, and a 0.5 kg weight. After an overnight transfer, the nylon membrane was air dried, and exposed to long wave UV for 3-5 min. RNA was size fractionated through agarose gels containing formaldehyde were transferred in the same manner except the gels were not denatured and neutralized.

#### 2.2.15. Southern and Northern Hybridization:

Hybridizations were performed essentially as described by Southern (1975). Nylon membranes were prehybridized for at least 1 h in 5X SSC, 5X Denhardt's solution, 50% formamide, 0.3%

SDS and 100 ug/ml denatured salmon sperm DNA. After addition of the radiolabeled probe, the hybridization mixture was incubated at 42°C for at least 8h. Filters were then washed in several changes of 2X SSC, 0.1% SDS at room temperature for 30-45 min and a final wash of 0.2X SSC, 0.1% SDS at 65°C for 15-30 min. Hybridization of the radioactive probe was visualized by exposing Kodak XAR-5 film to the filter at -70°C from 1 to 4 days in a cassette with 2 Lanex regular screens.

#### 2.2.16. Nuclease S1 Mapping:


Nuclease S1 mapping was performed essentially as described elsewhere (Berk and Sharp, 1977; Weaver and Weissman, 1979; Rudnicki et al., 1988b). RNA (20 ug) was resuspended in 20 ul of hybridization buffer containing 80% formamide, 40 mM PIPES (pH 6.5), 0.4 M NaCl, 5 mM EDTA and 50,000 cpm of probe. The mixture was covered with a drop of paraffin oil, heated at 70°C for 10 min then incubated overnight at 52°C. Then 0.3 ml of ice-cold digestion buffer containing 0.25 M NaCl, 0.03 M sodium acetate, 0.001 M ZnSO<sub>4</sub>, 20 ug/ml denatured salmon sperm DNA and 400 to 1000 u/ml of nuclease S1 was added to the hybridization mix. Digestion was allowed to proceed for 45 min at 45°C. The reaction was stopped by a phenol/chloroform (1:1) extraction. The heteroduplexes were recovered by ethanol precipitation and resuspended in sequencing loading buffer. The protected fragments were electrophoresed through 5% polyacrylamide-8 M urea sequencing gels and visualized by

autoradiography.

#### 2.2.17. Nick Translation Labeling of DNA:

Plasmid DNA was nick translated to specific activities of about  $10^8$  cpm per ug using the method of Rigby et al. (1977). In a volume of 50 ul, plasmid DNA (1 ug) was incubated in 50 mM Tris-Cl (pH 7.8), 5 mM  $MgCl_2$ , 10 mM 2-mercaptoethanol, 20 uM each of dATP, dGTP and dTTP, 50 uCi of [ $\alpha$ - $^{32}P$ ]dCTP (>3000 Ci/mM), 2.5 units DNA polymerase I and 50 pg DNase I for 45 min at 15°C. Unincorporated nucleotides were separated from labeled plasmid DNA by chromatography through a sephadex G-50 column.

#### 2.2.18. Multiprime Labeling of DNA:



Plasmid DNA fragments were labeled to specific activities of over  $10^9$  cpm per ug after the method of Feinberg and Vogelstein (1983). In a volume of 50 ul, denatured linearized plasmid DNA (25 ng) was incubated in 50 mM Tris-Cl (pH 7.8), 5 mM  $MgCl_2$ , 10 mM 2-mercaptoethanol, 20 uM each of dATP, dGTP and dTTP, 50 uCi of [ $\alpha$ - $^{32}P$ ]dCTP (>3000 Ci/mM), random hexanucleotides and 2 units of the Klenow fragment of DNA polymerase I for at least 3 h at room temperature. Labeled DNA fragments were used directly without separating the unincorporated nucleotides.

### 2.2.19. 5'-end Labeling of DNA:

DNA fragments or oligonucleotides were 5'-end labeled in a volume of 50  $\mu$ l containing 12.5 pM of 5' ends, 100 uCi of [ $\gamma$ - $^{32}$ P]ATP ( $>5000$  Ci/mM), 66 mM Tris-Cl (pH 7.6), 1 mM spermidine, 10 mM  $MgCl_2$ , 15 mM DTT and 4 units T4 polynucleotide kinase at 37°C for 30 min. The reaction was stopped by heating at 65°C for 15 min followed by a phenol/chloroform (1:1) extraction.

### 2.2.20. 3'-end Labeling of DNA:

DNA fragments with PstI termini were 3'-end labeled by replacement synthesis with T4 DNA polymerase. The reaction mixture (50  $\mu$ l) contained 50  $\mu$ M each of TTP, dATP and dGTP, 50 uCi [ $\alpha$ - $^{32}$ P]dCTP, 33 mM Tris-acetate, 66 mM potassium acetate, 10 mM magnesium acetate, 0.05 mM DTT, 0.1 mg/ml BSA and 2.5 units of T4 DNA polymerase. The mixture was first incubated without nucleotides for 5 min at 37°C and then for 20 min at 37°C after the addition of the nucleotides.

## 2.3. Cell Biological Methods:

The procedures outlined in this section are described in Rudnicki and McBurney (1987).

### 2.3.1. Routine Cell Culture:

All cell types, unless otherwise mentioned were cultured in alpha-modified minimal essential medium (MEM) (Gibco,

Mississauga, Canada) supplemented with 2.5% fetal bovine serum and 7.5% bovine serum (Bocknek Laboratories Inc., Rexdale, Ont., Canada). Embryonal carcinoma (EC) cell cultures were passaged every 48 h or less and were usually plated at a density of  $10^5$  cells per ml of medium.

Cells were removed from the plastic surface of the tissue culture dish by first washing the monolayer in PBS [0.8% NaCl, 0.02% KCL, 0.02%  $\text{KH}_2\text{PO}_4$  and 0.115%  $\text{NaHPO}_4$  (pH 7.4)] and then incubating for 5-10 min under T-EDTA [1 mM EDTA and 0.025% trypsin in PBS]. The cells were dispersed by vigorously pipetting up and down with a Pasteur pipette.

#### 2.3.2. Cell Lines:

The P19 cell line was isolated from a teratocarcinoma produced from transplantation of an embryo into an extra-uterine site in the C3H/He strain of mice. P19 are euploid and have a normal male karyotype (McBurney and Rogers, 1982).

The L6 cell line was produced by culturing and cloning of neonatal rat skeletal muscle satellite cells (Yaffe, 1968; Nadal-Ginard, 1978).

#### 2.3.3. Differentiation of EC Cells:

P19 cells were induced to differentiate into neurons, glia and smooth muscle by culturing the cells in petri grade dishes in the presence of  $5 \times 10^{-7}$  M retinoic acid for 2 days. The aggregates formed during this time were then plated back

onto tissue culture dishes. Cells were normally harvested for RNA analysis or fixed for immunofluorescence 10 days after the initiation of differentiation unless otherwise stated. Medium was changed every second day.

P19 cells were induced to differentiate into cultures containing cardiac muscle essentially as described above except the inducer drug used was 1% dimethyl sulfoxide (DMSO) and cells were kept in petri grade dishes for 4-5 days.

#### 2.3.4. Differentiation of L6 Myoblast Cells:

Rat L6 myoblasts were induced to differentiate into skeletal myotubes by exchanging the medium containing 10% bovine serum (3:1 mixture of bovine and fetal calf serum) in near confluent cultures with medium containing 5% horse serum. Fusion was evident within 3 days and cells normally harvested on day 7.

#### 2.3.5. Immunofluorescence:

Cells were either grown directly on coverslips or were grown on coverslips coated with 0.2% gelatin. Cells were fixed by immersing the coverslips first for 20 min in  $-20^{\circ}\text{C}$  methanol and second for 3 min in  $-20^{\circ}\text{C}$  acetone. Coverslips were then air dried prior to rehydration in PBS for at least 15 min.

Coverslips were incubated for 45 min at room temperature under 60  $\mu\text{l}$  of primary antibody (usually diluted 1/50 in PBS). The coverslips were washed 3 times for 5 min each in PBS and

then incubated with fluorochrome-conjugated anti-primary antibody (Dako Corporation, Sante Barbara, CA), usually diluted 1/50 in PBS, for 45 min at room temperature. The coverslips were washed again 3 times each for 5 min in PBS and then mounted upside down on a drop of mounting medium on a glass slide. The mounting medium contained 50% glycerol, 0.5% paraphenylene diamine buffered with PBS. The stained preparations were examined in a microscope equipped with epifluorescent optics. Ilford XPI 400 film was used for black and white photography and Kodak Ektachrome 400 colour slide film was used for colour photography.

The primary polyclonal antisera and monoclonal antibodies used in this thesis are listed in Table 2.1.

#### 2.3.6. Transfection and Selection in Cell Culture:

DNA transfections were performed by a modification (Wigler et al., 1979) of the Graham and van der Eb (1973) procedure. Plasmid DNA (10 ug) was ethanol precipitated and the pellet resuspended in 250 ul of 250 mM  $\text{CaCl}_2$ . This solution was added dropwise with vigorous agitation to 250 ul of a solution containing 280 mM NaCl, 50 mM HEPES (pH 7.10) and 1.5 mM sodium phosphate (pH 7.10). This solution was incubated at room temperature for 45 min before applying over a semi-confluent layer of cells plated the night before at a density of  $5 \times 10^5$  cells per 60 mm dish. After 6 h, the medium was removed and the cells incubated for 3 min in 15% glycerol in

Table 2.1. Reagents Used for Fluorescent Detection

<u>Reagent</u>	<u>Source</u>	<u>Specificity</u>	<u>Reference</u>
AEC3A1-9	mouse monoclonal	SSEA-1 antigen on EC cell surface	Harris et al., 1984
anti-A-MHC	mouse monoclonal	rat atrial-myosin heavy chain	Gorza et al., 1988
anti-A-MLC2	rabbit polyclonal	human ventricular-myosin light chain 2	G. Jackowski <sup>1</sup>
anti-alpha-actin	rabbit polyclonal	striated muscle alpha-actins	Bulinski et al., 1983
B4F8	mouse monoclonal	smooth and striated muscle alpha-actins	Lessard, 1988
CGA7	mouse monoclonal	smooth muscle actins	Gown et al., 1985
HUC1	mouse monoclonal	smooth and striated muscle alpha-actins	J. Lessard <sup>2</sup>
MF20	mouse monoclonal	myosin isoform present in all striated muscle	Bader et al., 1982
anti-ras	mouse monoclonal	H-ras p21 containing valine substitution at amino acid 12	Dupont, Canada
anti-SM1	mouse monoclonal	smooth muscle alpha-actin	Skalli et al., 1986
anti-TK	rabbit polyclonal	Herpes Simplex Virus thymidine kinase	Harr et al., 1985
TROMA-1	rat monoclonal	cytokeratin intermediate filaments	Kemler et al., 1981
anti-V-MHC	mouse monoclonal	human ventricular-myosin heavy chain	G. Jackowski <sup>1</sup>
anti-V-MLC1	rabbit polyclonal	sheep ventricular-myosin light chain 1	G. Jackowski <sup>1</sup>

1. G. Jackowski, in preparation.
2. J. Lessard, personal communication.

PBS. The plate was washed in PBS and the medium replaced. The cells were cultured for an additional 48 h and were then dispersed with T-EDTA and plated at a density of  $10^6$  cells per 100 mM dish. The next day, the medium was supplemented with 400 ug/ml antibiotic G418. Cells were cultured in the presence of antibiotic for 8 to 14 days. The surviving colonies were either picked with the aid of a pulled Pasteur pipette and expanded into cell lines or pooled and cultured en masse.

Chapter 3, Actin and Myosin Expression During De Novo  
Differentiation of Cardiac Muscle from Cultured  
P19 Embryonal Carcinoma Cells

3.1. Introduction:

Embryonal carcinoma (EC) cells are the multipotential stem cells of teratocarcinomas whose developmental potential closely resembles that of early embryonic cells. Since cultured EC cells can be induced in cell culture to differentiate into many specialized cell types, they provide an alternative system to study early developmental processes (Graham, 1977).

The P19 line of EC cells (McBurney and Rogers, 1982) develop into different spectra of cell types depending on the drug used to induce differentiation. After aggregation and exposure to retinoic acid (RA), P19 cells differentiate into neurons, glia and fibroblast-like cells (Jones-Villeneuve et al., 1982; 1983). When aggregated and exposed to dimethyl sulfoxide (DMSO), P19 cultures develop into a different spectrum of cell types which includes striated muscle (McBurney et al., 1982).

The majority of P19-derived muscle cells are mononucleate, contain muscle-specific isoforms of myosin and actin proteins, and often exhibit spontaneous rhythmic contractile activity (McBurney et al., 1982; Edwards et al., 1983). Electron microscopic examination of these myocytes revealed abundant immature myofibrils with occasional Z-bands

and the usual hexagonal arrangement of thick and thin filaments. Where two muscle cells abutted one another, junctional complexes were observed which resembled the intercalated disks of cardiac muscle (Smith et al., 1987). On the basis of these observations, we concluded the mononucleate myocytes within DMSO-treated P19 cultures were most probably cardiac muscle (McBurney et al., 1982; Edwards et al., 1983; Smith et al., 1987, Rudnicki et al., 1988b).

In this chapter, we further characterize the types of muscle genes expressed during the de novo differentiation of the mononucleate myocytes in DMSO-treated aggregates of P19 cells. Our results confirm the cardiac nature of these muscle cells and suggests that they are similar to embryonic cardiac myocytes.

### 3.2. Results:

#### 3.2.1. Detection of Myosin Proteins in DMSO-Treated Cultures:

The isoforms of myosin heavy chain (MHC) and myosin light chain (MLC) proteins found in the mammalian heart are expressed almost exclusively in cardiac tissue. In the fetal heart of small mammals, both atrial-myosin heavy chain (A-MHC) and ventricular-myosin heavy chain (V-MHC) are co-expressed whereas in the adult, A-MHC predominates (Lompre et al., 1981, 1984; Izumo et al., 1986; Pritzl and Zak, 1987). In the adult, the ventricular-myosin light chain 1 (V-MLC<sub>1</sub>) and atrial-myosin

Table 3.1. Summary of Muscle Antigens Detected by Indirect Immunofluorescence Experiments

Antibody Specificity	Myocyte Type		Source	Reference
	Flat and mononucleate	Bipolar and multinucleate		
1. A-MHC	+	-	mouse monoclonal	Gorza et al., 1983
2. V-MHC	+	-	mouse monoclonal	Jackowski*
3. A-MLC2	+	-	rabbit polyclonal	Jackowski*
4. V-MLC1	+	+	rabbit polyclonal	Jackowski*
5. C/S-MHC	+	+	mouse monoclonal	Bader et al., 1982

A-MHC, atrial-myosin heavy chain; V-MHC, ventricular-myosin heavy chain; A-MLC2, atrial-myosin light chain 2; V-MLC1, ventricular-myosin light chain 1; C/S-MHC, striated-muscle specific myosin heavy chain.

\* G. Jackowski, in preparation.

light chain 1 (A-MLC1) isoforms are localized to the ventricle and atria respectively. However, the A-MLC1 and V-MLC1 isoforms are co-expressed in fetal ventricles (reviewed in Barton and Buckingham, 1985). Thus, simultaneous co-expression of the different cardiac myosin isoforms is normally only observed in the fetal heart (reviewed in Buckingham et al., 1985, 1986). To determine whether cardiac-specific myosin isoforms were co-expressed in P19-derived myocytes, we made use of the antibodies listed in Table 3.1 to perform immunofluorescence double staining experiments.

Antibodies directed against A-MHC and V-MHC both reacted with myocytes with a flat mononucleate morphology in DMSO-treated cultures. Double labeling experiments indicated that A-MHC and V-MHC proteins were always co-expressed in these myocytes (Figure 3.1).

The antiserum reactive to A-MLC2 stained the flat mononucleate myocytes. In double labeling experiments with antibodies reactive with A-MHC (Figure 3.2, panels A, B and C), and V-MHC (Figure 3.3, panels A, B and C), the A-MLC2 protein was present in all cells that contained either cardiac MHC. Thus, the three cardiac A-MLC2, A-MHC and V-MHC protein isoforms were co-expressed in the flat mononucleate myocytes within DMSO-treated P19 cultures.

The monoclonal antibody MF20 (Eader et al., 1982), reacts with all forms of cardiac- and skeletal-muscle myosin heavy chain (C/S-MHC) proteins. In DMSO-treated P19 cultures,

Figure 3.1. Mononucleate myocytes contain both A-MHC and V-MHC proteins. Panel A is a phase-contrast field of cells stained with mouse monoclonal anti A-MHC antibody (B), and with biotinylated mouse monoclonal antibody reactive with V-MHC (C).. Indirect labeling was with rhodamine-conjugated anti-mouse IgG and a fluorescein-streptavidin complex. Bar represents 50 um.

A



B

C

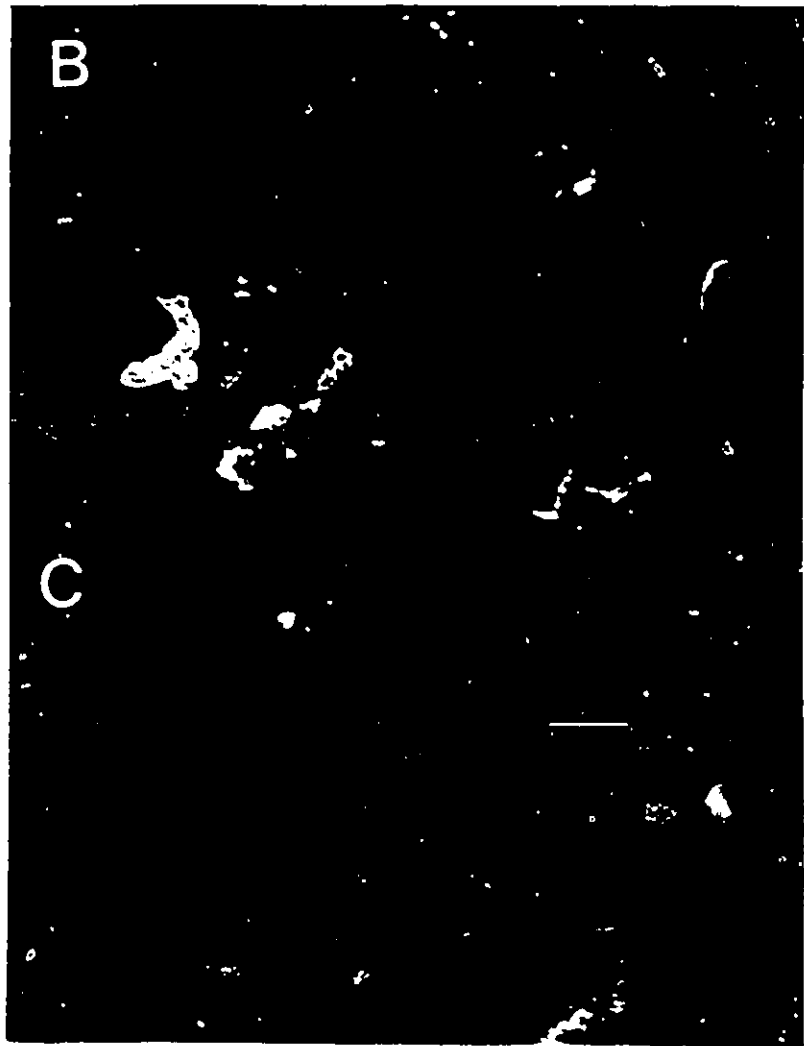


Figure 3.2. Co-expression of A-MLC2 and V-MLC1 proteins in mononucleate monocytes containing A-MHC protein. Panels A and D are phase-contrast fields of cells stained with either rabbit anti A-MLC2 antiserum (B) or rabbit anti V-MLC1 antiserum (E). The fields were also stained with a mouse monoclonal antibody reactive with A-MHC protein (C and F). Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgG. The arrows with the letters C and S point to mononucleate cardiac and multinucleate bipolar skeletal myocytes respectively. Bar represents 50 um.

A

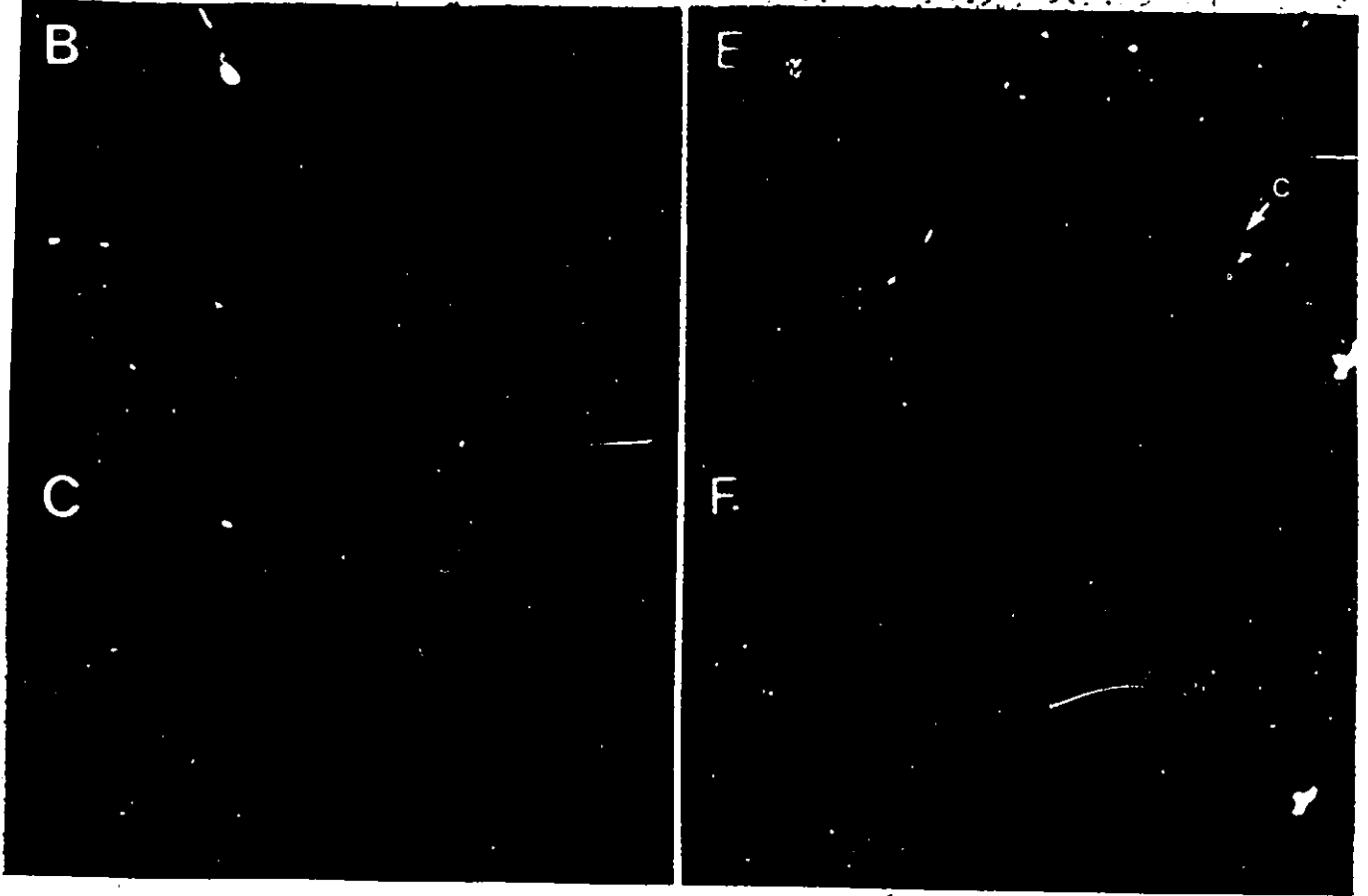
D

B

E

C

F



the anti C/S-MHC antibody labeled the flat mononucleate myocytes as well as a distinct population of bipolar multinucleate myocytes. In double labeling experiments, the A-MLC2 protein was found only in the C/S-MHC containing cells which were flat and mononucleate (Figure 3.4, panels A, B and C). The bipolar multinucleate cells, did not contain A-MLC2 protein (Figure 3.4, panels A, B and C). These results suggest that the mononucleate myocytes with a flat morphology were cardiac muscle cells and that the multinucleate myocytes with a bipolar morphology were skeletal muscle cells.

The V-MLC1 protein was found in both the flat mononucleate myocytes and the bipolar multinucleate myocytes which also labeled with the anti C/S-MHC antibody (Figures 3.2, 3.3 and 3.4, panels D, E and F). The reactivity of the anti V-MLC1 antiserum to skeletal muscle was most likely due to the identity between portions of the V-MLC1 and the skeletal muscle slow MLC1 proteins which are translated from differentially spliced mRNAs transcribed from the same gene (Barton and Buckingham, 1985).

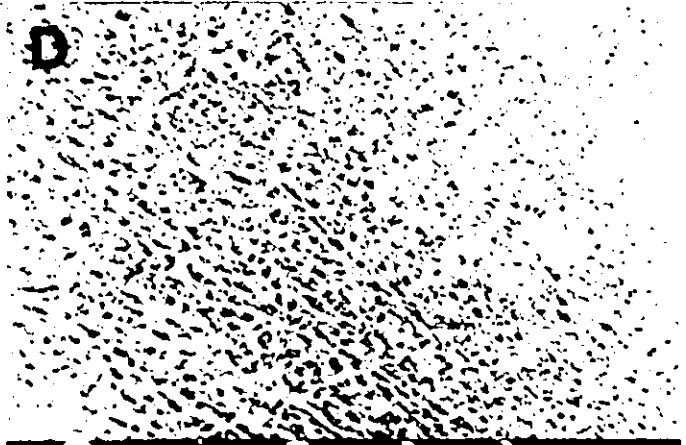
In 10 day old DMSO-treated P19 cultures, the flat mononucleate cardiac myocytes (V-MLC1+/V-MHC+) made up 95% of the striated muscle whereas the remaining 5% were bipolar multinucleate skeletal myocytes (V-MLC1+/V-MHC-). Thus, the major products of striated-muscle differentiation within DMSO-treated P19 cultures are cardiac myocytes.

Figure 3.3. Co-expression of A-MLC2 and V-MLC1 proteins in mononucleate monocytes containing V-MHC protein. Panels A and D are phase-contrast fields of cells stained with either rabbit anti A-MLC2 antiserum (B) or rabbit anti V-MLC1 antiserum (E). The fields were also stained with mouse monoclonal antibody (C and F) reactive with V-MHC protein. Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgG. The arrows with the letters C and S point to mononucleate cardiac and multinucleate bipolar skeletal myocytes respectively. Bar represents 50 um.

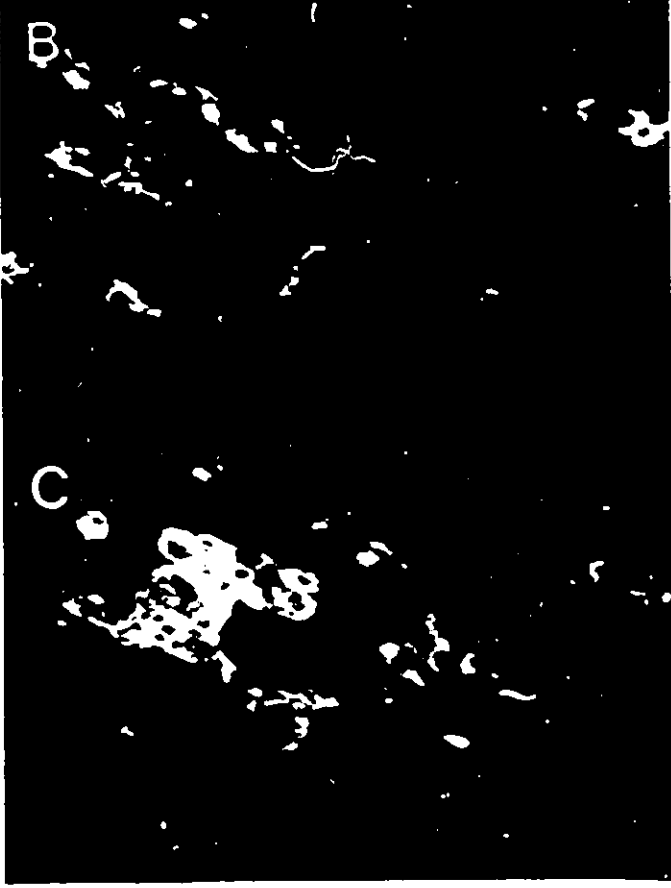
A



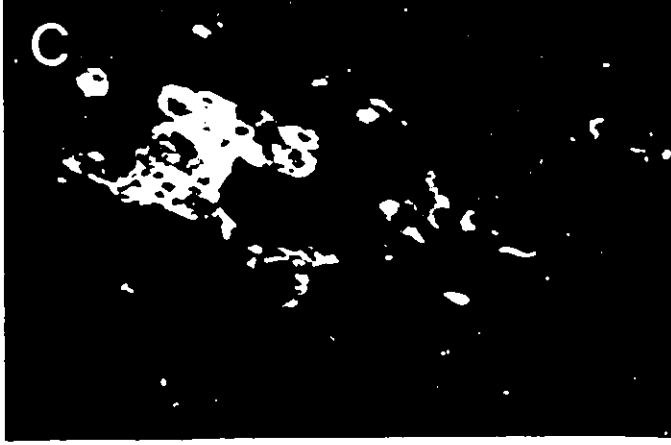
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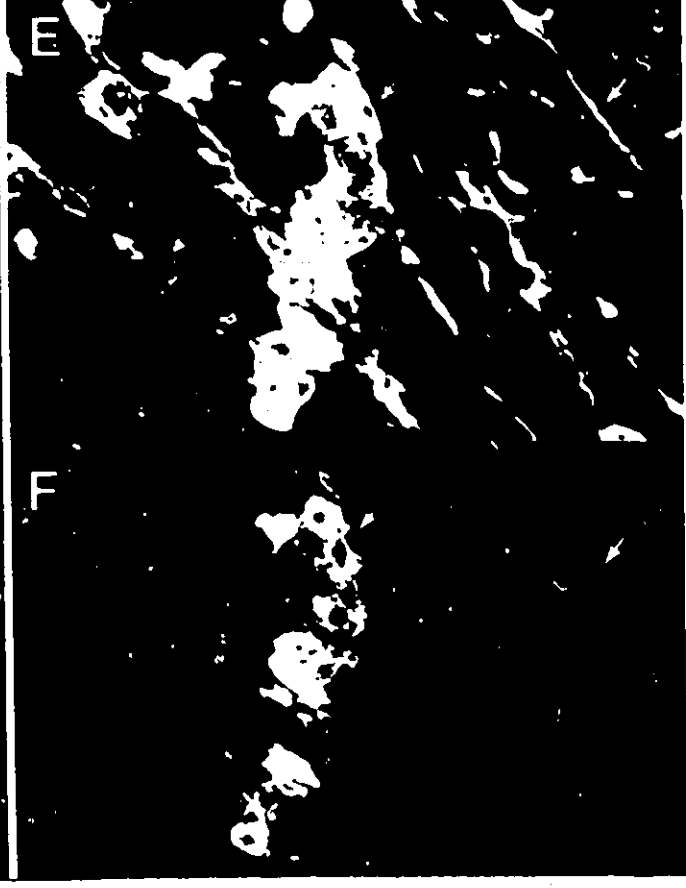
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C



E



F

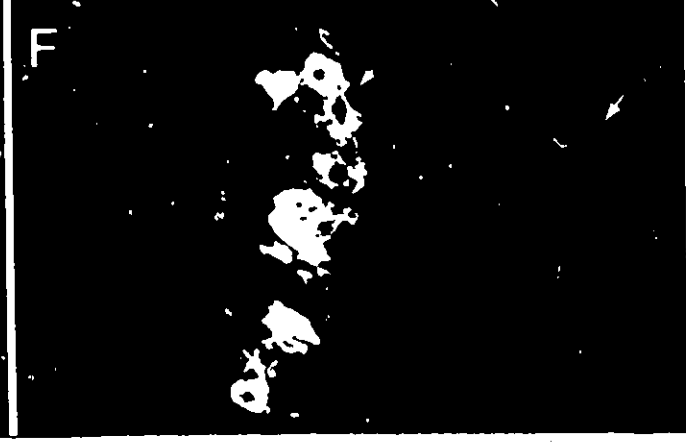
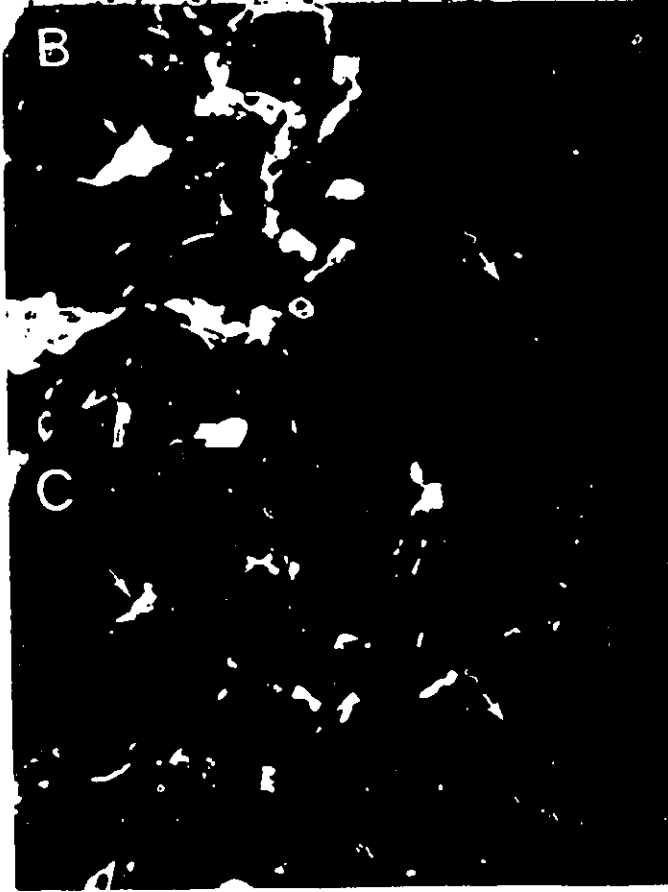


Figure 3.4. Presence of A-MLC2 and V-MLC1 proteins in striated muscle cells. Panels A and D are phase-contrast fields of cells stained with either rabbit anti A-MLC2 antiserum (B) or rabbit anti V-MLC1 antiserum (E). The fields were also stained with a mouse monoclonal antibody MF20 (C and F) reactive with M-MHC proteins (Bader et al., 1982). Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgG. The arrows with the letters C and S point to mononucleate cardiac and multinucleate bipolar skeletal myocytes respectively. Bar represents 50 um.

A

D



These results (summarized in Table 3.1) demonstrate that, in addition to C/S-MHC (Edwards et al., 1983), striated-muscle alpha-actin (Rudnicki et al., 1988), and desmin-containing intermediate filaments (Rudnicki and McBurney, unpublished), the majority of the myocytes in DMSO-treated P19 cultures co-express four isoforms of MHC and MLC proteins normally expressed in the heart. Therefore, the mononucleate muscle cells derived from DMSO-treated P19 cultures closely resemble the cardiac myocytes of the embryonic myocardium.

### 3.2.2. Accumulation of Myosin mRNAs During Cardiac Muscle Differentiation

To investigate the kinetics of myosin mRNA accumulation during the de novo differentiation of cardiac myocytes, we performed Northern blot analysis with myosin heavy and light chain cDNA probes (Figure 3.5). The relative amount of a specific mRNA was determined by densitometric scanning of the autoradiograms (Figure 3.6).

Total RNA for the Northern analysis was isolated at daily intervals throughout the 10 day differentiation regime. Rhythmically beating myocytes and cells which contained C/S-MHC protein first appeared on day 6. C/S-MHC mRNA also first appeared on day 6 and continued to increase in abundance until day 9 (Figures 3.5 A and 3.6 A). MLC1 mRNA was detected with the full length V-MLC1 cDNA probe and accumulated with about the same kinetics as the C/S-MHC mRNA (Figures 3.5 B and 3.6 B).

Figure 3.5. Expression of myosin genes during muscle differentiation. Total RNA, isolated at daily intervals throughout the DMSO-induced differentiation regime, was analysed by Northern hybridization to detect different myosin transcripts. (A) Hybridization to a myosin heavy chain (MHC) probe. This probe was the full length rat embryonic MHC cDNA (pMHC25, Medford et al., 1980). (B) Hybridization to the full length human V-MLC1 cDNA probe (G. Jackowski, in preparation). (C) Hybridization to the 0.6 kb PstI fragment containing the 3'-UT region of the human V-MLC1 cDNA. (D) Hybridization to the mouse alpha-tubulin cDNA (Lemishka et al., 1980). Lane 0 denotes RNA isolated from EC cells and lanes 1 to 10 denotes RNA isolated at daily intervals after initiation of differentiation (20 ug per lane). C denotes RNA isolated from adult mouse cardiac muscle (5 ug per lane). The location of the 28S and 18S rRNAs are indicated on the left of each panel.

**A**

C 0 1 2 3 4 5 6 7 8 9 10 C



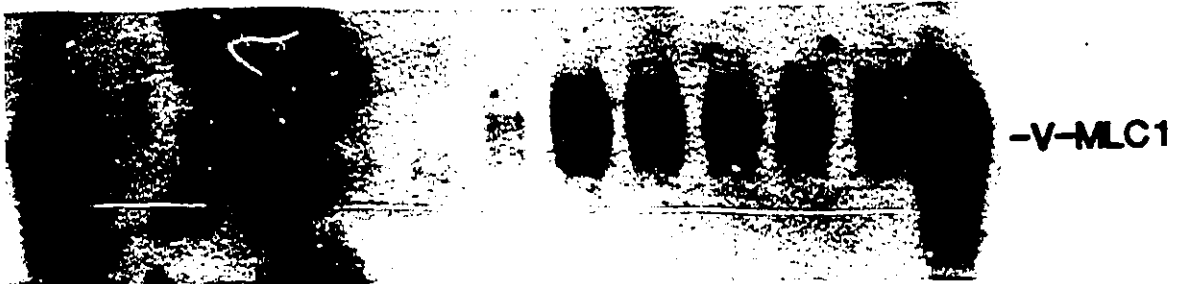
**B**

18S-



**C**

18S-

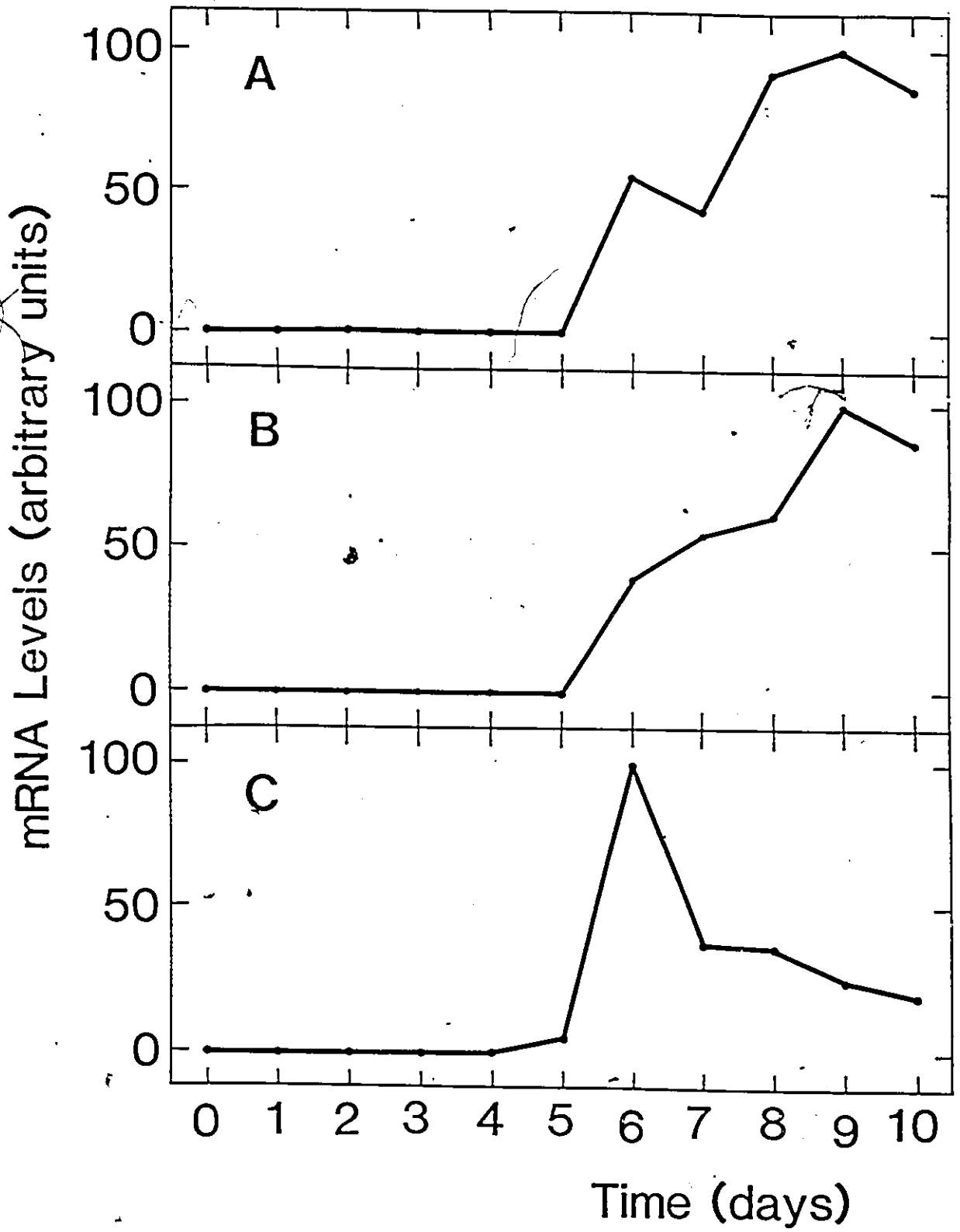


**D**

18S-



Figure 3.6. The accumulation of myosin transcripts during muscle differentiation. The relative levels of MHC(A), MLC1 (B), V-MLC1 (C), and tubulin mRNAs were determined by scanning densitometry of the autoradiograms shown in Figure 3.5. The level of each mRNA is expressed as a percentage of the maximum level of expression normalized to the level of tubulin mRNA.

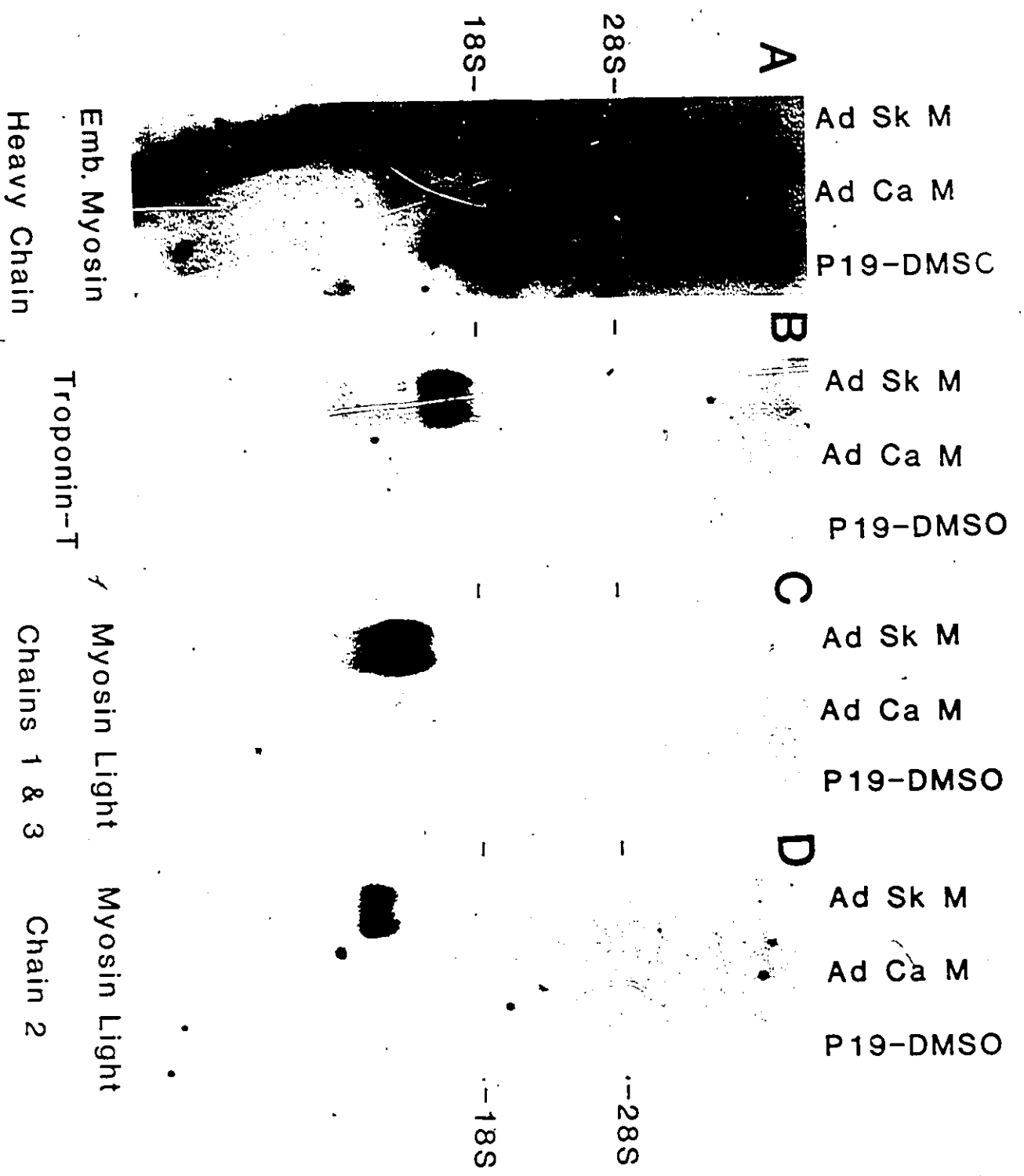


The kinetics of C/S-MHC and MLC1 mRNA accumulation were similar to the increase in proportion of C/S-MHC protein containing cells in differentiating cultures after DMSO-treatment (Edwards et al., 1983). In contrast, the ventricular myosin light chain 1 (V-MLC1) mRNA, as detected with a 3'-untranslated (3'-UT) region probe, also first appeared on day 6, but decreased rapidly thereafter to about 20% of the peak level by day 10 (Figures 3.5 C and 3.6 C).

The full length cDNA and 3'-UT V-MLC1 probes detected different patterns of MLC1 mRNA accumulation during DMSO-induced differentiation (compare Figure 3.6, B and C). We interpret this observation to suggest that the 3'-UT probe was hybridizing a subset of MLC1 mRNAs hybridized by the full length cDNA probe. Therefore, we suggest that muscle cells derived from DMSO-treated P19 cells may express the atrial-MLC1 and/or other MLC1 transcripts.

The level of tubulin mRNA remained constant throughout the differentiation regime (Figure 3.5 D) and its level was used to control for the amount of RNA in each lane. In addition, in RNA from DMSO-treated P19 cultures, we were unable to detect the fast MLC1/3, MLC2 and troponin-T mRNAs, which are normally expressed in skeletal muscle but not in cardiac muscle (Figure 3.7).

Figure 3.7. Muscle derived from P19 cells does not contain transcripts normally found in adult skeletal muscle. Total RNA, isolated from adult mouse heart and skeletal muscle, and DMSO-treated P19 cultures, was analysed by Northern blot hybridization for the presences of muscle-specific mRNAs. Ad Sk M, RNA (5 ug) from adult mouse skeletal muscle; Ad Ca M, RNA (5 ug) from adult mouse cardiac muscle; P19-DMSO, RNA (20 ug) from DMSO-treated P19 cultures 7 days after the initiation of differentiation. The location of the 28S and 18S rRNAs are indicated at the sides of the panels. Filters were hybridized to probes encoding; (A) the rat embryonic myosin heavy chain cDNA (pMHC25); (B) the rat troponin-T cDNA (pTNT-15); (C) the rat myosin light chains 1 and 3 cDNA (pLC84); and (D) the rat myosin light chain 2 cDNA (pLC-2). The faint band corresponding to the myosin heavy chain mRNA in DMSO-treated P19 cultures was detectable in longer exposures (see Figure 3.5 A), while myosin light chains 1 and 3, myosin light chain 2 and troponin-T transcripts were not.



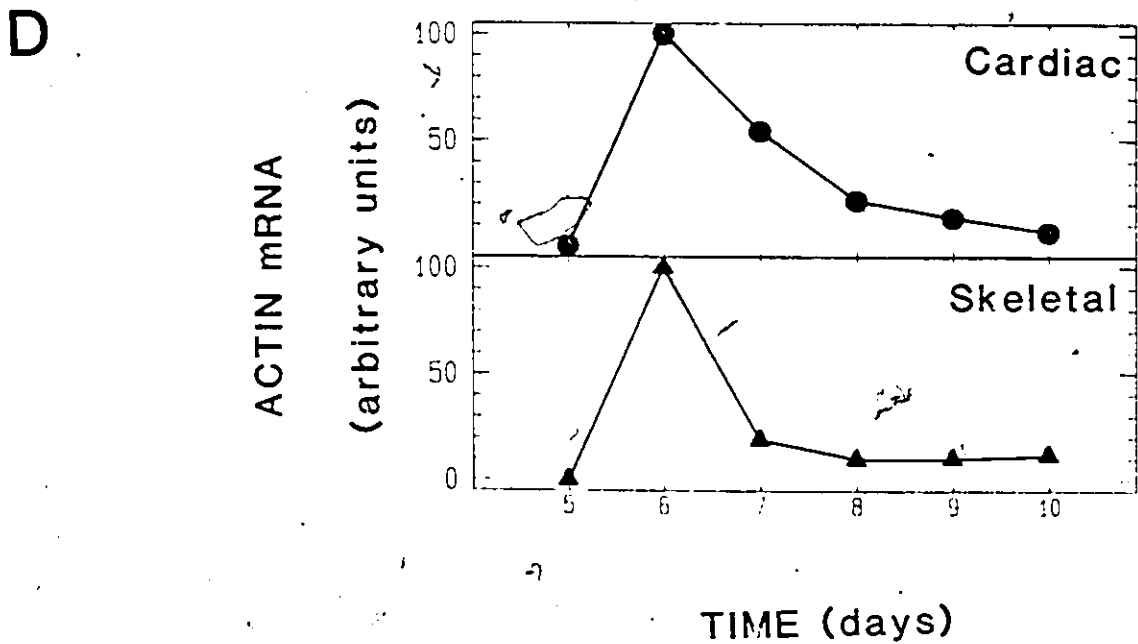
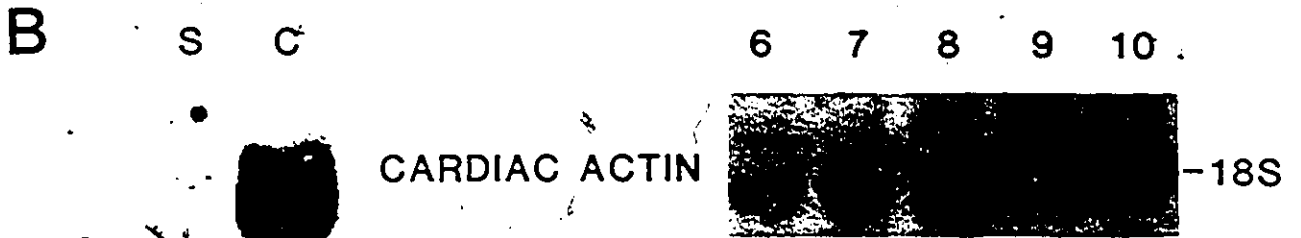
### 3.2.3. Concomitant Cardiac-Actin and Skeletal-Actin Expression During Cardiac Myocyte Differentiation:

In the adult mammalian heart, virtually all of the actin mRNA is of the cardiac type whereas in the late fetal heart, cardiac-actin and skeletal-actin mRNA are found at similar levels (Mayer et al., 1984; Nudel et al., 1984; Minty et al., 1982; Buckingham et al., 1986). To determine whether co-expression of cardiac and skeletal-actin occurred during the de novo differentiation of cardiac myocytes, we performed Northern blot analysis with actin isotype-specific probes on RNA isolated during the DMSO-induced muscle differentiation of P19 cells (Figure 3.8).

First, we detected both the 2.1 kb non-muscle and 1.7 kb muscle-actin mRNA isoforms with a probe which hybridizes all actin mRNA isoforms (Figure 3.8 A). The level of cytoskeletal-actin mRNAs (gamma and beta-actin) remained relatively constant during the 10 days examined and its level was used to control for the amount of mRNA in each lane. The 1.7 kb muscle-actin transcripts first appeared on day 6, and declined thereafter (Figure 3.8 A, lanes 0 to 10).

We next examined the relative levels of cardiac and skeletal actin mRNA levels using DNA probes derived from their 3'-untranslated regions (Figure 3.8, B and C). Both the cardiac and skeletal-actin mRNAs appeared abruptly on day 6 and decreased in level thereafter to about 10% of their peak levels by day 10 (Figure 3.8 B and C). In addition, since both probes were

Figure 3.8. Co-expression of cardiac-actin and skeletal-actin genes during cardiac muscle differentiation. Total RNA, isolated at daily intervals throughout the DMSO-induced differentiation regime, was analysed by Northern blot hybridization to different actin probes. Lanes 0 to 10 denote RNA isolated from EC cells and 1 to 10 days after initiation of differentiation respectively (20 ug per lane). S and C denote RNA isolated from adult mouse skeletal and cardiac muscle respectively (5 ug per lane). The location of the 18S rRNA is indicated on the right of each panel. (A) Hybridization to the 0.6 kb PstI fragment (see Figure 4.1), containing the last exon of the human cardiac-actin gene. This probe hybridizes both cytoskeletal (beta and gamma at 2.1 kb) and striated-muscle actins (alpha at 1.7 kb). (B) Hybridization to a cardiac-actin isotype specific probe. This probe was the 0.45 kb HaeIII-PstI fragment containing the 3'-UT region of the human cardiac actin gene (see Figure 4.1). (C) Hybridization to a skeletal-actin isotype specific probe. This probe was the 0.42 kb PstI-BamHI fragment from the 3'-UT region of the mouse skeletal-actin gene (Hu et al, 1986). (D) The relative levels of cardiac-actin and skeletal-actin mRNA determined by scanning densitometry of the autoradiograms shown in panels B and C. Cardiac-actin and skeletal-actin mRNA are expressed as a percentage of the maximum level of expression normalized to the level of cytoskeletal actin.



labeled to identical specific activities, we conclude that both cardiac and skeletal actin mRNAs were expressed at about equal levels. Therefore, both the cardiac-actin and skeletal-actin genes appear to be subject to similar developmental regulation during the de novo formation of DMSO-induced cardiac muscle.

### 3.3. Discussion:

P19 cells can be induced in cell culture to differentiate into a spectrum of cell types which includes striated muscle. We have determined the distribution of cardiac-specific myosin isoforms using monospecific antibodies and found that A-MLC2, V-MLC1, A-MHC and V-MHC proteins were co-expressed in P19-derived myocytes. These results, taken with our previous biochemical and ultrastructural studies (McBurney et al., 1982; Edwards et al., 1983; Smith et al., 1987; Rudnicki et al., 1988b), confirm the cardiac nature of these muscle cells and suggests that they may be analogous to the cells of the early embryonic myocardium.

In DMSO-treated P19 cultures, the pattern of cardiac-actin, skeletal-actin and V-MLC1 mRNA expression was similar in that the transcript abruptly appeared on day 6 and decreased rapidly thereafter. However, the level of C/S-MHC and MLC1 mRNA increased gradually from day 6 to peak in level at day 9 (compare Figures 3.6 and 3.8). Furthermore, the proportion of myocytes increased from about 4% on day 6 to about 15% by day 10

(Edwards et al., 1983; our data not shown). These results suggest that cardiac-actin, skeletal-actin and  $\alpha$ -MLC1 gene transcription may precede cardiac myocyte morphological differentiation. Similarly, asynchronous muscle-gene activation of transcription has also been observed during skeletal myoblast fusion in cell culture (Bains et al., 1984; Gunning et al., 1987). It may be necessary for actin and MLC proteins to be present in abundance early in myocyte differentiation in order to facilitate coordinated assembly of the sarcomeric contractile apparatus.

In the late vertebrate fetal heart, cardiac-actin and skeletal-actin are expressed at similar levels while in the adult, virtually all of the actin is of the cardiac type (Mayer et al., 1984; Nudel et al., 1984; Minty et al., 1982; Buckingham et al., 1986). In contrast, in fetal skeletal muscle, cardiac-actin is the major actin-gene product whereas in the adult, skeletal-actin becomes the predominant isoform (Bains et al., 1984; Gunning et al., 1987, and references therein). We observed that cardiac-actin and skeletal-actin gene expression was concomitantly activated and maintained at similar levels during the cardiac muscle differentiation in DMSO-treated cultures (Figure 3.8). Therefore, we suggest that cardiac-actin and skeletal-actin genes are subject to similar developmental regulation during the de novo differentiation of cardiac muscle.

In vertebrates, the myocardial tube is among the earliest organs to develop (DeHann, 1965). In the mouse embryo,

muscle-myosin has been detected as early as embryonic day 8 in cardiac muscle, and day 9.5 in skeletal myotomes (Jockusch-et al., 1984). In DMSO-treated P19 cultures, cardiac muscle appears at 5 to 6 days and skeletal muscle at 7 to 8 days (Edwards et al., 1983). Since EC cells are similar to the inner cell mass of the 3 day old embryo, the cardiac and skeletal muscle derived from DMSO-treated P19 cultures appear to develop according to the same schedule followed by these cell types in the embryo. Since no continuous lines of cardiac muscle have been established and since the differentiation of cardiac myocytes occurs in a relatively small population of cells in the embryo, the P19 cell-culture system appears to be unique for investigating the de novo differentiation of embryonic cardiac-muscle and for investigations concerning the activation and regulation of cardiac muscle-specific gene expression.

Chapter 4, Regulated Expression of a Transfected Human Cardiac-Actin Gene During Differentiation of P19 Cells

4.1. Introduction:

P19 cells can be induced to differentiate in vitro into different cell types depending on the drug used. After aggregation and treatment with dimethyl sulfoxide (DMSO), P19 cells differentiate into a spectrum of cells which includes cardiac muscle (Chapter 3; Rudnicki et al., 1988a). Since no continuous lines of cardiac muscle cells have been established and since primary cultures of cardiac muscle can only be maintained with difficulty for short periods, the P19 cell system is unique for investigating gene expression during the de novo differentiation of cardiac muscle.

We were interested in the tissue-specific regulation of gene expression during development. Our aim was to determine whether the P19 system would be suitable for gene transfer experiments to investigate cardiac-actin gene regulation. An isoform of actin, called cardiac-actin, is synthesized exclusively in cardiac and embryonic skeletal muscle cells. We have transfected the human cardiac-actin (CH-actin) gene into P19 cells and found the gene expressed with appropriate developmental and cell type specificity.

## 4.2. Results:

### 4.2.1. Introduction of the Human Cardiac Actin Gene into P19 Cells:

Our strategy was to transfect the human cardiac actin (CH-actin) gene into the P19 line of EC cells, obtain stable transformants and determine whether the CH-actin gene was appropriately regulated when the transformed cells were induced to differentiate into cardiac muscle and non-muscle cell types. The two plasmids used for transfection contained the neor gene, which confers resistance to the antibiotic G418, and the entire coding region of the CH-actin gene with either 5 kb (pNA8) or 0.8 kb (pNA-D1) of 5' flanking sequences (Figure 4.1 A).

To verify that the CH-actin gene was functional after subcloning into pSV2-neo, the plasmids pNA8 and pNA-D1 were introduced into mouse L cells using the calcium-phosphate precipitation method of DNA transfection (Graham and van der Eb, 1973). Antibiotic resistant G418 colonies of L cells were then pooled and total RNA isolated. Northern blot analysis indicated the presence of the cardiac-actin transcript of the expected size (1.7 kb) in RNA isolated from the transformed L cells (Figure 4.2), albeit at low levels. Therefore we concluded that the CH-actin gene was not altered by the subcloning procedure.

Plasmids were introduced into P19 cells using the same calcium-phosphate precipitation method of DNA transfection.

Figure 4.I. Construction of plasmids and structure of CH-actin gene. (A) The CH-actin gene (Engel et al., 1982; Hamada et al., 1982) along with 5.4 kb of 5' flanking sequence is contained within a 13 kb EcoRI fragment which was inserted into the EcoRI site of pSV2-neo (Southern and Berg, 1982) and is identified as pNA8. A second plasmid was constructed (pNA-DI) with sequences upstream of a BamHI site removed leaving 0.8 kb of flanking sequences upstream of the CH-actin transcription start site. Stippled areas denote CH-actin sequences, solid regions denote pBR322 sequences and striped regions denote the neor. (B) Relation of hybridization probes to the CH-actin gene. The 1.5 kb PstI 5'-fragment was used as a specific probe to identify P19(CH-actin) transformants in Southern blot hybridization experiments and the 0.45 kb HaeIII-PstI was used as a cardiac-actin isotype-specific probe in Northern blot hybridization experiments. The .6 kb PstI fragment was used in nuclease S1 mapping experiments and as a pan-specific actin probe in Northern blot hybridization experiments. Arrow indicates transcription, stippled areas denote coding exons and solid areas denote untranslated regions.

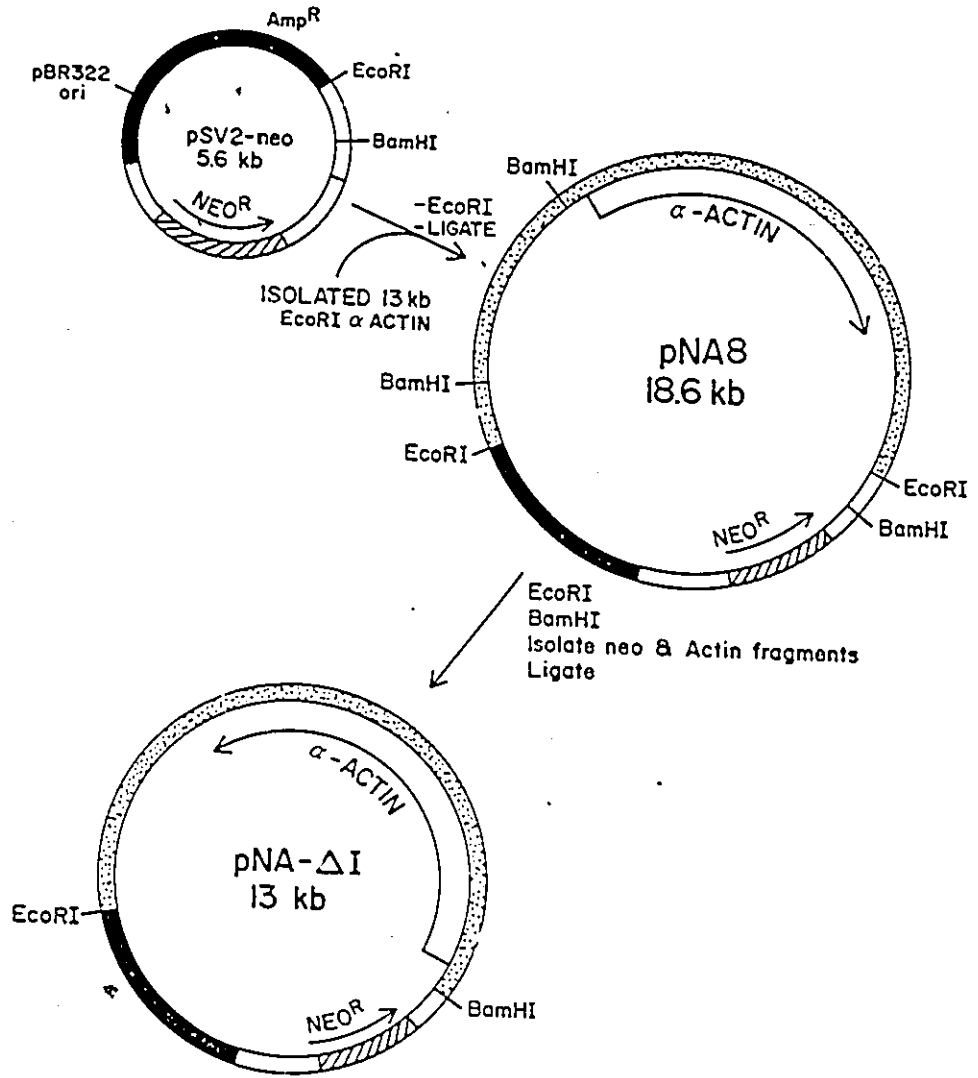
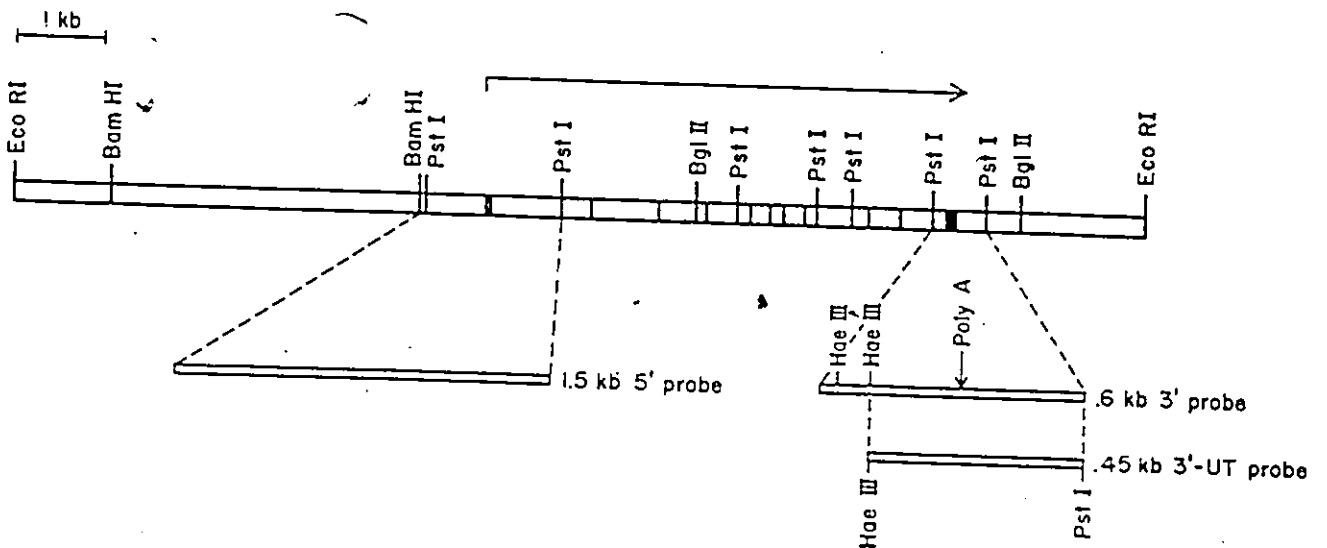
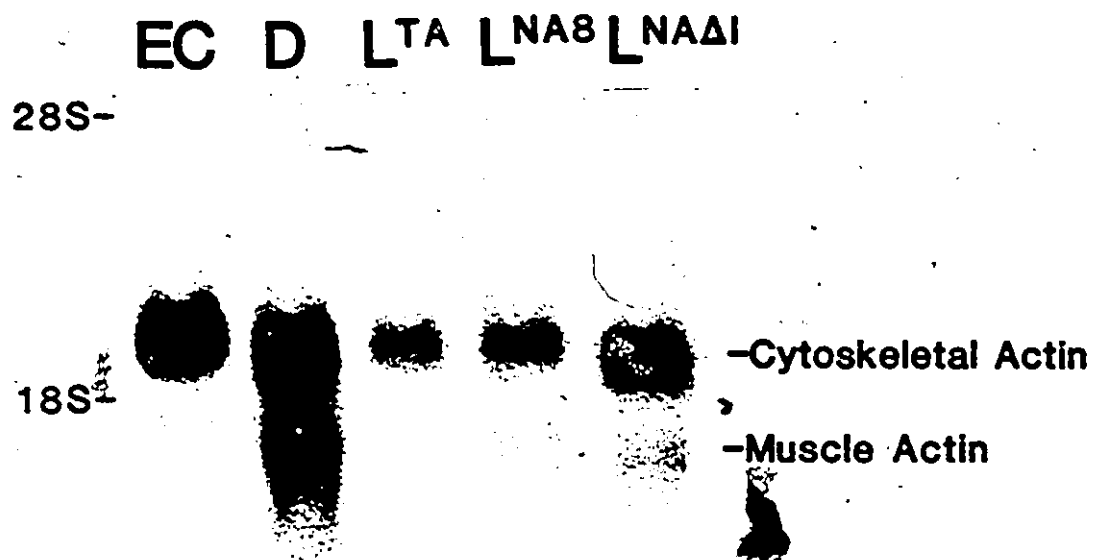
**A****B**

Figure 4.2. Transcription of the CH-Actin gene after transfection of plasmids pNA8 and pNA-DI into mouse L cells.

(A) Detection of actin mRNAs by Northern blot analysis with the 600 bp 3'-probe (Fig. 4.1). This probe hybridizes both cytoskeletal-actin (2000 nt) and muscle-actin (1700 nt) mRNAs.

(B) Detection of cardiac-actin mRNA by Northern blot analysis with the 450 bp 3'-UT region probe (Fig. 4.1) from the CH-actin gene. EC, RNA from undifferentiated P19 cells; D, RNA from DMSO treated P19 cultures containing cardiac muscle; LTA, RNA from untransfected mouse L cells; LNA8 and LNADI, RNA from L cells transfected with pNA8 and pNA-DI respectively. The migration of the rRNAs is indicated on the left as 28S and 18S.

**A**



**B**

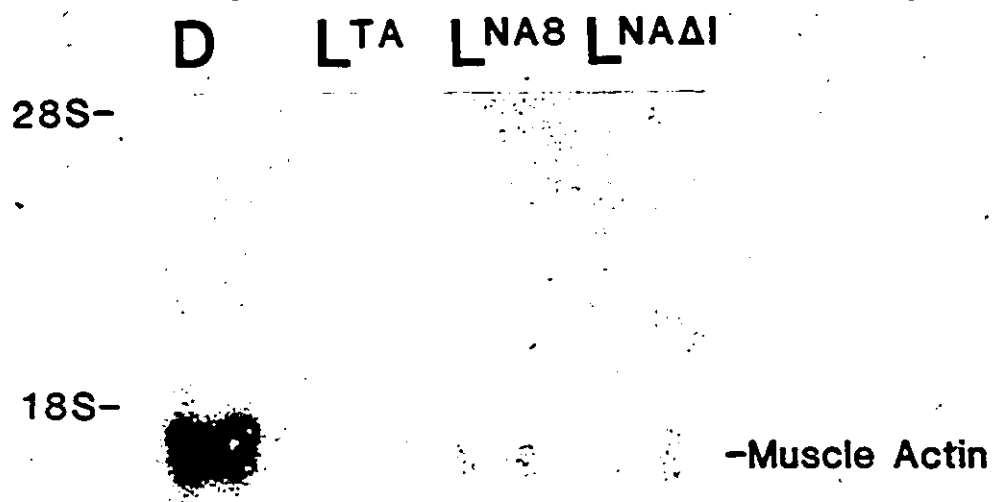


Table 4.1. Characteristics of P19(CH-Actin) Transformants

<u>CELL LINE</u>	<u>COPY<sup>A</sup> NUMBER</u>	<u>PERCENT MUSCLE CELLS<sup>B</sup> IN DMSO TREATED CULTURES</u>	<u>FOLD INCREASE IN<sup>C</sup> HUMAN CARDIAC ACTIN mRNA</u>
P19	0	15%	NA
P19A24	>200	10%	4.8
P19A35	45-55	11%	6.3
P19AD32	20-30	14%	3.8
P19AD43	45-55	ND	4.3
P19AD54	>200	30 %	2.8

A. Copy number was determined by dot blotting serial dilutions of DNA and comparison of level of hybridization with human genomic DNA.

B. Percent of muscle cells as determined by indirect immunofluorescence staining of dispersed cells with monoclonal antibody MF20 which specifically reacts with C/S-MHC protein (Bader et al., 1982).

C. The fold increase was determined by scintillation counting of nuclease S1 protected fragments cut out of the sequencing gel, the background ~~cpm~~ was subtracted and the level found in DMSO treated cultures was divided by the level found in EC cultures.

NA, not applicable; ND, not determined.

Antibiotic resistant colonies were then expanded into cell lines [designated P19(CH-actin)]. Southern blot analysis of 28 of these lines indicated that 23 contained between 1 and over 200 copies of the CH-actin gene (Figure 4.3 and Table 4.1). Each of the P19(CH-actin) cell lines differentiated into neurons, glia and fibroblast cells when aggregated and exposed to RA. For reasons not yet clear only half of these cell lines developed cardiac muscle when aggregated and exposed to DMSO. The proportion of muscle cells in DMSO treated cultures varied between 0 and 30% (Table 4.1), was a reproducible property of each clone and was unrelated to the number of copies of the transfected CH-actin gene (Table 4.1) or the level of neur gene expression (data not shown).

#### 4.2.2. Expression of the Human Cardiac Actin Gene in Transfected P19 Cells:

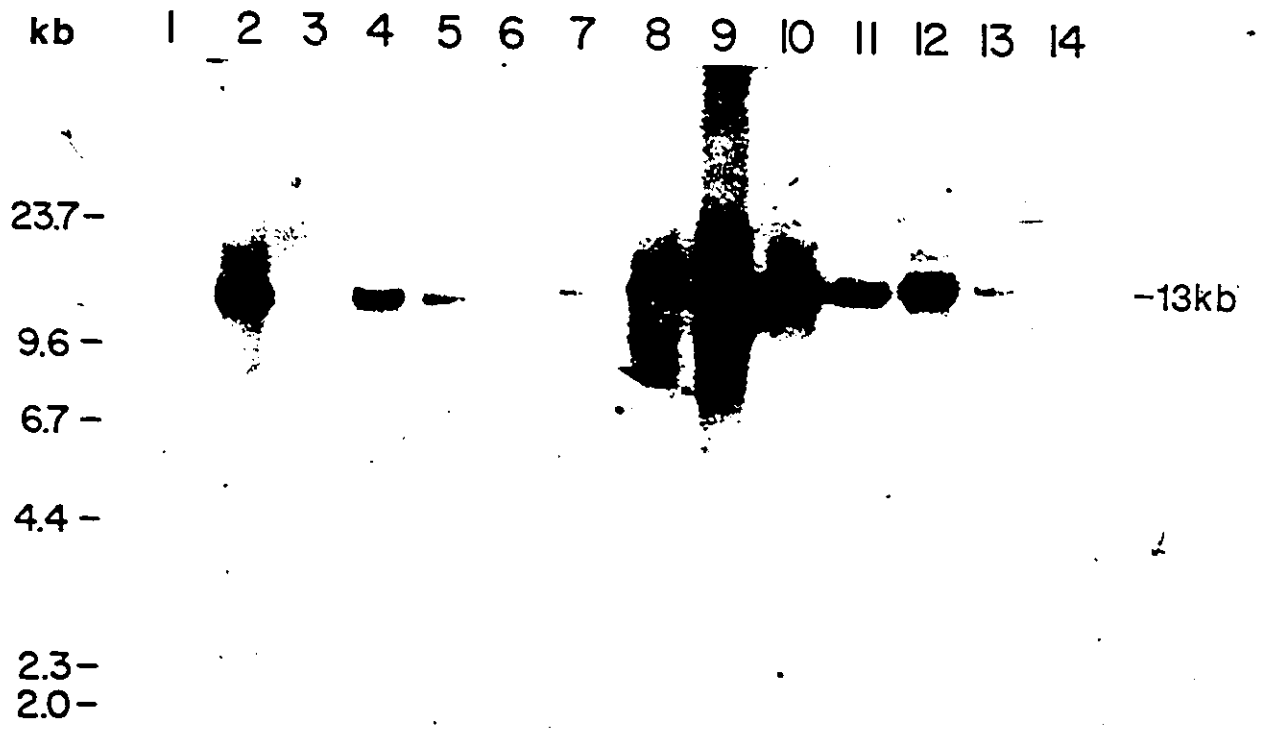
Northern blot analysis of selected P19(CH-actin) cell lines with actin probes was performed to determine whether the CH-actin gene was transcribed in transformants (Figure 4.4). Although this analysis could not discriminate between the mouse and human cardiac-actin transcripts, we can conclude that the non-muscle cells contained low levels of cardiac-actin mRNA of the expected size (1.7 kb).

We used a nuclease S1 mapping procedure to detect CH-actin mRNA in P19(CH-actin) transformants and to distinguish the CH-actin mRNA from transcripts originating

Figure 4.3. Presence of the CH-actin gene in transfected P19 EC cell lines. The CH-actin gene was detected in 28 stable transformants by Southern blot hybridization of EcoRI digested genomic DNA to the 1.5 kb non-coding 5'-probe (Figure 4.1).

(A) EC cell lines expanded from G418 resistant colonies following transfection with pNA8. Digestion with EcoRI releases the 13 kb fragment containing the CH-actin gene. Lanes 5, 9 and 12 correspond to cell lines P19A6, P19A24 and P19A35 respectively analysed in Fig. 4.6. (B) EC cell lines expanded from G418 resistant colonies following transfection with pNA-DI. EcoRI digestion releases a 13 kb fragment containing both the CH-actin and neur genes. Lanes 10, 13 and 14 correspond to cell lines P19AD32, P19AD43 and P19AD54 respectively analysed in Figure 4.6.

A



B

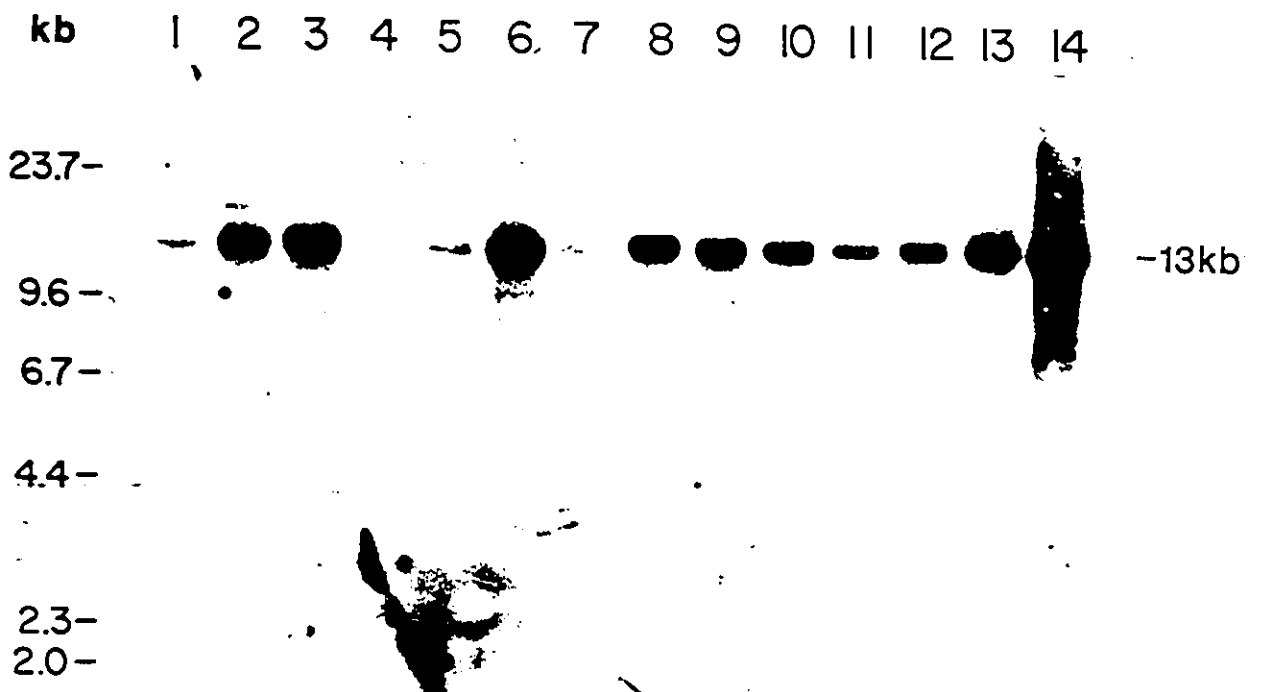


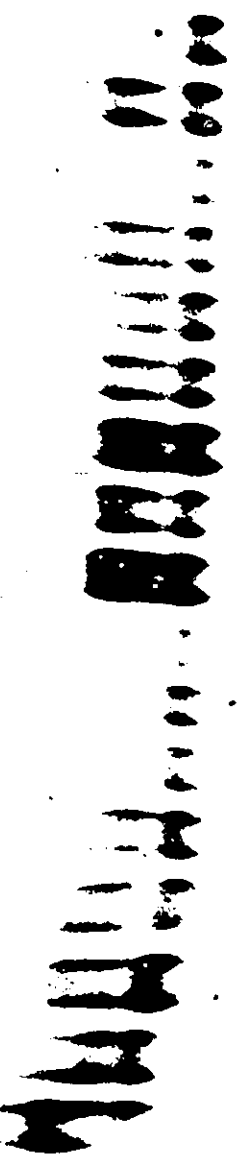
Figure 4.4. Presence of cardiac-actin mRNA in P19(CH-actin) transformants and their differentiated derivatives. Total RNA was isolated from P19 EC cells and their differentiated derivatives. RNA (20 ug) was electrophoresed through agarose gels, transferred to nylon membranes then hybridized to either a 600 bp 3'-probe (A) or the 450 bp 3'-UT region probe (B) from the CH-actin gene (Figure 4.1). The 600 bp 3'-probe hybridizes to cytoskeletal-actin, skeletal-actin and cardiac-actin mRNAs while the 450 bp 3'-UT region probe hybridizes specifically to cardiac-actin mRNAs. This analysis does not discriminate between CH-actin and CM-actin transcripts. EC, D and R denote undifferentiated P19 cells, DMSO-treated cultures containing cardiac muscle and RA-treated cultures containing neurons, glia and fibroblast-like cells respectively. Lane 1, untransfected P19; lane 2, P19A12; lane 3, P19A35; lane 4, P19AD31; lane 5, P19AD32 and lane 6, P19AD34. The migration of the rRNAs are indicated on the left as 28S and 18S.

A

28S -

EC D R EC D R EC D R EC D R EC D R EC D R

18S -



- $\alpha$  ACTIN  
- $\beta$  ACTIN

B

28S -

EC D R EC D R EC D R EC D R EC D R EC D R

18S -

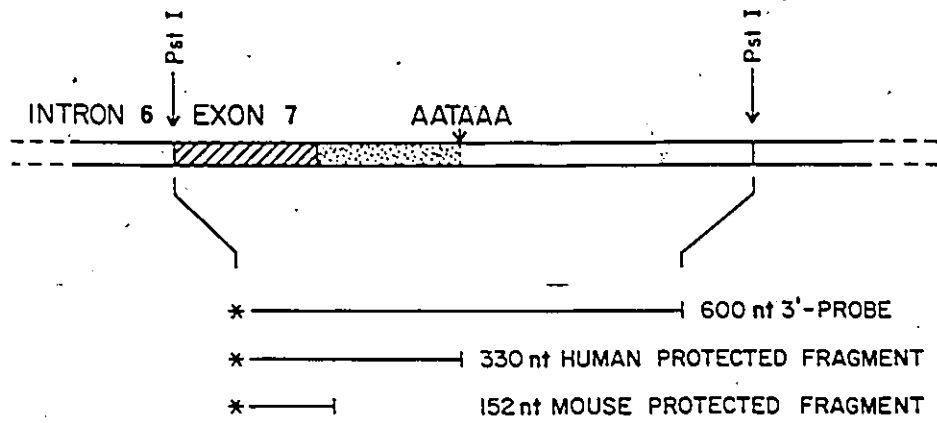
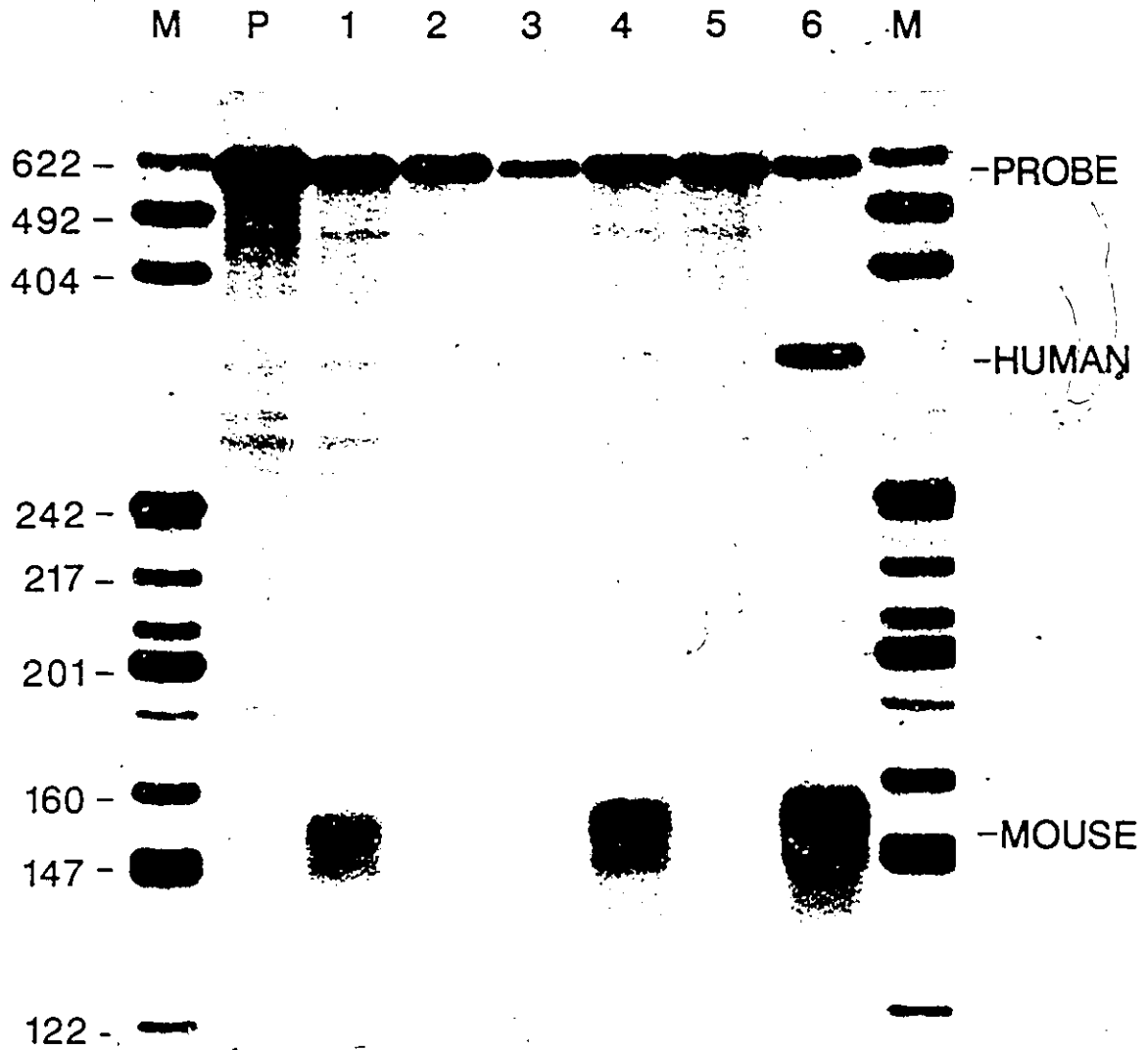


- $\alpha$  ACTIN

from the endogenous mouse cardiac actin (CM-actin) gene. The 600 nt probe used in these experiments spans the last exon of the CH-actin gene (Figures 4.1 B and 4.5 A). The CH-actin mRNA protected a 330 nt fragment from nuclease S1 digestion (Figure 4.5 B, lane 6) whereas the CM-actin mRNA protected fragments of between 152 and 140 nt (Figure 4.5 B, lanes 1, 4 and 6). None of the mRNAs encoding other actin isoforms protected a labeled region of the probe (Figure 4.5 B, lanes 2, 3 and 5). CM-actin mRNA was readily detected in DMSO treated P19 cultures (Figure 4.5 B, lanes 4 and 6) while no such transcript was detected in either EC cells or in RA treated cultures (Figure 4.5 B, lanes 3 and 5).

Nuclease S1 analysis of RNA from P19(CH-actin) cell lines indicated that the CH-actin mRNA but not the CM-actin mRNA was present in the EC cells. The level of CH-actin transcript was higher in DMSO treated cultures than that in EC and RA treated cultures for those transformants which differentiated into cardiac muscle and expressed CM-actin mRNA after exposure to DMSO (Figure 4.6, lanes 8, 11, 14, 17 and 20). This increase in CH-actin transcript after DMSO treatment was between 2.8 and 6.3 fold (Table 4.1). This increase in CH-actin mRNA may have been underestimated by at least 10 fold since muscle cells made up only 10-30% of the cells within DMSO treated cultures (Table 4.1), the level of CH-actin mRNA was determined on day 10 when the peak level of expression was on day 7 (Figure 4.7) and the level of CH-actin

Figure 4.5. Discrimination between CH-actin and CM-actin mRNAs by nuclease S1 mapping. (A) The 600 bp PstI fragment containing the last coding exon of the CH-actin gene (Figure 4.1) was 3' end labeled with T4 DNA polymerase, hybridized to RNA samples containing CH-actin and CM-actin mRNAs, and digested with nuclease S1. The CH-actin mRNA protects 330 nt whereas the CM-actin mRNA protects a maximum of 152 nt of the 3' end labeled 600 nt probe. Striped area denotes coding region, stippled area denotes 3' untranslated region and \* denotes label. (B) RNA from mouse striated muscle and P19 cells subjected to nuclease S1 analysis. M, size markers (indicated on the left in nucleotides) consisting of pAT153 digested with HpaII and 3' end labeled with T4 DNA polymerase; P, probe only; 1, mouse heart muscle; 2, mouse skeletal muscle; 3, undifferentiated P19 EC cells; 4, P19 cells induced to differentiate with DMSO into cultures which include cardiac muscle; 5, P19 cells induced to differentiate with RA into neurons, glia and fibroblast-like cells; 6, P19AD32 after DMSO treatment. Probe, Human and Mouse denote 600 nt input probe, 330 nt human protected fragment and 152 nt mouse protected fragments respectively.

**A****B**

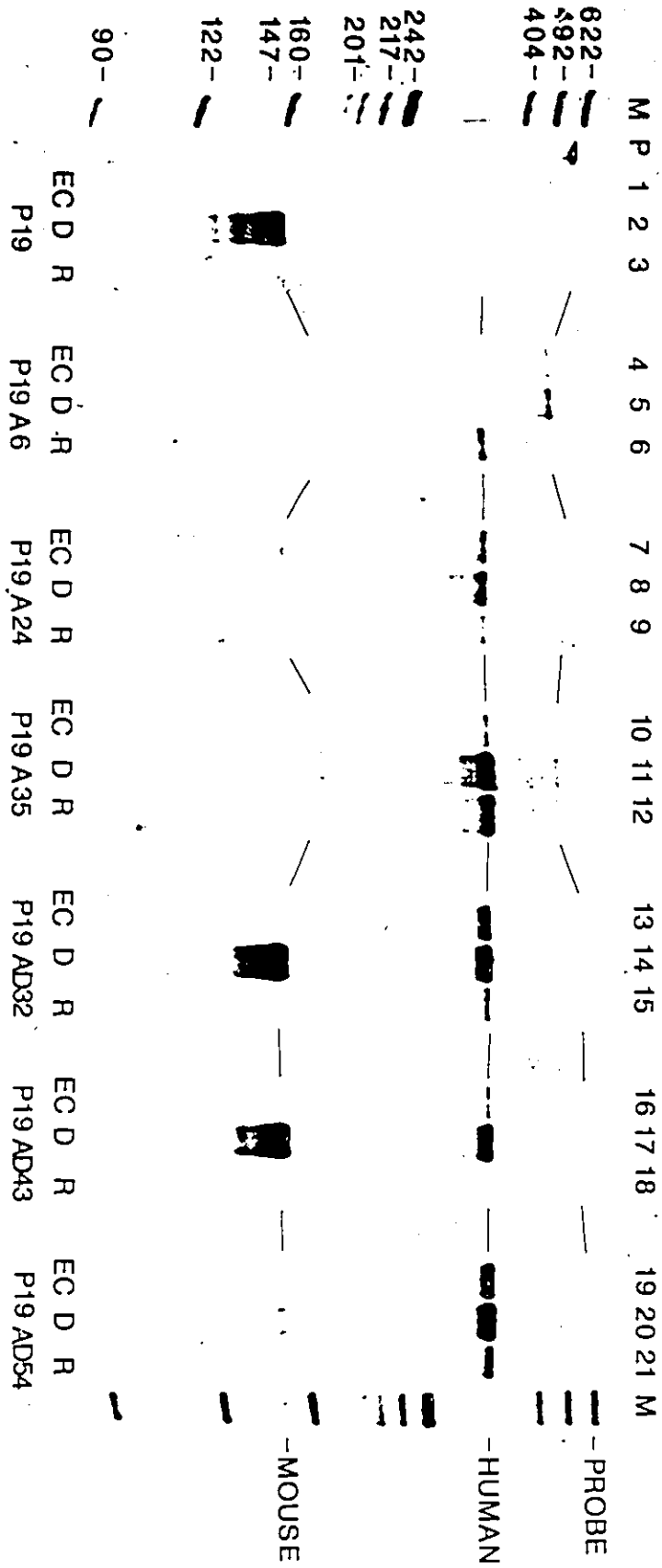
mRNA decreased in non-muscle cells within DMSO treated cultures (Figure 4.8).

The level of CH-actin mRNA in EC cells did not correlate with the level of neo mRNA (Figure 4.9) or with the copy number of the transfected gene (Table 4.1). In addition the length of the upstream region (5 kb and 0.8 kb) flanking the CH-actin gene did not appear to affect the levels of CH-actin mRNA in EC cells or their differentiated derivatives (Table 4.1 and Figure 4.6, compare P19An and P19ADn cell lines).

In two lines, P19A24 and P19AD54 with more than 200 copies of the CH-actin gene, only small amounts of CM-actin mRNA were present in DMSO treated cultures containing relatively large amounts of muscle (Figure 4.6, lanes 8 and 20). Higher levels of CM-actin were present after DMSO treatment of other cell lines, such as P19AD32, which developed similar amounts of muscle but which contained fewer copies of the CH-actin gene (Figure 4.6, lane 14; Table 4.1). A similar repression of the endogenous myosin light chain 2 (MLC2) gene in transgenic mice carrying rat MLC2 genes, has recently been reported (Shani et al., 1988). These results suggest that the CM-actin and CH-actin genes may compete for limiting amounts of transcription factor(s) in muscle cells.

Most RA treated cultures of P19(CH-actin) cells contained lower levels of CH-actin mRNA than those found in the corresponding EC cells (Figure 4.6, lanes 9, 15, 18 and 21).

Figure 4.6. Expression of the transfected CH-actin gene increased during cardiac muscle differentiation of clonal P19(CH-actin) cell lines. Cytoplasmic RNA isolated from EC cells and differentiated cells from DMSO- and RA-treated cultures from P19(CH-actin) cell lines was analysed for the presence of CH-actin and CM-actin mRNAs by nuclease S1 mapping as described in the legend to Figure 4.5. P19A and P19AD transformants were transfected with the plasmids pNA8 and pNA-DI respectively. Note that the level of CH-actin mRNA generally increased after DMSO treatment but not after RA treatment. EC, D and R denote RNA samples isolated from EC cells, DMSO-treated cultures and RA-treated cultures, respectively. For other abbreviations, see the legend to Figure 4.5.



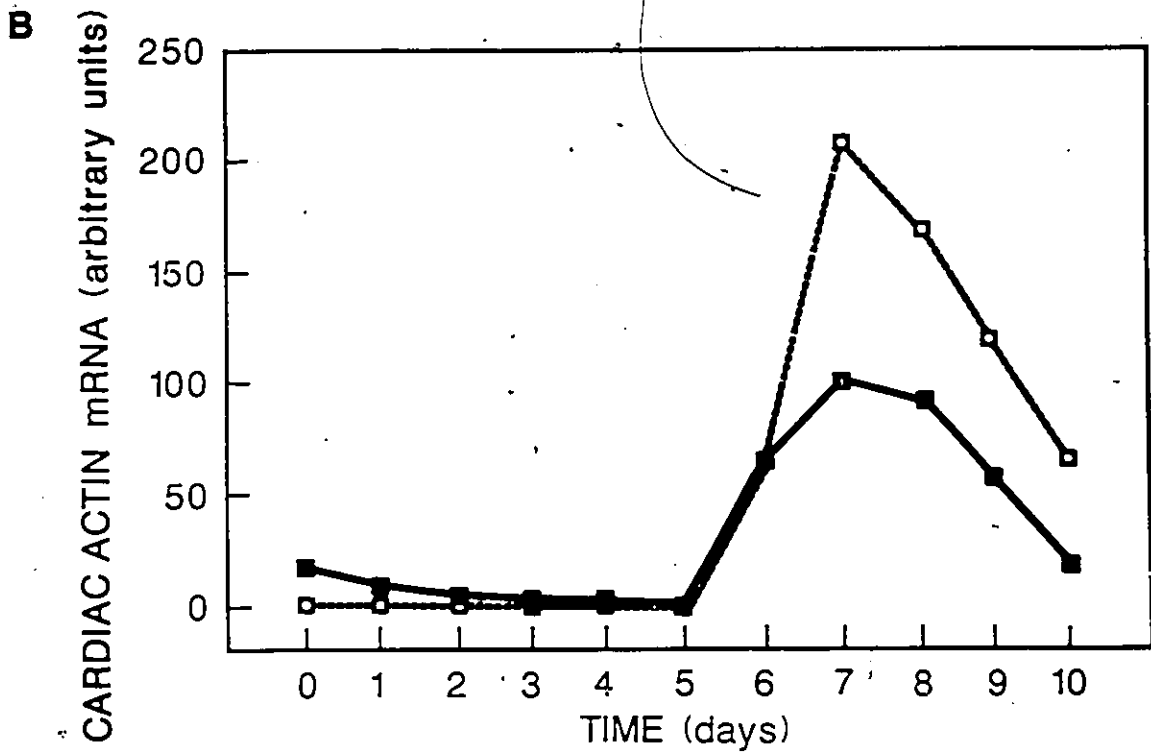
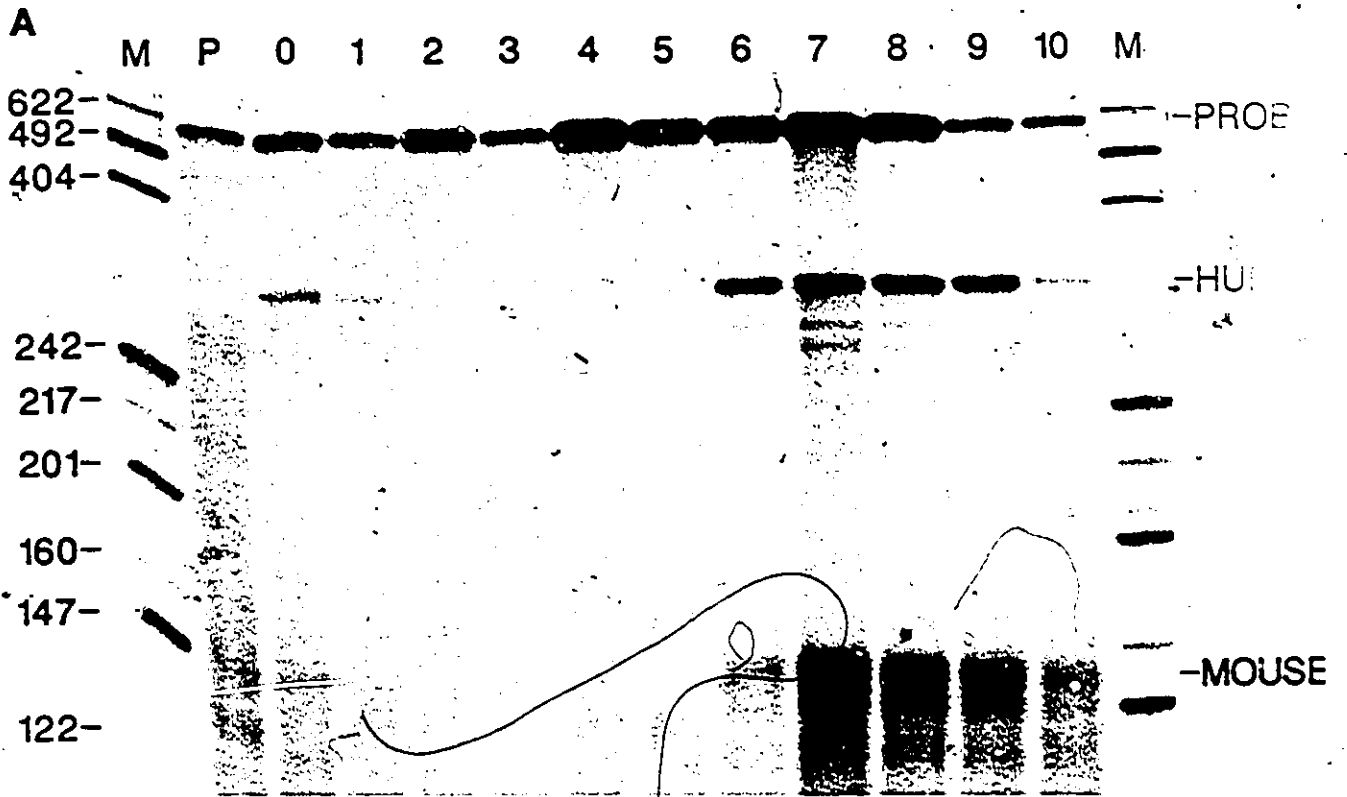
Two exceptions were observed in the 10 transformants examined. The CH-actin mRNA was detectable only after RA treatment of P19A6 cells (Figure 4.6, lane 6) while the level of CH-actin mRNA increased after RA treatment of P19A35 cells (Figure 4.6, lane 12). The unexpected behavior of the CH-actin gene in these two lines may be a consequence of rearrangement of the transfected gene or its integration into a region of the genome influenced strongly by neighbouring sequences.

#### 4.2.3. Developmental Regulation of the Human Cardiac Actin Gene During Cardiac Muscle Differentiation:

The kinetics of CH-actin gene expression during the development of cardiac muscle was examined to determine whether transcription of endogenous CM-actin and transfected CH-actin genes were subject to similar developmental regulation. The experiment was performed with cultures which consisted of over 50 pooled clones of P19(CH-actin) cells transfected with plasmid pNA-D1. This approach was taken in order to obtain the average of a large number of transfected clones.

Spontaneously beating cardiac muscle and cells reactive with the anti C/S-MHC antibody (MF20) first appeared on day 6 (Chapter 3; Rudnicki et al., 1988a). The CM-actin mRNA also became evident on day 6, increased rapidly to peak on day 7, and decreased slowly to about 30% of the maximum level by day 10 (Figure 4.7). This pattern of CM-actin expression was identical to that observed in untransfected DMSO-treated P19 cells.

Figure 4.7. Concomitant expression of transfected CH-actin and endogenous CM-actin genes during cardiac muscle differentiation. (A) Total RNA, from over 50 pooled P19(CH-actin) transformants derived after transfection with pNA-DI, was isolated at daily intervals throughout the DMSO-induced differentiation regime and analysed for the presence of CH-actin and CM-actin mRNAs by nuclease S1 mapping as described in the legend to Figure 4.5. At day 0, EC cells were transferred into petri grade dishes containing medium with 1% DMSO. After 5 days, aggregates were transferred into tissue culture-grade dishes without DMSO. Beating muscle first appeared on day 6. Lanes 0-10, RNA samples isolated on days 0 to 10; for abbreviations see the legend to Figure 4.5. (B) Relative level of CH-actin mRNA (solid line) and CM-actin mRNA (broken line) during DMSO-induced differentiation. The levels of C-actin mRNA were determined by scanning densitometry of the autoradiogram shown in panel A. The recorded peaks were then excised and weighed. C-actin mRNA is expressed in arbitrary units as a percent of the CH-actin mRNA level found on day 7.



The low level of CH-actin mRNA present in P19(CH-actin) cells decreased continuously during the first 5 days of the differentiation regime. The CH-actin mRNA level abruptly increased on day 6 in parallel with the CM-actin transcript, peaked on day 7 and decreased thereafter (Figure 4.7). These results suggest that the transfected CH-actin gene was subject to the same regulatory mechanisms which control the developmental expression of the endogenous CM-actin gene.

Because the simian virus 40 (SV40) promoter drives the neo gene in the plasmid carrying the CH-actin gene, it was possible that the peak in CH-actin mRNA seen in Figure 4.7 might be due to an effect of the SV40 enhancer. However, this seems unlikely because the level of neo mRNA actually decreased by about two fold during the course of DMSO-induced differentiation of transformed cells (Figure 4.9).

In DMSO-induced differentiation experiments, only about 10% of the cells develop into cardiac muscle. To determine the level of CH-actin mRNA in differentiated non-muscle cells, four fibroblast-like cell lines were isolated from cultures induced to differentiate with DMSO and their RNA assessed for the presence of the CH-actin transcript. No CH-actin mRNA was detected in two of three clonal fibroblast-like cell lines derived from P19AD43 (Figure 4.8, compare lane 1 with lanes 2, 3 and 4). In a non-clonal fibroblast-like cell line derived after DMSO treatment of clone P19AD54, the CH-actin mRNA was present at a level much lower than that found in the parental EC cells

Figure 4.8. Levels of CH-actin mRNA are reduced within differentiating non-muscle cells in DMSO-treated cultures. After DMSO treatment cells were passaged several times to obtain a muscle-free population of differentiated fibroblast-like cells. Total RNA, extracted from EC cells and fibroblast-like cells, was analysed for the presence of CH-actin mRNA by nuclease S1 mapping as described in the legend to Figure 4.5. C1, C2 and C3 were 3 clonal fibroblast-like cell lines derived after DMSO treatment of P19AD43 EC cells. MF was a non-clonal population of fibroblast-like cells with mixed form derived after DMSO treatment of P19AD54 EC cells. No CM-actin mRNA was detected in any of these samples. For other abbreviations see the legend to Figure 4.5.

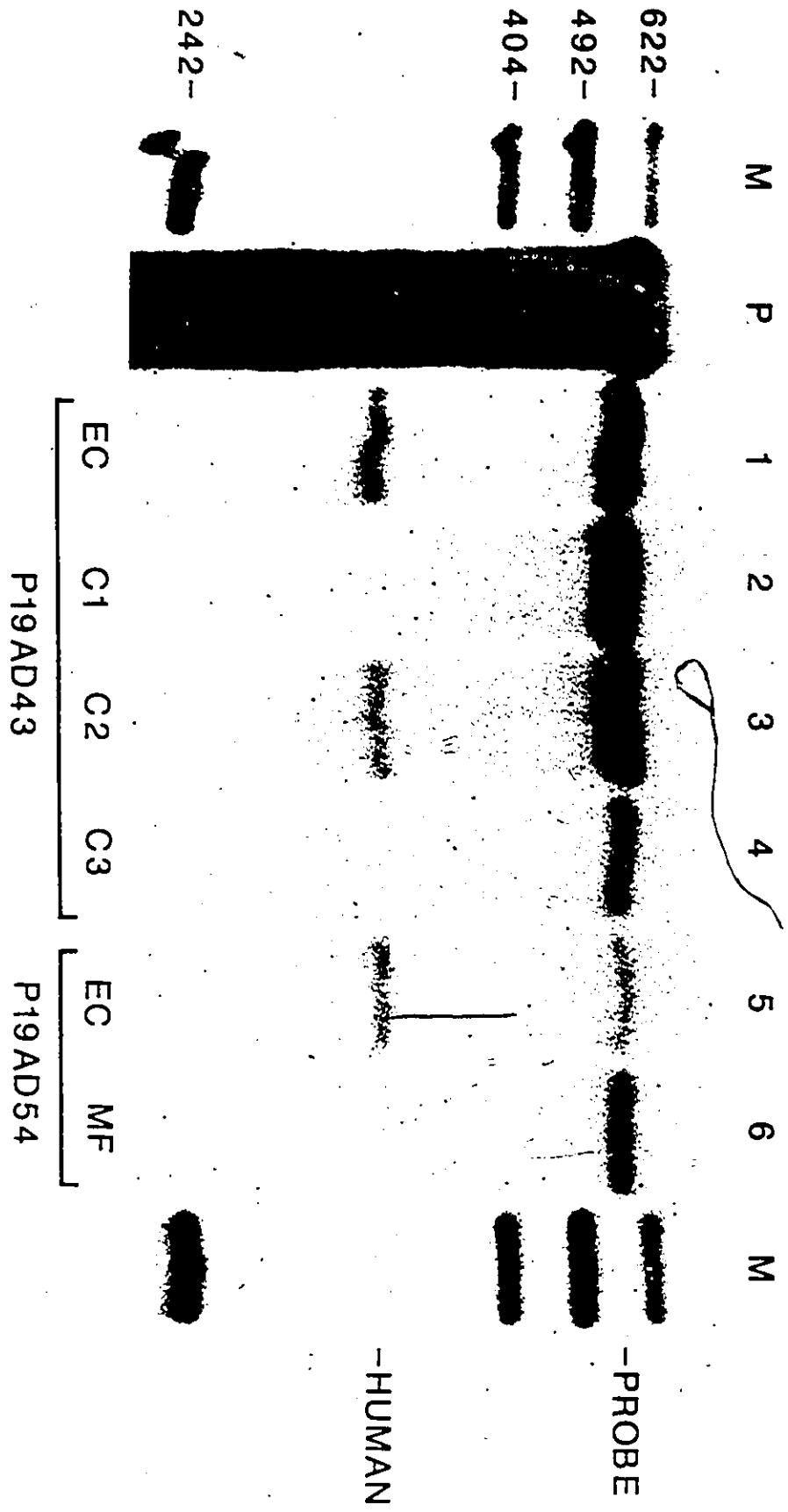


Figure 4.9. The level of neo mRNA decreases after DMSO-induced differentiation in P19(CH-actin) transformants. RNA (10 ug) was size fractionated by electrophoresis through agarose in the presence of formaldehyde, transferred to nylon membrane and hybridized to pSV2-neo. The probe was labeled with [ $\alpha$ - $^{32}$ P]dCTP to over  $10^9$  cpm/ug by multiprime labeling. The size of rRNAs are indicated as 28S and 18S. The position of the neo transcript is indicated with an arrow. (A) RNA isolated from P19AD32 cells and differentiated derivatives; EC, D and R denote embryonal carcinoma, DMSO treated cultures containing cardiac muscle, and RA treated cultures containing neurons, glia and fibroblast-like cells respectively. (B) RNA isolated from pooled P19(CH-actin) transformants at intervals during DMSO induced differentiation (see Fig. 4.7); EC, 3, 7, and 10 denote embryonal carcinoma, 3, 7, and 10 days after initiation of differentiation respectively. (C) RNA isolated from P19AD43 cells and non-muscle fibroblast-like cell lines derived after DMSO induced differentiation (see Fig 4.8); EC, C1, C2, and C3 denote embryonal carcinoma and clones 1 to 3 respectively.

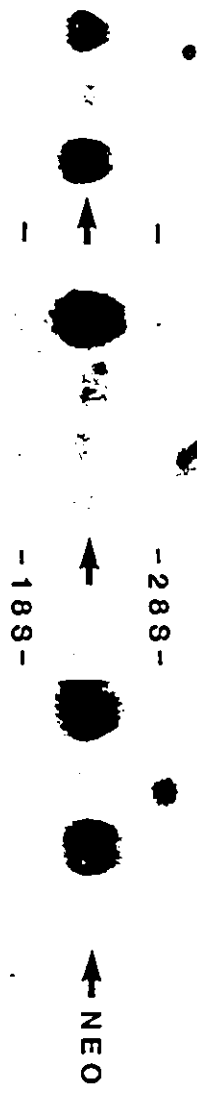
A

B

C

1 2 3 1 2 3 4 1 2 3 4

EC D R EC 3 7 10 EC C1 C2 C3

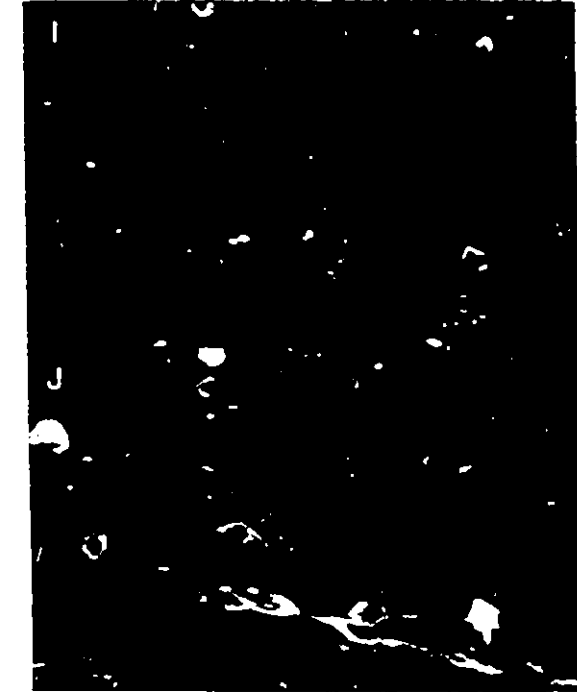
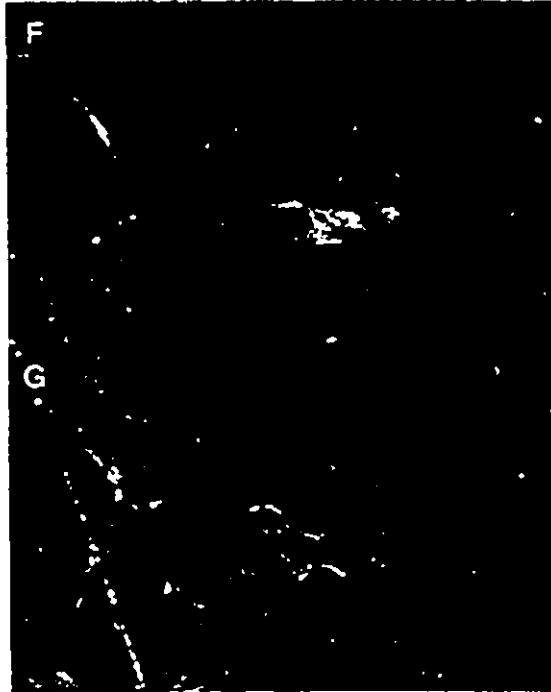
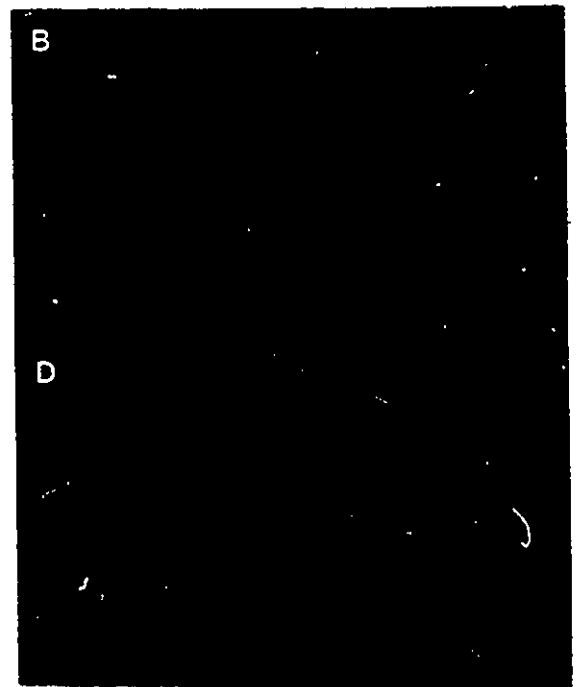
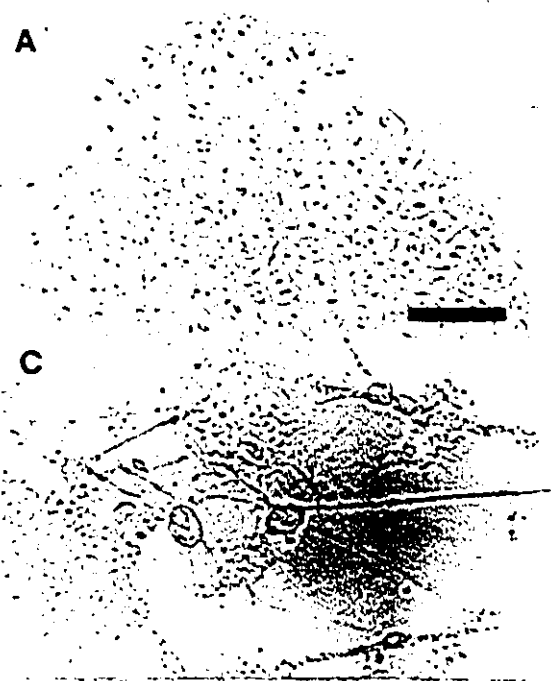


(Figure 4.8, compare lanes 5 and 6). None of these fibroblast-like cells were EC or muscle cells since they did not react with either anti-EC (AEC3A1-9, Harris et al., 1984) or anti-muscle myosin (MF20, Bader et al., 1982) antibodies. Interestingly, the C2 cell line which expressed some CH-actin mRNA (Figure 4.8) was the only differentiated cell line that also expressed neo mRNA (Figure 4.9). This suggests that the SV40 promoter may activate the CH-actin gene in some differentiated cell types. Nevertheless, the low level of CH-actin transcript present in EC cells becomes reduced even further in most of the non-muscle cells suggesting that both activating and repressing mechanisms are responsible for regulating the CH-actin promoter.

#### 4.2.4. Restriction of Alpha-Actin Protein to Muscle Cells

Detectable levels of CH-actin mRNA were present in some P19(CH-actin) cells, in RA treated cultures of some P19(CH-actin) cells and in non-muscle fibroblasts from DMSO treated cultures (Figures 4.4, 4.6 and 4.8). To determine whether the CH-actin mRNA was translated in these non-muscle cells we performed immunofluorescence experiments using a polyclonal antiserum which reacts with the cardiac and skeletal isoforms of actin (Bulinski et al., 1983). In four clonal lines of transformants (P19A24, P19A35, P19AD43 and P19AD54), virtually no cardiac-actin protein was detected in untreated and RA-treated cultures (Figure 4.10, panels A-D). Within

Figure 4.10. Restriction of the alpha-actin protein to muscle cells in P19(CH-actin) transformants. Indirect immunofluorescence was used to determine the distribution of cardiac-actin and skeletal-actin protein in P19AD43 cells. Both EC cells (panels A and B) and RA-treated cultures containing neurons, glia and fibroblast-like cells (panels C and D) expressed some CH-actin mRNA (Fig. 4.6) but did not contain detectable cardiac-actin protein (panels B and D). After DMSO treatment the majority of myocytes were cardiac muscle (E) but some skeletal muscle was also apparent (H). The muscle cells contained muscle-myosin (G and J) and cardiac- and/or skeletal-actin (F and I) proteins. A, C, E and H are phase-contrast fields. B, D, F and I are the corresponding fields stained with rabbit antiserum which reacts with cardiac-actin and skeletal-actin proteins (Bulinski et al., 1983). G and J were stained with the monoclonal-antibody MF20 (Bader et al., 1982) reactive with C/S-MHC protein. The reagents used in immunofluorescent experiments are listed in Table 2.1. Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgG. Bar represents 50 um.



DMSO-treated cultures, the cardiac-actin protein was not detected in cells that did not also contain C/S-MHC protein (Figure 4.10, panels E-J). In addition, no cardiac-actin protein was detected in the differentiated non-muscle cells from the four fibroblast-like lines derived after DMSO treatment of P19AD43' and P19AD54 (data not shown). These experiments suggest that the CH-actin protein is synthesized in non-muscle cells at levels below the limits of detection of our immunofluorescence technique, that the CH-actin protein is not incorporated into microfilaments, that the CH-actin protein is rapidly degraded in non-muscle cells, or that the CH-actin mRNA is not efficiently translated in non-muscle cells.

#### 4.3. Discussion

In this study, we have transfected the CH-actin gene into multipotential P19 cells and isolated stable transformants. Transcripts from the CH-actin gene were present at a low level in undifferentiated EC cells and in some differentiated non-muscle cells. However, a marked increase in the level of CH-actin mRNA occurred in parallel with transcripts from the endogenous CM-actin gene during cardiac muscle differentiation.

In EC cells and RA-treated cultures, transcription initiated from the CH-actin promoter generated low levels of

transcripts, although no CM-actin mRNA was detected. This inappropriate activity of the CH-actin promoter may be due to an effect of the vector sequences or to the proximity of the SV40 early promoter and enhancer used to drive the neo gene. However, the level of neo mRNA does not correlate with the level of CH-actin mRNA; thus a role for the SV40 enhancer seems unlikely. A more likely explanation for the low levels of CH-actin mRNA in EC cells derives from the presence in EC cells of a cellular product with E1A-like activity (Imperiale et al., 1984). Since the adenoviral E1A products enhance the expression of transfected cellular genes (Green et al., 1983), it seems likely that the E1A-like activity in EC cells may be responsible for activating expression of the CH-actin gene in transformed P19 cells. In addition, the chromatin structure of EC cells is unusually nuclease sensitive (Croce et al., 1981; Huebner et al, 1981), perhaps conferring an active configuration upon newly-introduced genes.

The CH-actin mRNA present in non-muscle cells did not give rise to detectable protein. Northern blot analysis of RNA from P19(CH-actin) transformants with a cardiac-actin isotype specific probe (the .45 kb HaeIII-PstI fragment, see Figure 4.1 A, containing the 3'-untranslated region, Gunning et al., 1983) indicated that the CH-actin mRNA in EC and RA treated cells was the expected size, 1.7 kb, and therefore likely correctly processed (Figure 4.5). Since the cardiac actin protein can be incorporated into cytoskeletal structures in mouse L cells

(Gunning et al., 1984), our failure to detect this protein in P19(CH-actin) transformants suggests that translation of the CH-actin transcript may be restricted to muscle cells. More direct evidence for translational regulation will be reported later (Appendix to Chapter 5).

Other tissue-specific genes have been transfected into mouse EC cells. Cloned globin and crystallin genes have been introduced into multipotential EC cells, but regulated expression could not be demonstrated, perhaps because the EC cells did not differentiate into those cell types expected to express high levels of the transfected gene (Kondoh et al., 1984; Pellicer et al., 1980; Wagner and Mintz, 1982). The F9 cell line, which differentiates only into extraembryonic endodermal cells (Strickland and Mahdavi, 1978), has been transfected with H-2 (Rosenthal et al., 1984), and alpha-fetoprotein (Scot et al., 1984) genes, and transcripts from these transfected genes did increase appropriately after transformed cells differentiated into endoderm.

The experiments reported here demonstrate that the transfected CH-actin gene was subject to appropriate developmental and tissue-specific regulation during differentiation of transfected P19 cells into cardiac muscle. Sequences located downstream of -800 bp from the CH-actin transcription start site were able to mediate this muscle-specific regulation and our evidence suggests that control is at both the transcriptional and translational levels.

Thus, transfected P19 cell cultures, like transgenic animals (Shani, 1985, 1986; Wilson et al, 1986; Geyer and Fryberg, 1986; Mohun et al., 1984, 1986), are a suitable biological test system for identifying those DNA sequences responsible for muscle-specific gene expression.

4.4. Chapter 4 Appendix: Regulated Expression of a Transfected  
Chimeric Actin-tk Gene during  
Differentiation of P19 Cells

4.4.1. Introduction:

This appendix reports the results of experiments performed in collaboration with Dr. Martha Ruben. Here, we describe experiments in which a chimeric actin-tk gene was stably transfected into P19 cells. We found that the cardiac-actin promoter functioned with appropriate cell-type specificity during the differentiation of P19 cells.

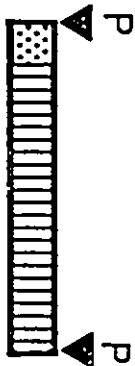
4.4.2. Results:

The differentiation-dependent changes in the levels of CH-actin mRNA in P19(CH-actin) transformants was likely due to regulation of CH-actin gene transcription (Figure 4.7). To investigate the CH-actin gene promoter in more detail, we constructed a chimeric gene in which the CH-actin gene promoter (-700 to +740 bp from the transcription start site) was linked to the herpes simplex virus thymidine kinase gene (HSV-tk) coding region (Figure 4.11 A). The chimeric actin-tk gene was co-transfected with pSV2-neo (Southern and Berg, 1982) into P19 cells. G418 resistant clones were subsequently selected and those containing the chimeric gene identified by Southern blot hybridization (data not shown).

Figure 4.11. Expression of the transfected actin-tk chimeric gene increased during muscle differentiation. (A) The actin-tk chimeric gene (pPTK) was constructed by linking the 1.5 kb PstI fragment (solid line) containing 700 bp of sequences upstream from the transcription start site, the first exon (44 bp) containing part of the 5'-untranslated region, and the first intron (700 bp) of the CH-actin gene to a fragment of the HSV genome (open region) carrying the coding region of the HSV-tk gene (striped region). The 800 bp PstI insert of pTK was used as a tk-specific probe in hybridization experiments. Arrow indicates transcription and exons are indicated as raised areas; P, PstI; E, EcoRI. Cont'd.

A

PT4



1.0 kb



pPTK

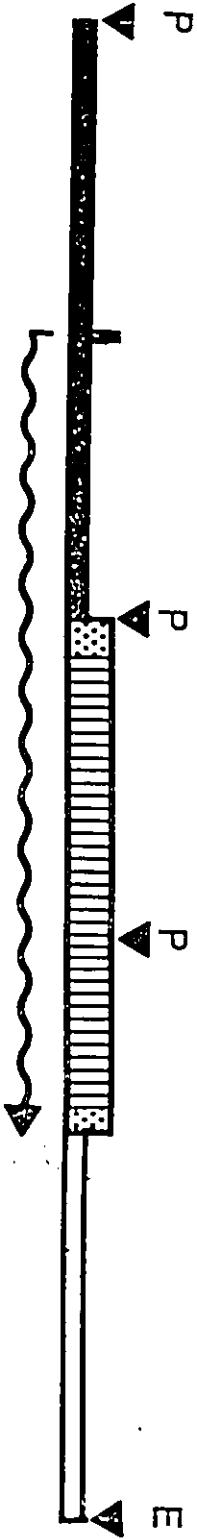


Figure 4.11. Cont'd. (B and C) Actin-tk and actin mRNAs were detected by Northern blot hybridization of total RNA isolated from P19(actin-tk) transformants to either tk-specific (B) or actin-specific (C) (0.6 kb PstI probe, Figure 4.1 B) probes. Shown here are two P19(actin-tk) clones. EC, D and R denote RNA isolated from EC cells, DMSO treated cultures and RA treated cultures respectively. P19P106 but not P19P2 contained the actin-tk mRNA, other bands are due to non-specific hybridization to rRNA. L6U3 is a rat cell line transformed with pPTK which expresses high levels of actin-tk mRNA.

B,

1 2 3 4 5 6 7 1 2 3 4 5 6 7



tk -

LGU3

	EC	D	R	EC	D	R	D	EC	D	R	EC	D	R
	P19P2			P19P106			P19	P19P2			P19P106		

C

- 28 S

-  $\beta$  ACTIN  
 - 18 S  
 -  $\alpha$  ACTIN



Northern blot analysis of P19(actin-tk) transformants indicated that in five independent clones, the low but detectable level of actin-tk mRNA present in EC cultures increased 3 to 13 times after DMSO treatment but not after RA treatment (Figure 4.11 B, lanes 5-7). This increase in actin-tk mRNA always coincided with the appearance of the 1.7 kb CM-actin transcripts in DMSO treated cultures (Figure 4.11 C, lane 6). Many of the P19(actin-tk) transformants did not express the chimeric gene under any conditions (Figure 4.11 B, lanes 2-4).

The HSV-TK protein translated from the actin-tk mRNA in DMSO-treated P19(actin-tk) transformants was detected by immunofluorescence by using a monospecific antibody (Haarr et al., 1985). The TK protein was located almost exclusively within the nuclei of cells which also contained muscle-myosin (Figure 4.12, panels A, B and C) and striated-muscle alpha-actin (Figure 4.12, panels D, E and F). Table 4.2 summarizes the results of these double staining experiments. In four P19(actin-tk) cell lines over 86% of the muscle cells contained TK protein and less than 2% of non-muscle cells in DMSO treated cultures (Table 4.2) and less than 0.1% of cells within EC cultures had detectable TK protein in their nuclei (data not shown). These rare TK containing cells within EC cultures may have spontaneously differentiated into muscle since they did not stain with anti-EC antibody (AEC3A1-9) and did stain with antibody (E4F8) reactive with all forms of muscle-actin.

Figure 4.12. Restriction of TK protein to muscle cells in P19(actin-tk) transformants. P19(actin-tk) transformants were treated with DMSO and after differentiation were dispersed and seeded onto coverslips prior to fixation and staining with rabbit anti-TK antiserum (Haarr et al., 1985) and either MF20 or B4F8, mouse monoclonals which react with C/S-MHC (Bader et al., 1982) and muscle-actin (Lessard, 1988) respectively. Shown here is P19P11, other results are summarized in Table 4.2. Panels A and D are phase fields. Cells which contained C/S-MHC protein (panel B) or alpha-actin protein (panel E) almost always also contained TK protein in their nuclei (panels C and F). Almost no non-muscle cells contained TK protein. The reagents used in immunofluorescent experiments are listed in Table 4.3. Second antibodies were fluorescein conjugated anti-rabbit and rhodamine conjugated anti-mouse IgGs. Bar represents 50 um in panels A, B and C and 30 um in panels D, E and F.

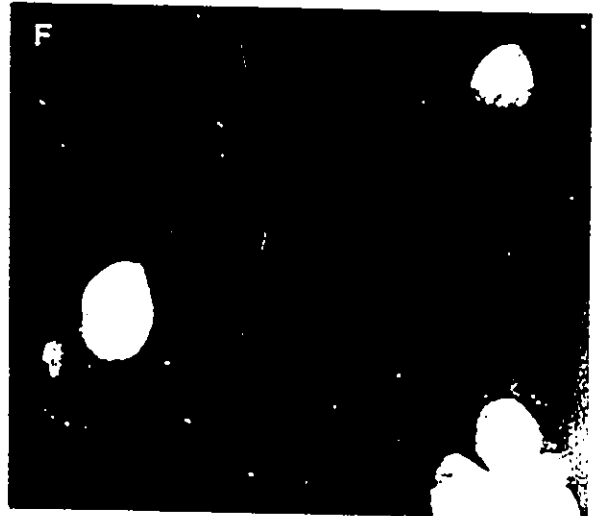
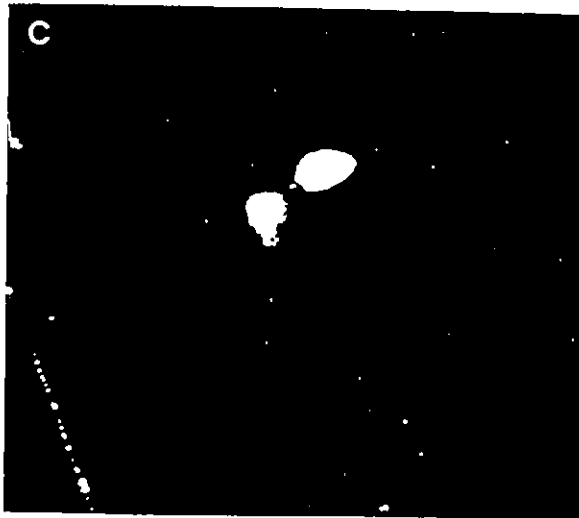
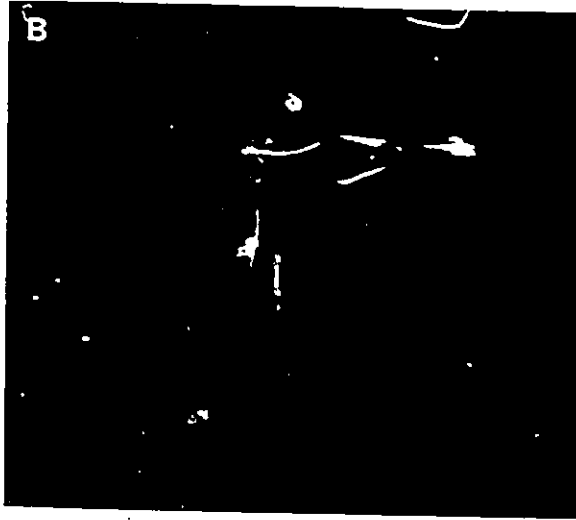
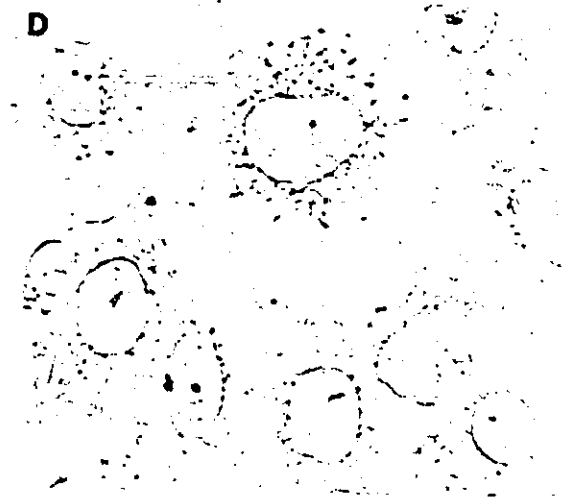
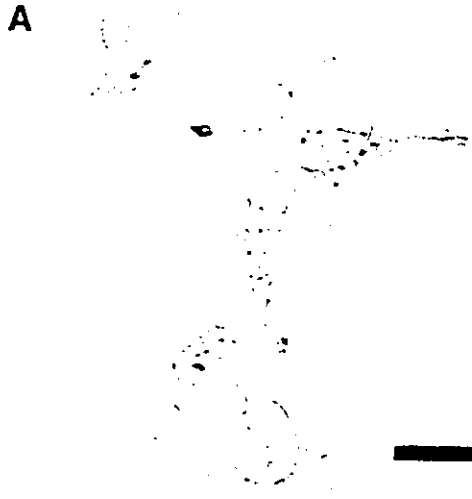


Table 4.2. Distribution of TK Protein in DMSO-Treated P19(actin-tk) Transformants

<u>EXP.</u>	<u>CELL LINE</u>	<u>CELLS SCORED</u>	<u>TK+ MUSC.</u>	<u>TK- MUSC.</u>	<u>TK+ NON-MUSC.</u>	<u>PERCENT TK+ MUSC.</u>	<u>PERCENT TK+ NON-MUSC.</u>	<u>FOLD INCREASE</u>
1	P19	364	17	17	0	NA	NA	NA
1	P19RP11	571	32	3	5	91%	14%	13.3
2		ND	102	18	19	85%	16%	
3		ND	85	23	19	79%	18%	
1	P19RP13	413	34	2	7	94%	17%	7.9
2		ND	71	9	14	89%	16%	
3		ND	29	8	5	78%	15%	
1	P19RP22	278	29	2	4	94%	12%	ND
1	P19RP26	301	53	5	8	91%	13%	4.1

A number of P19 cell lines stably transfected with pPTK were induced to differentiate with DMSO into cardiac muscle containing cultures. Cells were dispersed onto coverslips 6 days after initiation of differentiation and fixed and double stained for TK protein and C/S-MHC protein (MF20) as described in Figure 4.12. Percent TK+ Muscle is the percentage of muscle cells which contain detectable TK protein. Percent TK+ Non-muscle is the percentage of cells containing TK protein which are found in non-muscle cells. Fold Increase is the increase in the level of actin-tk mRNA over that found in the EC cells as determined by densitometry scanning of Northern blots. The peaks corresponding to the actin-tk mRNA were then excised and weighed. NA, not applicable; ND, not determined.

#### 4.4.3. Discussion:

In P19 cells stably transfected with the actin-tk chimeric gene, the TK protein was located almost exclusively in muscle cells. Thus the DNA sequences which direct the muscle cell-specific expression of the CH-actin gene lie between -700 and +740 bp from the transcription start site. These results suggest that the changes in the level of CH-actin mRNA (sections 4.1 to 4.3) are due to an increase in transcription of the CH-actin gene in cardiac muscle cells.

The activity of the CH-actin gene promoter has been previously investigated after transient transfection or stable transformation of mouse and rat skeletal myoblast cell lines (Miwa and Kedes, 1987; and references therein). In cells with integrated copies of the CH-actin promoter, the level of transcript generated by this promoter either did not increase or increased only modestly after differentiation of myoblasts into multinucleate myotubes (Hickey et al., 1986; Minty et al., 1986). The failure of skeletal myoblasts to efficiently regulate the CH-actin promoter may be due to the relatively advanced developmental stage of the cell lines used. Alternatively, the regulation of the CH-actin promoter may be a multistep process which requires "programming" by a cell which is a precursor to the muscle lineage. If such programming is required for the developmental regulation of CH-actin gene expression, the identification of the relevant regions of DNA

will require the use of a developing biological system such as is provided by the P19 cell culture system.

The experiments described in this appendix demonstrate that the transfected chimeric actin-tk gene was subject to appropriate tissue-specific regulation during differentiation of transfected P19 cells into cardiac muscle. Thus, sequences located between -700 and +740 bp from the CH-actin transcription start site must contain the regulatory sequences that mediate this muscle-specific regulation.

Chapter 5, Expression of the Human Cardiac Actin Gene in  
Differentiating Rat Skeletal Myoblasts

5.1. Introduction:

Multinucleate skeletal muscle develops from mononucleate proliferating myoblast cells which withdraw from the cell cycle, fuse to neighbouring myocytes, and express a variety of muscle-specific proteins (Yaffe, 1969; Konigsberg, 1971; Zalin, 1979). The initial appearance of many muscle-specific proteins is concomitant with the appearance of their mRNAs (Daubas et al., 1981) suggesting that muscle-specific gene expression is regulated primarily at the level of mRNA accumulation (Hastings and Emerson, 1982), probably by transcriptional control (Pearson and Epstein, 1982). To investigate the nature of muscle gene regulation, a number of investigators have transfected skeletal muscle genes into skeletal myoblast cell lines and, in some cases, the level of mRNA from the transfected gene was elevated after differentiation of myoblasts into myotubes (Melloul et al., 1984; Nudel et al., 1985; Jaynes et al., 1986).

We have previously transfected the human cardiac actin (CH-actin) gene into multipotential embryonal carcinoma (EC) cells and selected stable transformants. The levels of CH-actin mRNA rose dramatically in cultures of these EC cells during their differentiation into cardiac muscle (Chapter 4; Rudnicki et al., 1988b). The cardiac actin gene is normally

expressed in cardiac and embryonic skeletal muscle cells (Minty et al., 1982; Gunning et al., 1983). In order to investigate the expression of the CH-actin gene in cells differentiating along the skeletal muscle lineage, we transfected the CH-actin gene into rat L6 skeletal myoblasts (Yaffe, 1968). Contrary to our results using EC<sub>2</sub> cells, we found that myoblast cell lines containing stably integrated copies of the CH-actin gene [designated L6(CH-actin)] had CH-actin mRNA at comparable levels in myoblasts and myotubes. However, we were unable to detect cardiac-actin protein in the myoblasts. We interpret these results to suggest that the CH-actin mRNA was not efficiently translated in a non-muscle cell type.

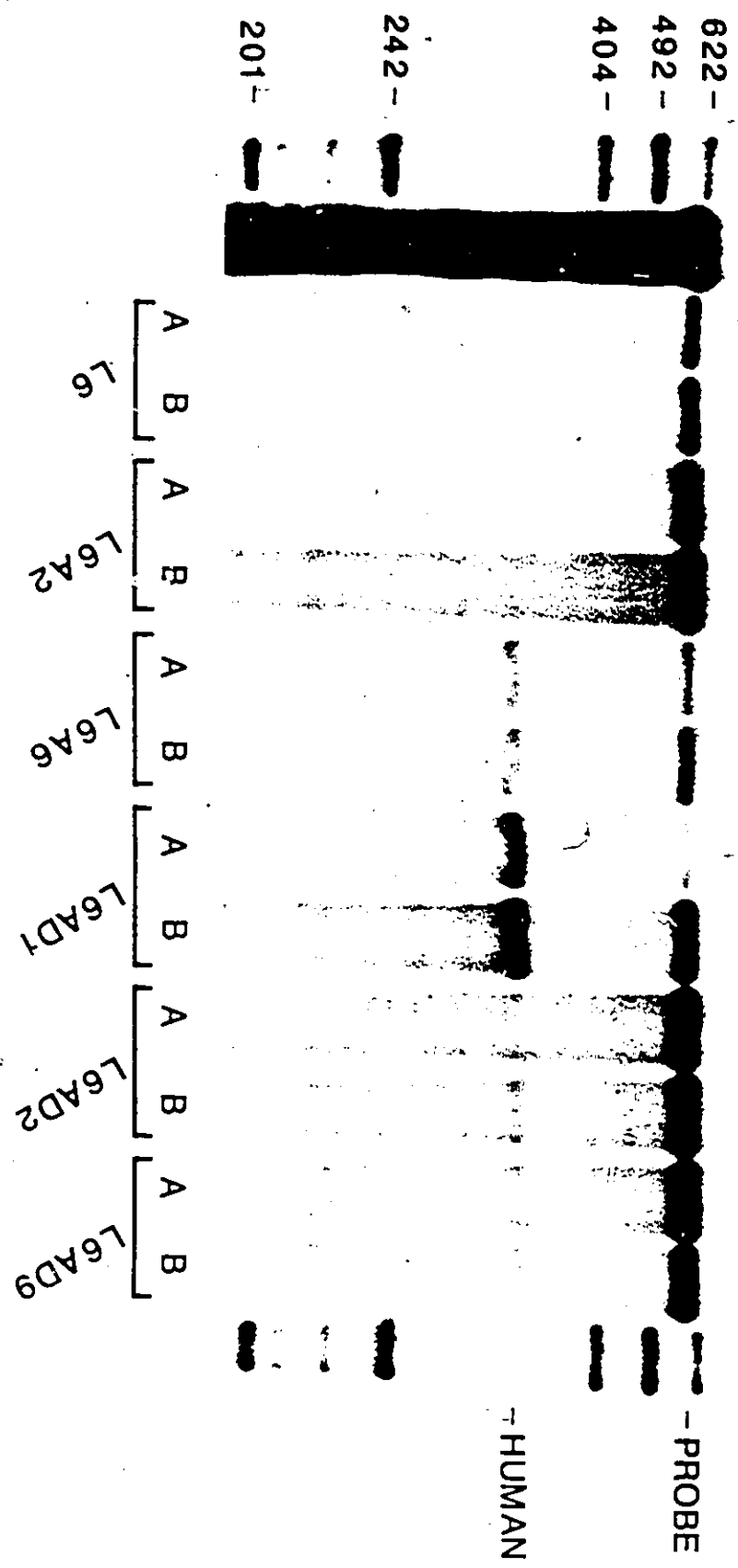
## 5.2. Results:

### 5.2.1. Detection of the Cardiac-Actin mRNA in L6(CH-actin) Transformants:

L6 cells were transfected with either of two plasmids carrying both the selectable neor gene and the CH-actin gene (Figure 4.1 A). These plasmids included 5.4 kb or 0.8 kb of sequence upstream of the transcription start site (plasmids pNA8 and pNA-D1, respectively). Clones of G418 resistant cells were isolated and the amount of CH-actin transcript in each clone was assessed by nuclease S1 mapping. Four of the 7 transformed L6 clones analysed expressed the CH-actin gene, but the levels

Figure 5.1. The levels of CH-actin transcript did not change after differentiation of L6 cells containing the CH-actin gene. To detect the CH-actin transcript in L6(CH-actin) transformants, the 0.6 kb PstI fragment (see Figure 4.1) containing the last coding exon of the CH-actin gene was 3'-end labeled with T4 DNA polymerase, hybridized to RNA samples from L6 clones carrying the CH-actin gene, and digested with nuclease S1. The CH-actin mRNA protected 330 nt of the 3' end of the probe. No rat skeletal-actin mRNA protected fragments were observed. Cell lines transfected with the pNA8 plasmid were designated L6An and cell lines containing the pNA-D1 construct were labeled L6ADn. M, size markers (indicated on the left in nucleotides) consisting of pAT153 digested with HpaII and 3'-end labeled with T4 DNA polymerase; P, probe only; A and B denote RNA isolated from mononucleate myoblasts and multinucleate myotubes, respectively; Probe and Human denote the 600 nt input probe and 330 nt fragment protected from digestion by the CH-actin transcript.

M P 1 2 3 4 5 6 7 8 9 10 11 12 M



of CH-actin RNA were variable from clone to clone (Figure 5.1). Under the stringent nuclease S1 digestion conditions used no rat muscle-actin protected fragments were observed.

The variability in the levels of CH-actin mRNA expressed in different transformants may stem from the presence of prokaryotic DNA adjacent to the CH-actin promoter as Konieczny and Emerson (1987) have noted a deleterious effect of plasmid vector sequences on the expression of a muscle gene promoter. Unexpectedly, the amount of CH-actin transcript did not increase more than 2-fold upon differentiation of these cells into multinucleate myotubes (Figure 5.1). Under the conditions of cell differentiation used, the level of rat striated muscle actin mRNA rises dramatically (Figure 5.4, panels B and D). Northern hybridization with with isotype-specific 3'-UT probes (see Chapter 3), indicated that untransfected fusing L6 myotubes expressed skeletal-actin but not cardiac-actin mRNA (data not shown).

Northern blot analysis (data not shown) of the RNAs from transformed clones of L6 cells indicated that the CH-actin transcripts in both myoblasts and in myotubes were 1.7 kb, the size expected for mature CH-actin mRNA (Gunning et al., 1983). This suggests that appropriate removal of the 6 introns from CH-actin transcripts occurred during RNA processing in both myoblasts and myotubes.

### 5.2.2. Detection of Cardiac-Actin Protein in L6(CH-actin) Transformants:

Cultures of transformed myoblast clones which contained the CH-actin transcript (L6AD1, L6AD2, L6AD9, L6A6) were examined by immunofluorescence to determine if these cells contained the CH-actin protein. We used the monoclonal antibody, B4F8, which reacts with all forms of muscle-specific actin including the human-cardiac and rat-skeletal isoforms (Lessard, 1988). While multinucleate myotubes stained brightly, myoblasts from all lines were uniformly unstained (Figure 5.2). Since the myotubes contain large amounts of rat skeletal actin, we can draw no conclusion regarding the presence of CH-actin protein in myotubes. However, our failure to detect CH-actin protein in any of the transformed myoblasts was surprising. The levels of CH-actin mRNA in the transformed myoblasts were comparable to the levels of mouse cardiac actin mRNA detected by the same procedure in differentiating cultures of mouse embryonal carcinoma cells (Chapter 4; Rudnicki et al., 1988b). In these latter cultures, the B4F8 antibody stains up to 30% of the cells very intensely. Thus, our failure to detect any CH-actin protein in transformed myoblasts is unlikely to be due to low sensitivity of the immunofluorescence procedure. Hence, the CH-actin mRNA in transformed myoblasts appears to give rise to very low levels of CH-actin protein.

Figure 5.2. Myoblasts which express CH-actin mRNA do not contain detectable cardiac-actin protein. The monoclonal antibody, B4F8 (Lessard, 1988) reacts with all muscle-specific isoforms of actin including the CH-actin protein. In exponentially growing cultures consisting primarily of mononucleate myoblasts of L6AD1 cells (panel A) all cells were unstained with B4F8 except for the single multinucleate myotube present in this field (panel B). The culture shown in panels C and D consisted primarily of multinucleate myotubes, all of which appear to contain the muscle-actin protein. Panels A and C are phase contrast photographs of the fluorescence fields shown in panels B and D. The bar represents 50  $\mu$ m.

C

A



### 5.3. Discussion:

The CH-actin gene was transfected into rat L6 skeletal myoblasts and stable transformants were isolated. The level of CH-actin transcript varied between clones but changed little during the differentiation of myoblasts into multinucleate myotubes. Thus, transcription of the transfected the CH-actin gene appeared not to increase significantly in differentiating L6 cells. In addition, our results indicate that differentiating L6 myotubes express the rat skeletal-actin and not the cardiac-actin gene. Therefore, we suggest that L6 cells are not an appropriate culture system to study CH-actin regulation.

As was the case in the non-muscle cells derived from DMSO-treated P19(CH-actin) cultures (Chapter 4), we were unable to detect cardiac-actin protein in undifferentiated L6(CH-actin) transformants. These results suggest that either the CH-actin protein is synthesized in non-muscle cells at levels below the limits detectable by immunofluorescence, that the CH-actin protein is rapidly degraded in non-muscle cells, that the CH-actin protein is not incorporated into microfilaments, or that the CH-actin mRNA is not efficiently translated in non-muscle cells. We present additional evidence for the regulation of translation of the CH-actin mRNA in the appendix that follows.

5.4. Chapter 5 Appendix: Expression of a Transfected Chimeric Actin-tk Gene in Stably Transformed Rat L6 Cells

5.4.1. Introduction:

This appendix reports the results of experiments performed in collaboration with Dr. Martha Ruben. To investigate the regulation of the CH-actin gene in cells differentiating along the skeletal muscle lineage, we transfected some modified CH-actin gene constructs into rat skeletal myoblasts (Figure 5.3). We found that myoblast cell lines containing stably integrated copies of the chimeric actin-tk gene [designated L6(actin-tk) had actin-tk mRNA at comparable levels in myoblasts and myotubes. Furthermore, our observations on the steady state levels of the TK protein suggest that the actin-tk mRNA is more efficiently translated in differentiating myotubes.

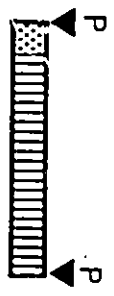
5.4.2. Results:

5.4.2.1. Detection of Actin-tk mRNA in L6(actin-tk) Transformants:

We prepared 3 chimeric genes (Figure 5.3) consisting of various amounts of the CH-actin promoter (3.1, 0.8, and 0.7 kb) plus the first non-coding exon (44 bp) and first intron (700 bp) of the CH-actin gene fused to the coding region of the

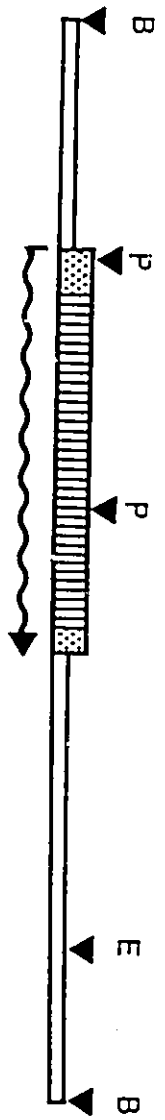
Figure 5.3. Structure of chimeric actin-tk genes. The actin-tk chimeric genes were constructed by linking restriction fragments from the CH-actin gene (solid region) containing various lengths of sequence upstream of the transcription start site, the 44 bp first exon (raised region) and 700 bp first intron to the coding region (striped region) of the tk gene. The CH-actin intron acceptor site was retained at the junction with the tk gene. The 800 bp PstI insert of pT4 was used as a tk-specific probe in Northern hybridization experiments. The wavy arrows indicate transcripts and exons are indicated as raised areas; P, PstI; E, EcoRI; B, BamHI.

pT4

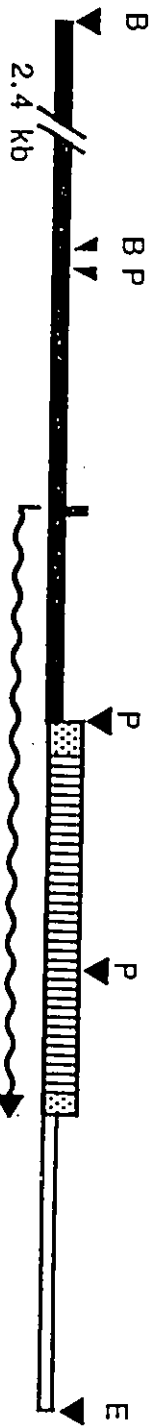


1.0 kb

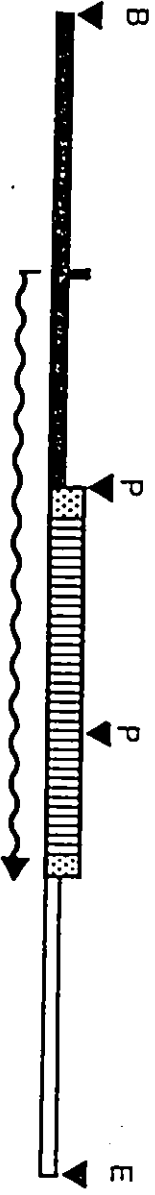
pX1



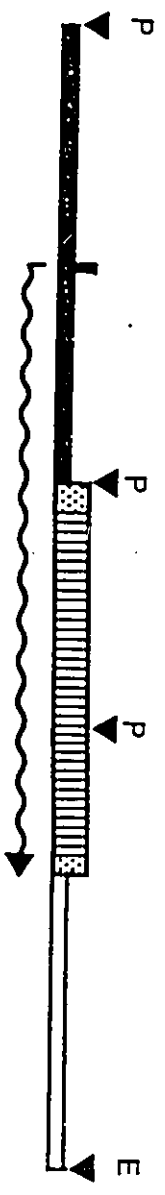
pUTK



pBTK



pPTK

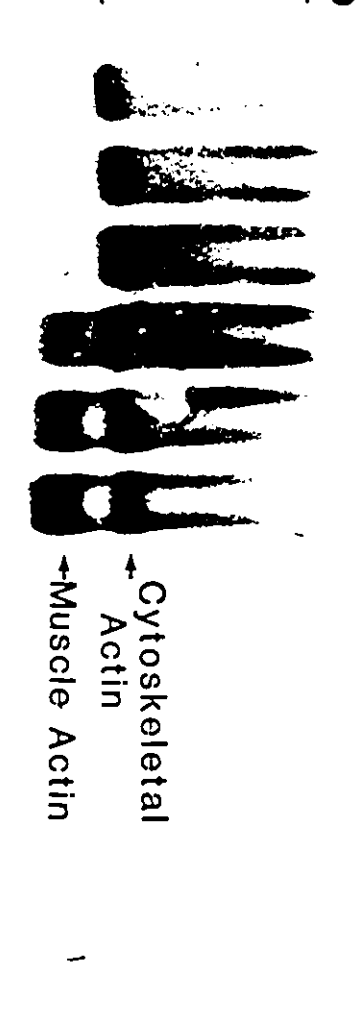
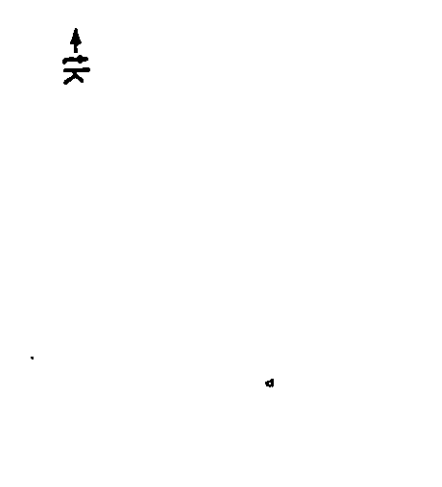
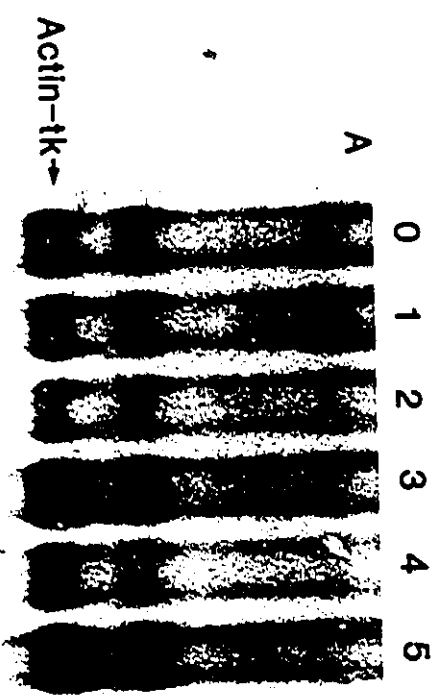


herpes simplex virus type I thymidine kinase (tk) gene. The chimeric gene should yield a transcript which, if properly spliced to remove the intron, would give rise to a chimeric mRNA consisting of 44 nt of non-coding sequence derived from the CH-actin 5'-untranslated (5'-UT) region followed by 1.8 kb of sequence from the tk gene including the TK coding region.

Six of the 9 clones of L6 cells transformed with the chimeric genes contained actin-tk mRNA (data not shown). The amounts of actin-tk mRNA varied from clone to clone but were not related to the number of copies of the chimeric gene or to the length of the CH-actin promoter sequence used to drive the chimeric gene (data not shown, see Ruben et al., 1988). In addition, the level of actin-tk transcript in each clone did not change significantly when the myoblasts differentiated into skeletal myotubes (data not shown, Figure 5.4 A).

Two transcripts appeared to be derived from the actin-tk genes. The more abundant was 1.8 kb, the size expected for the properly spliced actin-tk mRNA. The less abundant, more slowly migrating RNA species hybridized strongly to a probe for the CH-actin first intron (data not shown), indicating that this RNA species is the unspliced actin-tk transcript. Thus, the first intron of the CH-actin gene appears to be appropriately removed in the majority of the actin-tk pre-mRNAs.

Figure 5.4. The level of actin-tk mRNA changed little during the differentiation of L6U3 myoblasts into multinucleate myotubes. Total RNA was isolated at daily intervals from differentiating cultures of L6U3 (Panels A and B) and L6X22 (Panels C and D) cells. RNA was then electrophoresed in 1% agarose gels, transferred to nylon membranes, and hybridized to a tk-specific probe (pT4, Figure 5.3 A). These blots (panels A and C), were subsequently reprobred with the 600 bp PstI fragment of the CH-actin gene (Figure 4.1 A) which hybridizes to mRNA encoding cytoskeletal-actins and muscle-actins (panels B and D).



L6U3

L6X22

#### 5.4.2.2. Detection of TK Protein in L6(actin-tk)

##### Transformants:

To determine whether TK protein is expressed from the actin-tk mRNA, myoblasts and myotubes of two transformants containing high levels of the chimeric mRNA, L6U3 and L6P1, were examined by immunofluorescence using a polyclonal antiserum reactive with the TK protein (a gift from W. Summers, Yale University; Haarr et al., 1985). No TK protein was detected in the myoblasts of either line; however, when cells of both lines were induced to differentiate, specific nuclear staining became evident with the anti-TK antibody. The TK protein became readily detectable in nuclei of differentiating cells on day 3. At this time more than 30% of nuclei within these cultures stained with the anti-TK antibody (Table 5.1). Differentiation into myotubes occurred asynchronously and cells at various stages of differentiation were evident within the microscopic field shown in Figure 5.5. These differentiating cultures were stained for both TK protein and C/S-MHC protein (Bader et al., 1982). Both antigens were frequently present within the same myotubes but many myotubes, particularly those containing small numbers of nuclei, contained detectable TK but no C/MHC protein. The nuclei of myotubes containing small numbers of nuclei often stained very intensely with the anti-TK antibody while myotubes containing many nuclei were also stained but usually less intensely. All nuclei within a single myotube stained with similar intensities.

Table 5.1. Differentiating L6U3 cells express the TK protein

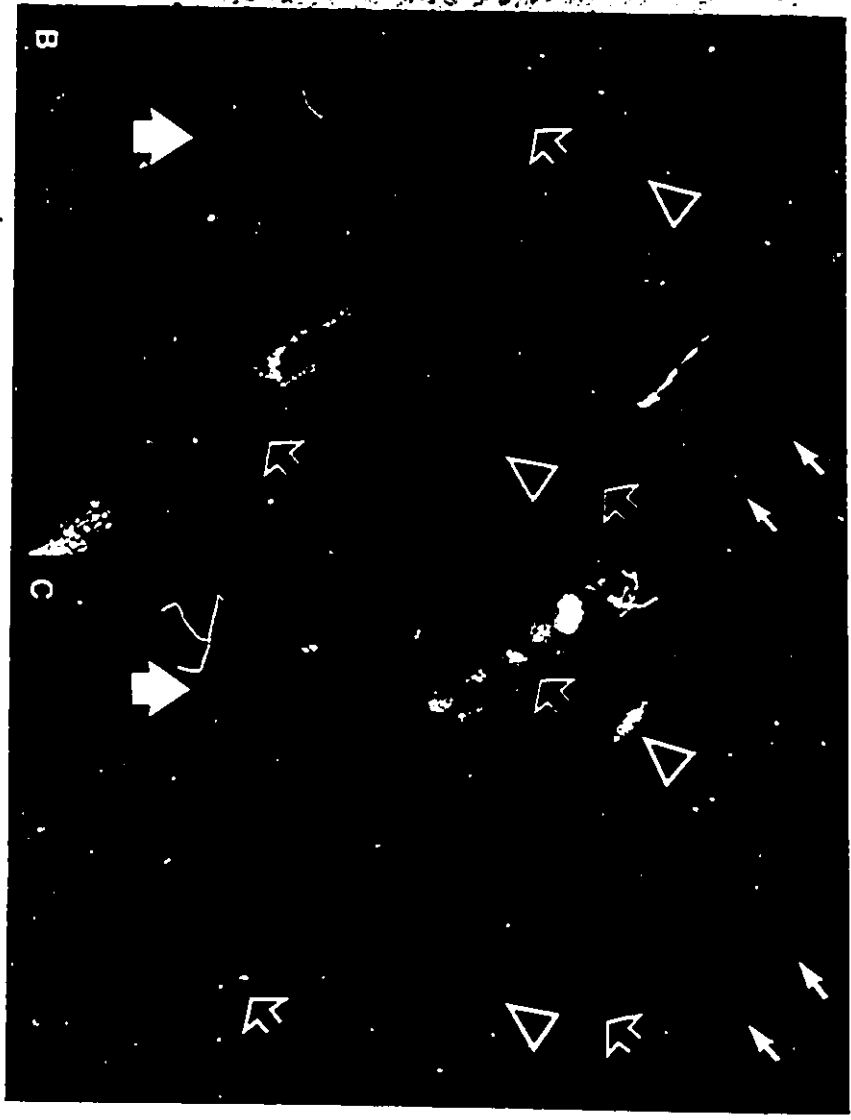
Day	% of cells with nuclear TK protein		
	<u>mononucleate</u>	<u>oligonucleate</u> <sup>A</sup>	<u>multinucleate</u> <sup>B</sup>
0	0.2	NA	NA
1	0.2	NA	NA
2	0.9	NA	NA
3	8.6	5.5	54.5
4	4.0	41.5	55.3
5	6.5	NA	52.2

A. Oligonucleate cells had 2 to 5 nuclei per cell.

B. Multinucleate cells had more than 5 nuclei per cell.

NA. Too few cells of this category were present to score.

Figure 5.5. Expression of the TK protein in differentiated L6(actin-tk) transformants. A culture of the L6U3 line 4 days after initiation of differentiation was fixed and stained for C/S-MHC protein (panel B) and TK (panel C) using monospecific antibodies. The same field is shown in phase contrast (panel A), rhodamine (panel B), and fluorescein (panel C) fluorescence. The small solid arrows point to 2 mononucleate myoblasts which did not stain with either antibody. The 3 open arrows indicate myotubes which stain with both antibodies while the 2 closed arrowheads and large solid arrow indicate cells containing TK protein but no C/S-MHC protein. Second antibodies were rhodamine conjugated anti-mouse and biotinylated anti-rabbit IgG. The biotin was then detected by binding of a fluorescein-streptavidin complex. Bar represents 50um.



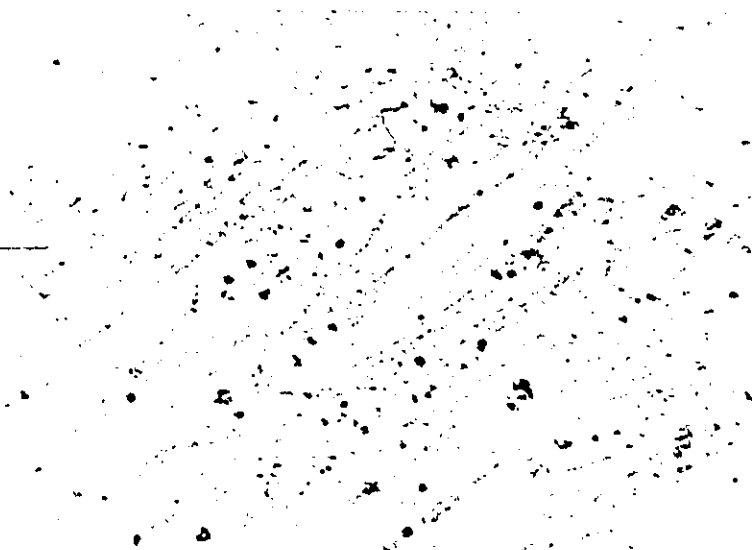
C

The marked increase in the number of cells containing TK protein appeared to occur in the absence of a significant increase in the amount of the actin-tk transcript (Figure 5.4 A). One possible explanation for the increased level of TK protein in myotubes is that the TK protein may be more stable in myotubes than in myoblasts. To investigate this possibility, we made use of the L6X22 cell line which was transformed with the pX1 plasmid (Figure 5.1 A) containing the intact tk gene with its own promoter. The amount of tk transcript in L6X22 cells decreased somewhat during differentiation (Figure 5.4 C) but was always present at concentrations at least as high as the actin-tk mRNA in L6U3 cells (determined by densitometric scanning of Northern blots such as those shown in Figure 5.4).

Immunofluorescence experiments with L6X22 cells revealed very low levels of the TK protein in the nuclei of both myoblasts and myotubes (Figure 5.6). Thus the selective accumulation of TK protein in myotubes appeared to occur only in those L6 clones containing the actin-tk mRNA and not in those containing the intact tk mRNA.

Figure 5.6. The TK protein was not abundant in differentiated L6X22 cells. L6X22 cells were induced to differentiate and after 5 days were fixed and stained for C/S-MHC protein (panel B) and TK (panel C) proteins as described in Figure 4.4. In contrast to L6U3 (Figure 5.5) and L6P1 (not shown) cells, the TK protein was below the limit of detection in differentiated myoblasts of L6X22 cells. Bar represents 50  $\mu$ m.

A



B



C

7

## 5.4.3. Discussion

The CH-actin and actin-tk genes were regulated at the level of mRNA accumulation in stably transfected embryonal carcinoma cells differentiating into cardiac muscle (Chapter 4; Rudnicki et al., 1988b). However, these same genes transfected into L6 cells yielded transcripts whose levels did not change significantly during myotube differentiation, suggesting that the CH-actin promoter was equally active in myoblasts and myotubes of this rat skeletal muscle cell line. Thus, the L6 cell line seems to be inappropriate for studies concerning the regulation of transcription of genes normally expressed only in embryonic skeletal or cardiac muscle.

In those clones of transformed L6 cells containing high levels of the CH-actin and actin-tk transcripts, the protein products were not detected before differentiation was initiated. All of our observations were of the steady-state levels of proteins detected by immunofluorescence, a procedure which may have relatively low sensitivity and for which quantification is difficult. Nevertheless, our evidence seems most consistent with the possibility that the CH-actin and actin-tk mRNAs were not efficiently translated in proliferating myoblasts. It is possible that sequences within the first intron, present within the chimeric actin-tk gene, somehow direct the transcript into a non-translatable intracellular compartment before or during

their removal by splicing. The processed actin-tk mRNA should contain only 44 nucleotides of CH-actin sequence, the first non-coding exon which comprises much of the 5'-UT region (Minty and Kedes, 1986). It seems more likely that this sequence is responsible for the differential translation of the actin-tk mRNA in L6 cells.

The TK protein was readily detected in myotubes containing actin-tk mRNA but was not detected in myotubes containing equal or higher concentrations of tk mRNA. Since the nucleotide sequences of these transcripts are identical except for the first 44 bases, these 44 nt appear to function in cis to activate translation in myotubes rather than to suppress translation in myoblasts.

In differentiating cultures of L6U3 and L6P1, many of the cells containing TK did not contain detectable C/S-MHC protein and the nuclei which stained most intensely for the TK protein were those from cells containing few (or only one) nuclei (see Figure 5.5). These observations suggest that, in differentiating cells, the TK protein initially appears earlier than C/S-MHC protein and, as differentiation proceeds, the abundance of the TK protein declines while the muscle specific proteins accumulate. If the 44 nt of the CH-actin 5'-UT region were regulating translation of the actin-tk mRNA, our interpretation of the kinetics of TK abundance would be that the factor(s) responsible for enhancing translation of muscle transcripts appears early in the myogenic program and, at these

early times, the actin-tk mRNA is readily translated because few other muscle-specific transcripts are present. As differentiation proceeds, more muscle transcripts accumulate and compete with the actin-tk mRNA for the translation factor(s) resulting in reduced rates of actin-tk mRNA translation.

5'-UT leader sequences are important in determining the rates of translation in vitro (Jobling and Gehrke, 1987) and in vivo (Johansen et al., 1984). This region of mRNA has also been implicated in translational regulation of viral (Logan and Shenk, 1984; Katz et al., 1986), heat shock (Hultmark et al., 1986; McGarry and Lindquist, 1985) and yeast mitochondrial transcripts (Costanzo and Fox, 1986; Thireos et al., 1986). The mechanisms by which the 5'-UT region could differentially regulate translation are not yet clear. One possibility is suggested by the work of McCarthy et al (1982) and Khandekar et al (1984) who found that the translation of chicken myosin mRNA was inhibited by small RNAs found in association with ribonuclear protein particles in skeletal and cardiac myoblasts. These RNAs can form RNA/RNA duplexes with the 3'- and 5'-UT regions of myosin mRNA suggesting that they may function as natural antisense RNAs (Heywood, 1986; Dasgupta et al., 1986). A similar mechanism may be responsible for the apparent translation control we have observed in L6 cells.

Endo and Nadal-Ginard (1987) have recently shown that differentiating L6 cells treated with EGTA to prevent fusion accumulate muscle-specific mRNAs but do not accumulate

muscle-specific proteins. These results suggest that expression of a large number of muscle gene products may be regulated at the translational level. In view of our results and the well known role of the 5'-UT region in regulating mRNA translation, it is tempting to speculate that the 5'-UT regions of transcripts from muscle genes share a sequence or structure which is responsible for regulating translation.

Chapter 6, Activated H-ras Expression Does Not Inhibit  
Differentiation of Cardiac and Skeletal Muscle from  
Embryonal Carcinoma Cells

6.1. Introduction:

The ras gene family encodes a set of proteins which are thought to be involved in the regulation of cell renewal and differentiation possibly by serving as mediators of growth factor induction of inositol phospholipid turnover (for reviews see Weinberg, 1985; Barbacid, 1987). The human H-ras oncogene isolated from a bladder carcinoma, contains a mutation that changes a glycine to a valine at amino acid position 12 (Shih and Weinberg, 1982). This activated H-ras oncogene can transform either cells from continuous lines (Taparowsky et al., 1982), or cells in primary cultures (Spandidos and Wilkie, 1984).

Activated ras oncogenes have been reported to either promote or inhibit the differentiation of certain cell types in vitro (Muller et al., 1983; Feramisco et al., 1984; Bar-sagi et al., 1985; Yoakum et al., 1985; Yuspa et al., 1985; Guerrero et al., 1986). In particular, some studies have suggested that activated ras oncogenes can prevent the differentiation of cultured lines of skeletal myoblasts (Olson et al., 1987; Payne et al., 1987).

P19 embryonal carcinoma (EC) cells are multipotential stem cells that can be induced to differentiate in cell culture. When P19 cells are aggregated and exposed to retinoic acid (RA), they differentiate into cultures which contain neurons, glia and fibroblast-like cells (Jones-Villeneuve et al., 1982; 1983). Previously, Bell has demonstrated that RA-treated P19 cells, stably transfected with an activated H-ras oncogene, differentiated into the same spectrum of cell types as the parental P19 cells (Bell et al., 1986). Within these RA-treated cultures, a subpopulation of differentiated fibroblast-like cells became transformed (immortal and anchorage-independent) with a low frequency. Nevertheless, these results suggested that it was unlikely that the H-ras gene plays a role in initiation of differentiation or in the choice of cell lineage.

After aggregation and exposure to dimethyl sulfoxide (DMSO), P19 cells differentiate into cultures which include 10% to 30% striated muscle cells, the majority of which resemble cells of the embryonic myocardium. The remaining muscle cells are skeletal myocytes that appear analogous to fetal skeletal myocytes (Chapter 3).

We set out to determine whether expression of the activated H-ras oncogene in transfected P19 cells would affect differentiation along the striated muscle lineages. To address this question, we transfected two plasmids containing the activated H-ras oncogene into P19 EC cells and selected for stable transformants.

## 6.2. Results:

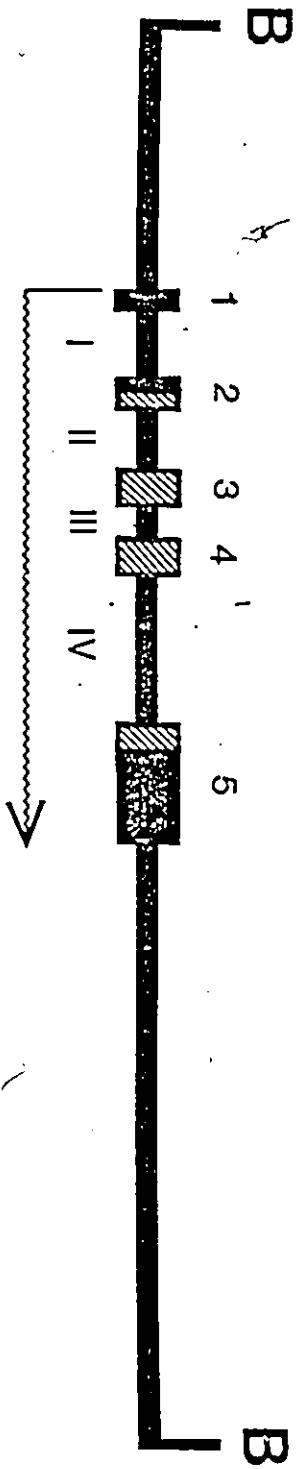
### 6.2.1. Construction and Activity of the H-ras Containing Plasmids:

The plasmid pEJ-neo (Bell et al., 1986) contains both the activated H-ras oncogene (Shih and Weinberg, 1982), and the neor gene (Figure 6.1). The plasmid pEJ(actin)-neo contains the human cardiac-actin (CH-actin) promoter upstream of the H-ras coding region (Figure 6.1). The CH-actin promoter sequences contain the regulatory regions required for myocyte-specific expression (Chapter 4; Rudnicki et al., 1988b).

To insure that the H-ras oncogene containing plasmids were capable of being expressed, the plasmids pEJ-neo and pEJ(actin)-neo were transfected into NIH/3T3 cells and the resulting number of transformed foci counted (Table 6.1). Plasmid pEJ(actin)-neo was 60% as active as pEJ-neo in transforming NIH/3T3 cells. Thus, functional ras protein was expressed from the H-ras genes in pEJ-neo and pEJ(actin)-neo.

Figure 6.1. Structure of the H-ras and chimeric actin-ras genes. The plasmid pEJ-neo contains the activated human H-ras gene subcloned into the BamHI site of pSV2-neo. In pEJ(actin)-neo, the first exon and intron of the H-ras gene were replaced with the 1.5 kb PstI fragment, containing 0.7 kb of upstream flanking sequence, the first exon (44 bp), and the first intron (0.7 kb) of the CH-actin gene. The PstI ends of the 1.5 kb fragment, and the SmaI site located 23 bp upstream of the H-ras ATG codon, were converted to EcoRI sites by the addition of linkers. Although in pEJ(actin)-neo, the splice acceptor site of the CH-actin first intron was lost, a consensus splice acceptor site located 14 bp upstream of the H-ras ATG codon was retained. Wavy lines with arrows indicate primary transcription. The five exons and four introns of the H-ras gene are numbered 1-5 and I-IV respectively. The first exon and intron of the CH-actin gene are numbered 1' and I' respectively. Solid areas of exons indicate untranslated regions and striped areas indicate coding regions. Both H-ras and CH-actin first introns are untranslated. B, BamHI sites; E, EcoRI sites.

PEJ-neo



PEJ(actin)-neo

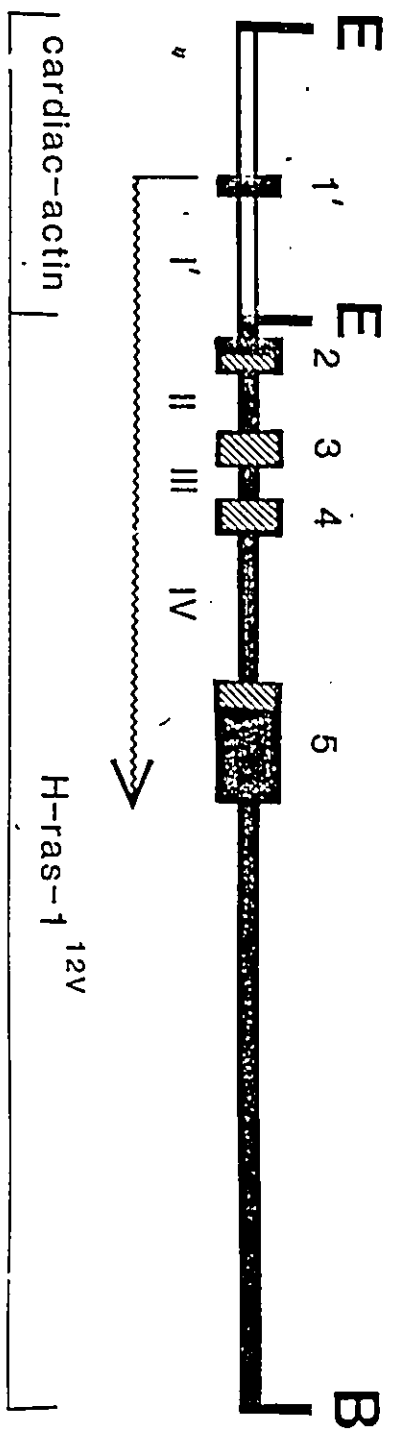


Table 6.1. NIH/3T3 Focus Formation Assay:

Transfection	Number of Foci
1. Untransfected	2
2. pEJ-neo	186
3. pEJ(actin)-neo	109

NIH/3T3 cells were transfected by the calcium-phosphate DNA precipitation method (Graham and van der Eb, 1973; Wigler et al., 1979) with 10 ug plasmid per 60 mm dish of subconfluent cultures. The next day,  $5 \times 10^5$  cells were plated into three 100 mm dishes. The foci were counted after 10 days.

Twenty transformed NIH/3T3 foci, formed following transfection with pEJ-neo and pEJ(actin)-neo, were pooled and expanded into cell lines. Northern blot analysis of total RNA isolated from these lines showed the presence of a low level of the actin-ras mRNA in NIH/3T3 cells transformed with pEJ(actin)-neo (Figure 6.2 A, lanes 1, 2 and 3). The sizes of the H-ras transcripts suggest that the transfected H-ras and actin-ras genes were correctly initiated and processed.

#### 6.2.2. Expression of the H-ras Containing Genes in Transfected P19 Cells:

Plasmids pEJ-neo and pEJ(actin)-neo were introduced into P19 cells by the calcium-phosphate precipitation method of DNA transfection (Graham and van der Eb, 1973; Wigler et al., 1979), and selected for neor expression in antibiotic G418. A P19 cell line stably transfected with pEJ-neo was designated P19(ras) whereas pooled P19 cells stably transfected with pEJ(actin)-neo were designated P19(actin-ras).

Untransfected P19 cells, the P19(ras) and P19(actin-ras) transformants formed colonies of EC cells on tissue-culture surfaces and in semi-solid medium (methyl cellulose) at similar efficiencies (Table 6.2). However, when cultured in methyl cellulose in the presence of RA, P19 gave rise to only a few small and slow growing colonies, whereas P19(ras) formed large anchorage-independent differentiated colonies at high frequency (Table 6.2 B; see Bell et al., 1986).

P19(actin-ras) transformants, when similarly cultured, gave rise to a similar yield of colonies as P19 (Table 6.2 B). These results suggest that the CH-actin promoter is not active in the cell lineage(s) transformed to anchorage-independence after RA-treatment of P19(ras) cells in semi-solid medium.

The P19(ras) transformant expressed high levels of H-ras mRNA and these levels remained unchanged during drug induced differentiation (data not shown). This cell line, when aggregated and exposed to dimethyl sulfoxide (DMSO) produced cultures which contained areas exhibiting spontaneous contractile activity and cells which stained with antibody MF20 (Bader et al., 1982), reactive with cardiac and skeletal muscle myosin heavy chain protein. Electron microscopic examination of DMSO-treated P19(ras) cultures (Performed by Dr. K. Reuhl), revealed myocytes containing abundant immature myofibrils with occasional Z-lines and the presence of intercalated disks between adjacent myocytes (not shown). Furthermore, bipolar skeletal myocytes were also evident (Figure 6.3). Thus, expression of the transfected activated H-ras gene in differentiating P19 cells did not appear to inhibit DMSO-induced striated muscle differentiation.

Table 6.2. Anchorage-Independent Growth

Cell Line	Plating (a) Efficiency	Colony number in (b)	
		<u>methyl cellulose</u>	
		-RA	+RA
1. P19	35%	39%	5%
2. P19( <u>ras</u> )	41%	44%	31%
3. P19( <u>actin-ras</u> )	56%	48%	6%

(a) To determine plating efficiency,  $10^3$  cells were seeded per 100 mm dish.

(b) Proportion of differentiated cells capable of growth in semi-solid medium. Dispersed cells ( $10^3$ ) were cultured in methyl cellulose with or without  $5 \times 10^{-7}$  M RA.

P19(ras) colonies were large and quick growing, P19(actin-ras) colonies were somewhat smaller and P19 colonies were quite small and did not increase in size.

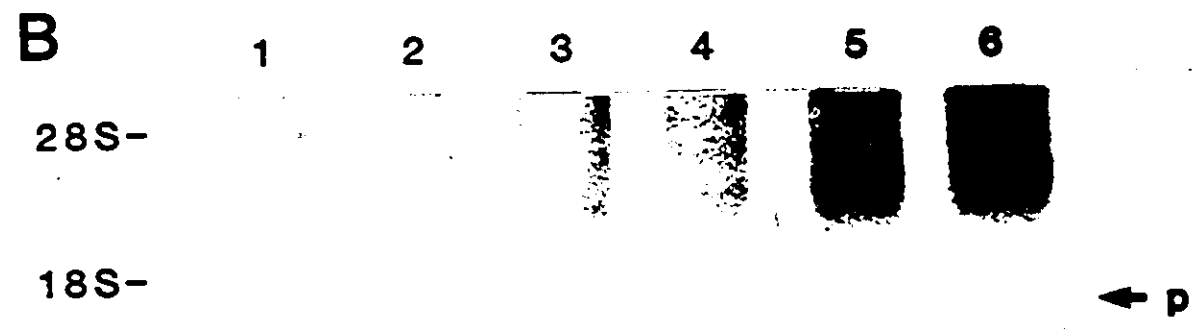
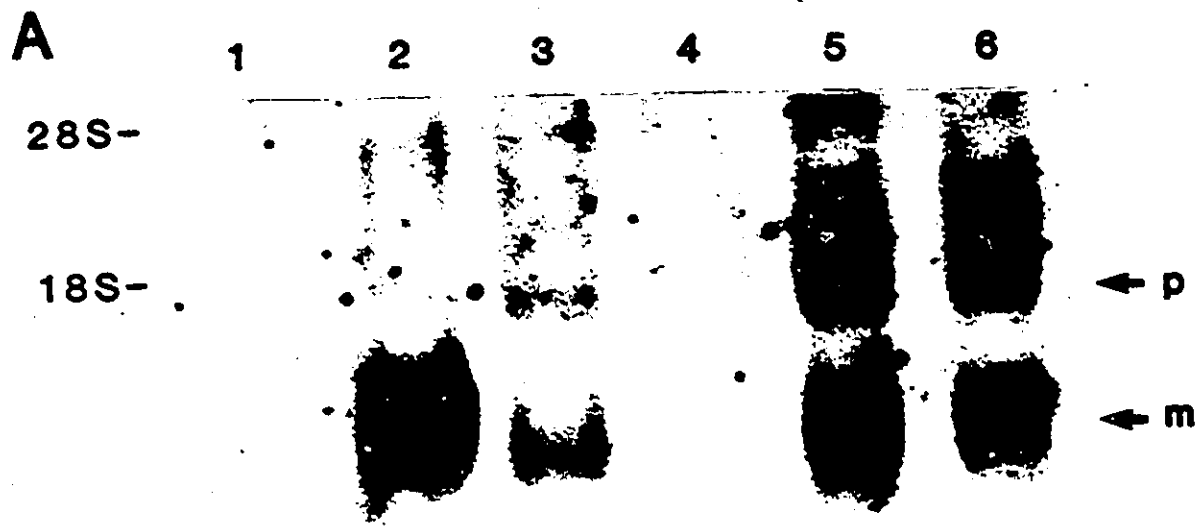
One explanation for the normal muscle differentiation we observed in P19(ras) cells was the possibility of low muscle cell-specific expression of the transfected H-ras oncogene. The CH-actin promoter contains sufficient sequence information to direct high level myocyte-specific transcription (Chapter 4; Rudnicki et al., 1988b). We therefore reasoned that the actin-ras chimeric gene construct should direct high levels of H-ras protein to myocytes of both the cardiac and skeletal type.

Total RNA was isolated from undifferentiated P19(actin-ras) transformants, and cultures 10 days after DMSO-treatment or RA-treatment. After differentiation, the RA-treated cultures contained neurons, glia and fibroblast-like cells (not shown), and the DMSO-treated cultures contained cardiac myocytes exhibiting spontaneous contractile activity; bipolar skeletal myocytes and mesenchyme-like cells after DMSO-treatment. Within DMSO-treated cultures, the 1.7 kb cardiac and skeletal-actin transcripts were readily detected by Northern hybridization (Figure 6.2, C). Thus, P19(actin-ras) transformants differentiated into the same spectra of cell types as the untransfected P19 line and the P19(ras) transformants.

Northern hybridization with a human H-ras specific probe (Figure 6.2, A, lanes 4, 5 and 6) indicated that a low level of actin-ras mRNA was present in the undifferentiated EC cells, that the level of this mRNA increased 12 fold in DMSO-treated cultures, and that this level only increased 3 fold after RA-treatment. The level of H-ras mRNA was normalized to the level of cytoskeletal-actin mRNAs, and both levels were determined by scanning densitometry of the autoradiogram shown in Figure 6.2. The small increase in actin-ras mRNA observed after RA treatment may be a consequence of expression of the transfected gene in a small proportion of non-muscle fibroblast-like cells. In any case, the northern analysis suggests that the CH-actin promoter was directing high-level transcription of the actin-ras gene in myocytes.

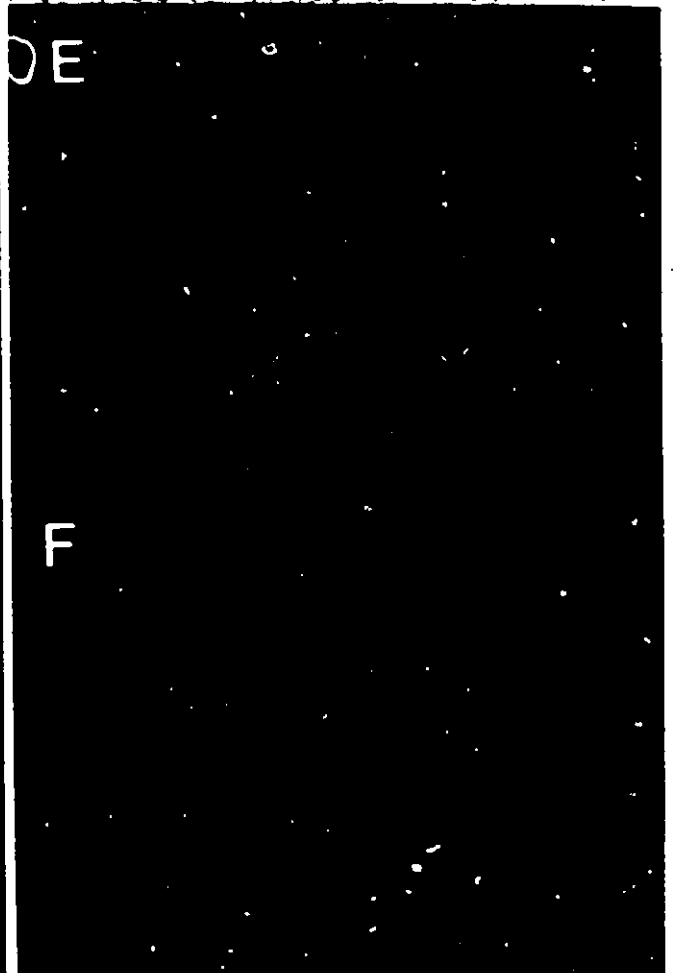
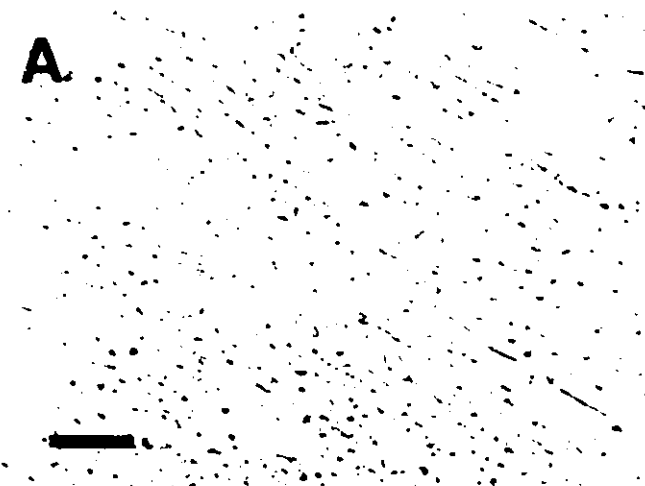
Two transcripts appeared to be derived from the chimeric actin-ras genes in P19(actin-ras) transformants. The smaller, more abundant transcript, was 1.05 kb, the size expected for the properly spliced actin-ras mRNA. The larger 1.7 kb transcript hybridized to a probe for the CH-actin first intron while the smaller 1.05 kb transcript did not (Figure 6.2 B). Thus, the larger less abundant transcript, appears to be an actin-ras mRNA species in which the H-ras introns but not the CH-actin first intron, have been spliced out. Nevertheless, the majority of the actin-ras transcripts appear to have the CH-actin first intron correctly removed.

Figure 6.2. Detection of H-ras mRNA by Northern hybridization analysis. The H-ras, actin-ras and actin transcripts were detected with either, (A) a human H-ras specific probe, (B) a CH-actin intron I specific probe, the 1.5 kb PstI fragment containing the promoter, first exon (44 bp) and first intron (0.7 kb) of the CH-actin gene, or (C) a probe which hybridizes all actin mRNAs, the 0.6 kb PstI fragment containing the last exon of the CH-actin gene (see Figure 4.1). Total mRNA (20 ug per lane) was isolated from; 1, NIH/3T3 cells; 2, NIH/3T3 transformed with pEJ-neo; 3, NIH/3T3 transformed with pEJ(actin)-neo, 4, undifferentiated P19(actin-ras) cells; 5, P19(actin-ras) cells after DMSO-induced differentiation which produces cultures that contain cardiac and skeletal muscle; and 6, P19(actin-ras) cells after RA-induced differentiation which produces cultures that contain neurons, glia and fibroblast-like cells. The positions of the pre actin-ras and mature actin-ras mRNAs are indicated on the right as p and m respectively. The positions of the 28S and 18S rRNAs are indicated on the left of each panel.



To examine the tissue-specific distribution of the activated H-ras protein in transfected P19 cultures, we performed immunofluorescence experiments with a monospecific antibody reactive with the activated H-ras protein containing a valine at amino acid position 12 (Figure 6.3). In DMSO-treated P19(ras) cells, the activated H-ras protein appeared to be present in low amounts in the plasma-membrane of most cells including myocytes (Figure 6.3, A, B and C). In DMSO-treated P19(actin-ras) cultures, the activated H-ras protein was located exclusively in cells that also contained an muscle-specific isoform of myosin light chain (Figure 6.3, D, E and F). The peripheral staining observed with the anti-myosin light chain antiserum was a consequence of formaldehyde fixation. In untransfected P19 cells and in undifferentiated and RA-treated P19(actin-ras) transformants, no staining with the antibody reactive with the activated H-ras protein was observed (data not shown).

Figure 6.3. Detection of the activated ras protein after muscle differentiation. P19(ras) (panels A, B and C), and P19(actin-ras) transformants (panels D, E and F), were induced to differentiate with DMSO, fixed and double stained with rabbit antiserum reactive with slow myosin light chain 1 protein (panels B and E) (G. Jackowski, personal communication), and mouse monoclonal antibodies reactive against the activated (valine at amino acid position 12) human H-ras protein (panels C and F) (DuPont, Mississauga, Canada). A and D are phase-contrast photographs of the corresponding fields. Second antibodies were texas red-conjugated anti-rabbit and biotinylated anti-mouse IgGs. The biotin was then detected by binding of a fluorescein-streptavidin complex. Bar represents 50 um.



### 6.3. Discussion:

Our data has demonstrated that neither ubiquitous expression nor high level muscle-specific expression of the activated H-ras oncogene blocked differentiation along the striated muscle lineages. This suggests that the mechanisms which activate muscle differentiation are unaffected by the presence of the activated H-ras protein in differentiating P19 cultures. These results are in contrast to other studies where activated H-ras expression has been shown to prevent the differentiation of continuous skeletal myoblast cell lines (Olson et al., 1987; Payne et al., 1987). The ability of the activated H-ras oncogene to interfere with myoblast differentiation may require the expression of other complementing factor(s).

The characteristics of skeletal myoblast cell lines that allow activated H-ras expression to interfere with the muscle differentiation program may be a consequence of the selection in cell culture for the continuous growth or immortality of the myoblast cells. Perhaps, these myoblast cell lines have abnormal patterns of cellular proto-oncogene expression which confer immortality. For example, the rat skeletal myoblast L6 cell line expresses high levels of c-myc, c-fos, N-ras and K-ras (Leibovitch et al., 1987). The mouse myogenic cell line C2, transfected with the activated H-ras oncogene, expressed 2 to 3

fold higher levels of the endogenous c-myc mRNA (Olson et al., 1987). The tumorigenic conversion of primary embryo fibroblasts requires the co-expression of ras and myc oncogenes (Land et al., 1983). In an analogous fashion, in immortal myoblast cells the activated H-ras oncogene may be complemented by expression of endogenous proto-oncogene(s) to block myocyte differentiation.

P19 cells, when induced to differentiate, give rise to anchorage-dependent mortal cells (Bell et al., 1986). In P19 transformants containing either the H-ras or actin-ras genes, normal muscle differentiation occurred after DMSO-treatment. Therefore, we suggest that the primary muscle progenitor cells derived from DMSO-treated P19 cultures, do not express the factor(s) which complement inhibition of differentiation in continuous myoblast cell lines expressing an activated H-ras oncogene. Thus, the multipotential P19 cell system, like transgenic mice (Stewart et al., 1984; Adams et al., 1985; Leder et al., 1986; Hanahan, 1986; Palmiter and Brinster, 1986; Quaife et al., 1987; Sinn et al., 1987), may be a more natural model to study the effects of oncogene expression on cell growth and differentiation.

Chapter 7, Isolation of Mesodermal Progenitor Stem Cell Lines  
from Differentiating Embryonal Carcinoma Cultures

7.1. Introduction:

P19 cells are multipotential EC cells that can give rise to many different types of differentiated cell types both in cell culture (Rudnicki and McBurney, 1987) and in vivo (Rossant and McBurney, 1982). After aggregation and exposure to dimethyl sulfoxide (DMSO), P19 differentiates into cells normally derived from the embryonic mesoderm. DMSO-induced cultures contain 10-30% cardiac myocytes, mesenchyme-like cells, and no neural derivatives (see Chapters 1 and 3).

In differentiating P19 cultures, the multipotential EC cells presumably become committed stem cells with limited potential prior to terminal differentiation. Some of these committed stem cells, like hematopoietic stem cells (Clarkson et al., 1978), or skeletal muscle satellite cells (Goldspink, 1974), may have some capacity for self-renewal in vitro.

In this chapter, we describe the isolation and characterization of two non-EC cell lines from P19 cultures induced to differentiate with DMSO. These cell lines are novel in that they appear to give rise to more than one cell type and thus may represent a stem cell with limited mesodermal developmental potential.

## 7.2. Results:

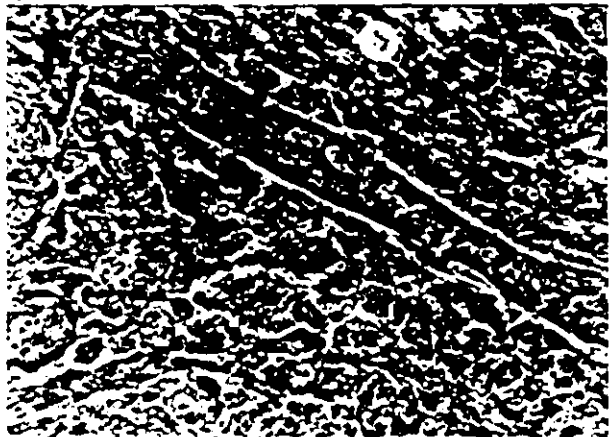
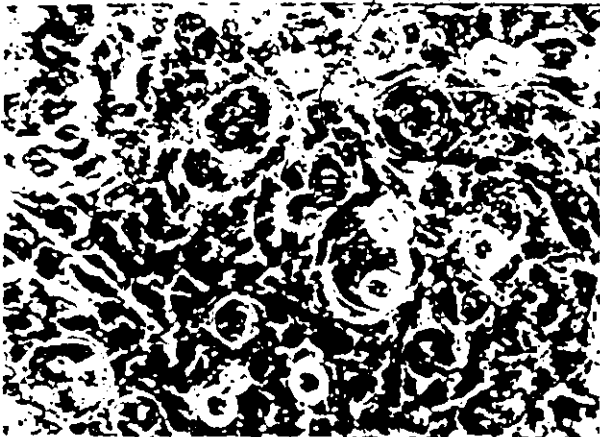
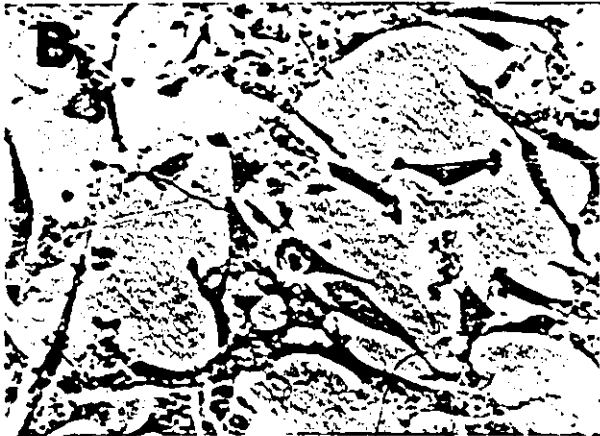
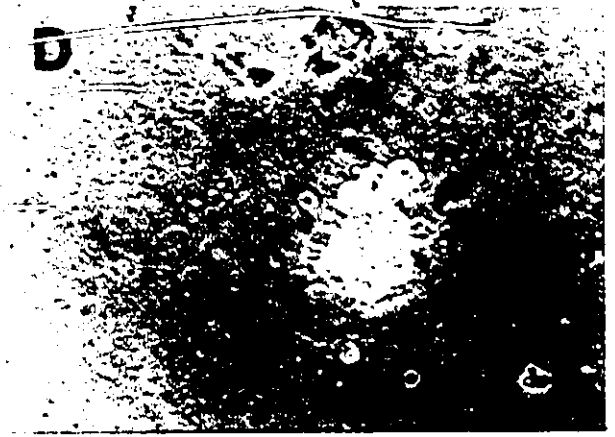
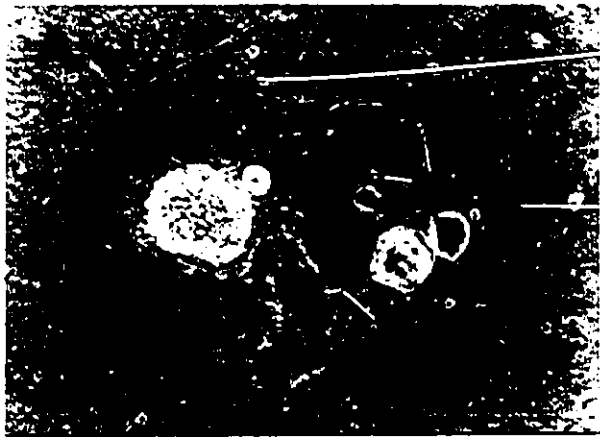
### 7.2.1. Isolation of Myogenic Cell Lines:

One of the characteristics of multipotential EC stem cells is the ability for anchorage-independent growth (Rodrigues et al., 1985; Bell et al., 1986). We were interested in cloning stem cells that had passed at least part way down the differentiation pathway and yet retained the capacity for self renewal. We assumed that these stem cells with limited potential might have at least some ability for anchorage-independent growth. We therefore selected for cells derived from differentiating P19 cultures which would grow in semi-solid medium.

P19 cells were induced to differentiate with DMSO using the standard regime. On day 7, beating muscle cells were evident. We dispersed these seven day old cultures into single cell suspensions, and cultured  $2 \times 10^3$  cells per ml in methyl cellulose. After 10 days of growth in methyl cellulose, about 1-2 per  $10^3$  cells seeded had formed small colonies (Figure 7.1, panels A and D). A number of these colonies were then picked and expanded into cell lines.

Eighteen cell lines with fibroblast-like morphology (Figure 7.1, panels B and E), were derived in this fashion. These cells were no longer EC cells since they did not react with the anti-EC antibody AEC3A1-9 (Harris et al., 1984). Seven of the lines after becoming confluent exhibited varying degrees

Figure 7.1. Isolation of continuous myogenic cell lines. P19 cells were aggregated and exposed to DMSO for five days after which the aggregates were transferred into tissue culture grade dishes. On the 7th day, cells were dispersed and seeded into methylcellulose. After 7-10 days anchorage-independent colonies were picked and expanded into cell lines. Two myogenic cell lines, MR322 (A, B and C) and MR16 (D, E and F), isolated in this way are shown here. All fields are phase-contrast. A and D are colonies after 8 days growth in methylcellulose. B and E are subconfluent cultures. C and F are post-confluent cultures containing myotubes. Bar represents 50 um.



of myotube formation and contractile activity. Two of the myogenic cell lines (designated as MR16 and MR322) were chosen for further investigation.

In confluent MR16 and MR322 cultures, cell fusion was observed and after a week, areas of contractile activity were evident (Figure 7.1, panels C and F). The cell lines formed myocytes with different morphologies. MR16 myocytes tended to be bipolar and formed long myotubes whereas MR322 myocytes tended to be rounded and only a few were bipolar. Both mononucleate and multinucleate myocytes were present in both cultures.

Both MR16 and MR322 formed colonies in methyl cellulose at an efficiency of about 22% of their plating efficiency on tissue culture plastic (Figure 7.1, panels A and D). Thus, these cell lines display a diminished ability for anchorage-independent growth as compared to the parental undifferentiated EC cells (Bell et al., 1986). In addition, MR16 and MR322 have been cultured for over 20 passages under subconfluent conditions without loss of developmental potential.

#### 7.2.2. Microscopic Examination:

The results reported in this section were performed in collaboration with Dr. Kenneth Reuhl. Light microscopic examination of thick sections of differentiated MR16 and MR322 cultures stained with toluidine blue revealed multiple layers of cells with different morphologies. The myocytes were identified by the presence of multiple nuclei within an enlarged dense

cell soma. The non-muscle cells were of a uniform size and contained spherical nuclei and a vacuolated cytoplasm. The extracellular space between these mesenchyme-like cells was filled with matrix material (not shown).

Electron microscopic examination of these cultures demonstrated the presence of extensive arrays of myofibrils within the myocytes that were often multinucleated, and the presence of fibrous matrix material in the intercellular space between the mesenchyme-like cells. The fibres within the matrix resembled the thin collagen fibrils found in developing cartilage (Hay, 1981; 1982). Furthermore, the non-muscle mesenchyme-like cells were vacuolated and contained well developed golgi (not shown).

Thus, differentiated MR16 and MR322 cultures contained at least two cell types, myocytes and an extracellular matrix (ECM) secreting mesenchyme-like cell. Moreover, since nine subclones of MR16, and six subclones of MR322 exhibited the same developmental potential, we suggest that the myocytes and mesenchyme-like cell are derived from a common stem cell.

#### 7.2.3. Detection of Myosin Proteins:

Since most of the myocytes within DMSO-treated P19 cultures are of the cardiac type (Chapter 3; Rudnicki et al., 1988a), it was possible that MR16 and MR322 may also give rise to cardiac myocytes. However, the presence of multinucleate myotubes within differentiating cultures

Figure 7.2. Detection of MLC proteins in MR16 myocytes. Panels A and D are phase contrast fields of post-confluent cultures of MR16 double stained with either rabbit anti A-MLC2 antiserum (B), or rabbit anti V-MLC1 antiserum (E), and mouse monoclonal antibody MF20 (C and F), reactive with C/S-MHC protein. Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgGs. Bar represents 50 um.

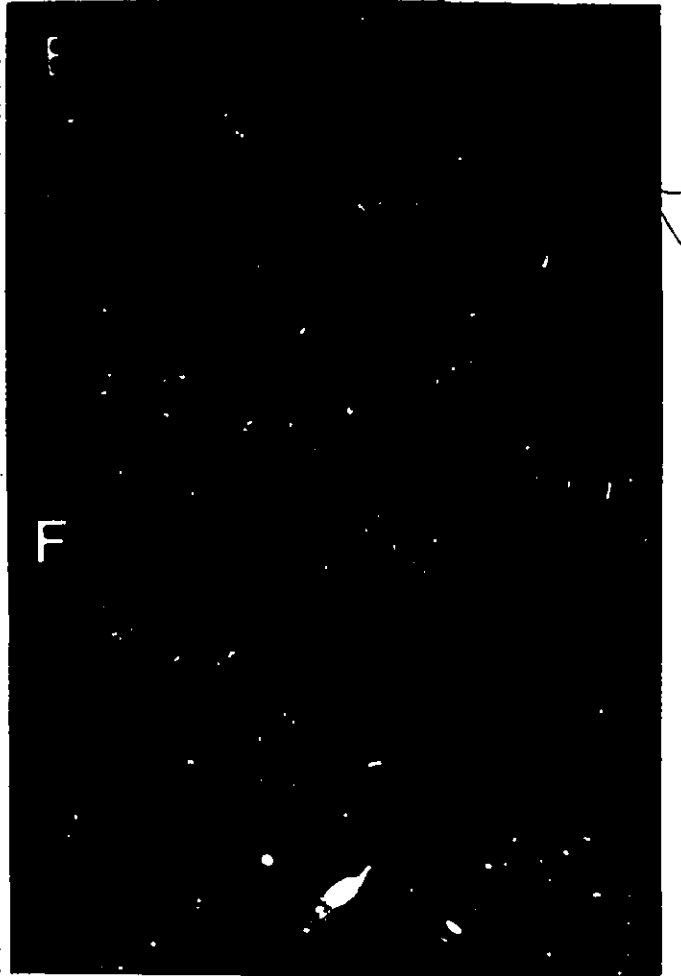
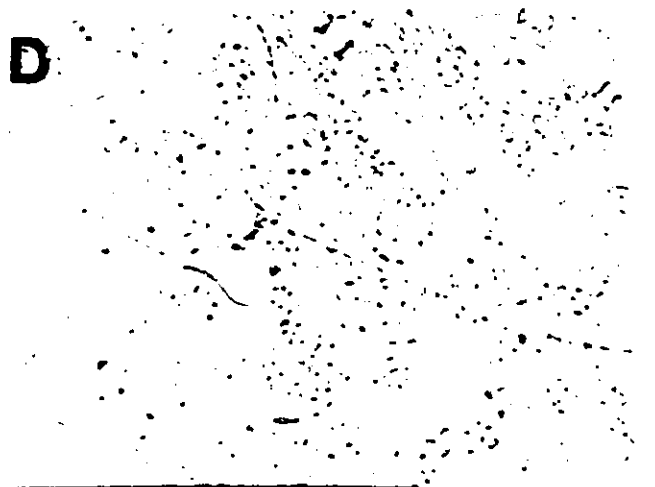
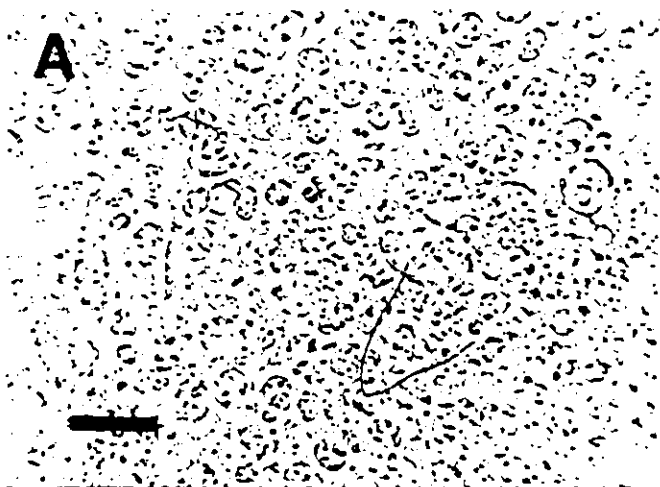


Figure 7.3. Detection of MLC proteins in MR322 myocytes. Panels A and D are phase contrast fields of post-confluent cultures of MR322 double stained with either rabbit anti A-MLC2 antiserum (B), or rabbit anti V-MLC1 antiserum (E), and mouse monoclonal antibody MF20 (C and F), reactive with C/S-MHC protein. Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgGs. Bar represents 50 um.

A



D

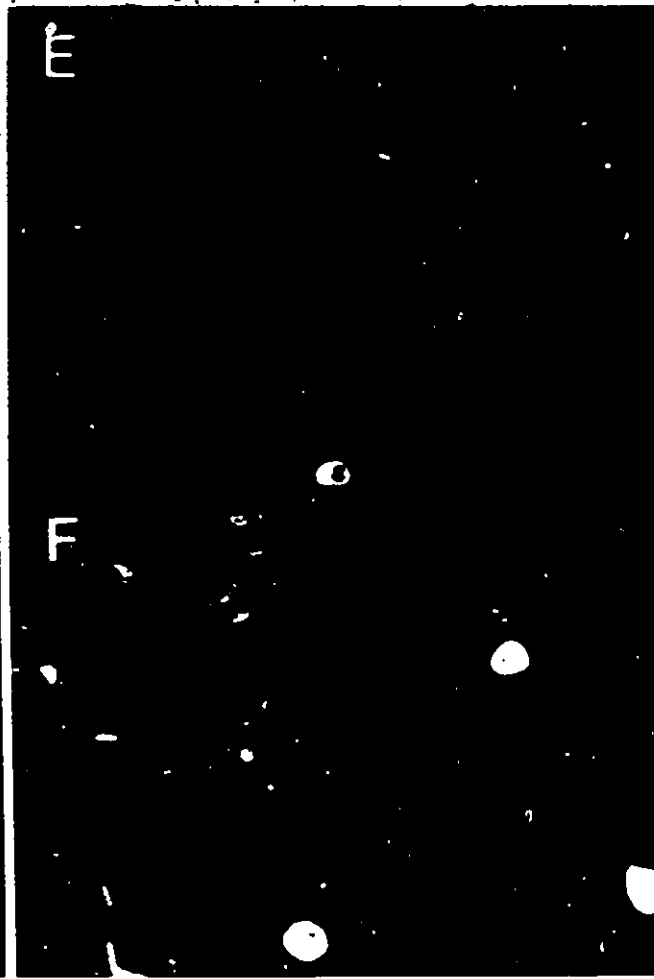


Table 7.1. Summary of Muscle Antigens Detected by Indirect Immunofluorescence Experiments:

Antibody Specificity	MR16	MR322	Reference
1. C/S-MHC	+	+	Bader et al., 1982
2. A-MLC2	-	-	G. Jackowski*
3. V-MLC1	+	+	G. Jackowski*
4. A-MHC	-	-	Gorza et al., 1988
5. V-MHC	+/-	+/-	G. Jackowski*

C/S-MHC, cardiac and skeletal muscle-specific myosin heavy chain; A-MLC2, atrial-myosin light chain 2; V-MLC1, ventricular-myosin light chain 1, A-MHC, atrial-myosin heavy chain; V-MHC, ventricular-myosin heavy chain.

\*G. Jackowski, in preparation.

suggested that the myocytes were of the skeletal type. To further characterize these muscle cells, we examined the expression of muscle genes by immunofluorescent staining using the antibodies listed in Table 7.1.

The mononucleate cardiac muscle cells in DMSO-treated P19 cultures co-expressed cardiac myosin proteins, atrial-myosin light chain 2 (A-MLC2), ventricular-MLC1 (V-MLC1), atrial-MHC (A-MHC), and ventricular-MHC (V-MHC). Furthermore, the bipolar multinucleate skeletal myocytes within these same cultures, only stained with anti V-MLC antiserum which cross reacts with skeletal muscle slow-MLC1 (Chapter 3; Rudnicki et al., 1988a).

In immunofluorescence experiments the myocytes within differentiated MR16 and MR322 cultures all labeled with the mouse monoclonal antibody MF20 (Bader et al., 1982), reactive with all forms of cardiac and skeletal muscle MHC protein (C/S-MHC) (Figures 7.2 and 7.3). The cardiac-specific A-MLC2 protein was not detected in any myocytes within differentiating MR16 and MR322 cultures (Figures 7.2 and 7.3, panels A, B and C). In contrast, the anti V-MLC1 antisera labeled all of the myocytes which also contained C/S-MHC protein (Figures 7.2 and 7.3, panels D, E and F).

The cardiac-specific A-MHC protein was not detected in any myocytes within differentiating MR16 and MR322 cultures (Figures 7.4 and 7.5, panels A, B and C). However, low levels of staining with anti V-MHC antibodies was observed in a subpopulation of the muscle cells (Figures 7.4 and 7.5, panels

Figure 7.4. Detection of MHC proteins in MR16 myocytes. Panels A and D are phase contrast fields of post-confluent cultures of MR16 double stained with rabbit anti V-MLC1 antiserum (B and E), and with mouse monoclonal antibodies reactive against either A-MHC (C), or V-MHC proteins (F). Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgGs. Bar represents 50  $\mu$ m.

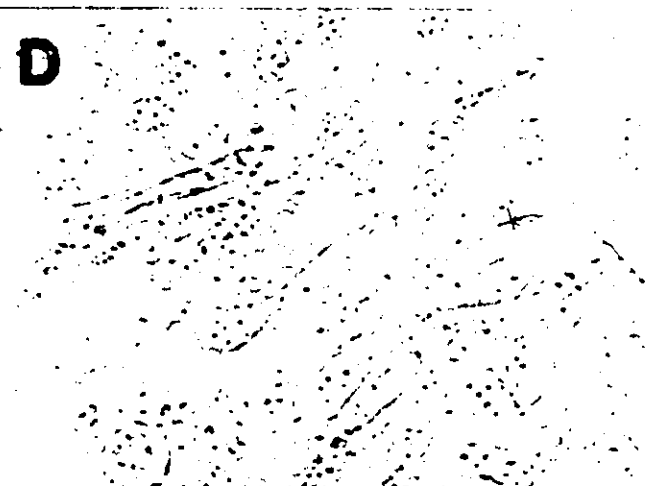
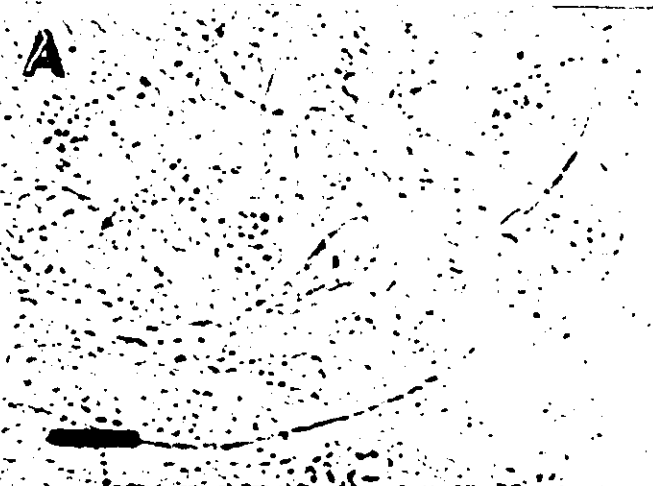
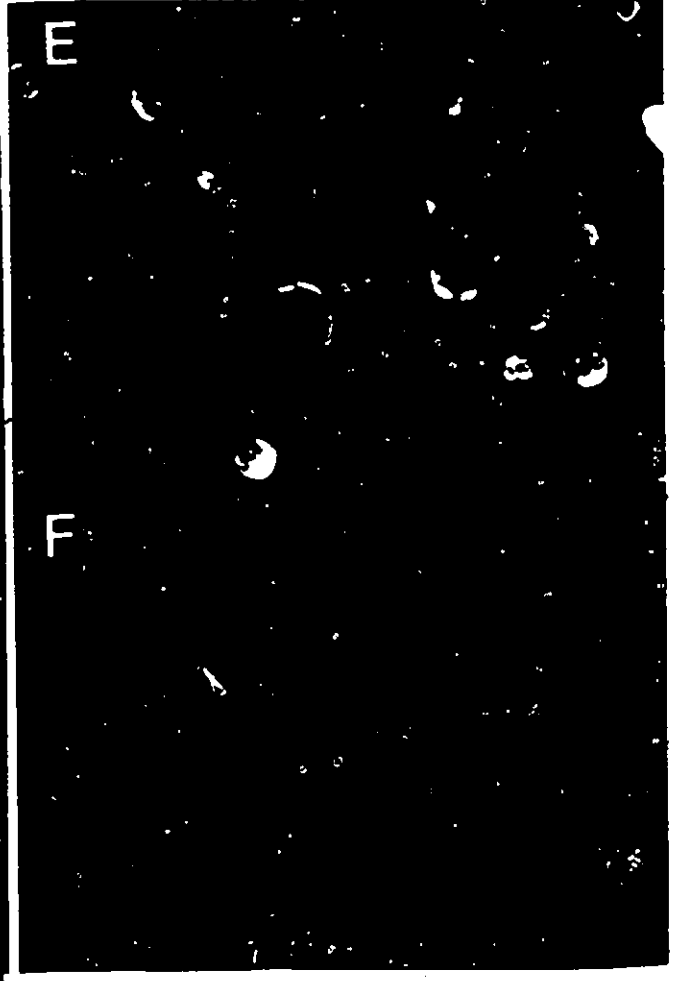
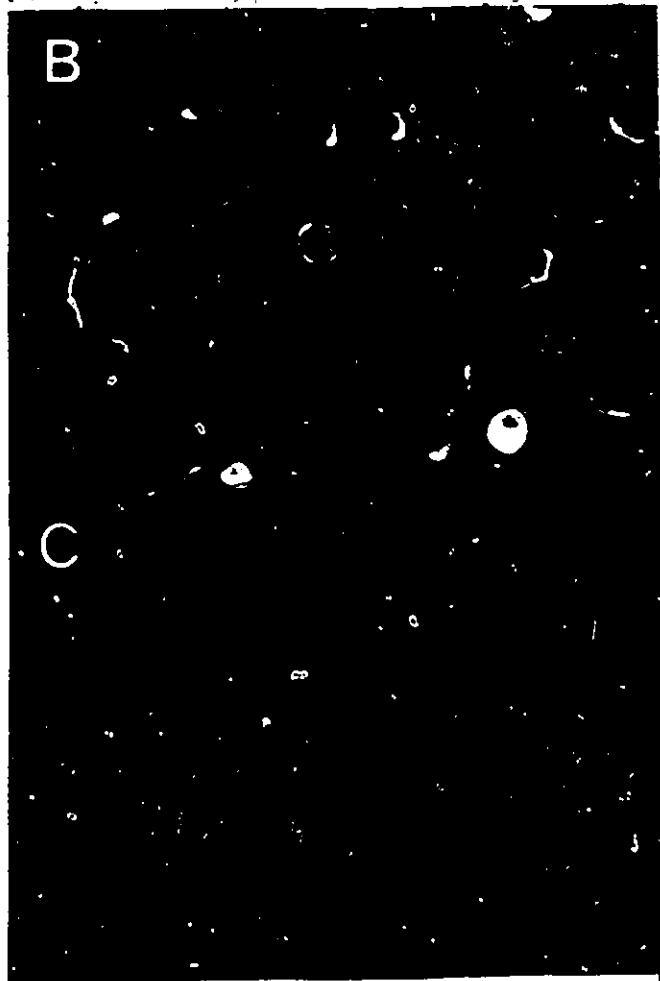
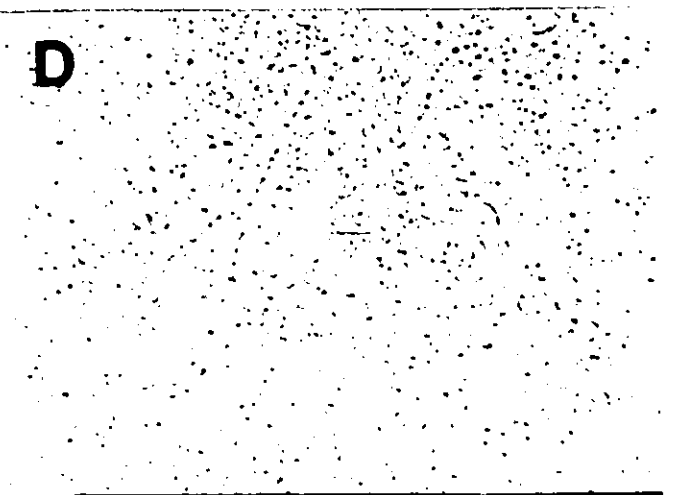
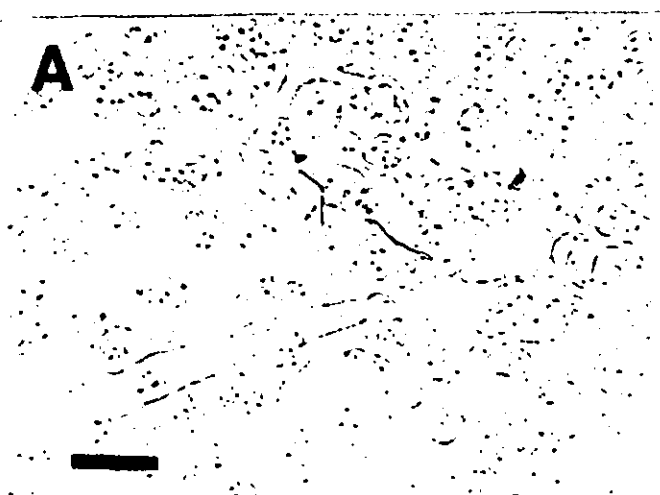


Figure 7.5. Detection of MHC proteins in MR322 myocytes. Panels A and D are phase contrast fields of post-confluent cultures of MR322 double stained with rabbit anti V-MLC1 antiserum (B and E), and with mouse monoclonal antibodies reactive against either A-MHC (C), or V-MHC proteins (F). Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgGs. Bar represents 50 um.



D, E and F). This staining may be a consequence of similarities between the V-MHC and the skeletal-muscle slow (type I)-MHC proteins (Buckingham et al., 1986).

These results (summarized in Table 7.1) demonstrate that the multinucleate myocytes in differentiating MR16 and MR322 cultures do not express the cardiac-specific A-MLC2 and A-MHC isoforms. Therefore, we suggest that these myocytes are dissimilar to the cardiac myocytes derived after DMSO-treatment of P19 cells and resemble embryonic skeletal muscle.

#### 7.3.4. Detection of Muscle-Specific mRNAs:

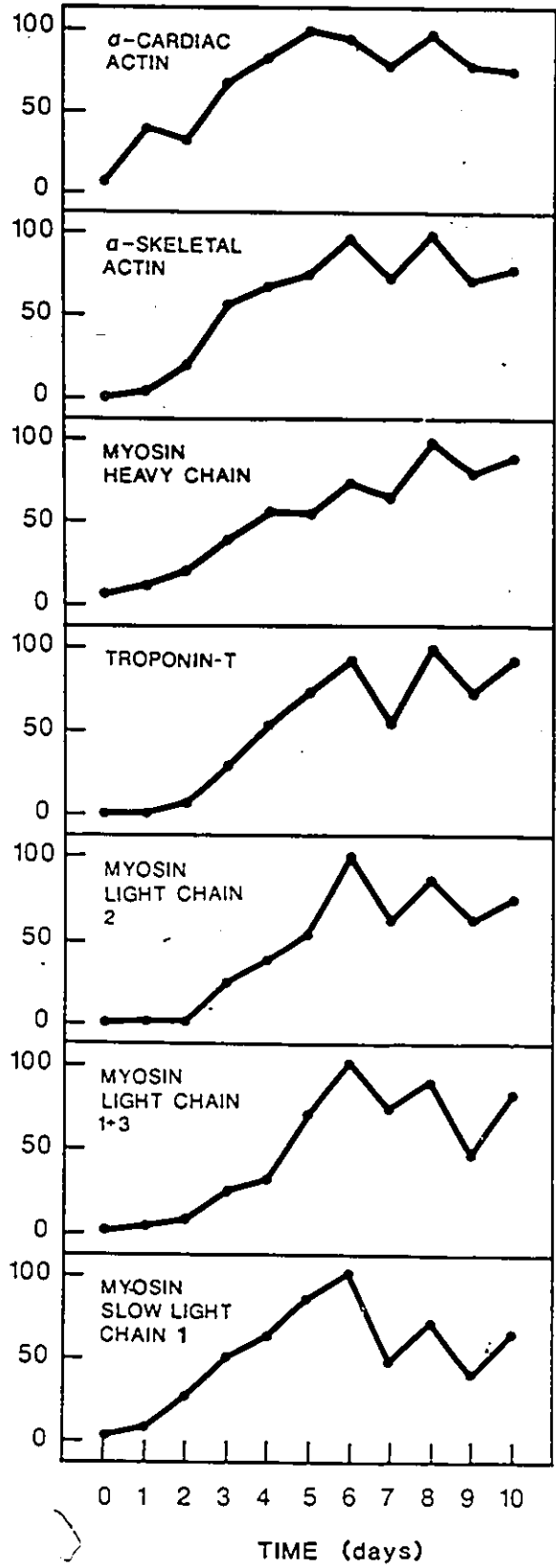
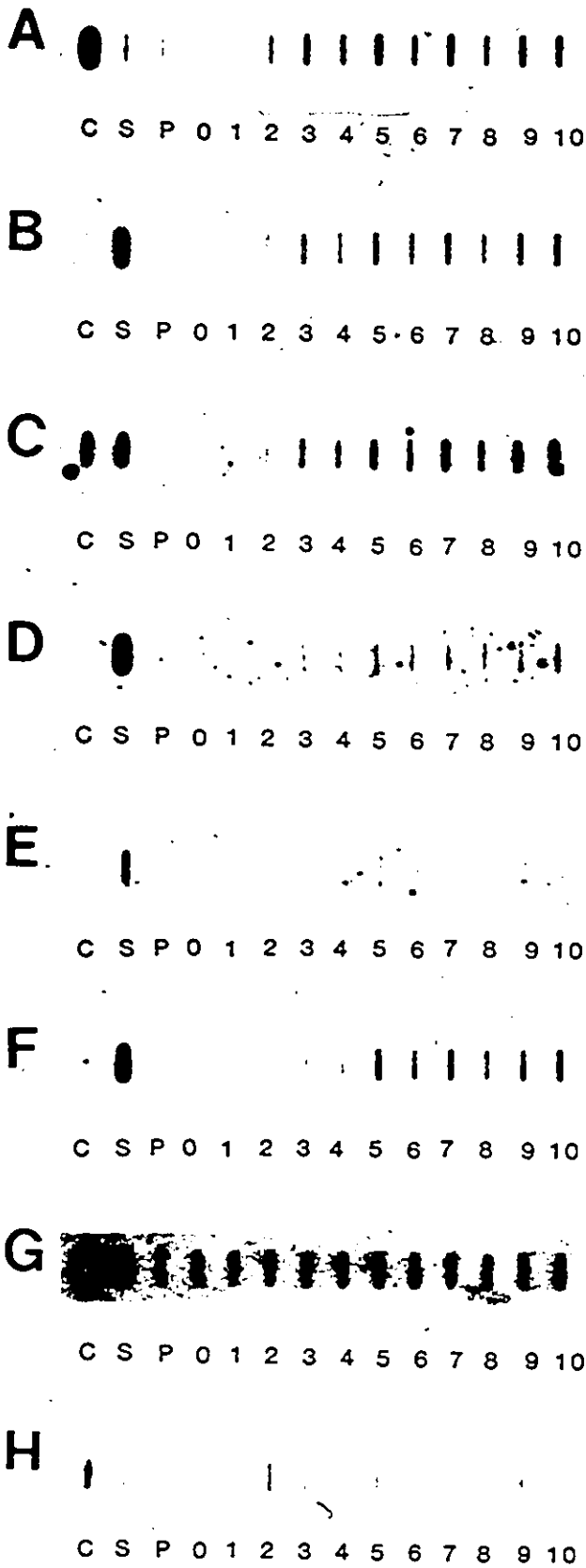
To further characterize the myocytes within differentiating MR16 and MR322 cultures, we examined the kinetics of accumulation of muscle-transcripts by slot blot analysis with a panel of DNA probes. The level of alpha-tubulin mRNA remained relatively constant and was used to control for the amount of RNA in each slot (Figures 7.6 H and 7.7 H).

Cardiac-actin and skeletal-actin transcripts were co-expressed and appeared to increase in level with time (Figures 7.6 and 7.7, A and B). The accumulation of the skeletal-actin mRNA appeared to lag slightly behind that of the cardiac actin mRNA. This pattern of accumulation was different from that observed during cardiac-myocyte differentiation where the cardiac- and skeletal-actin mRNAs appeared abruptly and decreased rapidly thereafter (Chapter 3; Rudnicki et al., 1988a).

Figure 7.6. Detection of muscle-specific transcripts within differentiating MR16 cultures. Slot blot analysis of total RNA, isolated at daily intervals after cultures had reached confluency, probed with a number of muscle-specific probes:

- (A) Hybridization to a cardiac-actin isotype specific probe.
- (B) Hybridization to a skeletal-actin isotype specific probe.
- (C) Hybridization to rat embryonic myosin heavy chain (MHC) cDNA.
- (D) Hybridization to the rat skeletal-muscle TNT cDNA.
- (E) Hybridization to the rat skeletal-muscle myosin light chain 2 (MLC2) cDNA.
- (F) Hybridization to the rat skeletal-muscle MLC1&3 cDNA.
- (G) Hybridization to the human V-MLC1 cDNA 3'-UT region which hybridizes the slow-MLC1 mRNA.
- (H) Hybridization to the mouse alpha-tubulin cDNA.

Total RNA was isolated from: C, mouse cardiac muscle; S, mouse skeletal muscle; P, undifferentiated P19 cells; 0 to 10, days during MR16 differentiation. The relative mRNA levels were determined by scanning densitometry of the autoradiograms and were plotted as the percentage of the maximum level of expression normalized to the level of tubulin mRNA.



mRNA LEVELS (arbitrary units)

TIME (days)

The level of MHC and slow-MLC1 transcripts increased during MR16 and MR322 differentiation (Figures 7.6 and 7.7, C and G). In P19 cultures induced to differentiate with DMSO, the level of MHC mRNA also increased with time but the V-MLC1 mRNA appeared abruptly and decreased rapidly thereafter (Chapter 3; Rudnicki et al., 1988a).

The skeletal-muscle specific transcripts, troponin-T, myosin light chain 2 and myosin light chains 1 and 3, were all present and accumulated with similar kinetics (Figures 7.6 and 7.7, D, E and F). These transcripts were not detected in DMSO-treated P19 cultures (Chapter 3; Rudnicki et al., 1988a).

We therefore conclude that MR16 and MR322 myocytes are dissimilar to the cardiac myocytes derived after DMSO-treatment of P19 cells. The results of the immunofluorescent staining experiments and the Northern hybridization analysis demonstrate that the myocytes within differentiating MR16 and MR322 cultures closely resemble fetal skeletal muscle cells.


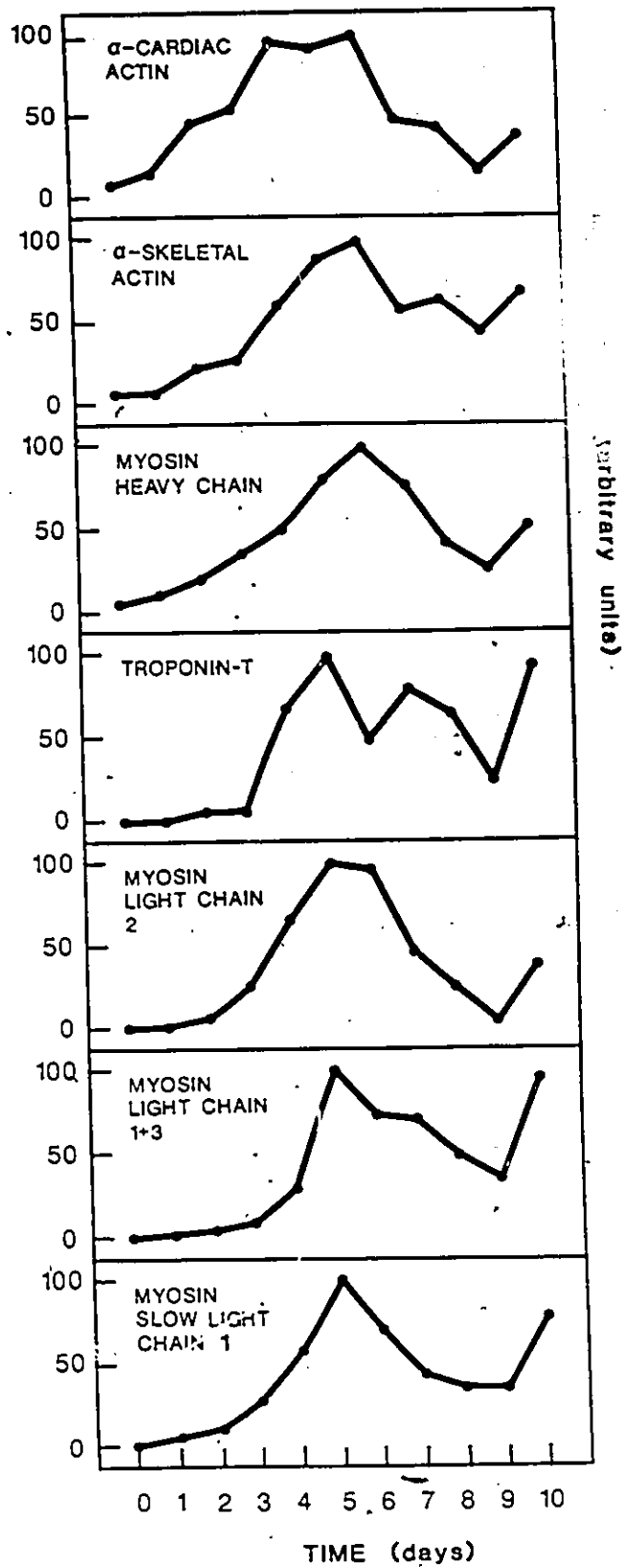
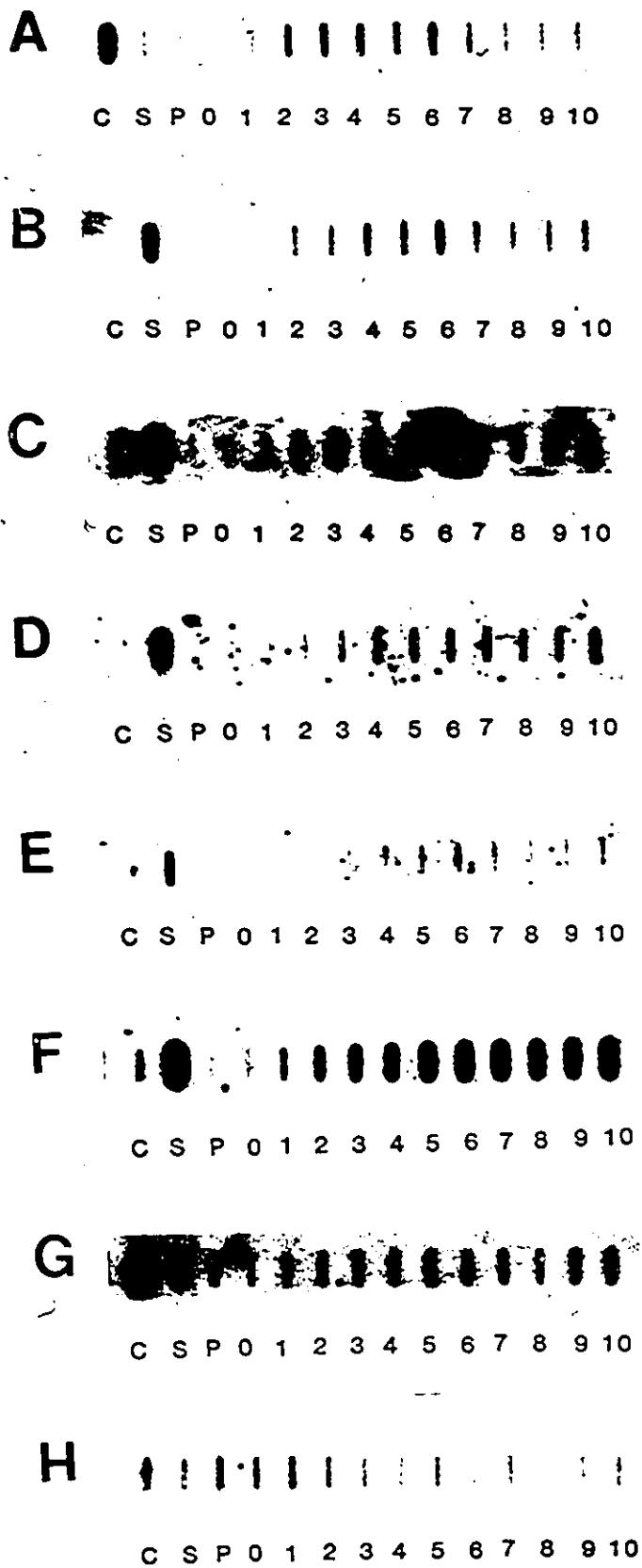


Figure 7.7. Detection of muscle-specific transcripts within differentiating MR322 cultures. Slot blot analysis of total RNA, isolated at daily intervals after cultures had reached confluency, probed with a number of muscle-specific probes:

- (A) Hybridization to a cardiac-actin isotype specific probe.
- (B) Hybridization to a skeletal-actin isotype specific probe.
- (C) Hybridization to rat embryonic myosin heavy chain (MHC) cDNA.
- (D) Hybridization to the rat skeletal-muscle TNT cDNA.
- (E) Hybridization to the rat skeletal-muscle myosin light chain 2 (MLC2) cDNA.
- (F) Hybridization to the rat skeletal-muscle MLC1&3 cDNA.
- (G) Hybridization to the human V-MLC1 cDNA 3'-UT region which hybridizes the slow-MLC1 mRNA.
- (H) Hybridization to the mouse alpha-tubulin cDNA.

Total RNA was isolated from: C, mouse cardiac muscle; S, mouse skeletal muscle; P, undifferentiated P19 cells; 0 to 10, days during MR322 differentiation. The relative mRNA levels were determined by scanning densitometry of the autoradiograms and were plotted as the percentage of the maximum level of expression normalized to the level of tubulin mRNA.



### 7.3. Discussion:

P19 EC cells, when induced to differentiate with DMSO, give rise to a spectrum of cells normally descended from the embryonic mesoderm. From these differentiated cultures, we selected for cells capable of anchorage-independent growth. In post-confluent cultures of two cell lines isolated in this manner (MR16 and MR322), a subpopulation of cells exhibited contractile activity and morphological differentiation typical of myogenic cells (Figure 7.1). Light and electron microscopic examination of sections of differentiated MR16 and MR322 cultures revealed the presence a multilayered array of multinucleate myocytes and ECM secreting mesenchyme-like cells. Since subclones of MR16 and MR322 exhibited the same developmental potential as the parental lines, we conclude that MR16 and MR322 are multipotential stem cells with a limited but multiple developmental repertoire.

Immunofluorescent staining experiments demonstrated the absence of the cardiac-specific isoforms A-MLC2 and A-MHC within MR16 and MR322 myocytes (Figures 7.2, to 7.5). Furthermore, Northern hybridization analysis showed the presence of the skeletal muscle-specific mRNAs, TNT, MLC2 and MLC1&3 (Figures 7.6 and 7.7). Since cardiac-actin and skeletal-actin mRNAs are co-expressed, and since the myocytes express the embryonic isoforms, slow-MLC1 and slow type I MHC, we suggest that the myocytes within MR16 and MR322 resemble embryonic skeletal

muscle cells.

During embryonic development, segmentation of the lateral mesodermal plate gives rise to the somites from which the myocyte and the sclerotome form. Cells derived from the sclerotome become either myoblasts or fibroblasts and chondroblasts according to their location (see Langman, 1981). It is tempting to speculate that the stem cell lines we have isolated from differentiating P19 cultures may be analogous to the mesodermal progenitor cells within the embryonic sclerotome. The myocytes may represent the primary myocytes derived from the embryonic somites. The mesenchyme-like cell secretes large amounts of ECM containing what appears to be thin collagen (type II) fibres, and thus may be analogous to fetal chondroblasts. Further characterization of the ECM secreting mesenchyme-like cells within these cultures may clarify this issue.

We have isolated two multipotential cell lines with similar characteristics. In post-confluent cultures, these lines differentiate into skeletal myocytes and ECM secreting mesenchyme-like cells. The experiments reported here demonstrate that non-EC stem cells can be isolated from EC-derived differentiated cultures. We suggest that the cell lines MR16 and MR322 may be analogous to a multipotential mesodermal progenitor cell and thus may prove useful in studies into cell differentiation and gene expression along the mesodermal lineages.

Chapter 8, Expression of Smooth Muscle Actins in Differentiated Cells derived from P19 Embryonal Carcinoma Cultures

8.1. Introduction:

In vertebrates, the actins are members of a multigene family with at least six different isoforms. These actins are located on different chromosomes and are subject to different tissue-specific and developmental regulation (see Chapter 1).

In general, two actins are co-expressed in a particular tissue, and the proportion of one to the other is dependent on cell-type and developmental stage. The alpha-actin genes, cardiac-actin and skeletal-actin, are expressed in heart and skeletal muscle. All non-muscle cells co-express the cytoskeletal beta-actin and gamma-actin genes. The smooth muscle tissues co-express the vascular alpha-actin and enteric gamma-actin genes. (Vandekerkhove and Weber, 1978; Chapter 1).

In this chapter we have further characterized the types of actins expressed within differentiating P19 cultures. Our observations suggest that a sub-population of the fibroblast-like cells within these cultures resemble fetal myofibroblastic cell types since they contain the smooth muscle-specific, vascular-actin protein.

## 8.2. Results:

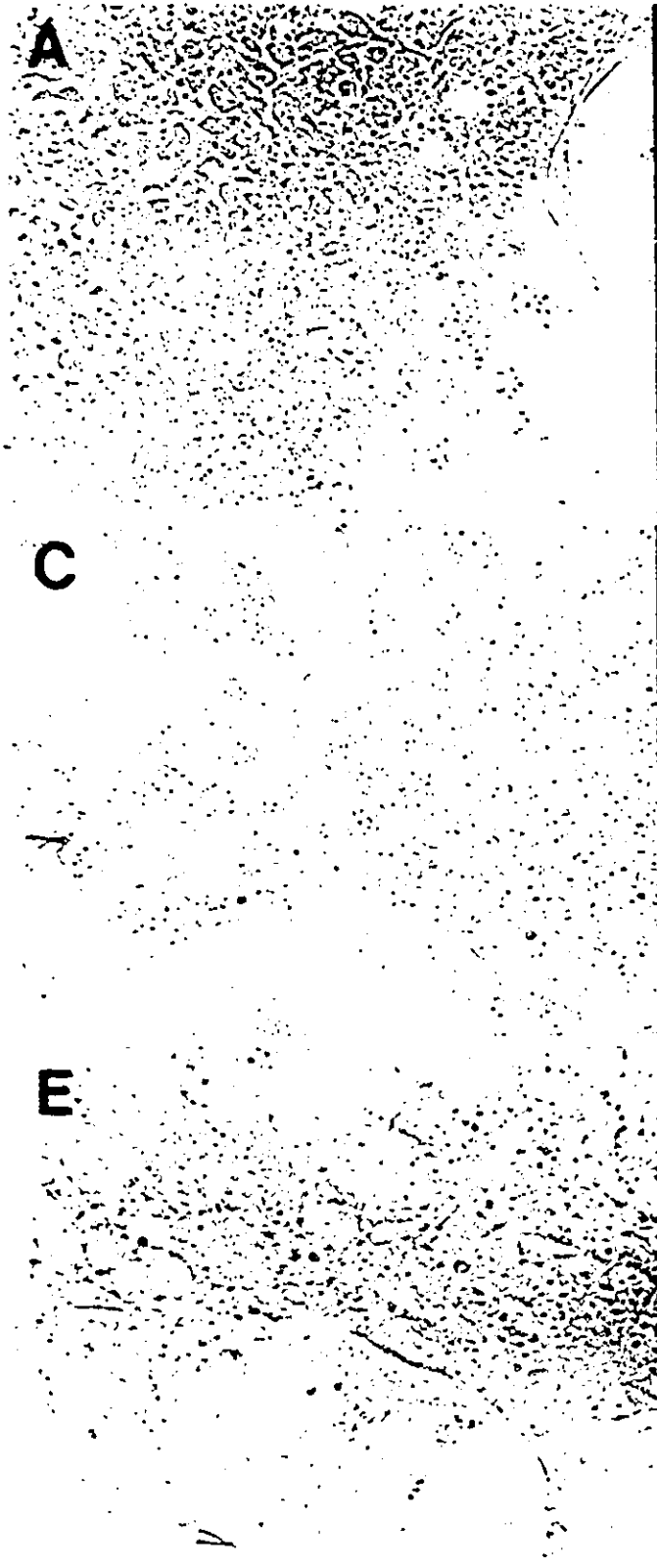
### 8.2.1. Detection of Striated and Smooth Muscle Actins:

The cardiac-actin and skeletal-actin genes are expressed only in striated muscle (Buckingham, 1986, and references therein). We have previously examined the distribution of cardiac-actin and skeletal-actin proteins in differentiated P19 cells by indirect immunofluorescent double staining with a rabbit anti striated muscle alpha-actin antisera (Bulinski et al., 1983) and mouse monoclonal antibody MF20, reactive with all isoforms of striated myosin heavy chain (C/S-MHC). In DMSO-treated P19 cultures, striated muscle alpha-actin proteins were only found in cells that also contained C/S-MHC protein (Chapter 4; Rudnicki et al., 1988b). In undifferentiated P19 cells and in RA-treated cultures, no cells reactive with either antibodies were observed. Northern hybridization analysis with cardiac-actin and skeletal-actin isotype-specific probes confirmed that these two mRNAs were co-expressed in P19-derived myocytes (Chapter 3; Rudnicki et al., 1988a).

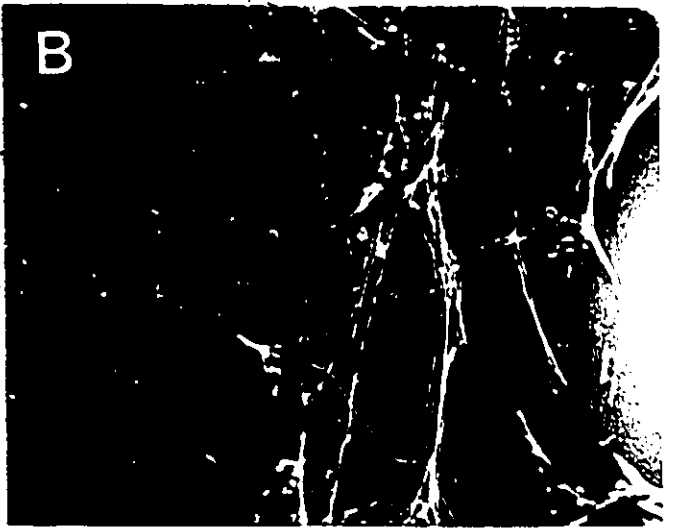
In examining the distribution of striated muscle alpha-actins, we also made use of the mouse monoclonal antibody B4F8, reactive with both striated and smooth muscle alpha-actins (Lessard, 1988). Within DMSO-treated cultures we unexpectedly observed a large number of cells staining with antibody B4F8 (Figure 8.1, A and B). The morphology of many of these cells was fibroblastic in appearance. Furthermore, in

Figure 8.1. Smooth-muscle actins in DMSO-treated cultures. Panels A, C and E are the phase-contrast fields of DMSO-induced P19 cells stained with; B, the mouse monoclonal antibody B4F8; D, the mouse monoclonal antibody CGA7; and F, the mouse monoclonal antibody HUC-1. The antibody specificities are listed in Table 8.1. The second antibody was fluorescein-conjugated anti-mouse IgG. Bar represents 50 um.

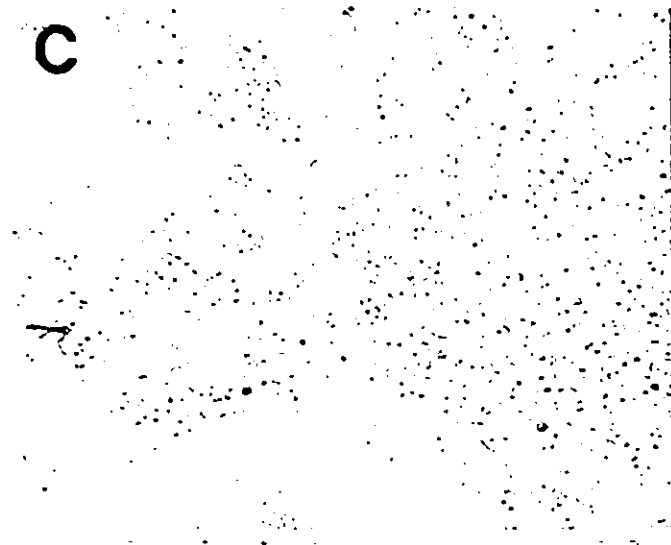
A



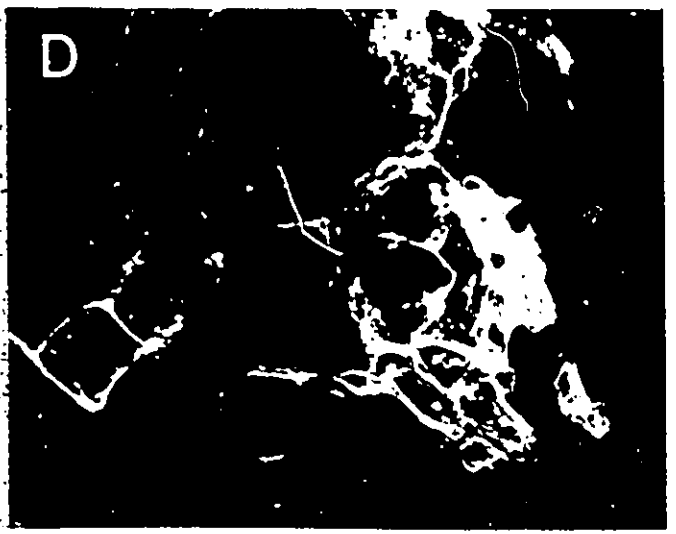
B



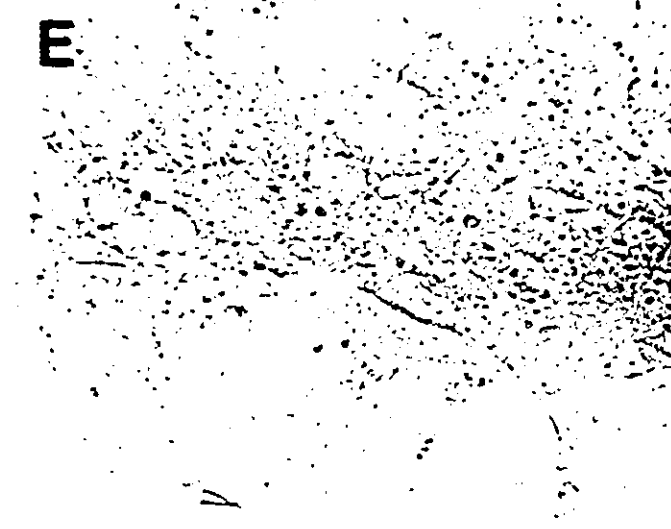
C



D



E



F

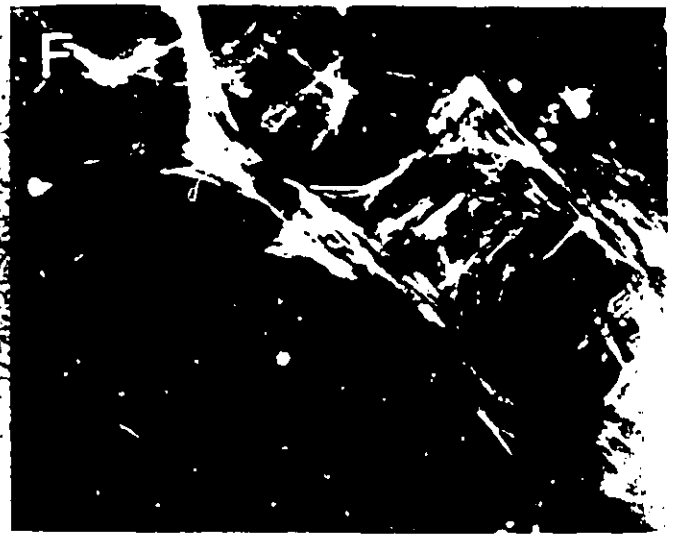
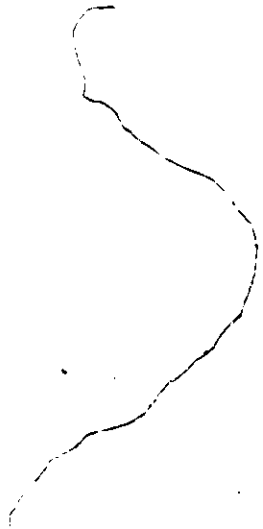


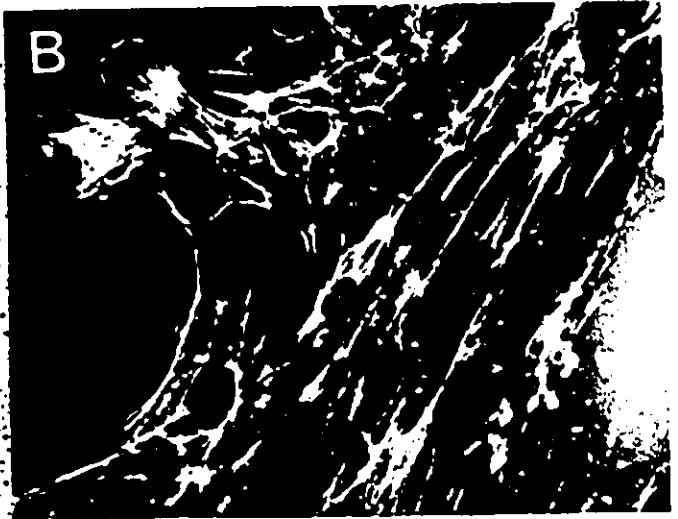
Figure 8.2. Smooth-muscle actins in RA-treated cultures. Panels A and C are the phase-contrast fields of RA-induced P19 cells stained with; B, the mouse monoclonal antibody B4F8; and D, the mouse monoclonal antibody CGA7. Panel E is a phase-contrast field of untreated cells after aggregation stained with mouse monoclonal antibody B4F8 (F). The antibody specificities are listed in Table 8.1. The second antibody was fluorescein-conjugated anti-mouse IgG. Bar represents 50  $\mu$ m.



A



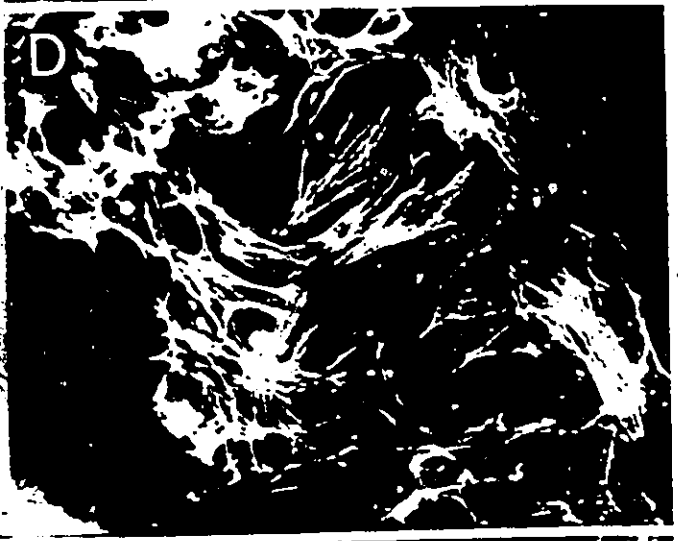
B



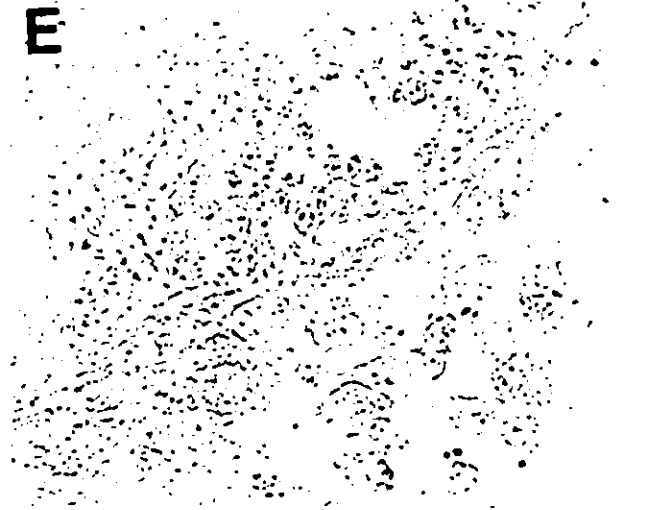
C



D



E



F



RA-treated cultures, which contain neurons, glia, fibroblast-like cells and no striated muscle, we observed a similar proportion of cells which stained with antibody B4F8 (Figure 8.2, A and B). In these cultures, the B4F8 antibody typically stained filaments arrayed as stress fibres. In addition, the mouse monoclonal antibody HUC-1, with the same reactivity as B4F8 (J. Lessard, in preparation), also stained cells within DMSO-treated (Figure 8.1, E and F) and RA-treated (data not shown) cultures. These results (summarized in Table 8.1) suggest that smooth muscle vascular alpha-actin may be expressed in some of the fibroblast-like cells within RA and DMSO-treated cultures.

#### 8.2.2. Detection of Smooth Muscle Actins:

To confirm that smooth muscle actins were indeed expressed in a sub-population of differentiating P19 cells we made use of anti-smooth muscle actin specific antibodies. The mouse monoclonal antibody CGA7 is reactive with both smooth muscle vascular alpha-actin and enteric gamma-actin proteins and not with the striated muscle alpha-actin proteins (Gown et al., 1985). Antibody CGA7 stained cells after DMSO-induced (Figure 8.1, C and D), and RA-induced (Figure 8.2, C and D) differentiation.

The mouse monoclonal antibody SM-1 reacts specifically with smooth muscle vascular alpha-actin protein (Skalli et al., 1986). We observed staining of cells with antibody SM-1 after

Table 8.1. Summary of Actins Detected by Indirect  
Immunofluorescence Experiments:

Antibody	Specificity	P19-DMSO			P19-RA	Reference
		Cardiac	Skeletal	Other		
1. C/S-actin	cardiac- and skeletal-actin	+	+	-	-	Bulinski et al., 1983
2. B4F8	smooth and striated muscle actins	+	+	+/-	+/-	Lessard, 1988.
3. CGA7	smooth muscle alpha- and gamma-actin	-	-	+/-	+/-	Gown et al., 1985
4. HUC1	smooth and striated muscle actins	+	+	+/-	+/-	Lessard*
5. SM-1	smooth muscle alpha-actin	-	-	+/-	+/-	Skalli et al., 1983

\*J. Lessard, personal communication.

For species and source of antibodies see Table 2.1.

DMSO-induced and RA-induced differentiation (Figure 8.3). Therefore, we conclude that a sub-population of cells within differentiated P19 cultures expressed smooth muscle vascular alpha-actin protein (results summarized in Table 8.1).

To further characterize the smooth-muscle alpha-actin containing cells within differentiated P19 cultures we performed double staining experiments with mouse monoclonal antibody CGA7, reactive with smooth muscle actins and rat monoclonal antibody TROMA-I, reactive with cytokeratin containing intermediate filaments (Kemler et al., 1981). Cytokeratin intermediate filaments are found in the epithelial cells which form on the outside of aggregates without drug exposure (Jones-Villeneuve et al., 1982) and after DMSO-treatment (Smith et al., 1987). Few cells which stain with TROMA-I are found after RA-treatment. In DMSO-treated cultures, smooth muscle actins were found in cells that also contained cytokeratin intermediate filaments. However, not all cells which stained with antibody CGA7 contained cytokeratin (Figure 8.4). In addition, the epithelial cells that formed around the periphery of untreated aggregates stained with antibody B4F8, reactive with vascular and striated alpha-actin proteins (Figure 8.2, E and F). These epithelial cells have previously been shown to resemble extra-embryonic endoderm (Smith et al., 1987).

Figure 8.3. Vascular alpha-actin in differentiated P19 cultures. Panels A and C are phase contrast fields of RA-induced (A and B) and DMSO-induced (C and D) P19 cells stained with the mouse monoclonal antibody SM-1 (B and D) reactive with vascular-actin protein. The second antibody was fluorescein-conjugated anti-mouse IgG. Bar represents 50 um.

A



C

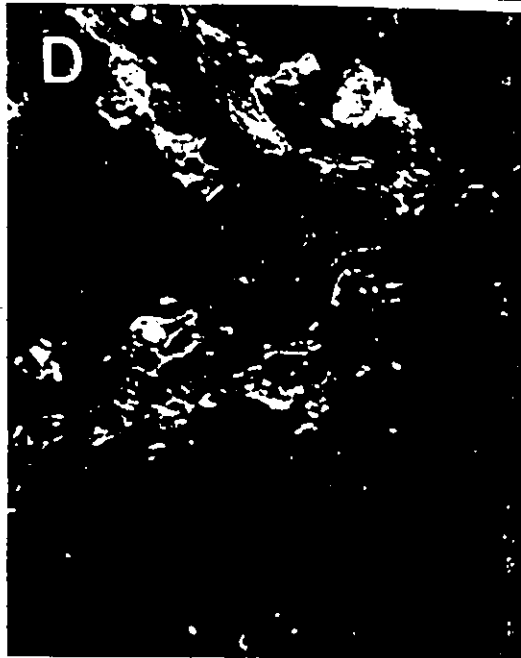
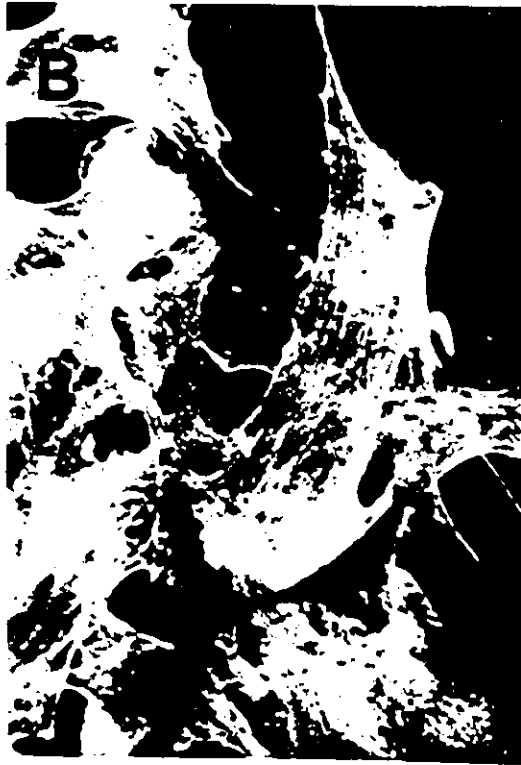
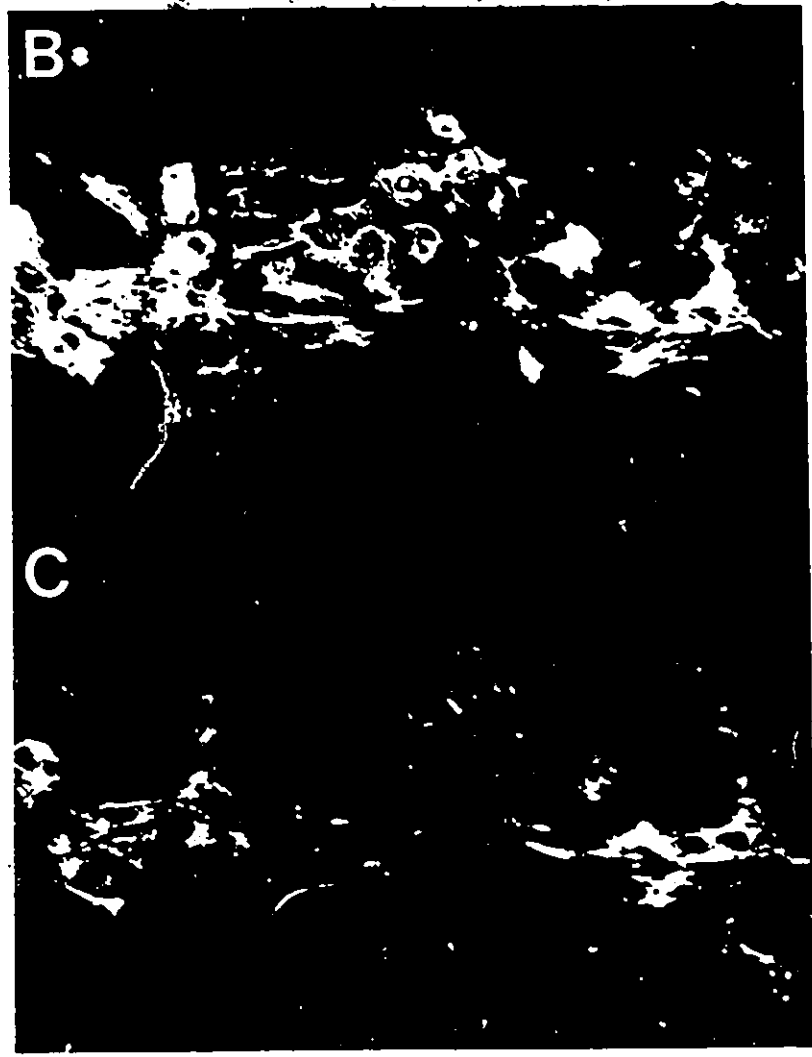


Figure 8.4. Epithelial cells contain smooth-muscle actin protein. Panel A is the phase-contrast field of DMSO-treated cells stained with B, rat monoclonal antibody TROMA-I, reactive with the cytokeratin containing intermediate filaments, and C the corresponding field stained with the mouse monoclonal antibody CGA7. Second antibodies were fluorescein-conjugated anti-rat and rhodamine anti-mouse IgG. Bar represents 50 um.



### 8.3. Discussion:

P19 embryonal carcinoma cells can be induced to differentiate into different spectra of cell types by aggregation and exposure to RA or DMSO. We have examined the distribution of striated muscle and smooth muscle actins by indirect immunofluorescence using monoclonal antibodies. We found smooth muscle vascular-actin containing cells after both RA and DMSO induced differentiation. In DMSO-treated cultures, the epithelial cells which contained smooth-muscle actin protein(s) also contained cytokeratin containing intermediate filaments. These epithelial cells closely resemble the extra-embryonic endoderm of the early embryo (Smith et al., 1987). However, some of the non-epithelial cells (TROMA-I<sup>-</sup>) in both RA-treated and DMSO-treated cultures, contained smooth muscle actin protein. Thus, at least two types of cells contained smooth muscle actin proteins in differentiated P19 cultures.

There are two smooth muscle actins, vascular alpha-actin and enteric gamma-actin. Although the smooth muscle actins are always co-expressed, the relative proportions of the actins is different in smooth muscle of different organs (Gabbiani et al., 1981; Skalli et al, 1984). In the rat, vascular alpha-actin protein is not detectable in the aorta until birth. However in the fetal rat, vascular alpha-actin protein is expressed in a

wide range of myofibroblastic cell types of possible epithelial and mesenchymal origin (Skalli et al., 1986).

In conclusion, we suggest that in addition to neurons and glia formed after RA-treatment, and striated-muscle after DMSO-treatment, differentiated P19 cultures include myofibroblastic cells. Thus, the P19 cell system may represent a useful culture model to study smooth muscle differentiation and gene expression.

## Chapter 9, General Conclusions

### 9.1. P19-Derived Cardiac Muscle:

After aggregation and exposure to DMSO, P19 cells differentiate into cultures which contain striated-muscle and other undefined cell types (McBurney et al., 1982). We found that the flat mononucleate myocytes within DMSO-treated cultures co-expressed the cardiac A-MLC2, V-MLC1, A-MHC and V-MHC proteins (Chapter 3; Rudnicki et al., 1988a). These results confirm that the flat mononucleate myocytes are indeed of the cardiac type and suggests that they resemble the cells of the embryonic myocardium. In Chapter 3, we also established that cardiac-actin and skeletal-actin genes are concomitantly activated and maintained at similar levels during the de novo differentiation of P19-derived cardiac myocytes.

The A-MHC and V-MHC proteins are co-expressed in the heart and the proportion of one to the other depends on developmental stage, muscle type and on hormonal influences. At birth, an increase in serum thyroid hormone has been shown to cause an increase in A-MHC gene transcription and a decrease in V-MHC gene transcription (Lompre et al., 1983; Izumo et al., 1986). Presumably, thyroid hormone affects cardiac-MHC transcription via a thyroid hormone binding protein which can interact with specific DNA sequences near or within the cardiac-MHC genes. The P19 cell culture system may be of some

use to investigate how thyroid hormone regulates cardiac-MHC transcription.

The first experiment would be to determine whether cardiac-MHC expression in DMSO-induced cells cultured was affected by the levels of thyroid hormone in the medium. The A-MHC and V-MHC transcripts can be detected in Northern hybridization experiments with isotype-specific probes generated from the 3'-UT region of their cDNAs (S. Schiaffino, personal communication). If thyroid hormone were affecting cardiac-MHC expression in P19-derived cardiac myocytes, the DNA sequences involved in thyroid hormone regulation of cardiac-MHC gene transcription could be identified using standard promoter analysis. These experiments would involve transfecting modified A-MHC and V-MHC constructs, preferably chimeric genes with various amounts of the MHC promoters driving a reporter gene, into P19 cells and deriving stable transformants (e.g. similar to methodology described in Chapter 4). The activity of the reporter genes would be assayed after DMSO-induced differentiation with or without thyroid hormone in the medium.

#### 9.2. Tissue-Specific Regulation of CH-Actin Transcription:

Transfection of cloned genes into cultured cells has allowed the identification of discrete DNA sequences which restrict the transcription of eukaryotic genes to particular cell types. This regulation appears to be exerted by sequences just upstream of the transcription start site (reviewed in

Reeves, 1984), and ~~by~~ regulatory elements such as enhancers (reviewed in Khoury and Gruss, 1983; Serfling et al., 1985). We were interested in identifying the regulatory sequences that control CH-actin gene transcription.

In Chapter 4 (see Rudnicki et al., 1988b), we demonstrated that the transfected CH-actin and the chimeric actin-tk genes were subject to appropriate developmental and tissue-specific regulation during the differentiation of P19 cells. Thus, our results suggested that sequences located between -700 bp and +740 bp from the CH-actin transcription start site mediate this regulation.

To further delineate the DNA sequences required for the regulation of CH-actin transcription, two approaches can be used. The first approach should be the standard promoter analysis where the CH-actin promoter and first intron are linked to an easily assayable reporter gene like chloramphenicol acetyl transferase (CAT) (Gorman et al., 1982). Serial upstream deletions into the promoter region as well as internal deletions within the first intron should allow the localization of the required regulatory elements. The first intron may contain sequences of interest since in the skeletal-actin gene it is highly conserved and contains an enhancer-like core sequence (Hu et al. 1986), and the beta-actin first intron has been shown to contain a serum responsive element (Kawamoto et al., 1988). It would be of interest to compare the activity of constructs in P19-derived cardiac muscle and in C2 skeletal muscle since these

two cell types display different patterns of cardiac-actin activation and maintenance of expression. Using such an approach, it may be possible to identify skeletal-muscle and cardiac-muscle specific regulatory elements.

A second approach to investigate CH-actin gene transcription would be to make use of the recently described protein-DNA mobility shift assay. This assay has proved useful in identifying trans-acting factors that interact with discrete DNA sequences (Dyran and Tjian, 1983; Sassone-Corsi and Borrelli, 1986). Once the sequences that interact with the trans-acting factors are known, the cDNAs encoding the specific DNA-binding proteins can be cloned. Recently, the cDNA encoding the DNA-binding protein that specifically binds enhancers in the major histocompatibility complex and immunoglobulin kappa chain genes has been cloned in the expression vector lambda gt11. The investigators employed a novel approach previously used to clone the cDNA encoding the DNA-binding protein Sp1 (Kadonaga et al., 1987). The specific binding of labeled oligonucleotide containing the enhancer sequence to the DNA-binding protein encoded in the expression vector allowed identification of the appropriate plaque (Singh et al., 1988). A similar strategy would allow direct cloning of the cDNAs that encode the proteins that interact with the CH-actin promoter.

These analyses should allow the dissection of the various activities of the regulatory sequences and trans-acting factors that regulate the developmental and tissue-specific

regulation of CH-actin gene transcription. In the future we hope this approach will help elucidate mechanisms relevant to an understanding of how genes are regulated during development and perhaps how genes can regulate development itself.

### 9.3. Tissue-Specific Regulation of CH-Actin Translation:

In differentiated P19(CH-actin) transformants no cardiac-actin protein was detected in non-muscle cells even though we could detect CH-actin mRNA. Similarly, the non-muscle cells within differentiated P19(actin-tk) transformants did not contain detectable TK protein (Chapter 4; Rudnicki et al., 1988b). In L6 cells transfected with either the full length CH-actin gene or with the actin-tk chimeric gene, no difference in the level of transcript derived from the CH-actin promoter was observed between myoblasts and myotubes. However, the TK protein was located exclusively in myotubes (Chapter 5; Ruben et al., 1988). We interpreted these results to suggest that CH-actin sequences from the first 44 nt of the 5'-UT region played a role in regulating translation of the CH-actin mRNA in muscle cells.

Examination of the 5' leader sequence of the human and mouse cardiac-actin mRNAs has revealed no obvious or conserved secondary structures (W. Colledge, personal communication). This raises some doubts as to whether the 5'-UT sequence is involved in regulating translation. To address whether the 5' leader sequence of the CH-actin mRNA can regulate translation,

a different gene construct could be tested in L6 cells. This construct would be analogous to the chimeric actin-tk gene except that a different reporter gene (like CAT) should be used. In addition, a modified chimeric actin-CAT gene can be tested that lacks the first intron of the CH-actin gene. If the leader sequence does confer regulated muscle-specific translation on a heterologous mRNA, then further studies, such as in vitro translation of synthetic mRNAs, could be pursued.

#### 9.4. Muscle Differentiation and the H-ras Oncogene:

Expression of transfected activated H-ras oncogenes in cultured skeletal myoblast cells has been shown to inhibit myogenic differentiation at a stage after withdrawal from the cell cycle (Olson et al., 1987; Payne et al., 1987). Our results demonstrated that in transfected P19 cell lines, neither ubiquitous nor high-level muscle-specific H-ras expression prevented striated-muscle differentiation (Chapter 6). Thus, the H-ras proto-oncogene may not be involved in the regulation of normal myocyte differentiation.

Skeletal myoblast differentiation is at least partially inhibited by expression of a transfected c-myc oncogene (Denis et al., 1987; Schneider et al., 1987). Interestingly, rhabdomyosarcomas of the heart can be induced in young avian embryos by infection of the MC29 retrovirus bearing the v-myc oncogene (Saule et al., 1987). Perhaps expression of a v-myc oncogene or co-expression of H-ras and v-myc oncogenes in P19

cells may inhibit cardiac myocyte differentiation. This may allow isolation of continuous transformed cell lines capable of activating the cardiac-actin promoter.

#### 9.5. Isolation of Stem Cells from Differentiating P19

##### Cultures:

In Chapter 7 we describe the isolation of non-EC stem cells from DMSO-treated P19 cultures. Two stem cell lines, MR16 and MR322, were characterized and both were capable of self-renewal and exhibited some ability for anchorage-independent growth. In post-confluent cultures, these cells differentiated into at least two cell types, skeletal myocytes, and ECM-secreting mesenchyme-like cells. Thus, these lines may be analogous to the multipotential stem cells of the early embryo that have become committed to the mesodermal lineage.

The methodology described in Chapter 7 may allow the isolation of other types of non-EC stem cells with different potentials from differentiating P19 cultures. It may be possible to isolate a neuro-ectodermal progenitor cell line from RA-treated P19 cells. In any case, the cell lines MR16 and MR322, may prove useful in studies concerning the commitment and differentiation of cells developing along the mesodermal lineage.

#### 9.6. Post Script:

Specific site-directed alteration of endogenous genes in mammalian cells is possible by gene-targeting, the homologous recombination of chromosomal DNA sequences with a transfected DNA sequence (Smithies et al., 1985; Thomas et al., 1986; Thomas and Capecchi, 1986). Recently, male embryo-derived stem (ES) cells have been used as the substrate for gene-targeting experiments where a plasmid containing both HPRT sequences and the neo gene was introduced into the HPRT gene (Thomas and Capecchi, 1987) and for correction of a defective HPRT gene (Doetschman et al., 1987). These experiments raise the possibility of replacing endogenous genes with genetically engineered versions in ES cells in cell culture and production of new mouse strains by introduction of the ES cells into mouse embryos. A similar approach can be used to produce ES cell lines with null mutations in genes with possible developmental roles.

Many genes, from homeobox-containing genes to cellular oncogenes, have been suggested to be involved in the regulation and control of cell proliferation and differentiation in development (for review see Muller, 1986). Transgenic mice have been produced which contain activated oncogenes or oncogenes under the regulation of tissue-specific promoters (Quaife et al., 1987; Sinn et al., 1987). However, the actual physiological activities of these proteins during normal development is still unclear. ES cell lines with null mutations

in genes such as these would be a valuable resource in approaching an understanding of their actual function during embryonic development.

The candidate gene should meet the following criteria; the gene must be cloned, the gene should have only a few or no pseudogenes and the gene must be transcribed in ES cells. Possible genes that would be good candidates for targeted insertional mutagenesis are src, fos, ras, myc and perhaps members of the hox gene family.

The gene-targeting vector would consist of 5'-untranslated sequences of the target gene, the coding region of the neor gene followed by the SV40 polyadenylation sequences. This vector can be introduced into ES cells by either transfection of a linearized plasmid construct, or infection with a disabled retrovirus incapable of integrating via its LTRs. Antibiotic G418 resistant ES cells presumably should be the result of an event whereby the promoterless neor gene has integrated into a transcriptionally active region of chromatin, in the correct orientation, yielding a transcript where the neor encoding sequences are translated in frame.

ES cell lines derived from antibiotic G418 resistant colonies can then be screened by Southern-blot analysis for clones containing the transfected gene integrated via homologous recombination into the target gene such that transcription is from the target gene promoter. One might expect this type of integration to prevent normal transcription of the target gene.

The low frequency of homologous recombination may necessitate the use of some novel screening and sib-selection procedures. The identification of the desired recombinants should be possible since the frequency of homologous to non-homologous recombination events has been reported to be as high as 1 in 80 (Song et al., 1987).

The modified ES lines can then be used to generate mouse cell lines heterozygous for the targeted mutation. The modified ES cells can be introduced back into an embryo, the embryo transferred into the uterus of a pseudopregnant mouse, and the resulting chimeric mice bred to produce new mouse strains (Evans et al., 1985; Bradley et al., 1987). Presumably, many of these mutations will be recessive lethals and the mouse strains therefore must be maintained as heterozygotes. These mice will then be subsequently bred to produce homozygous mice and the consequences of the mutation at a particular allele can then be assessed.

These approaches will help elucidate an understanding of mammalian embryonic development by generating molecularly defined mutants which affect embryogenesis.

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