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Cell Surface Phosphorylation And Myogenesis In L6 Rat Myoblasts

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CELL SURFACE PHOSPHORYLATION AND MYOGENESIS IN L6 RAT MYOBLASTS

by

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ABSTRACT

Cell surface components are important for myogenic differentiation. Studies with subconfluent day-2 cultures of rat myoblasts revealed that a cell surface 112 kDa protein was phosphorylated by a Ca^{++}, F^-, and Mg^{++}-dependent ecto-protein kinase (ecto-PK), and that adequate ATP was present on the cell surface for the efficient functioning of this ecto-PK. The following evidence suggests that both the 112 kDa protein and the ecto-PK may play important role(s) in the initiation of myogenesis. (i) The highest phosphorylation activity was observed in subconfluent cultures, i.e. before the onset of myogenesis. (ii) Treatment of cells with myogenesis inhibitors also resulted in a corresponding decrease in the phosphorylated 112 kDa protein (p112). (iii) The level of p112 in a conditional myogenesis-defective mutant corresponded with the cell's eventual ability to differentiate. (iv) A mutant defective in the ecto-PK (F72) was impaired in the phosphorylation of 112 kDa and in myogenesis. (v) A mutant containing only a residual level of 112 kDa protein (D1/S4) was defective in both p112 and myogenesis. (vi) Conditional myogenesis mutant D1 can not undergo myogenesis and the level of phosphorylation of the 112 kDa protein is very low when grown in 10% horse serum, however when grown in 1% horse serum D1 can fuse and the level of phosphorylation of the 112 kDa protein is normal. (vii) Since the level of p112 was normal in another myogenesis-defective mutant, the phosphorylation of this protein was not likely a consequence of myogenic differentiation.

Myogenic differentiation is comprised of a sequential cascade of multiple steps leading to the formation of multinucleated myotubes. Northern blot analyses with myogenic factor cDNAs and muscle-specific protein cDNAs showed

that mutants D1/S4 and F72 had normal Myf5 transcript level, but much lower transcript levels of Myf4, NCAM, MHC, MLC, and TnT than their parental L6 cells. Similar results were observed when L6 cells were treated with myogenesis inhibitors such as phloretin and 5-bromo-2-deoxyuridine (BrdUrd). These findings further suggested that the ecto-PK and 112 kDa protein might be involved in the early stage of myogenesis. When mutants D1/S4 and F72 were transfected with the *myf4* cDNA, the transcript levels of MHC, MLC, and TnT were elevated in both transfected cell lines. However the level of p112 and the transcript levels of NCAM and Myf5 were unaltered in these transfectants. These results suggested that the site of action of ecto-PK and 112 kDa protein might occur after Myf5, and before NCAM and Myf4 in the myogenic pathway.

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NOMENCLATURE

anti-PMV	rabbit antibodies raised against plasma membrane vesicles prepared from rat L6 myoblasts
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BrdUrd	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CHO cells	Chinese hamster ovary cells
CIP	calf intestinal alkaline phosphatase
CPK	creatine phosphokinase
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
DS-T4	D1/S4 cells transfected with PBS-M4
DS-Tc	D1/S4 cells transfected with PBS^+ vector only
ecto-PK	ecto-protein kinase
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
F-T4	F72 cells transfected with RSV-M4
F-T5	F72 cells transfected with RSV-M5
F-Tc	F72 cells transfected with pRc/RSV vector only
FCS	fetal calf serum
GuSCN	guanidine isothiocyanate
HAHT	high-affinity hexose transporter

HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid
MGL	β2-microglobulin
MLC	myosin light chain
MHC	myosin heavy chain
MRF	muscle regulatory factor
mRNA	messenger RNA
Myf	myogenic factor
NCAM	neural cell adhesion molecule
p112	phosphorylated cell surface 112 kDa protein
p112$^-$	myoblasts defective in p112
PBS-M4	PBS vector containing Myf4 cDNA
PMSF	phenylmethanesulphonyl fluoride
RNA	ribonucleic acid
RSV-M4	pRc/RSV vector containing Myf4 cDNA
RSV-M5	pRc/RSV vector containing Myf5 cDNA
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylaminde gel
TnT	troponin T

CHAPTER 1

INTRODUCTION

It has long been recognized that cell differentiation is controlled by highly specific regulatory mechanisms. This involves co-ordinated biochemical and morphological changes; while some changes are cell-autonomous, others are dependent on specific environmental stimuli.

Skeletal muscle is an important model for studying cellular and molecular events involved in cell determination and differentiation. From an experimental viewpoint, skeletal muscle cells are easy to grow in culture and the conversion of myoblasts into myotubes can be induced by growing in serum-deficient medium. The conversion of myoblasts into myotubes is called myogenic differentiation or myogenesis. Myogenesis involves proliferation, a withdrawal from the cell cycle, cell-cell recognition, synthesis of muscle-specific proteins, and membrane fusion. The myoblast system has the advantage that the fate of the cell can be easily followed. It provides a useful means to study the developmental control genes that regulate muscle determination and myogenic differentiation. It is also a good system for studying the initiation and sequence of events involved in the myogenic pathway.

1.1 MYOGENIC DIFFERENTIATION

Myogenic differentiation refers to a series of processes. Mononucleated myoblasts are the precursors of skeletal muscle cells. During the development of embryonic skeletal muscle, myoblasts proliferate for a period of time and then withdraw from the proliferative cycle. Under the appropriate conditions, they will fuse with one another in a specific way to form multinucleated skeletal muscle cells or myotubes. A number of distinct events have been identified in the myogenic pathway: (i) Biochemical differentiation. This is characterized by the induction of muscle-specific proteins and enzymes, such as myosin heavy chain (MHC), myosin light chain (MLC), troponin T (TnT), muscle creatine kinase (MCK), tropomyosin, desmin and acetylcholine receptor subunits. (ii) Total commitment. This occurs when cells cease DNA synthesis, and irreversibly withdraw from the cell cycle. (iii) Terminal differentiation. This involves the formation of multinucleated myotubes by membrane fusion.

Little is known about the molecular events that initiate and regulate myogenesis. Many factors have been shown to affect myotube formation: Ca^{++} concentration, concentration of growth factors, and expression of muscle-specific proteins. However, the cellular and molecular mechanisms by which such factors mediate their functions are not well understood.

Recently, several regulatory genes which control myogenesis have been discovered. The first one was *myoD*. It was isolated by subtraction-hybridization of cDNAs between 5-azacytidine-derived myogenic line and its parental 10T½ cells

(Davis et al., 1987). *MyoD* is expressed only in myoblasts and skeletal muscle tissue. Cardiac and smooth muscles, which express many of the same muscle-specific structure genes as skeletal muscle, do not express *myoD*. When the *myoD* cDNA is expressed in 10T½ fibroblasts, it converts 10T½ cells into myogenic cells (Davis et al., 1987).

Following the discovery of *myoD*, additional myogenic regulatory genes were subsequently identified. They are the myogenin gene (Wright et. al., 1989; Edmondson and Olson, 1989), human myogenic factor 3 gene (*myf3*) (Braun et al.,1989a), human *myf4* (Braun et al., 1989a) , human *myf5* (Braun et. al., 1989b), and human *myf6* (Braun et al., 1990). The *myf3* and *myf4* are the human homologues to the mouse *myoD* and myogenin gene respectively (Braun et al.,1989a). Together, the above factors comprise the *myoD* family of myogenic regulatory genes. The proteins coded by these myogenic regulatory genes are referred to as the myogenic factors. These factors are expressed exclusively in skeletal muscle cells.

It was originally thought that each of the above myogenic factors was sufficient to induce gene expression of muscle-specific proteins and subsequently myogenesis when expressed in a wide range of non-muscle cell lines (Stephen and Weintraub, 1991). However, when HepG2 cells were transfected with the *myoD* gene, the transcript levels of muscle-specific genes were not elevated in these transfectants, even though the MyoD transcript levels were elevated. Similar results were observed in CV1 cells and HeLa cells (Weintraub et al., 1989). The reason that

MyoD could not activate muscle-specific genes in HepG2 cells was thought to be due to the absence of factor(s) essential for MyoD activity (Schafer et al.,1990). In addition, the expression of any member of the MyoD family was thought to activate the expression of other members of the MyoD family (Thayer et. al., 1989; Edmondson and Olson, 1989; Braun et. al., 1989a). It was recently demonstrated that myogenin could induce the myocyte-specific enhancer-binding factor, MEF-2 (Edmondson et al., 1992). MEF-2 can function as an intermediary of myogenin autoactivation. Although quantitative differences in activity and time of embryonic expression are detectable, it is not clear whether these members of the MyoD family have biologically distinct functions. However, Braun et al. found that mice harbouring a knock-out *myf5* gene still have normal transcript levels of MyoD, myogenin and Myf6, and undergo normal muscle development (Braun et al., 1992). They also found that when a null mutation of MyoD was introduced into the germline of mice, the Myf5 transcript levels were elevated and the skeletal muscle was normal (Rudnicki et al., 1992).

The predicted amino acid sequences of these myogenic factors share a region of homology with a segment of MyoD. This region consists of about 70 amino acids, containing a basic domain which is homologous to the *myc* proteins. It also has the potential to form two amphipathic alpha-helices separated by a non-helical loop; in other words, these proteins contain a basic helix-loop-helix (HLH) motif (Murre et. al., 1989a). This basic domain confers DNA-binding activity and specificity (Davis et. al., 1990). This region is both necessary and sufficient for

biological activity and for sequence-specific DNA binding (Tapscott et al., 1988). The myogenic proteins are therefore referred to as DNA-binding basic HLH (bHLH) proteins with transcriptional regulatory functions.

These bHLH proteins form heterodimers with the gene product of a ubiquitously expressed bHLH gene, E2A (Murre et al., 1989b). The E2A protein is able to bind specifically to a gene with the same core CANNTG motif as the myogenic factors. It has also been demonstrated that MyoD can bind to two of the muscle creatine kinase (MCK) enhancer elements as well as the promoter region of the myosin light chain (MLC) 1/3 gene (Lassar et al., 1989). Myogenic factors have a relatively weak binding affinity to the CANNTG sites in the MCK enhancers. However, their binding affinity increases when they form the heterodimer with an E2A protein (Murre et. al., 1989b; Davis et. al., 1990; Brennan and Olson, 1990). The DNA binding affinity of myogenin was increased upon its interaction with other DNA binding proteins, such as nuclear factor 1, the myocyte-specific enhancer-binding factor 2, and cooperates with myogenic proteins 1 (COMP1) factor. Furthermore, the transcription of reporter constructs transiently expressed in differentiating muscle cells was also increased (Funk and Wright, 1992).

Another gene, *myd*, has also been identified. It can activate myogenesis when transfected into 10T½ cells. However, the structure of *myd* is unrelated to the MyoD family. The properties of this gene have not yet been characterized at the molecular level (Pinney et al., 1988).

1.2 INVOLVEMENT OF CELL SURFACE PROTEINS IN MYOGENESIS

In the embryo, the formation of multinucleated myotubes is associated with the basement membrane which consists of fibronectin, laminin and type IV collagen. During myogenesis it is essential that cells adhere to the extracellular matrix. Laminin can stimulate the differentiation and fusion of primary newborn mouse cells by interacting with cell surface components (Von de Mark and Ocalan, 1989). Integrins are cell surface receptors for extracellular matrix components such as fibronectin and laminin. Monoclonal antibodies raised against integrin receptors were found to block myogenesis in chicken embryo myoblasts (Boettiger et al., 1988).

The development of skeletal muscle is a complex process. At a specific time during development, the mononucleated myoblasts adhere to each other in a specific way and then fuse to form multinucleated myotubes. This process involves their plasma membranes and cell surface components.

1.2.1 Cell adhesion molecules (CAMs)

It has been recognized that cell-cell adhesion is essential to myotube formation, and that cell adhesion molecules play a important role in myogenesis. The cell-cell adhesion molecules (CAMs) can be divided functionally into Ca^{++}-dependent and Ca^{++}-independent molecules. Skeletal muscle cells are able to express both forms. CAMs are large intrinsic cell surface glycoproteins which are mobile in the plane of the membrane (Gall and Edelman, 1981; Pollerberg et al., 1986).

1.2.1.1 Neural cell adhesion molecules (NCAM)

One of the primary CAMs is the neural cell adhesion molecule (NCAM) which is Ca^{++}-independent. NCAM was originally characterized in nervous tissue, however, it is also expressed in other cell types. Several isoforms of NCAM exist: a large polypeptide, a small polypeptide, and a small surface polypeptide. All three isoforms originate from alternative splicing of one single gene (Edelman and Crossin, 1991). The different polypeptide lengths are due to developmental and tissue-specific alternative RNA splicing. On the N-terminus, each polypeptide has five successive domains homologous to the domains of the immunoglobulin superfamily (Cunningham, 1987). Following these five domains, there are repeat regions which resemble the fibronectin type III repeats. Both large and small polypeptide chains are transmembrane polypeptides, but they have different cytoplasmic domains. The small surface polypeptide does not contain the cytoplasmic domain, it is linked to the cell surface by a phosphatidylinositol intermediate (Edelman and Crossin, 1991).

In muscle, the expression of NCAM isoforms is closely regulated during myogenesis *in vivo* and *in vitro*. The expression is correlated precisely with terminal myoblast differentiation and myotube formation. Three different NCAM transcripts were detected in myoblasts. The major transcript present in proliferating myoblasts corresponds to the transmembrane polypeptide while the major isoform expressed in myotubes is the lipid-linked small surface polypeptide (Covault et al., 1986; Moore et al., 1987).

cultured myoblasts, the initiation of biochemical changes may occur as early as day 1. Thus, it is doubtful whether the above-mentioned cell phosphorylation can play any role in the initiation of myogenesis.

1.3 OBJECTIVES

It may be apparent from the previous discussion that myogenesis is a very complicated process which includes many molecular and cellular events. A number of factors and molecules are involved in regulating and in carrying out these changes. For example, myogenic factors are responsible for activating the expression of various muscle-specific proteins. Cell surface proteins, such as CAMs, are essential for both biochemical and morphological differentiation. Little is presently known about the sequential steps of myogenesis and the regulation of upstream events involved in initiating the entire process of myogenesis.

The objectives of my research project were (i) to characterize cell surface phosphorylation in day-2 culture, (ii) to determine the relationship between cell surface phosphorylation and myogenesis, and (iii) to try to determine the site of action of cell surface phosphorylation in the myogenic pathway. The cell line used is rat L6 myoblasts (Yaffe, 1968).

The first part of this project involved determining the extent of cell surface phosphorylation in day-2 cultures of rat L6 myoblasts and the kinetic properties of the phosphorylation reaction. Attempts were also made to characterize the ecto-PK and the substrate protein involved.

immunological specificities. At least four cadherins have been characterized: the epithelial (E), neuronal (N), placental (P), and muscle (M) cadherins (Miyatani et al., 1989; Ringwald et al., 1987; Nose et al., 1987; Donalies et al., 1991). Although they are the products of different genes, the amino acid sequence showed 50% or greater identity. They also share similar functions and biochemical properties. Cadherins are transmembrane glycoproteins with typical signal sequences and three to four regions of internal homology. Each internal homology consists of about 110 amino acids, but it does not resemble immunoglobulin as the NCAMs do. The cytoplasmic domains are thought to interact with the cytoskeletal elements (Kemler and Ozawa, 1989).

N-cadherin is expressed throughout myogenesis in primary cultures of embryonic chick skeletal muscle. Antibodies to N-cadherin inhibit Ca^{++}-dependent myocyte interaction and the rate of cell fusion (Knudsen et al., 1990). The inhibition of myoblast fusion by anti-cadherin antibodies was also observed in rat L6 myoblasts, and increased cadherin transcript levels were observed during differentiation (Pouliot et al., 1990).

The properties of the M-cadherin (muscle cadherin) were recently examined in differentiating muscle cells. This M-cadherin transcript was present at low levels in myoblasts and was up-regulated during myogenesis (Donalies et al., 1991).

1.2.2 Cell surface phosphorylation

Protein phosphorylation is one of the most effective ways of altering the function and the structure of proteins. It is also a reversible process which allows for regulatory control in many cell processes. Phosphorylation plays a very important role in the regulation of numerous biological processes (Krebs, 1987). While protein kinases are predominantly found inside the cells, protein kinases as well as their substrate proteins are also found on the cell surface.

Cell surface protein kinase (ecto-PK) activity has been detected in cultured 3T3 cells (Mastro and Rozengurt, 1976). Their substrate proteins were also located on the cell surface. This protein kinase was responsible for the phosphorylation of serine and threonine residues. When compared with quiescent 3T3 cells, both the serum stimulated 3T3 cells and SV40-transformed 3T3 cells had increased levels of cell surface phosphorylation (Mastro and Rozengurt, 1976). Ecto-PK activity and substrate protein were also found in guinea pig macrophages (O'Donnell, 1978), HeLa cells (Kubler et al., 1982), mouse lymphoma cells (Kubler et al. 1989), Chinese hamster ovary cells (Kubler et al. 1989), and goat epididymal spermatozoa (Dey and Majumder, 1990). It is interesting to note that cell surface phosphorylation was increased significantly after complete differentiation of neural cells (Ehrlich et al., 1986). In fact, NCAMs are found to be phosphorylated by the ecto-PK, suggesting that the N-CAM function may be modified by ecto-PK.

Although several cell surface proteins have been shown to be phosphorylated by ecto-PK, the correlation between cell surface phosphorylation and

its role in regulating cellular events and in transmitting external stimuli have not been established with any certainty.

Protein phosphorylation and dephosphorylation are involved in signal transduction in eukaryotic cells. For example, insulin can stimulate the utilization of carbohydrate (including the uptake of glucose into cells), protein synthesis, and lipid synthesis (in fat cells). Insulin activates the phosphorylation of the β-subunit of the insulin receptor kinase, and the phosphorylation of a 48 kDa membrane protein. The phosphorylation of these protein substrates is an early step in signal transmission (Haring et al., 1987). The phosphorylation of membrane proteins plays an important role in receiving extracellular stimulation and transmitting the signal into the cells. It is possible that the myogenic differentiation program may be initiated by a similar signal transduction mechanism.

Since NCAMs are involved in myogenesis and they can be phosphorylated by ecto-PK, it is possible that ecto-PK may be involved in myogenesis by regulating the NCAM activity. Ecto-PK and its endogenous substrate proteins have recently been demonstrated in myoblasts. The specific phosphorylation of a cell surface 48 kDa protein by an ecto-PK was thought to be required for myoblast fusion (Lognonne and Wahrmann, 1986, 1988, 1990). It is important to note that their studies were carried out with day-4 or confluent cultures. Since biochemical changes should occur before morphological changes during myogenesis, one would expect biochemical changes should take place at a very early stage of myogenesis. For

cultured myoblasts, the initiation of biochemical changes may occur as early as day 1. Thus, it is doubtful whether the above-mentioned cell phosphorylation can play any role in the initiation of myogenesis.

1.3 OBJECTIVES

It may be apparent from the previous discussion that myogenesis is a very complicated process which includes many molecular and cellular events. A number of factors and molecules are involved in regulating and in carrying out these changes. For example, myogenic factors are responsible for activating the expression of various muscle-specific proteins. Cell surface proteins, such as CAMs, are essential for both biochemical and morphological differentiation. Little is presently known about the sequential steps of myogenesis and the regulation of upstream events involved in initiating the entire process of myogenesis.

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The first part of this project involved determining the extent of cell surface phosphorylation in day-2 cultures of rat L6 myoblasts and the kinetic properties of the phosphorylation reaction. Attempts were also made to characterize the ecto-PK and the substrate protein involved.

The second part of the project has examined the relationship between the cell surface phosphorylation and myogenesis. One of the approaches used to determine the components involved in myogenesis was to use myogenesis inhibitors to possibly reveal whether cell surface phosphorylation is affected in these myogenesis-impaired cells. However, this approach cannot be used to define conclusively the components involved in myogenesis, as most of the inhibitors can act on more than one target site. Another approach used was to compare the properties of the myogenesis-defective mutants with their myogenesis-competent parental cells (Cates et al., 1984). These studies were done to help reveal the components involved in myogenesis.

The third part of my project was to determine the possible site of action of cell surface phosphorylation in the myogenic pathway. In these studies the transcript levels of muscle-specific proteins, the cell adhesion molecule (NCAM), and myogenic factors in myogenesis-impaired cells have been determined in relation to cell surface phosphorylation. To further study the site of action of cell surface phosphorylation, myogenic factor cDNAs were transfected into non-fusing cells. The expression of other myogenic factors, muscle-specific proteins, and the level of cell surface phosphorylation in these transfectants were monitored to provide some information about the sequence of events during myogenesis.

The above studies were carried out to obtain useful information in our understanding the initiation and the events involved in myogenesis.

CHAPTER 2

PHOSPHORYLATION OF A CELL SURFACE 112 kDa PROTEIN BY AN ECTO-PROTEIN KINASE IN RAT L6 MYOBLASTS

2.1 INTRODUCTION

While ATP is considered largely as an intracellular energy source, extracellular ATP has been shown to play important regulatory roles in differentiation, transduction of external stimuli, and cell-cell interaction (Filippini et al., 1990; Gordon, 1986; Burnstock, 1981). Extracellular ATP, even when present in micromolar concentrations, can cause depolarization and changes in membrane permeability in mast cells, mononuclear and polymorphonuclear phagocytes, hepatocytes and a number of transformed cell lines (Gomperts, 1985; Charest et al., 1985; Becker & Henson, 1975; Weisman et al., 1984; Chahwala & Cantely, 1984; Dubyak & DeYoung, 1985; Steinberg & Silverstein, 1987). Extracellular ATP is thought to interact directly with specific cell surface proteins. Of the ecto-enzymes examined, ecto-protein kinase(s) are the likely candidates for mediating some of the observed phenomena, as phosphorylation can alter the structure and function of proteins in a reversible manner (Kubler et al., 1986).

Ecto-protein kinases, as well as their substrate proteins, have been detected in a variety of mammalian plasma membranes. They catalyze the transfer of terminal phosphate of [γ-^{32}P]ATP onto endogenous cell surface proteins, as well as onto exogenously added macromolecules. Generally speaking,

ecto-protein kinases can be classified according to their dependency on cAMP. cAMP-dependent ecto-protein kinases have been detected in HeLa cells, CHO cells, mouse lymphoma S49 cells, and in spermatozoa (Kubler et al., 1989; Majumder, 1978; Dey & Majumder, 1990). Maximum activation of phosphorylation is usually achieved with 5μM cAMP; whereas similar activation cannot be observed with cGMP. The Km value of the spermatozoa cAMP-dependent ecto-protein kinase for ATP is around 500μM (Dey & Majumder, 1990). cAMP-independent ecto-protein kinases are found in transformed 3T3 cells, adipocytes, HeLa cells, neural cells, fibroblasts, macrophages and myoblasts (Mastro & Rozengurt, 1976; Weisman et al., 1984; Kang et al., 1979; Kubler et al., 1982; Ehrlich et al., 1986; Remold-O'Donnell, 1978; Lognonne & Wahrmann, 1986, 1988). They are generally detected using ATP concentrations ranging from 10^{-6} to 10^{-5}M. Some reactions are dependent on Ca^{2+} and/or Mg^{2+} ions. Where determined, the Km values of these protein kinases for ATP are in the 10^{-6} to 10^{-5}M range (Mastro & Rozengurt, 1976; Kang et al., 1979). In most cases, a number of endogenous cell surface proteins, ranging from 40 kDa to 250 kDa, are phosphorylated by these protein kinases. Most of the ecto-protein kinases examined are serine/threonine protein kinases.

A cAMP-independent ecto-protein kinase was detected in confluent day-4 culture of rat L6 myoblasts (Senechal et al., 1982; Lognonne & Wahrmann, 1986,1988,1990). Using 5μM ATP as the substrate, the overall phosphorylation of cell surface proteins of fusion-competent myoblasts was activated 2-4 fold by

2mM $CaCl_2$; about twenty proteins, ranging from 15 kDa to 250 kDa were phosphorylated. Amongst these proteins, the phosphorylation of a 48 kDa protein was activated markedly in undifferentiated myoblasts and was reduced significantly in myotubes. The phosphorylation of this protein was hardly detected in non-fusing mutants.

This chapter describes the presence of an ecto-protein kinase present predominantly in subconfluent undifferentiated rat L6 myoblasts. A cell surface 112 kDa protein was consistently phosphorylated by this ecto-protein kinase.

2.2 METHODS AND MATERIALS

2.2.1 Cell lines and culture media

Yaffe's L6 rat skeletal myoblast line (Yaffe, 1968) was maintained in Alpha medium (Flow Laboratories) supplemented with 10% (v/v) horse serum (Flow Laboratories) and gentamicin (50μg/ml; Gibco) as described by D'Amore (D'Amore & Lo 1986). Transfers were routinely made every 3 days (before fusion); 0.1% trypsin was used to detach cells from the plates. Cells were counted with a Coulter Counter. For routine phosphorylation assays, cells were seeded at 8×10^4 cells per 60mm plate.

2.2.2 Phosphorylation conditions.

For routine phosphorylation the following conditions were chosen.

Day-2 rat myoblast cultures were washed twice with 5ml of buffer A (15mM Hepes, 5mM $MgCl_2$, 10mM $CaCl_2$, 8g/litre NaCl, 0.5mM PMSF, and 2μg/ml leupeptin). Cells were then incubated with 3ml of buffer A for 30 min. After incubation, buffer A was replaced with 1.2ml of the phosphorylation buffer (buffer A with 0.03μM γ-^{32}P-ATP and 2mM NaF). Phosphorylation was allowed to proceed for 8 min. The phosphorylation reaction was terminated by washing the cells once with 5ml of buffer A containing 10μM ATP, and then twice with 5ml of buffer A. Cells were then lysed with 0.3ml of the lysis buffer (10mM Tris-HCl, pH 7.5, 0.15M NaCl, 1% Triton X-100, 1mM EGTA, 2μg/ml leupeptin, and 0.5mM PMSF); 0.1ml of the 4X SDS sample buffer was then added to each sample. After boiling for 5 min., samples were loaded onto an 8.5% SDS-polyacrylamide gel. In each set of experiments, the same amount of protein was loaded onto each lane. The molecular weight markers used were: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soya bean trypsin inhibitor, 20 kDa; and α-lactalbumin, 14.4 kDa. Protein bands were stained with Coomassie blue. After drying, the gel was exposed to a Kodak X-OMAT AR film. All experiments were repeated at least three times. The level of p112 was found to be very consistent in all cases.

The kinetic properties of the ecto-protein kinase in phosphorylating the 112 kDa protein were determined using γ-^{32}P-ATP at concentrations ranging from 0.0023μM to 0.020μM; the same amount of radioactivity was added to different concentrations of the unlabelled ATP. The reaction was carried out at

21°C. After separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the 112 kDa band was excised from the gel, digested with 250µl of 30% H_2O_2 at 80°C for 4 hrs. The amount of radioactivity was determined by liquid scintillation counting in scintillation fluid supplemented with 0.7% acetic acid. The level of the phosphorylated 112 kDa protein (p112) at 2, 3, 4, 5 min. after the addition of γ-^{32}P-ATP was used to calculate the rate of phosphorylation. The data presented are results of representative experiments which were repeated at least twice.

2.2.3 Analysis of phosphoamino Acids

Phosphoamino acid analysis was carried out as described by Litchfield and Ball, 1987, with some modifications. After separation by SDS-PAGE, the [^{32}P]-labelled 112 kDa protein was excised from the gel. This was then incubated with shaking in 3ml of 50mM ammonium bicarbonate containing 0.1% SDS and 5% 2-mercaptoethanol for 16 hrs. at 37°C. The solubilized protein was precipitated in the presence of 10% trichloroacetic acid and 12µg/ml bovine serum albumin. The precipitated protein was washed with ice cold 80% ethanol, and then hydrolysed in 6N HCl for 2 hrs at 110°C. The hydrolysed sample was then lyophilized, and washed twice with distilled water. After drying, the hydrolysate was dissolved in 10µl of pH 1.9 buffer (2.5% formic acid (88% stock) and 7.8% glacial acetic acid) containing unlabelled phosphoamino acids (phosphoserine, phosphothreonine and phosphotyrosine at 0.15mg/ml each). The sample was then applied to a 10 x 10cm cellulose-coated TLC plate. The

sample was electrophoresed in the first dimension using the pH 1.9 buffer towards the anode at 1250 volts for 25 min. After which, the sample was electrophoresed in the second dimension using the pH 3.5 buffer (0.5% pyridine and 5.0% glacial acetic acid) at 1250 volts for 15 min. The plate was then dried, stained with ninhydrin (0.2% in acetone) and developed in a 55°C oven for 1 hr to visualize standard phosphoamino acids, and then exposed to a Kodak X-OMAT AR film to detect the ^{32}P-labelled phosphoamino acids. The experiment was repeated three times.

2.2.4 Alkaline phosphatase treatment

Cells were phosphorylated with γ-^{32}P-ATP under standard conditions. After washing with buffer A, cells were treated with or without alkaline phosphatase for 1 or 2 min. After which, cells were washed once with 5ml of buffer A, and then lysed with 0.3ml of the lysis buffer. After boiling with the SDS sample buffer for 5 min., samples were subjected to SDS-PAGE. After drying, the gel was exposed to a Kodak X-OMAT AR film. The level of p112 was then determined by densitometry.

2.2.5 Hydroxylamine treatment

Hydroxylamine treatment was used to determine the presence of the acyl-phosphate linkage in the ^{32}P-labelled 112 kDa protein (Hokin et al., 1965). After separation by SDS-PAGE, the ^{32}P-labelled 112 kDa protein was excised from the gel, and incubated with shaking in 3ml of 50mM ammonium bicarbonate

containing 0.1% SDS and 5% 2-mercaptoethanol for 16 hrs. at 37°C. The solubilized protein was then precipitated with 10% trichloroacetic acid containing 12μg/ml bovine serum albumin. The precipitated protein was washed with ice-cold 80% ethanol, and dissolved in 0.1ml water. This was then incubated in 0.2ml of 0.1M acetate buffer (pH 5.4) containing 0.8M hydroxylamine at room temperature for 10 min. Hydroxylamine was prepared in the cold just before use by adding 2 parts of 8N NaOH to 5 parts of 4N hydroxylamine hydrochloride. The control sample was treated with 0.64M NaCl instead of hydroxylamine. After the reaction, samples were subjected to SDS-PAGE. The amount of radioactivity associated with the control and the treated 112 kDa protein was determined by methods as described earlier.

2.2.6 Materials

α-^{32}P-ATP and γ-^{32}P-ATP were purchased from ICN Biochemicals, Canada Ltd. Hydroxylamine hydrochloride, alkaline phosphatase, and trypsin were purchased from Sigma Chemical Co.. All other chemicals were obtained from commercial sources and were of the highest available purity.

2.3 RESULTS

2.3.1 Phosphorylation of a 112 kDa protein by extracellular ATP

When a day-2 culture of rat L6 myoblasts, originally seeded at 8 x 10^4 cells per 60 mm plate, was incubated with 0.03μM γ-^{32}P-ATP at 21°C in the phosphorylation buffer, a significant amount of radioactivity was found to be

associated with these cells, even after washing with unlabelled ATP (10μM). Analysis of these cells by SDS-PAGE revealed that a 112 kDa protein was consistently labelled by the γ-^{32}P-ATP (Fig. 2.1). The phosphorylated 48 kDa protein found in confluent rat myoblasts (Lognonne & Wahrmann, 1986, 1988, 1990) could not be detected in the present study. A number of proteins ranging from 40 to 50 kDa were phosphorylated. However, these proteins were not consistently phosphorylated, the extent of phosphorylation of these lower molecular weight proteins varied considerably from experiment to experiment; whereas the level of the phosphorylated 112 kDa protein (p112) remained relatively constant within each set of experiments. Thus, only the level of p112 was monitored in this investigation.

Unlike other ecto-protein kinases, the phosphorylation of the 112 kDa protein by extracellular ATP was detectable only when the reaction was carried out in the presence of Ca^{2+}, F^-, and Mg^{2+} (Fig. 2.2) (Chang et al., 1974; Mastro & Rozengurt, 1976; Kubler et al., 1982, 1989). The requirements of various ions were tested over a range of concentrations. The optimal concentrations of Ca^{2+}, F^-, and Mg^{2+} required were 10mM, 2mM, and 5mM, respectively. In three separate experiments, phosphorylation of the 112 kDa protein could not be observed in reactions carried out in 0, 1 or 20mM Ca^{2+}, phosphorylation could be observed only at 10mM Ca^{2+}. The absence of p112 at higher Ca^{2+} and F^- concentrations was probably due to precipitation of CaF_2. This stringent Ca^{2+} requirement was not observed with the intracellular protein

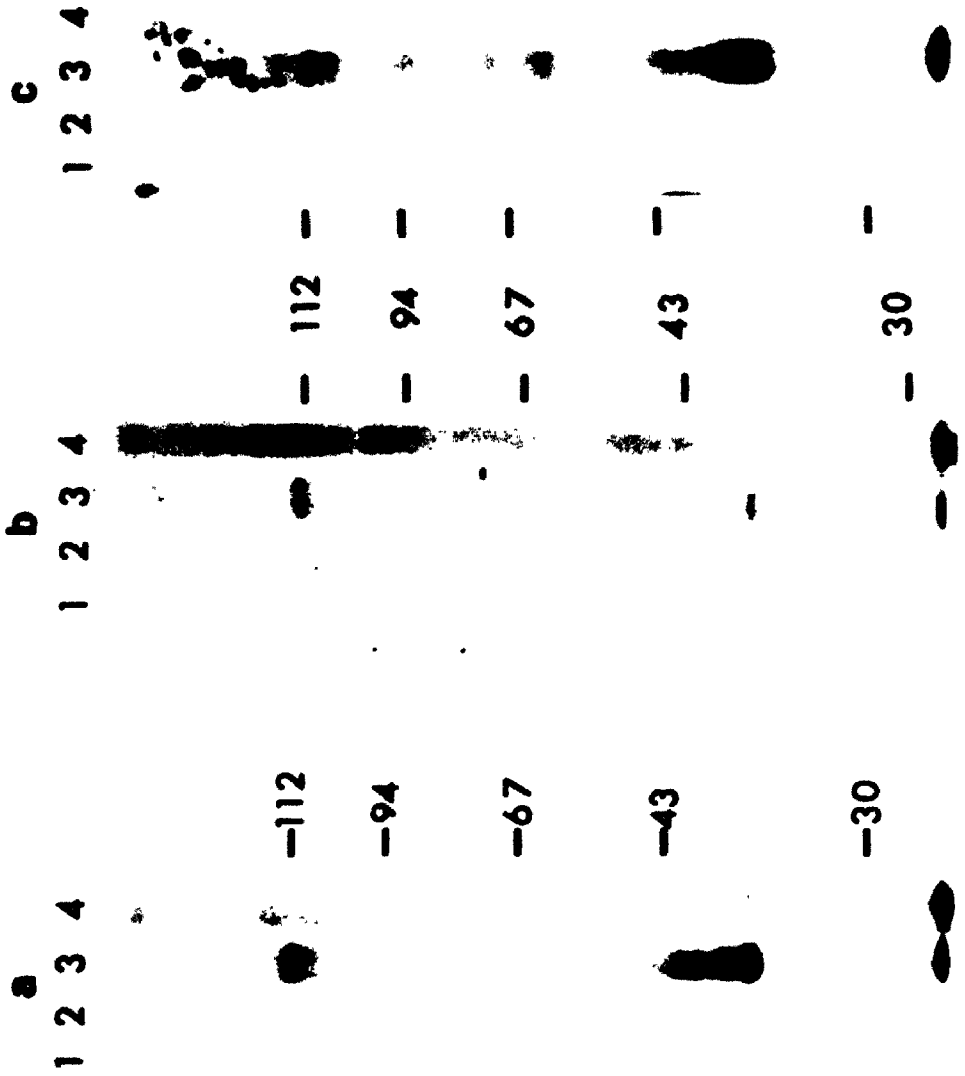
Figure 2.1 Phosphorylation of an 112 kDa protein by extracellular ATP.

The phosphorylation of cell surface proteins by extracellular ATP was carried out as described in the text. The concentration of γ-^{32}P-ATP used was 0.03μM. The phosphorylation reaction was carried out in the presence of 10mM $CaCl_2$, 5mM $MgCl_2$ and 2mM NaF. This figure shows the rate of phosphorylation of the 112 kDa protein. The numbers on top of each lane indicate the time (in minutes) of the reaction. The molecular weight markers used were: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa.


1 2 3 4 5 6 7 8
112
94
67
43
30

Figure 2.2 Requirement of Ca^{2+}, F^-, and Mg^{2+} ions for the phosphorylation of the 112 kDa protein.

Phosphorylation studies were carried out under standard condition, except that the concentrations of Ca^{2+}, F^-, and Mg^{2+} were varied as indicated. In all cases, the reaction was carried out at 21°C for 8 min. Panel a indicates the effect of varying $CaCl_2$ concentrations. Lanes 1, 2, 3, and 4 indicate reactions carried out in the presence of 0, 1, 10, and 20mM of $CaCl_2$, respectively. The concentrations of $MgCl_2$ and NaF were maintained at 5mM and 2mM, respectively. Panel b indicates the requirement of $MgCl_2$ for phosphorylation. Lanes 1, 2, 3, and 4 indicate phosphorylation carried out in the presence of 0, 1, 5, and 10mM $MgCl_2$, respectively. The concentrations of $CaCl_2$ and NaF were maintained at 10mM and 2mM, respectively. Panel c indicates the effect of fluoride on the phosphorylation of the 112 kDa protein. Lanes 1, 2, 3, and 4 indicate reactions carried out in the presence of 0, 1, 2, and 3mM NaF, respectively. The concentrations of $CaCl_2$ and $MgCl_2$ were maintained at 10mM and 5mM, respectively. The molecular weight markers used were: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa.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kinases. It should be noted that a millimolar range of Ca^{2+} was also required for optimal activation of the ecto-protein kinase found in confluent rat myoblasts (Lognonne & Wahrmann, 1986). It is presently not certain whether NaF is required to inhibit dephosphorylation of p112 by phosphatases, or to activate the ecto-protein kinase involved. The level of p112 was increased by pretreatment of cells with protease inhibitors such as leupeptin and PMSF for 30 min (Fig. 2.3a). This suggested that the rate of degradation of the protein was relatively fast. The extent of phosphorylation was found to increase with temperature (Fig. 2.3b). Subsequent experiments were carried out at 21°C, even though the p112 level was higher at 37°C; this was intended to minimize the chances of internalization of ATP.

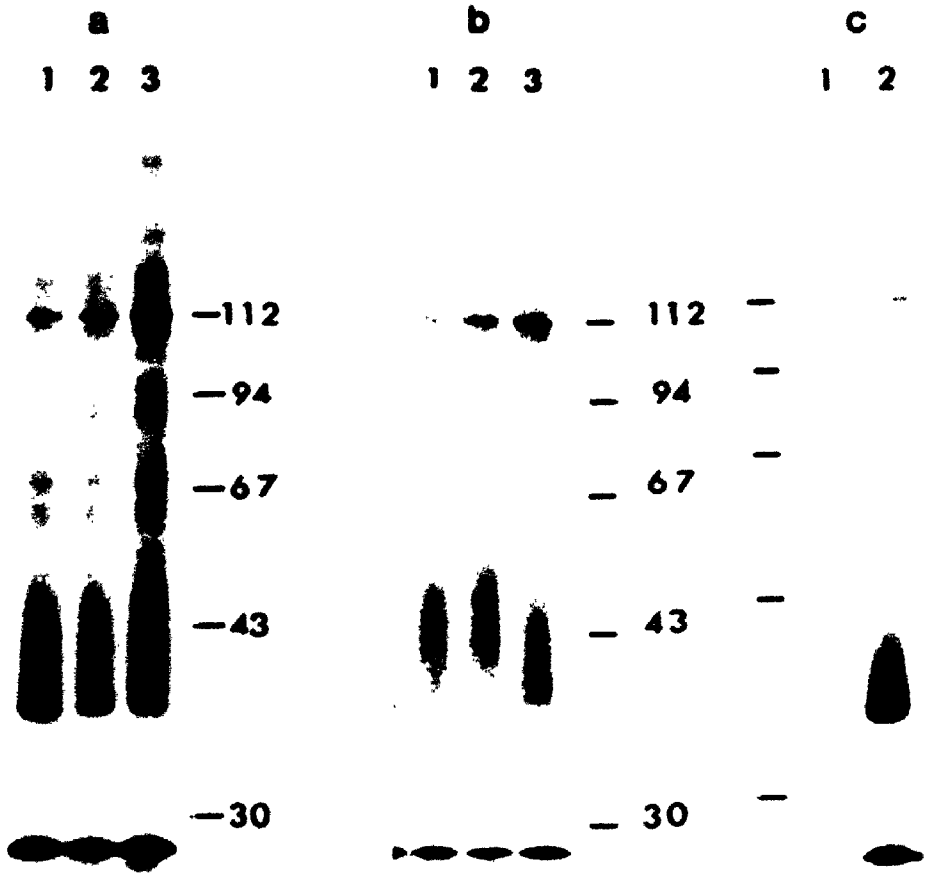
2.3.2 Nature of the phosphorylated 112 kDa protein

Attempts were made to determine the nature of the association of phosphate with the 112 kDa protein. Three pieces of evidence indicated that p112 was not formed by tight binding of the entire ATP molecule to the 112 kDa protein. First, the 112 kDa protein could not be labelled with α-^{32}P-ATP (Fig. 2.3c). Second, after phosphorylation with γ-^{32}P-ATP, the radioactivity associated with p112 could not be displaced with 10μM of non-radioactive ATP. Third, the radioactivity associated with p112 remained attached to the protein even after SDS-PAGE.

Acyl phosphate was found to be a phosphorylated intermediate upon

Figure 2.3 Nature of the phosphorylation reaction.

The phosphorylation reaction was carried out as described in the text. The reaction was carried out for 8 min. Panel a indicates the effect of preincubation of the cells in the presence of protease inhibitors. The reaction was carried out at 21°C. Lane 1 indicates phosphorylation of the cells without preincubation with buffer A. Lane 2 indicates phosphorylation of the cells preincubated for 30 min with buffer A lacking any protease inhibitors. Lane 3 indicates phosphorylation of the cells preincubated for 30 min with buffer A (which contains 0.5mM PMSF and 2μg/ml of leupeptin). Panel b shows the effect of temperature on phosphorylation. These experiments were carried out with cells preincubated for 30 min with buffer A. Lanes 1, 2 and 3 indicate reaction carried out at 4°, 21°, and 37°C, respectively. Panel c shows the phosphorylation reaction using α-^{32}P-ATP (lane 1), and γ-^{32}P-ATP (lane 2).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1 2

incubation of the brain (Na^+,K^+)-dependent ATPase with γ-^{32}P-ATP (Hokin et al., 1965). Attempts were therefore made to determine the presence of acyl phosphate in p112. The following evidence precluded the presence of acyl phosphate in the p112 examined. First, before separation by SDS-PAGE, the sample was boiled in the SDS-sample buffer for 5 min, this treatment should destroy the acyl phosphate bond (Hokin et al., 1965; Mastro & Rozengurt, 1976). Second, treatment of p112 with 0.8M hydroxylamine, pH 5.4 for 30 min did not reduce the amount of [^{32}P]-phosphate incorporated in p112. The hydroxylamine-treated p112 retained 95% of the [^{32}P]-phosphate associated with p112.

Further characterization of the phosphorylation reaction involved a determination of the amino acid phosphorylated. After eluting from the SDS-gel, p112 was precipitated with cold trichloroacetic acid, washed, lyophilized, and hydrolysed in 6N HCl for 2 hrs at 110°C. The identity of the phosphoamino acid was then determined by two dimensional electrophoresis on TLC plates. Serine was the only amino acid phosphorylated (Fig. 2.4). Thus this suggested the involvement of a serine protein kinase in the phosphorylation of the 112 kDa protein.

2.3.3 Kinetic properties of the phosphorylation reaction

The rate of phosphorylation of the 112 kDa protein by the ecto-protein kinase remained linear for at least 8 min (Fig. 2.5a). Using different ATP concentrations, the levels of p112 at 2, 3, 4, 5 min after the addition of γ-

Figure 2.4 Phosphoamino acid from the phosphorylated 112 kDa protein.

After separation by SDS-PAGE, the [^{32}P]-labelled 112 kDa protein was excised from the gel. After elution from the gel, the solubilized protein was hydrolysed and the phosphoamino acids were then separated by two-dimensional electrophoresis on TLC plates, as described in Methods and Materials. After electrophoresis, the plate was dried, stained with ninhydrin to visualize standard phosphoamino acids, and then exposed to a Kodak X-OMAT AR film to detect the ^{32}P-labelled phosphoamino acids. The standard phosphoamino acids used were phosphoserine, phosphothreonine and phosphotyrosine. 0, 1, and 2 denote the origin, the direction for the first dimension, and the direction for the second dimension of electrophoresis, respectively. PS, PT, and PY indicate the positions of the standard phosphoserine, phosphothreonine, and phosphotyrosine, respectively.

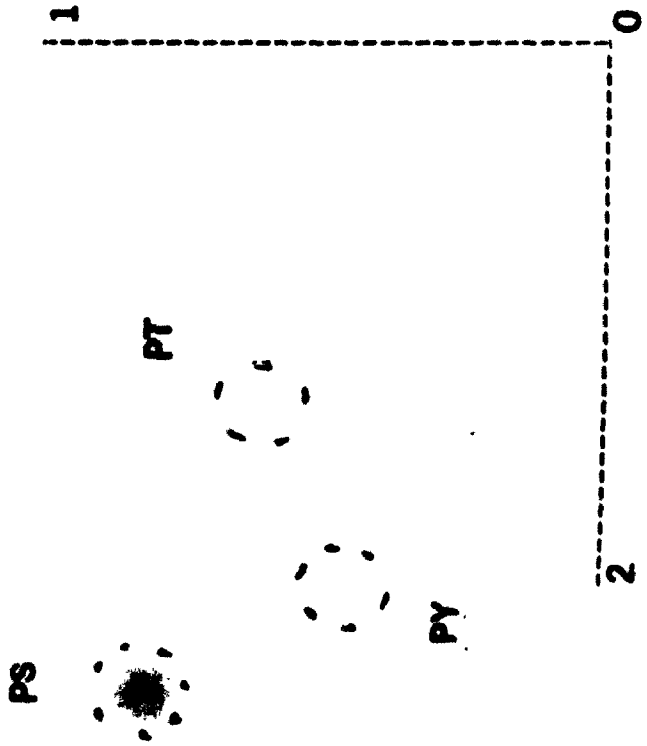
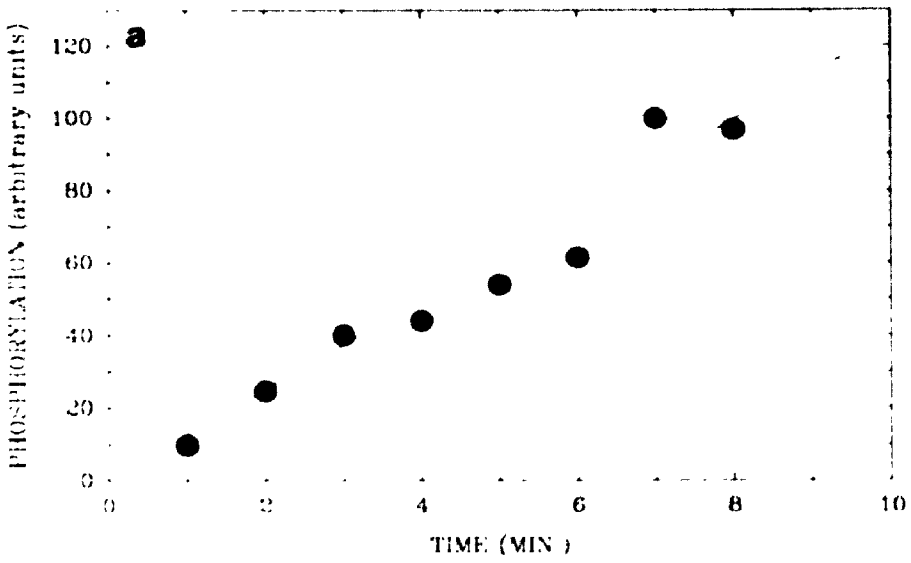
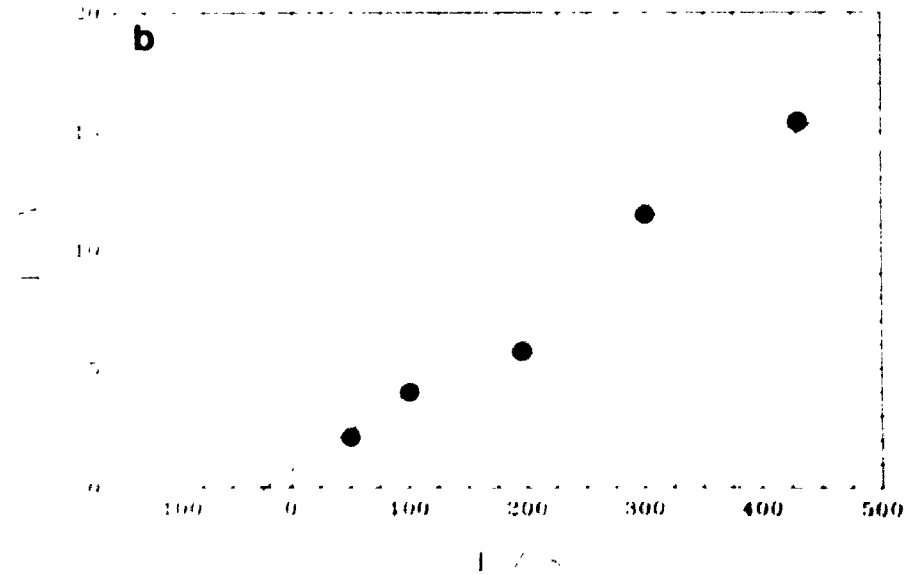

1
0
2
PT
PY
PS

Figure 2.5 Phosphorylation kinetics of the 112 kDa protein.

Phosphorylation of cell surface proteins by extracellular ATP was carried out under standard condition, as described in the text. The reaction was carried out at 21°C. Panel a shows phosphorylation of the 112 kDa protein as a function of time. The concentration of γ-^{32}P-ATP used was 0.03 μM. After separation by SDS-PAGE, the gel was then exposed to a Kodak X-OMAT AR film. This panel shows the quantitation of the level of the phosphorylated 112 kDa protein by densitometry. Autoradiograms used were within the linear range of the film. Panel b shows the kinetics of the phosphorylation reaction. The methods used for determining the kinetic properties of the ecto-protein kinase were descnoed in the text. The rate of phosphorylation (V) is expressed as pmoles/10^5cells/min. S refers to the concentrations of ATP (in μM) used. The data presented were results of representative experiments which were repeated at least twice. Results were found to be very consistent in all cases.


a
PHOSPHORYLATION (arbitrary units)
120
100
80
60
40
20
0
0
2
4
6
8
10
TIME (MIN)


b
100
0
100
200
300
400
500

^{32}P-ATP were used to calculate the rate of phosphorylation (Fig. 2.5b). Since the reaction was carried out at 21°C, the amount of ATP secreted by the cells should be negligible during this very short period of time.

The apparent Km and Vmax values for the phosphorylation of the 112 kDa protein were 0.04μM, and 1.67 x 10^{-4}pmol/10^5 cells/min, respectively. In other words, 1 x 10^3 molecules of the 112 kDa protein per cell could be phosphorylated in one minute under our experimental condition. While the kinetic properties of the previously described rat myoblast ecto-protein kinase have not been determined (Senechal et al., 1982; Lognonne & Wahrmann, 1986, 1988), the present enzyme has significantly higher affinity for ATP than the ecto-protein kinases found in other cell types (Dey & Majumder, 1990; Mastro & Rozengurt, 1976; Kang et al., 1979).

2.3.4 Profiles of the phosphorylated proteins

Quite a number of intracellular protein kinases have been characterized; some are activated by cAMP or cGMP whereas others do not require specific ions for their activities (Krebs, 1986; Edelman et al., 1987; Kikkawa et al., 1989). So if γ-^{32}P-ATP can enter the cells, a large number of proteins should be phosphorylated; this profile should be distinct from that brought about by the ecto-protein kinase. Phosphorylation studies were therefore carried out with intact whole cells and with cells that had been permeabilized with 1% Triton X-100 or with 10μg/ml trypsin. When grown under our

Figure 2.6 Integrity of the rat L6 myoblast plasma membrane

Integrity of the plasma membrane of the rat L6 myoblasts was evaluated by the permeability of the fluorescent dye, ethidium bromide, into the cells. Rat myoblast monolayers were first washed with buffer A, and then incubated with buffer A containing 0.5μg/ml ethidium bromide for 10 min. The presence of ethidium bromide inside the cells was then examined using a fluorescent microscope. Panels A and C show the cell morphology (as shown by phase contrast microscopy) of untreated myoblasts and myoblasts treated with 0.01% Triton X-100 for 5 min, respectively. Panels B and D show the staining of the nuclei by ethidium bromide in untreated myoblasts and in myoblasts permeabilized with 0.01% Triton X-100, respectively.

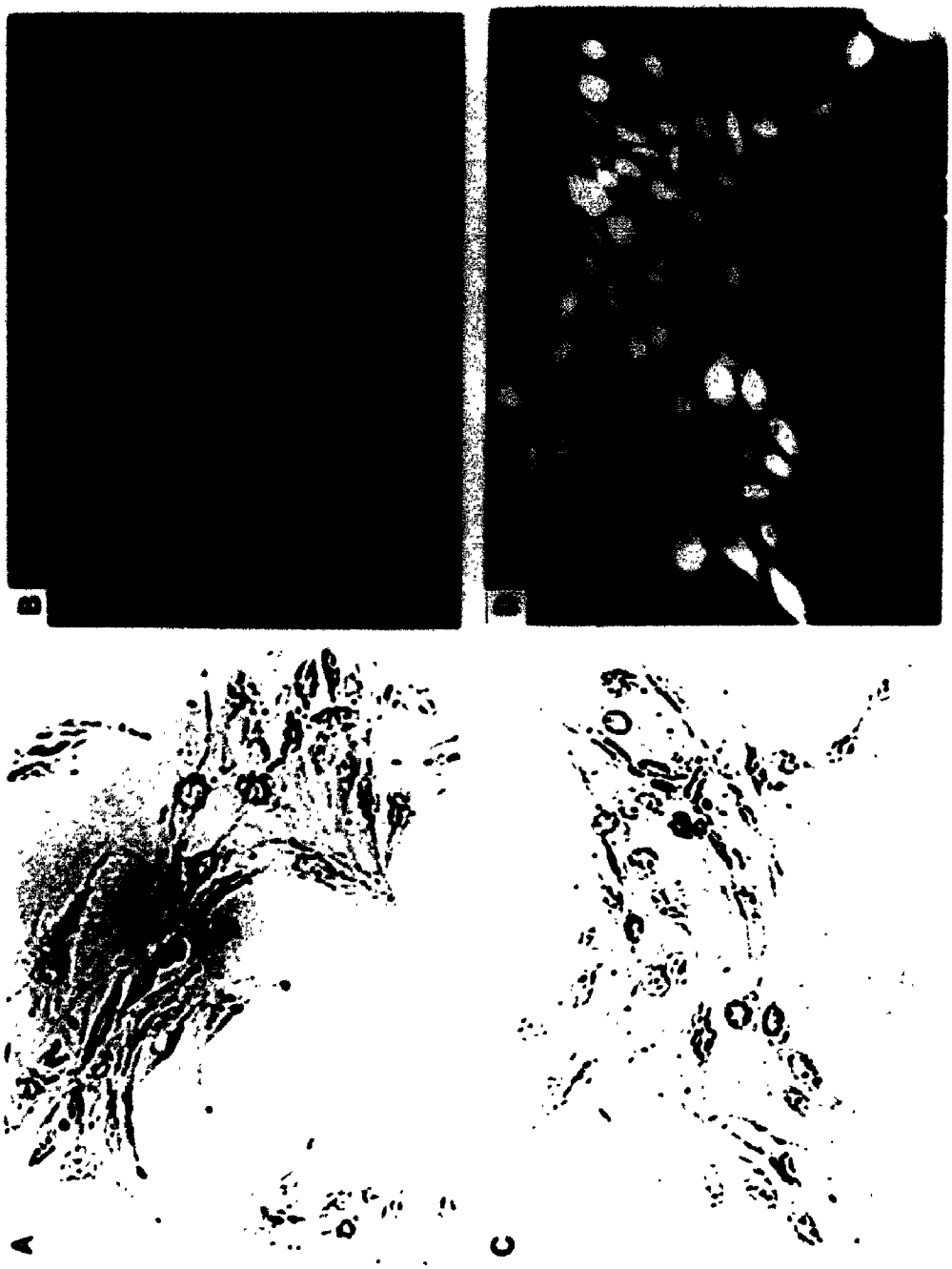


Figure 2.7 Phospho-protein profiles of intact and permeabilized cells.

Cells were first permeabilized by treatment with 1% Triton X-100 for 5 min., or with 1μg/ml trypsin for 30 min. Cell surface phosphorylation studies were then carried out under the standard condition, as described in the text. The reaction was carried out for 8 min. at 21°C. The concentration of γ-^{32}P-ATP used was 0.03μM. This figure shows the profiles of phosphorylated proteins in intact cells (Panel a, lane 1 and Panel b, lane 1), in cells previously lysed by 1% Triton X-100 (Panel a, lane 2), and in cells permeabilized with 10μg/ml trypsin (Panel b, lane 2).

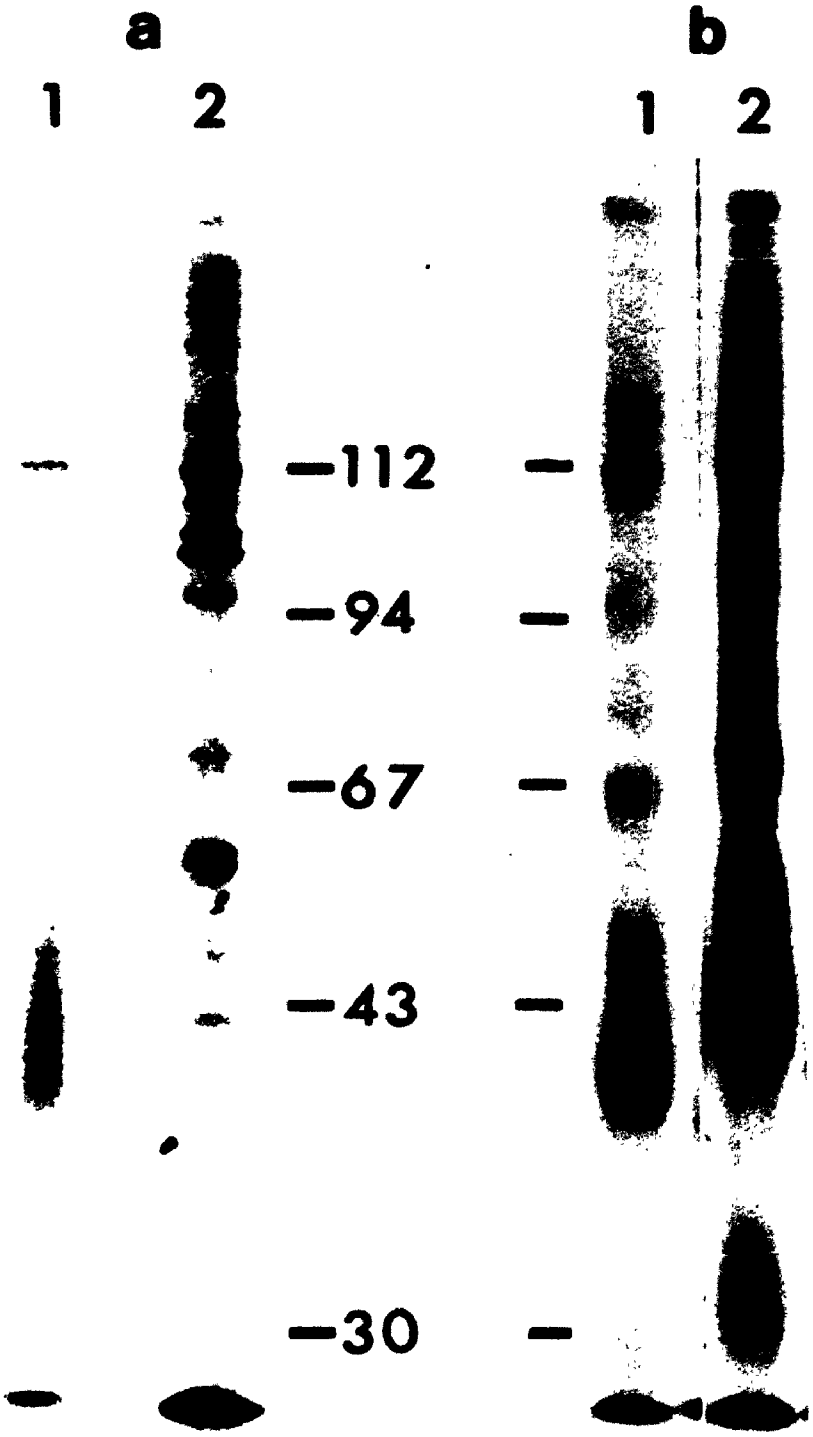

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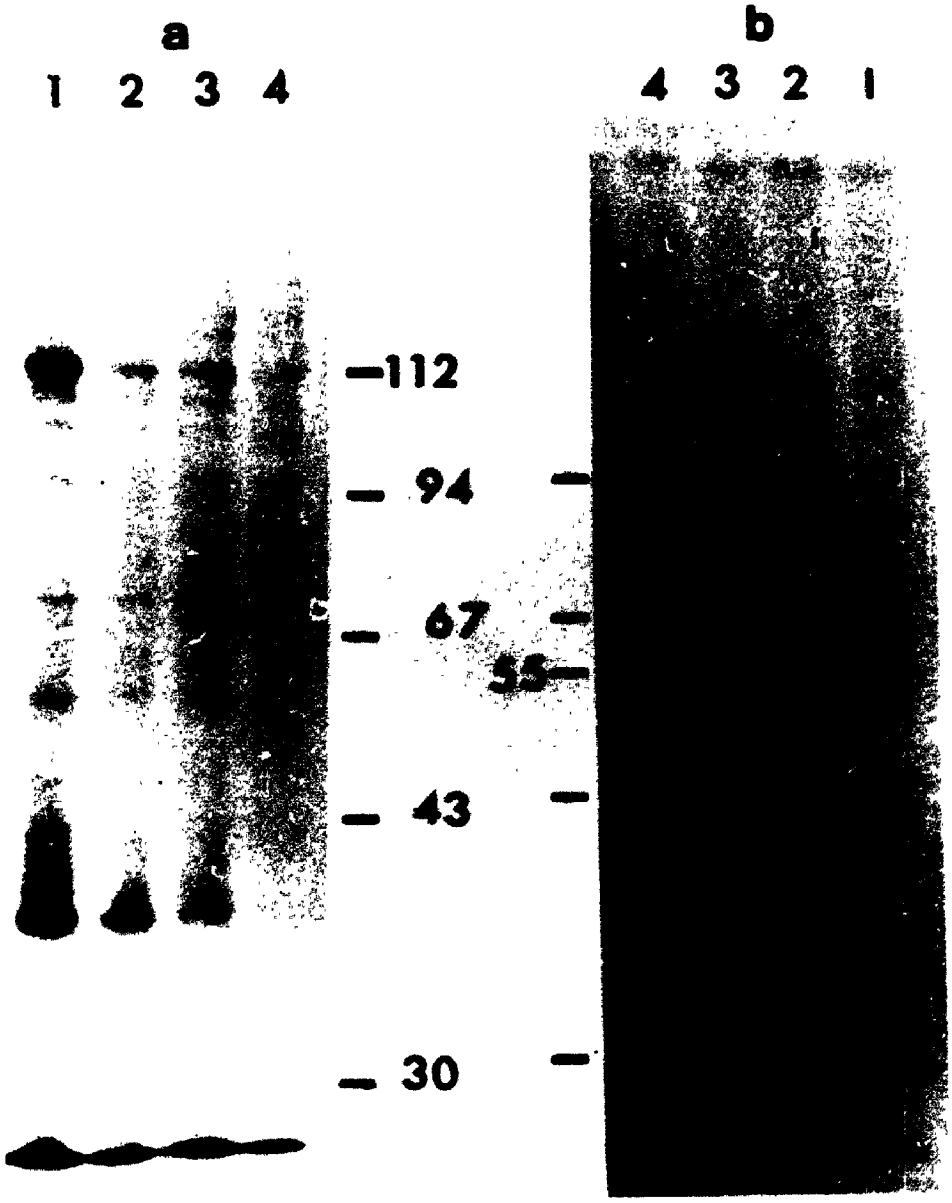
Figure 2.8 Effect of exogenously added fetal calf serum on the phosphorylation of the 112 kDa protein.

Cell surface phosphorylation assays were carried out under standard conditions, except that 3μl of heat-inactivated fetal calf serum was included in 1.2ml of the phosphorylation buffer. Panel a shows the effect of heat-inactivated fetal calf serum on the phosphorylation of the 112 kDa protein. Lanes 1, 2, 3, and 4 show the effect of 0%, 0.05%, 0.1% and 0.5% fetal calf serum on the phosphorylation of the 112 kDa protein. Panel b indicates the phosphorylation of fetal calf serum proteins upon incubation with the cells. After phosphorylation, 280μl of the supernatant from each well was added to 93μl of the 4X SDS sample buffer. After boiling in SDS-sample buffer for 5 min, 100μl of this sample was applied onto the SDS polyacrylamide gel. Lanes 1, 2, 3, and 4 show the phosphorylation of a 55 kDa fetal calf serum protein by cells incubated with 0%, 0.05%, 0.1% and 0.5% fetal calf serum, respectively.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experimental conditions, rat L6 myoblasts were normally not permeable to ethidium bromide (Fig. 2.6B). These cells were rendered permeable to this fluorescent dye only after treatment with Triton X-100 (Fig. 2.6D). The phosphoprotein profiles of intact and permeabilized cells differed significantly (Fig. 2.7). While the 112 kDa protein was specifically phosphorylated in intact cells, a large number of proteins was phosphorylated in the permeabilized cells. This indicated that the exogenously added ATP could not enter intact cells. In agreement with this finding, cAMP and cGMP (which should activate a number of intracellular protein kinases) did not alter phosphoprotein profile generated by the exogenously added γ-^{32}P-ATP (data not shown). In other words, the exogenously added ATP could not gain access to the intracellular cyclic nucleotide-dependent protein kinases. A comparison of the phosphoprotein profiles at different temperatures also revealed that ATP did not enter the cells at 37°C (Fig. 2.3b).

2.3.5 Inhibition of cell surface phosphorylation by exogenously added proteins

If the catalytic site of the protein kinase is accessible to the external environment, then exogenously added proteins may compete with the 112 kDa protein for this site, thus preventing phosphorylation of the 112 kDa protein. Exogenously added heat-inactivated fetal calf serum (FCS) was found to inhibit phosphorylation of the 112 kDa protein (Fig. 2.8a). Since FCS could not dephosphorylate p112 when added to the phosphorylated cells, the heat-inactivated FCS was not likely to contain phosphatase activity. As shown in

Figure 2.8b, the exogenously added FCS proteins were phosphorylated by γ-^{32}P-ATP, albeit not very efficiently, when incubated in the presence of the cells. Similarly, a 75 kDa horse serum protein could also be phosphorylated by the ecto-protein kinase. These findings indicated that the catalytic site of the protein kinase was accessible to the external environment.

2.3.6 Effect of exogenously added hydrolytic enzymes.

Information on the location of the 112 kDa protein can be obtained through the use of non-penetrating modifying reagents. Mild trypsinization has been shown to abolish cell surface phosphorylation of 3T3 cells (Mastro & Rozengurt, 1976). In the present study, cells were treated with trypsin prior to the phosphorylation reaction (Table 2.1). Treatment with 0.1 and 0.5μg/ml of trypsin for 30 min. reduced the amount of p112 by 23% and 54%, respectively. This indicated that the 112 kDa protein and/or the ecto-protein kinase were exposed on the cell surface, even before the phosphorylation reaction.

If p112 is exposed on the cell surface, then it may be dephosphorylated by exogenously added alkaline phosphatase. After the phosphorylation reaction, cells were treated with 0.1 or 1μg/ml of alkaline phosphatase for one or two minutes. As shown in Table 2.1, 0.1μg/ml alkaline phosphatase reduced the phosphorylation of the 112 kDa protein by 31% and 49% in 1 and 2 min, respectively; whereas 1μg/ml alkaline phosphatase dephosphorylated 79% of p112 in 1 min. Thus this shows that the phospho-

TABLE 2.1. Effect of exogenously added hydrolytic enzymes on cell surface phosphorylation

In the case of trypsin treatment, cells were preincubated with different concentrations of the enzyme for 30 min. before the phosphorylation reaction. After washing, cell surface phosphorylation was then carried out under standard condition. Alkaline phosphatase treatment was carried out with cells that have been subjected to the phosphorylation reaction. After washing with buffer A, the treated cells were lysed and processed by standard conditions. After separation by SDS-PAGE, the gel was then exposed to a Kodak X-OMAT AR film. The level of p112 was then determined by densitometry. The data presented were the average of three separate experiments; results were very consistent in all cases.

TREATMENT WITH HYDROLYTIC ENZYMES	ENZYME CONCENTRATION (µg / ml)	TIME OF INCUBATION (min.)	PERCENTAGE OF P112 DETECTED
Trypsin	---	30	100
	0.1	30	77
	0.5	30	46
Alkaline phosphatase	---	1	100
	0.1	1	69
	0.1	2	51
	1.0	1	21

amino acids in p112 are accessible to the exogenously added alkaline phosphatase.

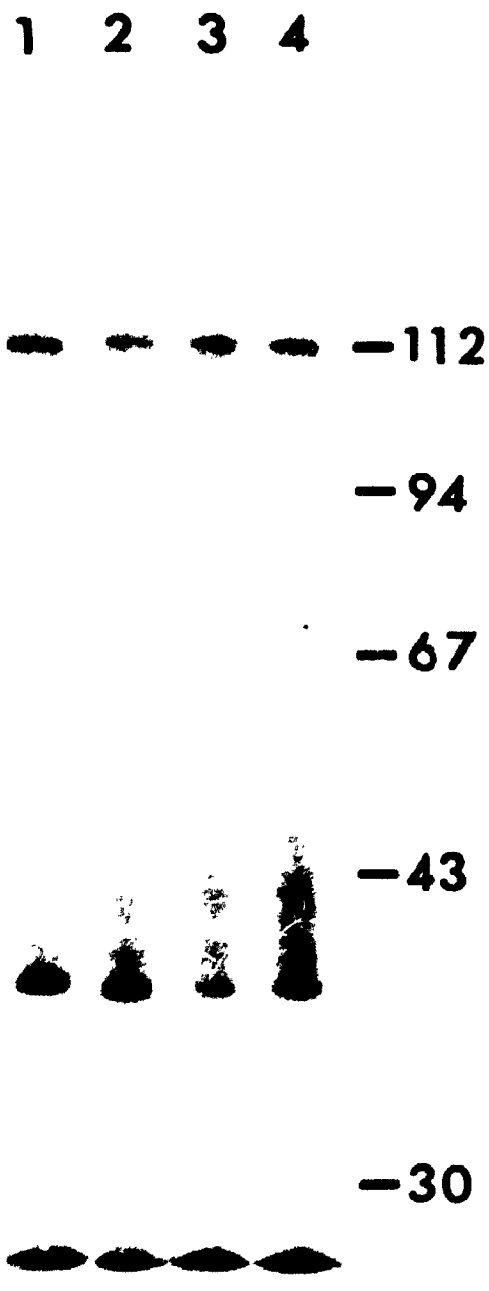
2.3.7 Nature of the cell surface 112 kDa protein and the ecto-protein kinase

Having determined the surface location of the phosphorylation site of the 112 kDa protein, the next question concerns the nature of the ecto-protein kinase and the 112 kDa protein. If either one of these proteins is a peripheral protein, then removal of these proteins should reduce the level of p112. We have previously shown that peripheral proteins on rat myoblasts could be removed by treatment with 0.4M glycine buffer (pH 2.0), 0.5M borate buffer (pH 10), or with 1M NaCl (Lo & Duronio, 1984b). As shown in Fig. 2.9, such treatments had no effect on the level of p112. This implied that the 112 kDa protein and the ecto-protein kinase were either very tightly bound peripheral proteins, amphitropic proteins, or integral proteins (Burn, 1988; Ferguson & Williams, 1988).

It was interesting to note that the 112 kDa protein was phosphorylated to a greater extent in cells previously permeabilized by Triton-X 100, or by 10μg/ml trypsin (Fig. 2.7). This increase in p112 was accompanied by increased phosphorylation of many other proteins. This suggested that the above-mentioned 112 kDa protein might be a transmembrane protein. However, it was also possible that another protein with a similar mobility on SDS gel might be phosphorylated on the inner membrane surface.

Figure 2.9 The 112 kDa protein and the ecto-protein kinase are not loosely bound peripheral proteins on the cell surface.

Rat L6 myoblasts were first washed with different solutions for 5 min. to dissociate loosely bound cell surface peripheral proteins. After which cells were phosphorylated and processed under standard conditions. Lanes 1, 2, 3, and 4 indicate cells washed with 1N NaCl, control cells, cells washed with 0.4M glycine buffer (pH 2.0), and cells washed with 0.5M borate buffer (pH 10.0), respectively.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2.4 DISCUSSION

The present investigation reports the phosphorylation of a rat myoblast cell surface protein by extracellular ATP. Upon incubation of undifferentiated rat myoblasts with γ-^{32}P-ATP, an 112 kDa protein was consistently phosphorylated under our experimental conditions (Figs. 2.1 and 2.2). The phosphorylation of this protein was detected with very low ATP concentrations (0.03μM), and with myoblasts grown at relatively low density (around 10^5 cells per 60 mm plate).

The following findings suggested that the 112 kDa protein was phosphorylated by extracellular γ-^{32}P-ATP. First, phosphorylation reactions were carried out with 0.03μM ATP for 8 min at 21 ^{o}C. Under this condition, the negatively charged ATP should not penetrate the plasma membrane. Second, the intracellular ATP concentration in rat L6 myoblast was around 24mM (D'Amore & Lo, 1986a). If there was no compartmentation of the intracellular ATP, extracellular ATP (0.03μM) was not likely to diffuse against its concentration gradient into the cells. Third, the phosphorylated 112 kDa protein (p112) could not be radioactively labelled by 0.03μM γ-^{32}P-ATP when 10μM of unlabelled ATP was included in the phosphorylation buffer. Thus even if γ-^{32}P-ATP could enter the cytoplasm, the specific activity of ATP would be so low that specific labelling should not be observed (assuming equilibration of the newly internalized ATP with the intracellular ATP pool). It should be noted that the above findings did not eliminate the possibility that ATP might be transported

across the membrane by specific proteins, thus making it accessible to the intracellular protein kinases (Krebs, 1986; Taylor, 1990). However, the phosphoprotein profiles in intact and permeabilized cells (Fig. 2.7), and the lack of effect by cyclic nucleotides suggested that the exogenously added ATP was unable to enter the cells.

The next question concerns the association of [^{32}P]-phosphate with the 112 kDa protein. Our data precluded the mere binding of ATP to the 112 kDa protein and the formation of acyl phosphate linkage in p112. (i) While the binding of ATP to its receptors should normally be completed in less than 1 min., the rate of phosphorylation of the 112 kDa protein remained linear for at least 8 min. (Fig. 2.5). (ii) If the whole ATP molecule was bound to the protein, α-^{32}P-ATP should be just as effective as γ-^{32}P-ATP in labelling the protein. However, α-^{32}P-ATP was unable to label the 112 kDa protein (Fig. 2.3). (iii) Non-covalently bound ATP should be dissociated from the protein by SDS-PAGE, HCl hydrolysis, and trichloroacetic acid precipitation. Obviously, this was not the case (Fig. 2.4). (iv) The amount of [^{32}P]-phosphate associated with p112 was not affected by boiling in SDS-sample buffer and by hydroxylamine treatment (Hokin et al., 1965). This suggested that the acyl-phosphate linkage was not present in the p112 excised from the gel. The nature of the phosphorylation reaction was revealed by phosphoamino acid analysis which indicated the possible involvement of a serine protein kinase (Fig. 2.4).

Direct indication of the location of the phosphorylation site comes from studies using exogenously added macromolecules. First, the phosphorylation of the 112 protein could be inhibited by heat-inactivated fetal calf serum (Fig. 2.8a). Second, a 55 kDa fetal calf serum protein and a 75 kDa horse serum protein could be phosphorylated by rat myoblasts upon incubation with γ-^{32}P-ATP (Fig. 2.8b). Unlike histone or phosvitin (Chiang et al., 1979; Kubler et al., 1989), both serum proteins should not disrupt the cell membrane, as they are normally included in the growth medium (Klip et al., 1982; Lo & Duronio, 1984a; Mesmer & Lo, 1989). These studies showed that the phosphorylation reaction must occur on the cell surface. This conclusion was corroborated by experiments using exogenously added hydrolytic enzymes. First, the level of p112 was reduced by pretreatment of cells with very low trypsin concentrations (0.1 - 0.5μg/ml) (Table 2.1). Since the rat myoblast plasma membrane was not disrupted at these trypsin concentrations (Lo & Duronio, 1984b; D'Amore et al., 1986), only cell surface proteins should be affected. Second, treatment of the phosphorylated cells with alkaline phosphatase resulted in a significant reduction of the [^{32}P]-labelled p112 (Table 2.1). Since alkaline phosphatase cannot penetrate the membrane, the phosphorylated serine residues of the 112 kDa protein must be exposed to the external environment.

Although the above findings show that the catalytic site of the protein kinase is accessible to the external environment, it remains to be proven that the protein kinase itself normally resides on the cell surface. It is possible

that this protein kinase may be a cytoplasmic enzyme released from damaged cells, and binds to the surface of intact cells after its release. The following findings suggest that this is not likely the case. First, the level of p112 was not altered by prewashing cells with high salt, acidic or alkaline buffers, thus suggesting the protein kinase was not bound to the cell surface by ionic interaction (Fig. 2.9). Second, the protein kinase involved exhibited properties distinct from those of the intracellular protein kinases. Third, if the protein kinases were released from damaged cells, their activities should increase with cell density, thus leading to an elevated p112 level in confluent cultures. However, data presented in Chapter 3 showed that the level of p112 actually decreased with increasing cell density. Fourth, cell lysis could not be detected in day-2 cultures of rat L6 myoblasts (Fig. 2.6), which exhibited significant phosphorylation of the 112 kDa protein. Fifth, a comparison of the ATP, AMP and ADP concentrations in the extra- and intra-cellular spaces precluded the occurrence of cell lysis during normal growth conditions (Chapter 3). The above findings therefore suggested that the protein kinase involved was not normally a cytoplasmic enzyme.

Kinetic analysis of the phosphorylation of the 112 kDa protein indicated that the Km value for ATP was 0.04μM and the Vmax value for p112 was 1.67 x 10^{-4}pmoles/ 10^5 cells/min. (Fig. 2.5). The Km value for ATP was significantly different from those of the cyclic nucleotide-independent ecto-protein kinases found in 3T3 cells, adipocytes, HeLa cells, fibroblasts and macrophages

(Mastro & Rozengurt, 1976; Kang, et al., 1979; Kubler et al., 1982; Chiang et al., 1979; Remold-O'Donnell, 1978), and also from those of the cyclic nucleotide-dependent ecto-protein kinases found in spermatozoa (Dey & Majumder, 1990).

Similar to those present in normal and transformed fibroblasts (Mastro & Rozengurt, 1976) and in HeLa cells (Kubler, et al., 1982), the ecto-protein kinase examined in this study is a phosphoserine protein kinase. While ten or more proteins were phosphorylated by the ecto-protein kinases present in 3T3 cells, macrophages, HeLa cells, and adipocytes (Mastro & Rozengurt, 1976; Kang, et al., 1979; Kubler et al., 1982; Chiang et al., 1979; Remold-O'Donnell, 1978), only the 112 kDa protein was consistently phosphorylated by the rat myoblast ecto-protein kinase. It should be noted that much higher ATP concentrations (ranging from 0.5 to 50μM) were used in previous studies.

The ecto-protein kinase examined in this study also differed significantly from the previously reported rat myoblast ecto-protein kinase (Lognonne & Wahrmann 1986,1988,1990). The latter was detected in day 4 to day 6 cultures (confluent monolayers) using 5μM ATP; whereas the present ecto-protein kinase was detected in subconfluent day-2 cultures, using 0.03μM ATP. While more than 20 proteins (ranging from 15 kDa to 250 kDa) were phosphorylated by the ecto-protein kinase from confluent cells, only the 112 kDa protein was consistently phosphorylated by the ecto-protein kinase from subconfluent cells in the present study. The latter protein kinase was dependent

on F^- ions either for activation of ecto-PK or for inhibition of phosphatases; whereas the former did not require F^- ions for its activity. On the other hand, both rat myoblast ecto-protein kinases were cyclic nucleotide independent phosphoserine protein kinases, and required Mg^{2+} and Ca^{2+} ions for their activities.

To summarize, a cell surface phosphoserine protein kinase was found in subconfluent rat L6 myoblasts. This enzyme was involved in the phosphorylation of a cell surface 112 kDa protein.

CHAPTER 3

INVOLVEMENT OF A CELL SURFACE PROTEIN AND AN ECTO-PROTEIN KINASE IN MYOGENESIS

3.1 INTRODUCTION

A number of distinct sequential events are involved in the formation of multinucleated myotubes from proliferating myoblasts (Wakelam, 1985; Schneider & Olson, 1988; Florini & Magri, 1989): (i) Biochemical differentiation. This involves the induction of muscle-specific proteins such as α-cardiac and α-skeletal actin, myosin heavy and light chains, tropomyosin, troponin-T, desmin, the muscle isoenzyme of creatine kinase, acetylcholine esterase, and nicotinic acetylcholine receptor, and voltage-gated Na^+ and Ca^{2+} channels (Gunning et al., 1987; Endo & Nadal-Ginard, 1987). The induction of these muscle-specific proteins can be reversed by growth stimulation (Nguyen et al., 1983). (ii) Total commitment. This occurs when cells cease DNA synthesis, and irreversibly withdraw from the cell cycle. These post-mitotic mononucleated cells are irreversibly committed to subsequent myogenesis, but have not yet begun to fuse. (iii) Terminal differentiation. This involves formation of multinucleated myotubes by fusion. In the past two decades, enormous efforts have been made to elucidate the myogenesis pathway. The identification of more than 40 different myogenesis inhibitors suggests that myogenesis is comprised of a sequential cascade of multiple steps leading to terminal differentiation, and interference with

any one step would abolish differentiation (Schneider & Olson, 1988; Endo & Nadal-Ginarad, 1987). One of the problems associated with these inhibitor studies is that most inhibitors can act on more than one target site; this makes it difficult to define conclusively the components involved in myogenesis.

Another approach to determine the components involved in myogenesis is to identify the alterations found in myogenesis-defective mutants (Cates et al., 1984). Our laboratory has previously examined the properties of mutants defective in myogenesis. Mutant D1/S4 was isolated from rat L6 myoblasts by its resistance to cell detachment caused by anti-L6 antibodies (D'Amore & Lo, 1988). While this antibody can activate hexose transport in its parental L6 myoblasts (Lo & Duronio, 1984a,b), it has no effect on hexose transport by this mutant (D'Amore & Lo, 1988). It was subsequently found that the anti-L6 antibody interacted with a 112 kDa plasma membrane protein, and this protein was missing in mutant D1/S4. The second myogenesis-defective mutant examined was mutant F72, which was isolated from L6 myoblasts by its ability to grow in the presence of toxic 2-deoxy-2-fluoro-D-glucose (D'Amore et al., 1986b). While not much was known about its primary genetic defect, this mutant was impaired in the high affinity hexose transport system (HAHT); even though cytochalasin B binding studies showed that it contained a normal level of the HAHT transporter (Chen, 1989). Mutant F72 also contained a normal level of the 112 kDa protein (D'Amore & Lo, 1988). Mutant D23 was isolated from L6 myoblasts by its ability to grow in the presence of 2-deoxy-D-glucose

(D'Amore et al., 1986b). This mutant was defective in the HAHT transporter and also in myogenesis (Chen & Lo, 1988; Kudo & Lo, 1990). In view of their inability to fuse, these mutants may conceivably serve as useful tools in dissecting the events involved in myogenesis.

The present investigation examined the factors involved in the initiation of myogenesis. Previous mutant analyses have implicated the possible involvement of an ecto-protein kinase in myogenesis (Lognonne & Wahrmann, 1986, 1988, 1990). However, it was not clear whether this ecto-protein kinase was involved in the initiation of myogenesis, as these studies were carried out with day-4 confluent cultures. As indicated in Chapter 2 an ecto-protein kinase is present in subconfluent day-2 rat L6 myoblast cultures (Chapter 2); the natural endogenous substrate protein for this ecto-protein kinase is a cell surface 112 kDa protein. Since initiation of biochemical differentiation should occur at an early stage of the growth phase, the possibility exists that the newly discovered ecto-protein kinase may be involved in the initiation of myogenesis. Indeed, data presented in this chapter suggest that the functioning of the ecto-protein kinase and the 112 kDa protein may be required for myogenesis.

3.2 METHODS AND MATERIALS

3.2.1 Cell lines and culture media

Yaffe's L6 rat skeletal myoblast line (Yaffe, 1968) was maintained in Alpha medium (Flow Laboratories) supplemented with 10% (v/v) horse serum

(Flow Laboratories) and gentamicin (50μg/ml; Gibco), as previously described in 2.2.1 of Chapter 2.

3.2.2 Intracellular level of ATP

The intracellular ATP level was determined as described by D'Amore (D'Amore & Lo 1986a). Cells were first washed twice with 10ml ice-cold phosphate-buffered saline and solubilized in 1ml of 8M guanidine hydrochloride. 20μl of this suspension was diluted 20-fold with water; 20μl of this dilution were then used for ATP determination by the luciferase-luciferin assay (Linklater et al., 1985). Luminescence was measured on a Lumac Model 2010 Luminometer (Mandel). The amount of ATP in unknown samples was determined using the linear portion of a standard curve (i.e. 0.01 - 0.05nmol ATP). Protein determinations were made by the method of Lowry et al. (1951), using bovine serum albumin as a standard. All determinations were carried out in triplicate, and the experiment was repeated twice. Results were consistent in both cases.

3.2.3 Levels of newly synthesized ATP

Newly synthesized ATP was labelled by incubating rat myoblasts with [^{32}P]-phosphate for two hours. Rat L6 myoblasts were grown in normal growth medium for two days to a density of 4.4 x 10^5 cells per 60mm plate. After being washed and kept in phosphate- and serum-free medium for 30 min., cells were then labelled with 50μCi/ml [^{32}P]-H_3PO_4 for 2 hrs at 37°C. After keeping

the labelling medium, cells were washed with 15mM Hepes buffer (pH 7.5) twice. Each plate was then incubated with 1ml of 15mM Hepes buffer (pH 7.5) with or without trypsin (3μg/ml) for 5 or 10 min at 37 °C. At the end of the incubation period, the supernatant was collected and centrifuged to remove any detached cells. The adenine nucleotides in all samples were identified and quantitated by the method as described by Norman et al. 1974. All samples (labelling medium and supernatant from various incubations) were adjusted to pH 3-4 with 17.5% perchloric acid (1μl/100μl sample). To each 100μl sample, 1μl of each of the following nucleotide carriers was added: 0.1M ATP, 0.1M ADP, and 0.1M AMP. After which, 20μl of each sample was spotted onto a silica gel TLC plate. Plates were developed in a tank containing 100 ml of the solvent system comprised of isobutyl alcohol-amyl alcohol-ethoxyethanol-ammonia-deionized water (15:10:30:15:25). The solvent system was matured for at least 48 hrs before use, and added to the tank 1 hr before introduction of plates (Norman et al., 1974). Optimal separation of the adenine nucleotides was achieved by running the plates in the solvent twice. The adenine nucleotides were visualized as blue spots in UV light. The blue spots were cut out and counted in scintillation fluid. The above determinations were carried out in duplicate; and the experiment was repeated twice.

3.2.4 Phosphorylation of the cell surface 112 kDa protein

The phosphorylation of cell surface proteins was carried out by methods as described in Section 2.2.2.

3.2.5 Ecto-protein kinase activity

The phosphorylation of exogenously added horse serum proteins was also used to assess the ecto-protein kinase activities. Cell surface phosphorylation assays were carried out essentially as described earlier, except that 3µl horse serum was included in 1.2ml of the phosphorylation buffer. After phosphorylation, 280µl of the supernatant from each well was added to 93µl of the 4X SDS sample buffer. After boiling in sample buffer for 5 min., 100µl of this sample was loaded onto the SDS polyacrylamide gel. Each lane was loaded with the same amount of protein. After drying, the gel was exposed to a Kodak X-OMAT AR film. The level of the phosphorylated 75 kDa horse serum protein was determined by densitometry. Autoradiograms used were within the linear range of the film. All phosphorylation studies were carried out in triplicate; and each experiment was repeated at least twice. The figures presented the results of representative experiments. Results were found to be consistent in all cases.

3.2.6 Level of the 112 kDa cell surface protein

The level of the 112 kDa cell surface protein was determined by immunoblotting studies using rabbit antibodies raised against plasma membrane vesicles prepared from rat L6 myoblasts (anti-PMV) (D'Amore & Lo, 1988). Total cell extracts (150-200µg) from cells grown under different conditions were subjected to SDS-PAGE. After which, proteins were electrophoretically transferred from the gel onto nitrocellulose membranes (0.45µm) using a transfer buffer containing 25mM Tris, 192mM glycine (pH 8.3), 20% methanol, and 0.1%

SDS (Towbin et al., 1979) at 55 volt for 2 hrs. The paper was incubated with 20% fetal calf serum in Tris buffer containing 150mM NaCl, 10mM Tris-HCl (pH 8.0), and 0.05% Tween 20 for at least 5 hrs. at 23°C to block nonspecific protein binding. After washing the paper once with the same Tris buffer, the paper was incubated with anti-PMV or preimmune serum for 14 hrs. at 23°C; this was then followed by washing with the Tris buffer 3 times, 5-10 min. for each washing. The immune complexes were detected with ^{125}I labelled protein A. After removal of unbound ^{125}I-protein A by washing three times with Tris buffer, blots were dried and exposed to Kodak X-OMAT AR film with intensifying screen at -80°C. The level of the 112 kDa protein was determined by densitometry. Immunoblotting studies were carried out in duplicate, and the experiment was repeated three times. Results were consistent in all cases.

3.2.7 Determination of fusion index and nuclei density

Cells were plated in six-well Falcon plates in Alpha medium. On different days after subculturing, cells were treated with 1mM $ZnSO_4$ in 20% DMSO to swell the nuclei, and then fixed in 2.5% glutaraldehyde in phosphate-buffered saline (Chen & Lo, 1989). Cells were then stained with 6% Giemsa. The fusion index was determined by calculating the ratio of the number of nuclei in myotubes to the total number of nuclei per field (Morris & Cole, 1972; Kudo & Lo, 1990). Only structures containing at least three nuclei were considered as myotubes. Usually each field contained at least 50 nuclei. Ten fields were counted for each set of determinations.

3.2.8 Materials

[^{32}P]-H_3PO_4 and γ-^{32}P-ATP were purchased from ICN Biochemicals, Canada Ltd. Phloretin and 5-bromo-2'-deoxyuridine (BrdUrd) were purchased from Sigma Chemical Co. All other chemicals were obtained from commercial sources and were of the highest available purity.

3.3 RESULTS

3.3.1 Level of extracellular ATP excreted by rat myoblast L6.

As indicated in Chapter 2, an ecto-protein kinase was involved in the phosphorylation of a cell surface 112 kDa protein in subconfluent rat L6 myoblasts, and the Km value of this enzyme for ATP was 40nM. Before examining the regulation and possible roles of these proteins, it is important to determine whether there is adequate ATP on the cell surface for efficient functioning of this ecto-protein kinase. While extracellular ATP concentrations in purinergic nerve terminals, circulating blood, and endothelial cells have been determined, no information is available on the amount of ATP excreted by cultured skeletal myoblasts (White, 1985; Pearson & Gordon, 1979; Born & Kratzer, 1984; Gordon, 1986). In the present investigation, myoblasts were first labelled with [^{32}P]-phosphate for two hours. The concentration of [^{32}P]-ATP present in the growth medium was found to be around 3nM (Table 3.1). Based on an estimated intracellular volume of 0.22μl/10^5 cells (D'Amore & Lo, 1986a), the intracellular concentration of ATP was calculated to be 24mM. This was similar to the previously determined values in rat myoblasts (D'Amore & Lo,

Table 3.1 Concentrations of nucleoties excreted by rat myoblasts

Newly synthesized nucleotides were labelled by incubating rat L6 myoblasts with [^{32}P]-phosphate for two hours. The amounts of intracellular ATP and newly synthesized nucleotides in both intracellular and extracellular spaces were determined by the procedures as described in "Methods and Materials". The intracellular volume of rat L6 myoblasts grown under our experimental conditions was 0.22 μl/10^5 cells (D'Amore & Lo, 1986a). Using this value, the intracellular concentrations of [^{32}P]-ATP and ATP were calculated to be 32 μM and 24 mM, respectively. The calculation of the ATP concentration in the extracellular space was based on the assumption that the newly synthesized ATP equilibrated with the intracellular ATP pool before excretion.

	CONCENTRATION OF NEWLY SYNTHESIZED NUCLEOTIDES (μM)			ESTIMATED CONCENTRATION OF ATP (μM)
	AMP	ADP	ATP	ATP
INTRACELLULAR SPACE	38.10	62.81	31.92	24000.00
GROWTH MEDIUM	0	0.002	0.003	2.26
HEPES BUFFER 5 min	0.001	0.015	0.034	25.56
HEPES BUFFER 10 min	0.002	0.020	0.037	27.82
HEPES BUFFER+TRYPSIN 10 min	0.003	0.022	0.038	28.57

1986a) and in human myoblasts (Mesmer & Lo, 1989, 1990). The levels of [^{32}P]-ATP, [^{32}P]-ADP and [^{32}P]-AMP were calculated to be 32μM, 63μM and 38μM, respectively. It was interesting to note that the ratios of these newly synthesized nucleotides differed from those of the steady state cellular nucleotide levels. If the newly synthesized ATP could equilibrate with the intracellular ATP pool at the end of the incubation period, the ATP concentration in the growth medium would then be 2.26μM.

Since extracellular ATP is subjected to hydrolysis, the amount excreted by the cells may be higher than that present in the growth medium after 2 hrs of incubation. As shown in Table 3.1, the concentrations of [^{32}P]-ATP excreted from the cells at 5 and 10 min. after washing were 34 and 37nM, respectively. If there was equilibration with the intracellular ATP pool, the extracellular ATP concentration would then be around 26μM. Studies using trypsin to inactivate cell surface hydrolytic enzymes (Pearson & Gordon, 1979) suggested that there was negligible hydrolysis of the extracellular ATP at 10 min. after washing (Table 3.1). These studies showed that even if the newly synthesized ATP could not equilibrate with the intracellular ATP pool, there was still adequate cell surface ATP for efficient operation of the ecto-protein kinase.

A comparison of the ^{32}P-labelled nucleotides in the extra- and intracellular spaces revealed that the extracellular nucleotides were excreted by specific mechanisms. Similar percentages of all three adenine nucleotides should

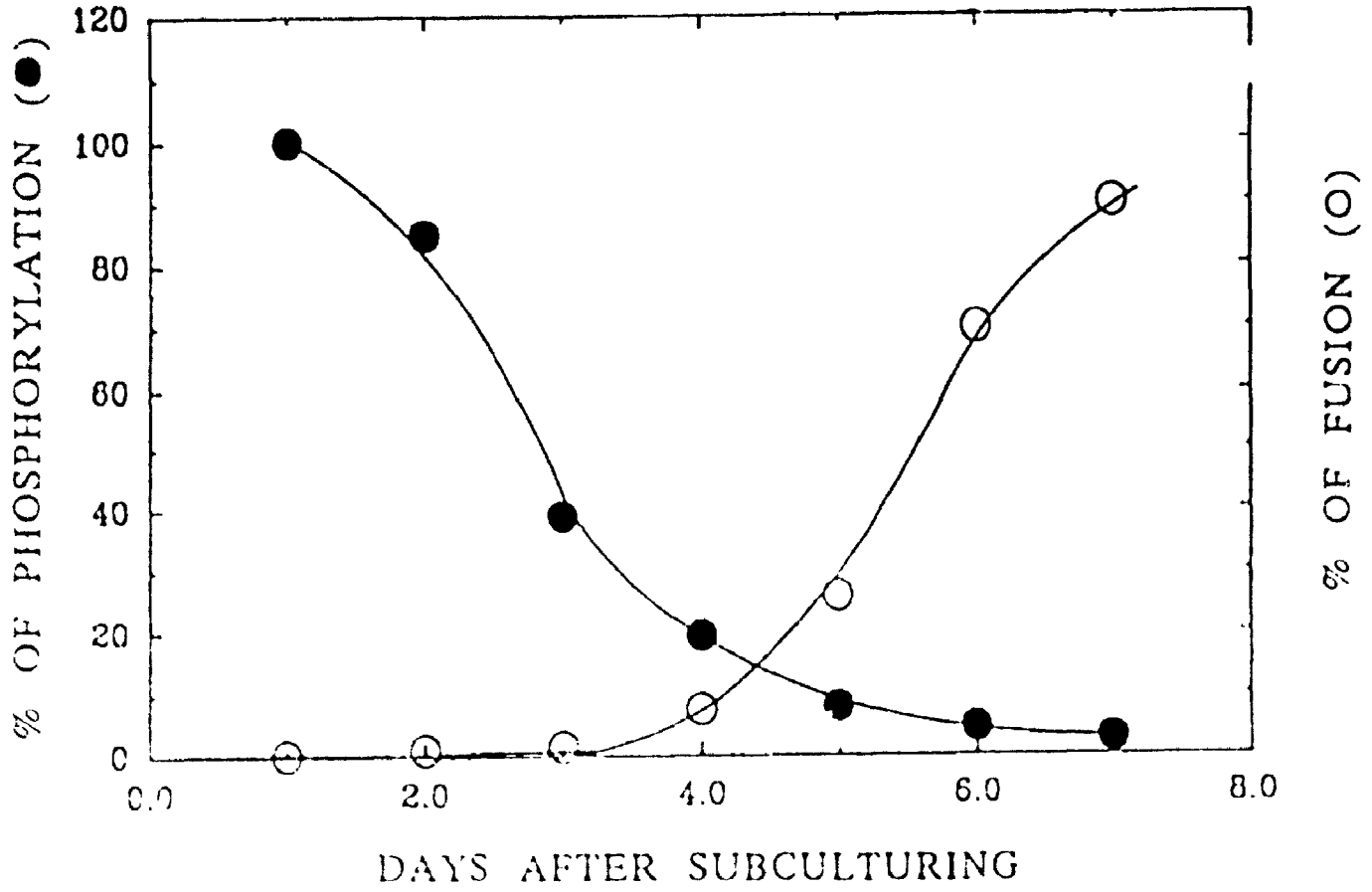
be released into the extracellular space if these nucleotides were externalized as a result of cell lysis. We found that 67.48 pmoles of ATP were synthesized by 4.4 x 10^5 cells in two hours. About 54% of this newly synthesized ATP was excreted out of the cells at 10 min. after washing; whereas only 25% and 5% of the newly synthesized ADP and AMP were excreted during the same period of time. Thus similar to cAMP (Fehr et al., 1990; Brunton & Heasley, 1988), extracellular ATP was selectively excreted by the cells, and not via damaged cell membranes. It was interesting to note that when cultured porcine aortic endothelial cells were labelled with ^{3}H-adenosine for 1 hr, up to 50% of the ^{3}H-ATP was released from the cells (Pearson & Gordon, 1979). Moreover, the newly synthesized ATP was able to equilibrate with the intracellular ATP pool within 1 hr. In summary, the findings indicate that there is a constant and adequate supply of ATP on the cell surface for efficient operation of the newly discovered ecto-protein kinase.

3.3.2 Effect of myogenesis and cell density on the phosphorylation of the 112 kDa protein

As shown in Chapter 2, cell surface phosphorylation activity can be monitored by determining the level of the phosphorylated 112 kDa protein (p112). Changes in the phosphorylation activities at different developmental stages of the cells may shed some light on the roles and regulation of the ecto-protein kinase, the 112 kDa protein, and/or p112. Phosphorylation assays were carried out with cells grown to different stages of myogenesis (Fig. 3.1). Rat L6

Figure 3.1 Effect of growth stages on the phosphorylation of the 112 kDa protein.

Rat L6 myoblast was grown in 10% horse serum for different days after culturing. Fusion index and the phosphorylation of the 112 kDa protein were determined by procedures as indicated in the text. The extent of cell fusion (O) was determined by calculating the ratio of the number of nuclei in myotubes to the total number of nuclei per field (Morris & Cole, 1972). The level of the phosphorylated 112 kDa protein (p112) was determined by densitometry of the autoradiogram. The level of p112 (●) in various cultures was expressed as percentage of that found in day-1 culture.


% OF PHOSPHORYLATION (●)
% OF FUSION (○)
120
100
80
60
40
20
0
0.0
2.0
4.0
6.0
8.0
DAYS AFTER SUBCULTURING

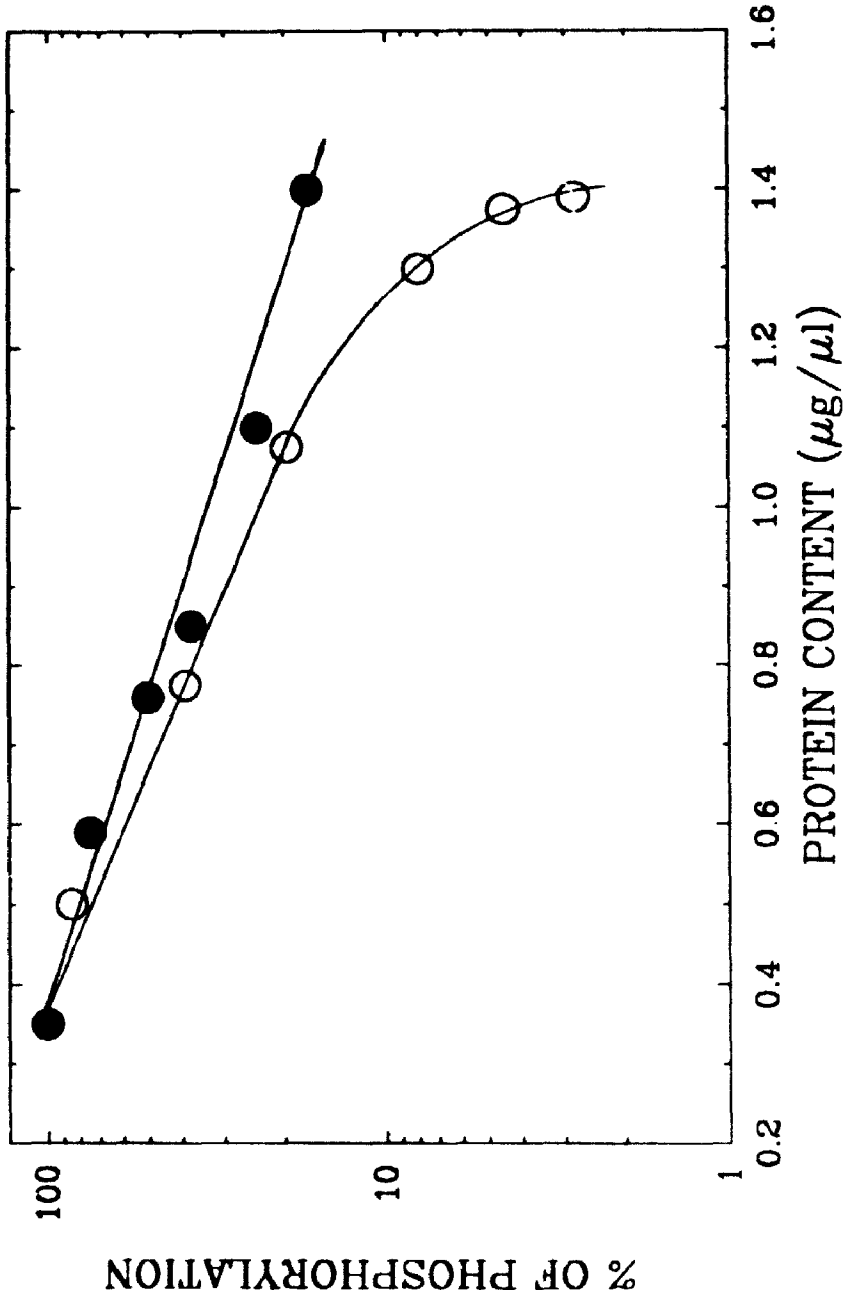
myoblasts began to line up and form multinucleated myotubes on day 4 after subculturing, and fusion was essentially complete on day 7 (Fig. 3.1) (Chen & Lo, 1989; Kudo & Lo, 1990). The level of p112 was found to decrease dramatically with increasing days after subculturing. Using the p112 level of day-1 culture as 100%, about 85% and 40% activity were observed on day-2 and day-3 cultures, respectively (Fig. 3.1). Negligible phosphorylation activity was observed upon formation of multinucleated myotubes (day-5 to day-7 cultures).

In order to determine whether the reduction of p112 was due to a mere increase in cell density, cells were seeded at different densities and grown for two days. Myotubes were not detected in these cultures, even with protein contents as high as 1.4μg/μl. The p112 level in these myoblasts was found to be inversely proportional to the cell density (as indicated by the protein concentration) (Fig. 3.2). About 50% reduction was observed when the cell density was doubled. Similar decrease in ecto-protein kinase activities at higher cell densities was also observed with other cell types (Mastro & Rozengurt, 1976).

A comparison of the cell density and myogenesis effects revealed that the p112 level was reduced to a much greater extent upon formation of myotubes (Fig. 3.2). Before the appearance of myotubes (cultures with protein contents from 0.3 to 0.77μg/μl), the reduction of p112 in cultures grown for different days was similar to that of the cell density effect. However, upon formation of myotubes (day-4 to day-7 cultures, i.e. cultures with protein contents

Figure 3.2 Effect of cell density and myogenesis on the phosphorylation of the 112 kDa protein.

In order to determine the effect of cell density, independent of myogenesis, on the phosphorylation of the 112 kDa protein, cells were seeded at different densities and grown for two days. Cell surface phosphorylation by these cultures was then carried out by procedures as described in the text. The level of p112 was determined by densitometry of the autoradiogram. The level of p112 in a culture with a protein content of 0.3μg/μl was taken as 100% percent. Solid circles indicate level of p112 in day-2 cells seeded at different densities. It was important to note that myotubes could not be observed in these cultures. Open circles indicate level of p112 in cells seeded at the same density, but grown to different stages of myogenesis. The open circles at 1.07, 1.3, 1.38 and 1.4μg/μl protein corresponded to the level of p112 in day-4, day-5, day-6 and day-7 cultures, respectively. The fusion indices of these cultures were 7%, 23%, 72%, and 92%, respectively (Fig. 3.1).


% OF PHOSPHORYLATION
100
10
1
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
PROTEIN CONTENT (μg/μl)

from 1.07-1.4μg/μl), the p112 level was dramatically reduced even without much change in the protein content of the cultures (Fig. 3.2). For example, at a protein content of 1.4 μg/μl, the p112 level in myotubes was 4 times lower than that in myoblasts. Thus the cell surface phosphorylation activity was affected by myogenesis.

The effect of myogenesis on the p112 level was further examined by using cultures exhibiting different rates of fusion. The concentration of horse serum in the growth medium was found to affect the rate of fusion (Table 3.2). When grown in normal (10%) horse serum, L6 cells exhibited 7% and 23% fusion with day-4 and day-5 cultures, respectively; however 21% and 65% fusion were observed with the corresponding cultures grown in 1% horse serum. In other words, myoblasts grown in 1% horse serum have a faster rate of fusion. If the p112 level is affected by myogenesis, then day-3 cultures of L6 cells grown in 1% horse serum should have a lower p112 level than those grown in 10% horse serum. Indeed, this was found to be the case; only 33% of the normal p112 level was observed in L6 culture grown in 1% horse serum (Table 3.2), even though the number of nuclei per plate was quite similar. A plausible implication of this observation is that ecto-protein kinase is no longer required after myogenesis has been initiated.

3.3.3 Is the phosphorylation of the 112 kDa protein required for myogenesis

The relationship between the phosphorylation of the 112 kDa

Table 3.2 Level of p112 in rat myoblasts exhibiting different rates of myogenesis.

The conditional myogenesis-defective mutant D1 and its parental L6 cells were initially grown in 10% horse serum for 1 day, and then subcultured in 1% or 10% horse serum. The level of p112 in the day-3 cultures were then determined by methods as described in the text. The level of p112 in L6 myoblasts grown in 10% horse serum was taken as 100%. Fusion indice were determined for day-4 and day-5 cultures by procedures as described by Kudo & Lo (1990).

GROWTH CONDITIONS		FUSION INDEX		LEVEL OF THE PHOSPHORYLATED 112 KDA . ROTEIN
CULTURE	HORSE SERUM CONCENTRATION	DAY 4 CULTURE	DAY 5 CULTURE	DAY 3 CULTURE
1. Cultures with normal rates of myogenesis				
L6	10%	7%	23%	100%
D1	1%	12%	27%	93%
2. Culture impaired in myogenesis				
D1	10%	0%	0%	26%
3. Culture with a faster rate of myogenesis				
L6	1%	21%	65%	33%

protein and myogenesis was examined using various chemical reagents. Although their primary sites of action have not been defined, cytochalasin B (CB), 5-bromo-2'-deoxy-uridine (BrdUrd) and phloretin are potent inhibitors of myogenesis (Kudo & Lo, 1990; Chen & Lo, 1989; Endo & Nadal-Ginard, 1987; Delain & Wahrmann, 1975). Unfortunately, both CB and BrdUrd can also act on other proteins with similar affinities (Kobler et al., 1990; Chen & Lo, 1988; Sanwal, 1979; Pearson, 1981; Ray et al., 1987). Thus they cannot be used as definitive tools in examining the relationship between myogenesis and cell surface phosphorylation.

It has been recently demonstrated that phloretin, but not phlorizin, inhibited myogenesis at concentrations that did not affect most other cellular functions (Kudo & Lo, 1990; De Jonge et al., 1983; Snow et al., 1978; Shefcyk et al., 1983; Movius et al., 1989). The effects of these reagents on cell surface phosphorylation were therefore determined. As indicated in Table 3.3, phosphorylation of the 112 kDa protein was inhibited by 25 and 50μM phloretin; this was accompanied by the eventual inhibition of myogenesis; whereas 50μM phlorizin had only minimal effects on these two processes. Although its site of action was not clear, BrdUrd was also found to affect both phosphorylation and myogenesis. Thus the phosphorylation of the 112 kDa protein and the ability to differentiate seemed to be affected in a similar manner.

3.3.4 Studies with a conditional myogenesis-defective mutant

Table 3.3 Inhibition of the phosphorylation of the 112 kDa protein and myogenesis by chemical reagents.

On day-2 after subculturing, rat L6 myoblasts were incubated with various chemical reagents. After 24 hrs of incubation, cells were washed, and cell surface phosphorylation was carried out by the procedures as described in the text. The level of the phosphorylated 112 kDa protein was quantitated by densitometry of the autoradiogram. The level of p112 in the untreated cells was taken as 100%. Fusion indices of day-6 rat L6 myoblasts treated with the above-mentioned chemical reagents were determined by the procedure as described by Kudo and Lo (1990).

TREATMENT	PHOSPHORYLATION OF THE 112 KDA PROTEIN IN DAY-3 CULTURE	FUSION INDEX OF DAY-6 CULTURE
Control	100%	85%
Phlorizin (50 μM)	87.2%	72%
Phloretin (25 μM)	40.7%	36%
Phloretin (50 μM)	36.1%	15%
BrdUrd (7.5 μM)	35.6%	0%

The relationship between myogenesis and the phosphorylation of the 112 kDa protein was further explored using a conditional myogenesis-defective mutant. Mutant D1, a concanavalin A-resistant mutant, was impaired in both biochemical and morphological differentiation when grown in 10% horse serum (Chen & Lo, 1989; Zeuner et al., 1988) (Table 3.2). However, it was able to differentiate when cultured in 1% horse serum. Thus this mutant can serve as a unique tool in examining the relationship between myogenesis and phosphorylation, without the use of chemical reagents.

In the present investigation, both L6 and D1 cells were initially grown in 10% horse serum for 1 day, and then subcultured in 1% or 10% horse serum for two more days. Cell surface phosphorylation studies revealed that similar p112 levels were present in both myogenesis-competent cells, viz. L6 grown in 10% horse serum and D1 grown in 1% horse serum. However, only 26% of the normal p112 level was observed in myogenesis-impaired cells (i.e. D1 grown in 10% horse serum). Thus there is a close correlation between the p112 level in undifferentiated myoblasts and the cells' eventual ability to fuse.

3.3.5 A mutant defective in the 112 kDa protein was impaired in myogenesis

Since at least two components are involved in the observed cell surface phosphorylation, it is not clear which component is essential for myogenesis. Mutant D1/S4 was previously shown to be defective in a cell surface 112 kDa plasma membrane protein (D'Amore & Lo, 1988). Cell surface

phosphorylation studies revealed that this mutant was also defective in p112 (Fig. 3.3). This suggested that the cell surface 112 kDa protein might be the endogenous substrate protein of the ecto-protein kinase. Since this mutant is also defective in myogenesis (Fig. 3.4), it may be surmised that the 112 kDa protein is required for myogenesis, and p112 is not just a side product of the phosphorylation reaction.

3.3.6 Mutant defective in the ecto-protein kinase was also impaired in myogenesis

In our search for mutants defective in the ecto-protein kinase, various rat myoblast mutants were screened for the ability to phosphorylate the 112 kDa protein. These studies revealed that mutant F72 contained only a small amount of p112 (Fig. 3.3). Unlike mutant D1/S4, mutant F72 contained a normal level of the 112 kDa protein (D'Amore & Lo, 1988). It is therefore conceivable that this mutant may be defective in the ecto-protein kinase responsible for the phosphorylation of the 112 kDa protein.

Attempts were therefore made to assay for the ecto-protein kinase activity independent of the 112 kDa protein. Among various exogenously added proteins tested, a 75 kDa horse serum protein was found to be phosphorylated by L6 cells under the optimal assaying condition for the ecto-protein kinase, i.e. in the presence of NaF, Mg^{2+}, and Ca^{2+} (Fig. 3.5). Mutant D1/S4 (lane 4) and its parental L6 cells (lane 3) could phosphorylate this 75 kDa protein to a similar

Figure 3.3 Level of the phosphorylated 112 kDa protein in various myogenesis-defective mutants.

Day-2 cultures of various myogenesis-defective mutants and their parental L6 myoblasts were used in these studies. Cell surface phosphorylation studies were carried out as described in the text. Lanes 1, 2, 3, and 4 indicate phosphorylated protein profiles in mutants D1/S4, F72, D23, and the parental L6 cells. The same amount of protein was loaded onto each lane. The molecular weight standards used were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

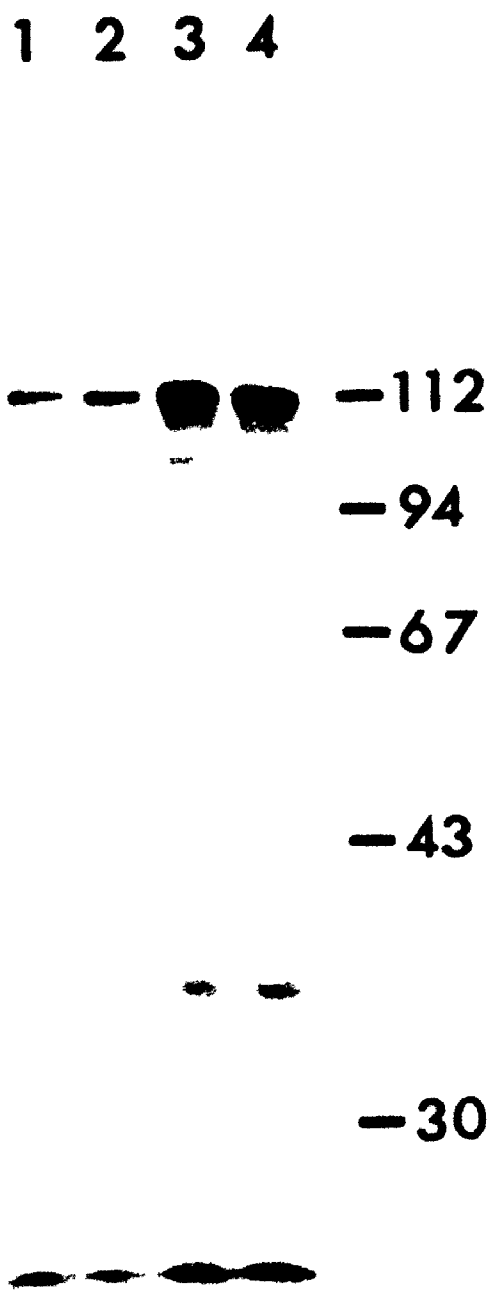

1 2 3 4
112
94
67
43
30

Figure 3.4 Morphology of L6, F72 and D1/S4 myoblasts on day 6 after subculturing.

Myoblasts were plated at a density of 10^5 cells/100 mm plate. After subculturing for 6 days, cells were treated with 1mM $ZnSO_4$ in 20% DMSO to swell the nuclei, and then fixed in 2.5% glutaraldehyde. Cells were then stained with 6% Giemsa. L6 indicates the formation of multinucleated myotubes in the Day-6 L6 culture. F72 and D1S4 show that mutants F72 and D1/S4 were unable to fuse even on day 6 after subculturing.

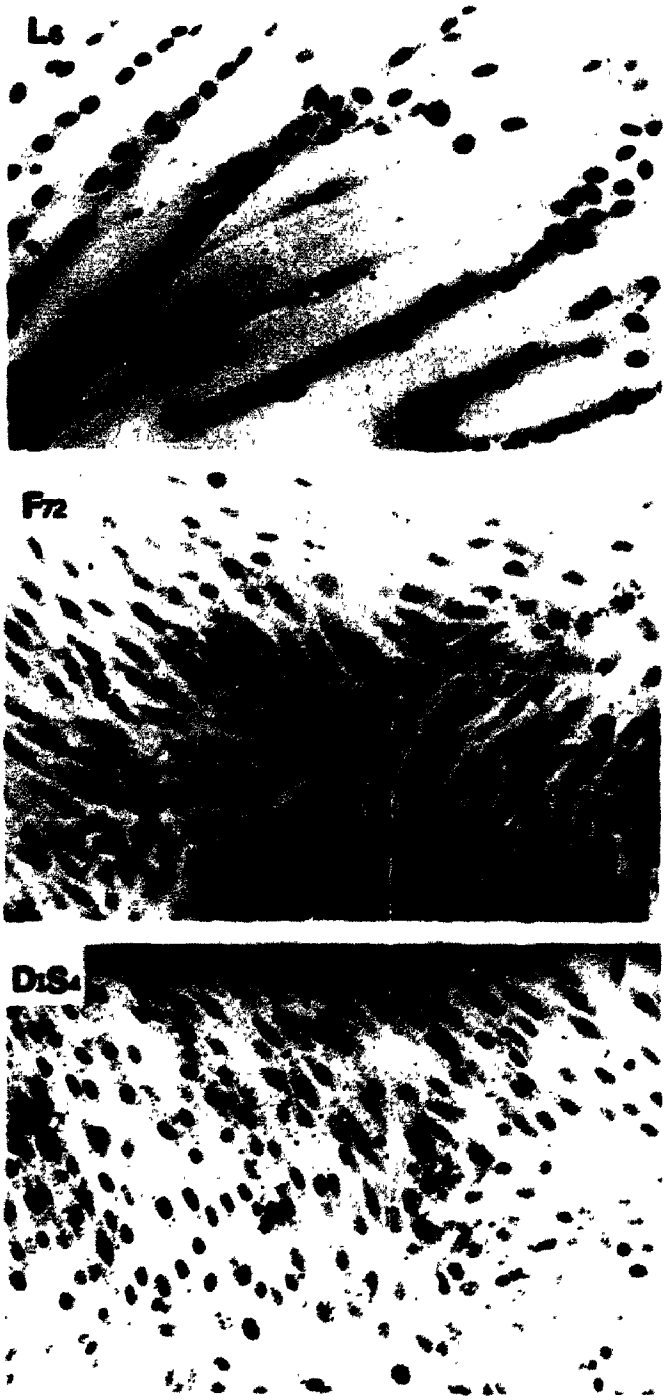
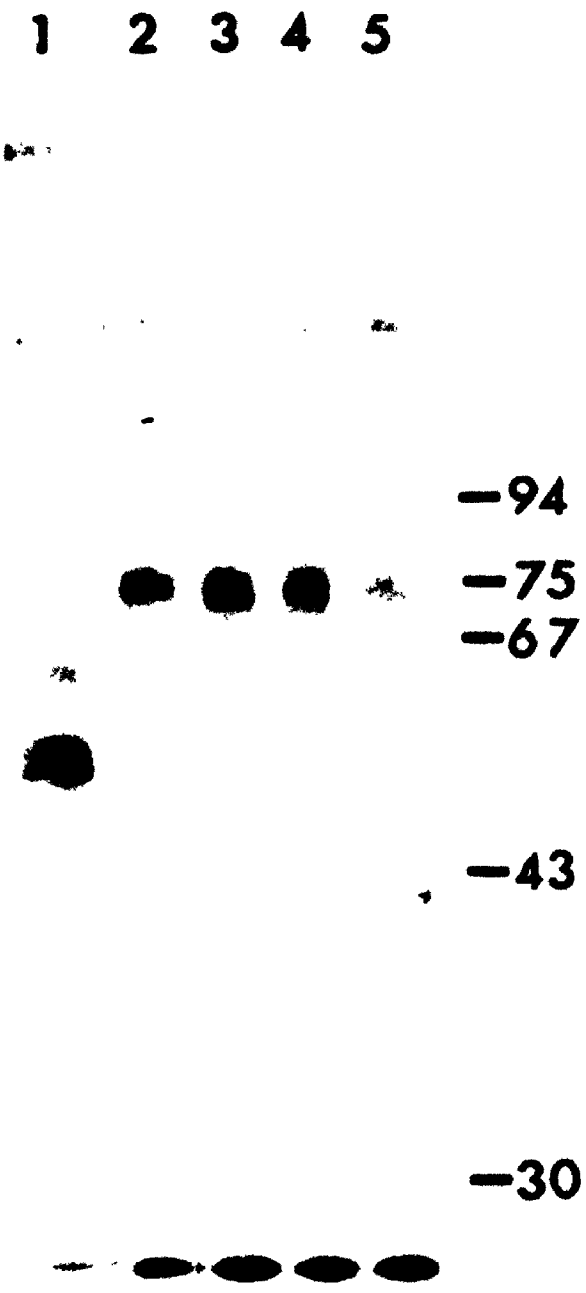

L6
F72
D1S4

Figure 3.5 Phosphorylation of a 75 kDa horse serum protein by myogenesis-defective mutants.

Exogenously added horse serum was used to measure ecto-protein kinase activity. The phosphorylation of horse serum proteins was determined as described in the text. Lane 1 indicates the phosphoprotein profile by L6 cells in the absence of NaF. This shows that the 75 kDa protein was not phosphorylated in the absence of NaF. Lanes 2, 3, 4 and 5 indicate the phosphoprotein profiles by mutant D23, L6, D1/S4, and F72, respectively. The same amount of protein was loaded onto each lane. The molecular weight standards used were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).


1 2 3 4 5
-94
-75
-67
-43
-30

extent, whereas mutant F72 (lane 5) was unable to phosphorylate this protein. Thus in agreement with data presented in Fig. 3.3, mutant F72 is defective in the ecto-protein kinase. It is important to note that this mutant is also defective in myogenesis (Fig. 3.4). Thus the absence of ecto-protein kinase seems to correlate with the cells' inability to differentiate.

3.3.7 Not all myogenesis-defective mutants are defective in p112

The question that arises from the above studies is whether the phosphorylation of the 112 kDa protein is involved in triggering myogenesis or is a mere consequence of myogenesis. If the latter is the case, then mutants impaired in myogenesis should all have much reduced level of p112. These possibilities were examined using another myogenesis-defective mutant. Mutant D23 was defective in myogenesis, and in the high affinity hexose transport component (D'Amore et al., 1986b; D'Amore & Lo, 1986b; Chen & Lo, 1988; Kudo & Lo, 1990). The ability of this mutant to phosphorylate the 112 kDa protein was therefore examined. This mutant was found to retain the normal ability to phosphorylate the 112 kDa protein and the exogenously added 75 kDa horse serum protein (Figs. 3.3 and 3.5), even though it was defective in myogenesis. This finding is important in demonstrating that the phosphorylation of the 112 kDa protein is not a consequence of biochemical differentiation, but rather it may be one of the important steps involved in myogenesis.

3.3.8 Levels of the 112 kDa protein, and ecto-protein kinase in myotubes and

in cells impaired in myogenesis

So far we have demonstrated a close correlation between p112 and myogenesis. This alteration in p112 could be due to changes in the level of the 112 kDa protein and/or the ecto-protein kinase activity. Attempts were therefore made to determine the levels of these two proteins. The level of the 112 kDa protein was determined by immunoblotting studies using antibodies raised against plasma membrane vesicles prepared from rat L6 myoblasts (D'Amore & Lo, 1988), whereas that of the ecto-protein kinase was quantitated using exogenously added horse serum (Fig. 3.5). These two proteins were reduced by 47% and 26%, respectively in the myogenesis-impaired cells (Table 3.4). A comparison with the level of p112 in these cells (Table 3.4) suggested that decreases in these two proteins could account for the changes in p112 level. In view of the dramatic decrease in the 112 kDa protein, it was unlikely that the reduction in p112 was due to changes in the level of ATP excreted by the cells, or due to alterations in the amount of 'cold' phosphate already present in the 112 kDa protein.

In the case of the fused myotubes, only 17% and 23% of the respective levels of ecto-protein kinase and the 112 kDa protein were detected, compared with myoblasts. (Table 3.4). Unlike the effects of the myogenesis inhibitors, both proteins were reduced to a similar extent in the myotubes. Again, these decreases could account for the much reduced level of p112 (4.5%) observed in the myotubes. This finding therefore shows that myogenesis results

Table 3.4 Levels of the 112 kDa protein, ecto-protein kinase and p112 in cells impaired in myogenesis and in myotubes.

Rat myoblast L6 cells were used in this study. Cells were treated with BrdUrd or with phloretin as described in Table 3.3. Ecto-protein kinase activity was determined using horse serum as the exogenously added substrate, as described in Fig. 3.5. The level of the phosphorylated 75 kDa horse serum protein was determined by densitometry. The level of the 112 kDa protein was determined by immunoblotting studies using antiserum raised against plasma membrane vesicles prepared from L6 cells (D'Amore & Lo, 1988). The level of the phosphorylated 112 kDa protein was determined as described in Fig. 3.1. The extent of cell fusion was determined by calculating the ratio of the number of nuclei in myotubes to the total number of nuclei per field (Kudo & Lo, 1990).

Cultures	Ecto-protein kinase activity	Level of the 112 kDa protein	Phosphorylation of the 112 kDa protein	Fusion index
A. DAY-3 CULTURE				
Control	100%	100%	100%	0%
BrdUrd (7.5 μM)	73%	53%	35.6%	0%
Phloretin (50 μM)	75%	53%	36.1%	0%
B. DAY-6 CULTURE	17%	23%	4.5%	70%

in a dramatic reduction of both protein kinase and the cell surface 112 kDa protein.

3.4 DISCUSSION

It has been shown in Chapter 2 that a cell surface 112 kDa protein in rat L6 myoblasts can be phosphorylated by extracellular ATP. An ecto-protein kinase is thought to be responsible for this process. The Km value of this enzyme for ATP was 0.04μM, and serine residues of the 112 kDa protein were phosphorylated. While the location of the catalytic site of the ecto-protein kinase and the phosphorylation site of the 112 kDa protein have been determined, not much is known on the physiological roles and identity of these proteins.

In order for the ecto-protein kinase to carry out its function, sufficient extracellular ATP should be available to this enzyme. After two hours of labelling with [^{32}P]-phosphate, the amount of newly synthesized ATP (as indicated by [^{32}P]-ATP) present in the growth medium, and excreted by the cells at 10 min after washing were 0.003μM and 0.036μM, respectively (Table 3.1). If the newly synthesized ATP equilibrated with the intracellular ATP pool before excretion, the ATP concentration in the growth medium and excreted by the cells would then be 2.26μM and 26μM, respectively (Table 3.1). Similar to cultured porcine aortic endothelial cells (Pearson & Gordon, 1979), about 54% of the newly synthesized ATP was excreted at 10 min. after washing. These findings indicate that there is a constant and adequate supply of cell surface ATP for

efficient functioning of the ecto-protein kinase, even if the newly synthesized ATP cannot equilibrate with the general intracellular ATP pool. A comparison of the ratios of the extracellular and intracellular amounts of ATP, ADP and AMP revealed that ATP was selectively excreted from the cells, and was not released as a result of cell lysis.

The following evidence suggested that both the exogenously added 75 kDa horse serum protein and the endogenous cell surface 112 kDa protein might be phosphorylated by the same ecto-protein kinase. (i) NaF was required for the phosphorylation of these two proteins (Fig. 3.5; Chen & Lo, 1991). (ii) Multinucleated myotubes exhibited a much reduced ability to phosphorylate these two proteins (Table 3.4). (iii) Growth of cells 7.5μM BrdUrd, or 50μM phloretin reduced the cells' ability to phosphorylate these two proteins (Table 3.4). (iv) The ecto-protein kinase defective mutant, F72, was unable to phosphorylate these two proteins (Figs. 3.3 and 3.5). Thus these studies showed that the exogenously added horse serum 75 kDa protein can serve as a substrate protein for the ecto-protein kinase. This provides another means for assaying the ecto-protein kinase activity, independent of the 112 kDa protein.

The phosphorylation of the cell surface 112 kDa protein by ecto-protein kinase was affected by the physiological states of the myoblasts. Similar to ecto-protein kinases found in other cell types (Mastro & Rozengurt, 1976), the level of the phosphorylated 112 kDa protein (p112) in mononucleated myoblasts

present investigation to examine the temporal order of expression of the myogenic components.

4.2 METHODS AND MATERIALS

4.2.1 Cell lines and culture media

Yaffe's L6 rat skeletal myoblast line (Yaffe, 1968) was maintained in Alpha medium (Flow Laboratories) supplemented with 10% (v/v) horse serum (Flow Laboratories) and gentamicin (50 µg/ml; Gibco) as described by D'Amore (D'Amore & Lo, 1986). Transfers were routinely made every 3 days (before fusion); 0.1% trypsin was used to detach cells from the plates. Cells were counted using a Coulter Counter. Unless indicated otherwise, cells were routinely seeded at a density of 1 x 10^6 cells per 20 x 150 mm Nunc plate. Mutants D1 and D1/S4 were generously provided by B.D. Sanwal, Department of Biochemistry, University of Western Ontario, London, Ontario, Canada (D'Amore & Lo, 1988; Zeuner et al., 1988; Chen & Lo, 1989). Mutant F72 was maintained on fructose medium supplemented with 0.05 mM 2-deoxy-2-fluoro-D-glucose (D'Amore et al., 1986).

4.2.2 Isolation of total RNA from rat myoblasts

Total cellular RNA was extracted from day-2 to day-6 cultures using the method as described by Chomczynski (Chomczynski & Sacchi, 1987). After washing with sterile phosphate buffered saline, 4.5 ml of the lysis buffer (4M GuSCN, 0.5% sarcosyl, 0.1 M ß-mercaptoethanol, and 25 mM Na citrate, pH 7.0)

in the level of extracellular ATP, or the amount of unlabelled phospho-amino acids in the protein.

The observed cell density effect suggests that the ecto-protein kinase, the 112 kDa protein, and/or p112 may play active roles in subconfluent cells, whereas they are not as active in confluent cultures. Since biochemical differentiation occurs at an early stage of the growth phase, these proteins may be required for the initiation of myogenesis. Two different approaches were used to test this possibility. The first approach examines the effect of myogenesis inhibitors on the level of p112. Treatment of cells with phloretin or BrdUrd resulted in a reduction of the p112 level and in the eventual abolishment of myogenesis; however, phlorizin had only minimal effects on these two processes (Table 3.3). The ecto-protein kinase and the 112 kDa protein were reduced by 26% and 47%, respectively in these myogenesis-impaired cells (Table 3.4). It should be noted that these proteins were affected to a different extent. The reduction in the amounts of these proteins could account for the reduced level of p112 in these cells. Thus the levels of ecto-protein kinase, the 112 kDa protein and p112 level seem to reflect the cells' eventual ability to form myotubes.

The second approach relied on the use of a conditional myogenesis-defective mutant (Chen & Lo, 1989). When grown under the myogenesis-competent conditions (Table 3.2), both mutant D1 (grow in 1% horse serum) and

its parental L6 cells (grown in 10% horse serum) contained similar levels of p112 (Table 3.2). However, only 26% of the normal p112 level was observed when the mutant was grown under the myogenesis-incompetent conditions (i.e. in 10% horse serum) (Table 3.2). This again demonstrates the relationship between the p112 level in undifferentiated myoblasts and the cells' eventual ability to differentiate.

The relationship between myogenesis and p112 was further explored using isogenic mutants defective in the 112 kDa protein. Even though mutant D1/S4 exhibited the normal ecto-protein kinase activity (Fig. 3.5), only a small amount of p112 could be detected in this mutant. Thus, in agreement with previous findings (D'Amore & Lo, 1988), mutant D1/S4 was altered in the structural or regulatory gene for the 112 kDa protein. Since this mutant was also impaired in myogenesis (Fig. 3.4), it is therefore conceivable that myogenesis may be dependent on the presence of the 112 kDa protein.

The other mutant that exhibited a very low level of p112 was the mutant F72 (Fig. 3.3). Immunoblotting studies showed that this mutant contained the normal amount of the 112 kDa protein (D'Amore & Lo, 1988). This mutant was also unable to phosphorylate the 75 kDa horse serum protein (Fig. 3.5) and the 112 kDa protein (Fig. 3.3). Thus this mutant might be altered in the structural or regulatory gene for the ecto-protein kinase. The fact that this mutant is also impaired in myogenesis (Fig. 3.4) suggests that myogenesis

may also be dependent on ecto-protein kinase.

Since mutants D1/S4 and F72 exhibited different levels of the 112 kDa protein and the ecto-protein kinase, and they were selected by completely different methods from rat L6 myoblasts (D'Amore et al., 1986; D'Amore & Lo, 1988), it was unlikely that they were defective in the same structural gene, or the same regulatory element. The finding that both mutants are defective in myogenesis, suggests that the genetic elements defective in these mutants are involved in myogenesis. Thus, the 112 kDa protein and the ecto-protein kinase may directly or indirectly be associated with the myogenic pathway.

Another question that arises from the above studies is whether the reduction in the amount of p112 is the cause, or the result of the inactivation of myogenesis. It has been previously shown that the mutant D23 is defective in myogenic differentiation (Kudo & Lo, 1990). The finding that this mutant was unaltered in its ability to phosphorylate both the endogenous 112 kDa protein and the exogenous 75 kDa horse serum protein (Figs. 3.3 and 3.5), and that optimal phosphorylation activity was observed with subconfluent L6 myoblasts suggested that the phosphorylation of the 112 kDa protein is not a secondary event resulting from biochemical differentiation.

To summarize, this study showed that the ecto-protein kinase is able to function efficiently utilizing the ATP excreted from the cells. The

phosphorylation of the cell surface 112 kDa protein by this ecto-protein kinase was affected by the cell density and the developmental stage of the cells. The highest level of p112 was detected in subconfluent mononucleated myoblasts. Studies with myogenesis-defective mutants showed that the expression of ecto-protein kinase and the 112 kDa protein correlated with the ability to form myotubes. Reduction of the p112 level by myogenesis inhibitors or in myogenesis-incompetent cells might result in the eventual abolishment of myogenesis.

CHAPTER 4

USE OF p112-DEFICIENT MYOBLASTS TO DETERMINE THE TEMPORAL ORDER OF EXPRESSION OF MYOGENIC COMPONENTS

4.1 INTRODUCTION

A number of distinct sequential events are involved in myogenic differentiation. These include biochemical differentiation, total commitment, and morphological differentiation (Endo and Nadal-Ginard, 1987). Biochemical differentiation is characterized by altered expression of a number of unlinked genes for muscle-specific proteins such as myosin light (MLC) and heavy (MHC) chains, tropomyosin, troponin T (TnT), desmin, muscle-specific creatine kinase, acetylcholine esterase and nicotinic acetylcholine receptor, and the voltage-gated Na^+ and Ca^{2+} channels etc. (Nguyen et al., 1983; Emerson et al., 1986; Breitbart et al., 1987; Endo and Nadal-Ginard, 1987; Ewton et al., 1988; Kaufman and Foster, 1988). The common feature shared by these muscle-specific genes is the presence of the CANNTG motif (or referred to as the E-box conserved sequence) in their enhancer regions (Olson, 1990; Emerson, 1990). The transcription of these genes is activated by the binding of hetero-oligomeric complexes of the myogenic and non-myogenic DNA binding basic helix-loop-helix (bHLH) proteins to the CANNTG motif (Lassar et al., 1989,1991; Farmer et al., 1992; Lin & Konieczny, 1992).

A family of structurally related proteins, MyoD, Myf5,

Myf4/myogenin, and MRF4/herculin/Myf6, is able to convert embryonic fibroblasts to stable myogenic cell lineages. The latter can be maintained as proliferating myoblasts or can be induced to form multinucleated myotubes (Davis et al., 1987; Wright et al., 1989; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Milner and Wold, 1990; Emerson, 1990; Olson, 1990; Weintraub et al., 1991; Fujisawa-Sehara et al., 1992). MyoD1 and Myf5 are thought to be responsible for myogenic determination. MyoD1 or Myf5, but not both, are expressed constitutively in many muscle cell lines, and in newly differentiated muscle (Davis et al., 1987; Braun et al., 1989 a,b). Forced expression of MyoD was found to activate muscle-specific genes in a variety of cell types (Weintraub et al., 1989). MyoD was thought to regulate the Myf5 mRNA in a negative manner (Rudnicki, et al., 1992). Myf4/myogenin is normally repressed in non-differentiating myoblasts; however, it is rapidly up-regulated during biochemical differentiation (Braun et al., 1989a; Edmondson & Olson, 1989; Wright et al., 1989; Fujisawa-Sehara et al., 1992). Studies with a myogenin antisense oligonucleotide revealed that myogenin was essential for myogenesis (Florini & Ewton, 1990). MRF4/Myf6/herculin is not expressed in most established muscle cell lines and is abundant in muscles of neonatal animals. It is probably involved in the later stage of muscle maturation (Rhodes & Konieczny, 1989; Miner & Wold, 1990; Braun et al., 1990; Yutzey et al. 1990; Hinterberger et al., 1991; Block & Miller, 1992).

Cell surface proteins are also thought to play a role in myogenesis

(Champaneria et al, 1989; Engel & White, 1990; Knudsen, 1990). The expression of the neural cell adhesion molecule (NCAM) (Moore & Walsh, 1985; Moore et al., 1987; Knudsen et al. 1989, 1990a; Dickson, et al., 1990), cadherin (Knudsen et al., 1990b; Pouliot et al. 1990; Donalies et al.1991), fibronectin and its receptor (Chung & Kang, 1990), integrin (Menko and Boettiger, 1987) and the rat myoblast high affinity hexose transport system (Chen & Lo, 1989; Kudo & Lo, 1990) were found to be closely associated with myogenic differentiation. Presumably these components take part in biochemical differentiation, or in cell-cell interaction prior to membrane fusion.

Two other cell surface proteins, an ecto-protein kinase and a 112 kDa protein were previously shown to be associated with myogenesis (Chapter 3). Inactivation of either one of these two proteins resulted in the inability of myoblasts to form myotubes. First, rat L6 myoblasts grown in the presence of phloretin or 5-bromo-2'-deoxyuridine (BrdUrd) not only exhibited much reduced levels of the ecto-protein kinase, the 112 kDa protein, and the phosphorylated 112 kDa protein (p112), but were also impaired in myogenesis. Second, reduction of p112 in a conditional mutant (D1) was accompanied by the cells' eventual inability to differentiate. Third, a mutant (F72) deficient in the ecto-protein kinase, but not in the 112 kDa protein, was defective in myogenesis. Finally, a mutant (D1/S4) harbouring a much reduced level of the 112 kDa protein, but normal ecto-protein kinase activity, was also unable to form myotubes. Using these p112-deficient myoblasts, attempts were made in the

present investigation to examine the temporal order of expression of the myogenic components.

4.2 METHODS AND MATERIALS

4.2.1 Cell lines and culture media

Yaffe's L6 rat skeletal myoblast line (Yaffe, 1968) was maintained in Alpha medium (Flow Laboratories) supplemented with 10% (v/v) horse serum (Flow Laboratories) and gentamicin (50 μg/ml; Gibco) as described by D'Amore (D'Amore & Lo, 1986). Transfers were routinely made every 3 days (before fusion); 0.1% trypsin was used to detach cells from the plates. Cells were counted using a Coulter Counter. Unless indicated otherwise, cells were routinely seeded at a density of 1 x 10^6 cells per 20 x 150 mm Nunc plate. Mutants D1 and D1/S4 were generously provided by B.D. Sanwal, Department of Biochemistry, University of Western Ontario, London, Ontario, Canada (D'Amore & Lo, 1988; Zeuner et al., 1988; Chen & Lo, 1989). Mutant F72 was maintained on fructose medium supplemented with 0.05 mM 2-deoxy-2-fluoro-D-glucose (D'Amore et al., 1986).

4.2.2 Isolation of total RNA from rat myoblasts

Total cellular RNA was extracted from day-2 to day-6 cultures using the method as described by Chomczynski (Chomczynski & Sacchi, 1987). After washing with sterile phosphate buffered saline, 4.5 ml of the lysis buffer (4M GuSCN, 0.5% sarcosyl, 0.1 M ß-mercaptoethanol, and 25 mM Na citrate, pH 7.0)

was added to each plate. Cells were scraped off with a rubber policeman, and homogenized 10 times on ice with a Dounce homogenizer (pestle A). 0.45 ml of 2M Na acetate (pH 4.0), 4.5 ml of water saturated phenol, and 1.0 ml of $CHCl_3$/isoamyl alcohol (49:1) were then added to each 4.5 ml of the homogenate; this was then mixed vigorously for 10 sec., and cooled on ice for 15 min. Samples were then centrifuged at 2500 x g for 10 min. at room temperature in a table-top centrifuge. The aqueous phase was transferred to a new tube, and precipitated with one volume of isopropanol at -20°C for one hour. Samples were then centrifuged at 11,000 x g in a Beckman JA 20.1 rotor at 4°C for 20 min. The pellet was resuspended in 0.3 ml of lysis buffer and precipitated with two volumes of ethanol in a microcentrifuge tube overnight at -20°C. The total RNA was pelleted by centrifuging at 14,000 x g at 4°C for 15 min. After washing with 80% ethanol, the RNA was again pelleted by centrifugation. Finally, the total RNA was dried under vacuum for 5 min, and dissolved in 200 µl of diethylpyrocarbonate (DEPC)-treated sterile water.

4.2.3 Preparation of cDNA probe

Plasmids harbouring cDNAs for various myogenic factors were purchased from the Repository of Human DNA Probes and Libraries, American Type Culture Collection, Rockville, MD:. the Myf3 (human MyoD1), Myf4 (human myogenin), Myf5, and Myf6 (human MRF4) cDNAs were originally provided by H.H. Arnold (Braun et al., 1989 a,b). The mouse NCAM cDNA (clone pM1.3) was originally obtained from C. Goridis (Goridis et al., 1985);

whereas the myosin light (MLC) and heavy (MHC) chain, and the troponin T (TnT) cDNAs were originally from B. Nadal-Ginard (Endo & Nadal-Ginard, 1987). The ß2-microglobulin and E-cadherin cDNAs were originally obtained from F. Daniel and M. Takeichi (Daniel et al., 1983; Takeichi, 1988). Plasmid DNA was isolated using the QIAGEN plasmid kit (QIAGEN, Germany). Different restriction enzymes were used for the isolation of the cDNA inserts: Eco RI for Myf3, Myf4, Myf5 and Myf6; Pst I for MLC, MHC and TnT. Plasmid DNA was incubated with the appropriate restriction enzyme for 2 hrs at 37°C. The inserts were separated by electrophoresis through a 1 % agarose gel and purified by the Gene Clean Kit (Bio/Can Scientific Inc) using the protocol recommended by the supplier. The purified insert was then labelled with α-^{32}P-dCTP using the Prime-a-Gene kit (Promega).

4.2.4 Northern gel blotting studies

For RNA blotting, 20 µg/lane of total RNA was denatured with formaldehyde (Sambrook et al., 1989). An RNA ladder (BRL) was used for the standard molecular weight marker. After separation by electrophoresis through a 1 % formaldehyde agarose gel, the RNAs were transferred to a Biotrans nylon membrane (ICN Biochemicals) by a vacuum blotting system (VacuGene^{TM}XL, Pharmacia). The nylon membrane was then baked for 2 hrs. at 80°C in a vacuum oven. This was then hybridized with the appropriate labelled cDNA probes. The blotted and fixed membrane was prehybridized at 37°C for at least 2 hrs in a solution containing 45% formamide, 5X SSC, 2X Denhardt's solution,

20 mM sodium phosphate buffer, pH 6.5, 0.1% sodium dodecyl sulfate, and 100 μg/ml denatured herring sperm DNA (Maniatis et al., 1982). Hybridization with the labelled cDNA probe was carried out at 37°C overnight in the prehybridization solution containing 10% dextran sulfate, and 1 x 10^6 cpm/ml of ^{32}P-labelled insert. After washing in 2X SSC and 0.1% sodium dodecyl sulfate two times at room temperature for 15 min. and then at 42°C for 1 hr., the blot was exposed to Kodak XAR-5 film for 1 - 4 days at -84°C. The bands in the autoradiogram were quantitated using the JAVA Video Analysis Software, Jandel Scientific. Since the transcript levels of the ß2-microglobulin and E-cadherin remained unaltered during myogenesis, they were used for normalizing the amount of myogenesis-associated transcripts in each sample. All experiments were repeated at least twice with two different RNA preparations. Experiments were very consistent.

4.2.5 Determination of fusion index

Cells were plated in six-well Falcon plates in Alpha medium with or without myogenesis inhibitors (BrdUrd or phloretin). On different days after subculturing, cells were treated with 1 mM $ZnSO_4$ in 20% DMSO to swell their nuclei, and then fixed in 2.5% glutaraldehyde in phosphate buffered saline (Chen & Lo, 1989). Cells were then stained with 6% Giemsa. Fusion index was determined by calculating the ratio of the number of nuclei in myotubes to the total number of nuclei per field (Morris & Cole, 1972). Only structures containing at least three nuclei were considered as myotubes; usually each field

contained at least 50 nuclei. Ten fields were counted for each set of determinations.

4.2.6 Materials

Various restriction enzymes, Prime-a-Gene kit and molecular weight markers were purchased from Promega. Phloretin and BrdUrd were purchased from Sigma Chemical Co.. All other chemicals were obtained from commercial sources and were of the highest available purity.

4.3 RESULTS

4.3.1 Expression of muscle-specific regulatory factors in chemically-induced p112-defective myoblasts

As indicated in Chapters 2 and 3 phosphorylation of a cell surface 112 kDa protein by an ecto-protein kinase might be essential for myogenesis (Chapters 2 and 3). In order to determine the temporal order of expression of various myogenic components, the present investigation examined the transcript levels of three different groups of myogenesis-associated genes in cells possessing much reduced levels of the phosphorylated 112 kDa protein (p112).

The first group includes the myogenic bHLH proteins (Olson, 1990; Emerson, 1990; Lassar et al., 1991; Weintraub et al., 1991). Labelled Myf3, Myf4, Myf5 and Myf6 cDNAs were used to probe the rat L6 myoblast total RNAs in northern blot analysis. This study showed that the Myf3 and Myf6

cDNAs could not recognize any transcripts in L6 myoblasts or in L6 myotubes (Fig. 4.1) . However, mRNAs recognizable by the Myf4 and Myf5 cDNA probes could be detected in the rat L6 muscle cells (Fig. 4.1). Taking the level present in day-2 culture as 100%, about 50% and 80% increase in the Myf4 transcript was observed in day-3 and day-4 cultures, respectively (Fig. 4.2B), whereas about 40% and 50% reduction of the Myf5 transcript was observed in day-3 and day-4 cultures, respectively (Fig. 4.2A). These studies showed that the Myf4 and Myf5 transcripts were regulated in opposite ways during myogenesis. Under our experimental conditions, multinucleated myotubes were first observed in day-4 culture, and the fusion indices reached 27% and 70% on day-5 and day-6, respectively (Fig. 3.2 of chapter 3). In other words, changes in the Myf4 and Myf5 transcript levels occurred before the appearance of myotubes. Although growth conditions were different, comparable changes in the Myf4 and Myf5 transcripts were also observed by other workers (Hinterberger et al., 1991).

When L6 myoblasts were grown in the presence of BrdUrd or phloretin, only 35% of the normal p112 level could be detected in day-3 cultures (Chapter 3). The fusion indices of BrdUrd- and phloretin-grown day-6 cultures were 0% and 15%, respectively (Fig. 4.2A; Chen & Lo, 1989; Kudo & Lo, 1990). Northern blot analysis showed that the decrease in p112 was accompanied by a similar reduction in the Myf4 transcript; about 30% of the control Myf4 mRNA level was observed in these cells (Fig. 4.2B). On the other hand, the rate of decline in the Myf5 mRNA was not altered in these chemically-induced p112$^-$

Figure 4.1 Levels of transcripts for various myogenic factors in rat L6 myoblasts on different days after subculturing.

Total cellular RNA was extracted from day-2 to day-6 cultures of rat L6 myoblast. Northern blot analysis was carried out as described in the text. Each lane was loaded with 20 μg RNA. RNA from each culture was probed with ^{32}P-labelled human *myf3, myf4, myf5*, and *myf6* cDNAs, respectively. The number on top of each lane indicates the number of days after subculturing.

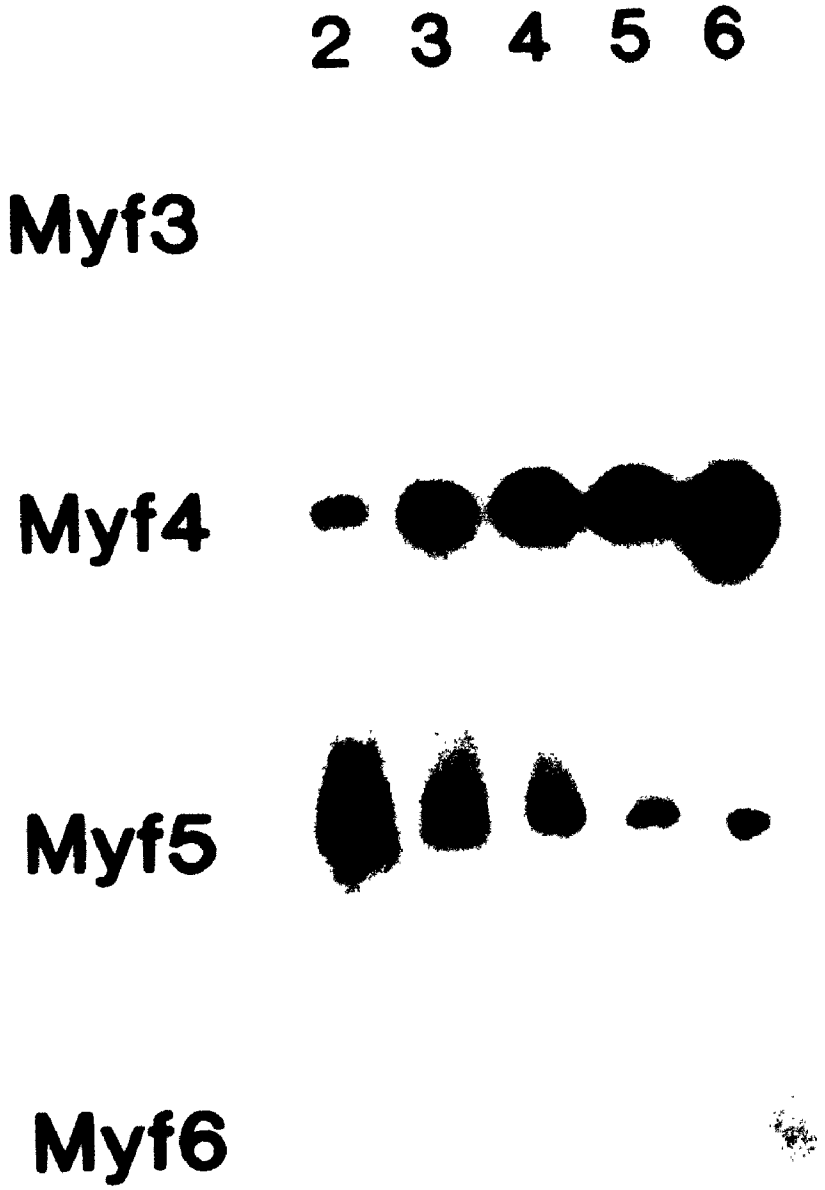
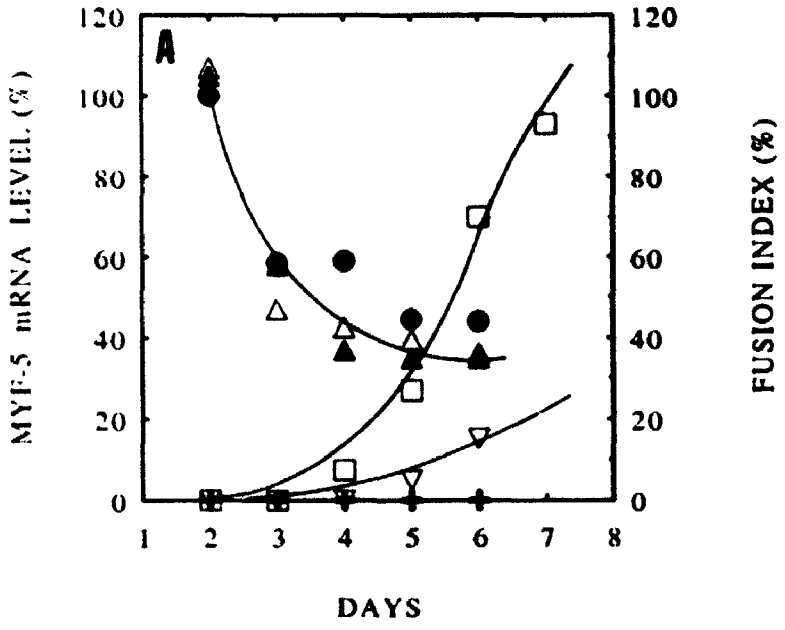
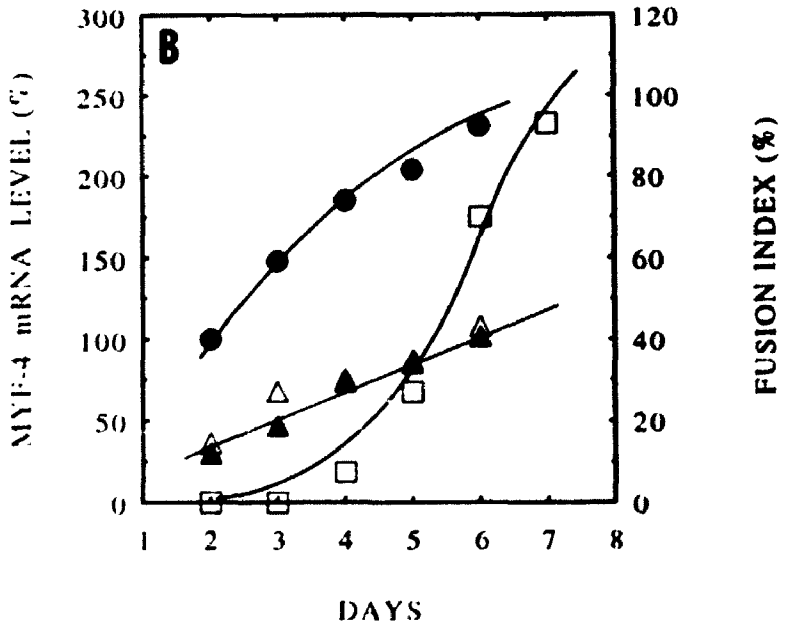

2 3 4 5 6
Myf3
Myf4
Myf5
Myf6

Figure 4.2 Levels of Myf4 and Myf5 mRNAs in BrdUrd- and phloretin-grown rat L6 myoblasts

Total cellular RNA was extracted from day-2 to day-6 cultures of rat L6 myoblasts grown in the presence or absence of 50 μM phloretin or 7.5 μM BrdUrd. Each lane was loaded with 20 μg RNA. The mRNA levels of Myf4, Myf5, and ß2-microglobulin were determined by northern blot studies using the appropriate labelled cDNAs. The bands in the autoradiogram were quantitated using the JAVA Video Analysis Software, Jandel Scientific. The Myf4 and Myf5 mRNAs in each sample were normalized according to the amount of ß2-microglobulin mRNA present in the same sample. Transcript levels present in day-2 culture of untreated L6 myoblast were used as 100%. Panels A and B show the levels of the Myf5 and Myf4 mRNAs, respectively. ●, ▲, and △ denote mRNA present in control, BrdUrd- and phloretin-grown cells, respectively. Fusion index was determined by calculating the percentage of the total nuclei present in myotubes. □, +, and ▽ denote the fusion indices of untreated, BrdUrd- and phloretin- grown rat L6 myoblasts, respectively.


A
MYF-5 mRNA LEVEL (%)
FUSION INDEX (%)
DAYS
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8


B
MYF-4 mRNA LEVEL (%)
FUSION INDEX (%)
DAYS
300
250
200
150
100
50
0
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8

myoblasts (Fig. 4.2A). If these two myogenic factors are involved in the same myogenic pathway, then BrdUrd and phloretin probably act at sites downstream from Myf5 and upstream from Myf4.

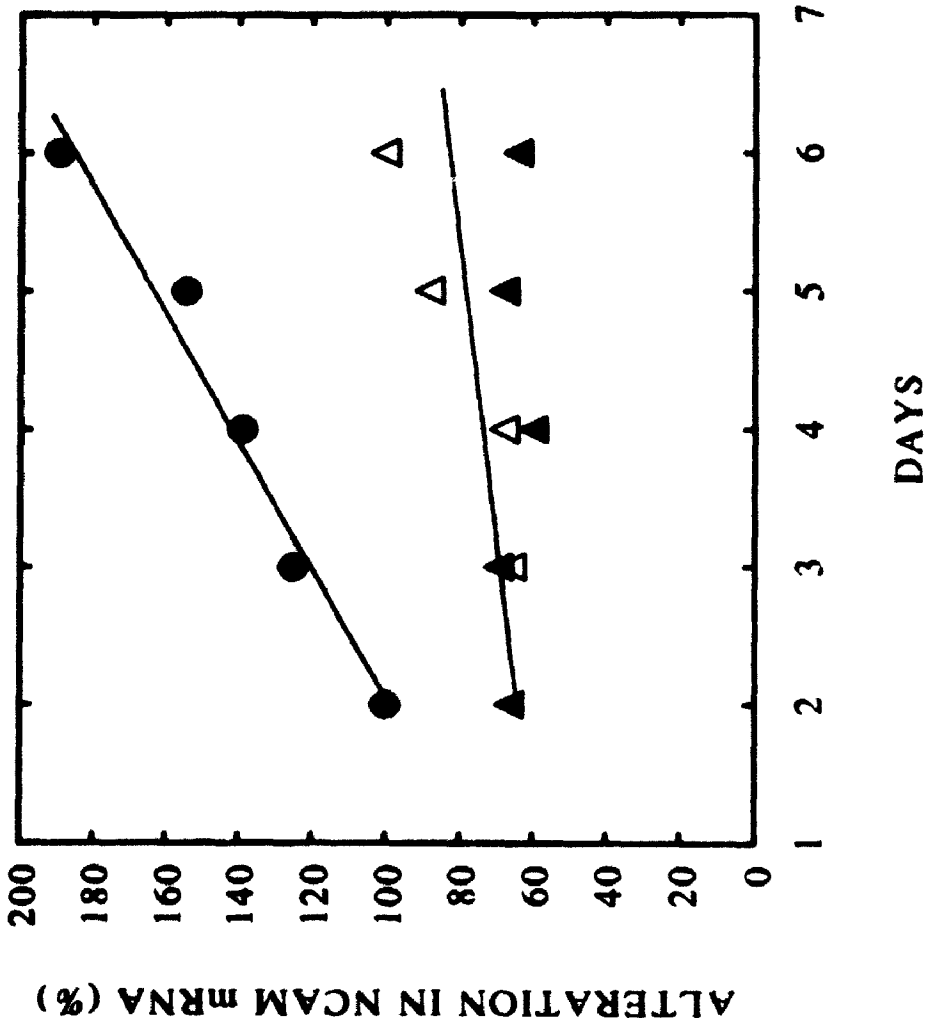
4.3.2 Expression of NCAM and muscle-specific genes in the chemically-induced p112-defective myoblasts

Studies with mouse myoblasts revealed that the pattern of NCAM expression was tightly regulated during myogenesis (Moore et al., 1987). Mouse myoblasts transfected with the NCAM cDNA exhibited an enhanced level of muscle-specific enzyme and a faster rate of fusion (Dickson et al., 1990). The level of the NCAM mRNA in rat myoblasts was therefore examined (Fig. 4.3). Similar to the mouse muscle cell lines G8-1 and C2 (Moore et al., 1987), three transcripts, 6.7 kb, 4.8 kb, and 3 kb, were recognized by the NCAM cDNA (data not shown); of these, the 3 kb transcript was the most prominent one. This 3 kb NCAM mRNA was elevated about two fold during myogenesis, and this increase was initiated before the appearance of myotubes (Fig. 4.3). Unlike the $p112^+$ L6 cells, the 3 kb NCAM mRNA was hardly elevated in the BrdUrd- and phloretin-grown cells.

The transcript levels of muscle-specific genes were also examined. The MHC, MLC and TnT mRNA levels in untreated L6 myoblasts were increased dramatically during myogenesis (Endo & Nadal-Ginard, 1987; Fig. 4.4). A comparison of the transcript levels in day-2 and day-6 cultures revealed 7-,27-,

Figure 4.3 NCAM mRNA level in BrdUrd- and phloretin-grown rat L6 myoblasts.

Northern blot analysis and quantitation of mRNA in the total RNA extracts from control and myogenesis inhibitor-treated cultures were carried out as described in Fig. 4.2. Labelled NCAM cDNA was used to probe the specific mRNA. The 3 kb NCAM mRNA level present in day-2 culture of untreated L6 cells was used as 100%. ●, ▲, and △ denote NCAM mRNA in control, BrdUrd- and phloretin-grown cells, respectively.


ALTERATION IN NCAM mRNA (%)
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
DAYS

and 11- fold increases of MLC, MHC, and TnT mRNAs, respectively (Fig. 4.4). This was distinctly different from the 2-fold increase observed with the Myf4 and NCAM mRNAs (Figs. 4.2 and 4.3). While the fastest rate of increase of the Myf4 mRNA occurred between days-2 and -4 (Fig. 4.2), the fastest rate of increase of the muscle-specific transcripts was observed between day-4 to day-6 cultures (Fig. 4.4). In other words, there was a lag period before the actual increase in these transcripts. This suggested that the increases in the muscle-specific mRNAs were subsequent to the increase in the Myf4 mRNA.

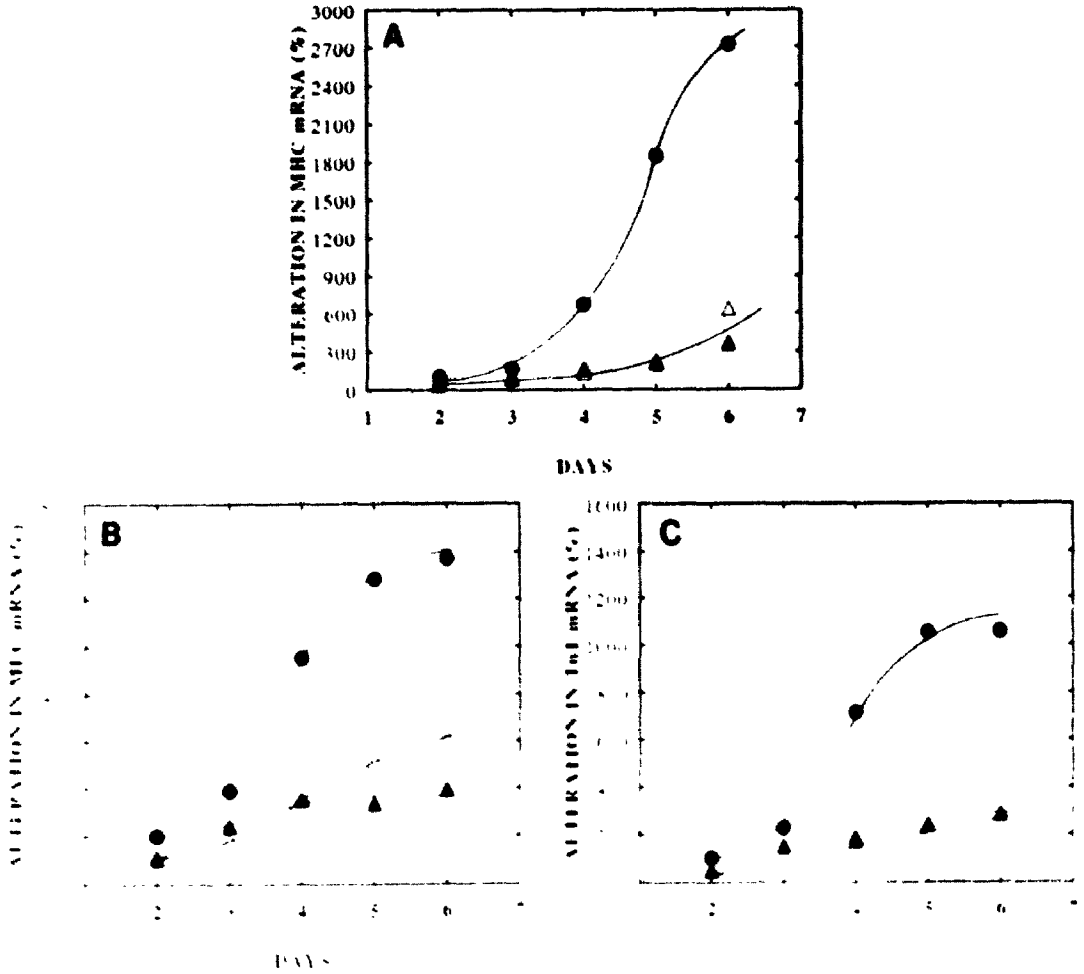
The levels of the muscle-specific transcripts in the BrdUrd- or phloretin- grown L6 myoblasts were also determined (Fig. 4.4). The reduction in p112 in these cells was accompanied by decreases in the MLC, MHC, and TnT mRNAs. Since transcription of these muscle-specific genes is activated by the myogenic factors, the reduction of these muscle-specific transcripts was probably due to the decrease in the Myf4 mRNA.

4.3.3 Transcript levels of myogenesis-associated genes in a conditional p112-defective mutant

The other approach to examine the site of involvement of p112 was to use a conditional p112-defective mutant. When mutant D1 was grown in 1% and 10% horse serum, about 93% and 26% of the normal p112 level was detected in these myoblasts, respectively (Chapter 3). Furthermore, this mutant was able to form myotubes when grown in 1%, but not in 10%, horse serum

Figure 4.4 Transcript levels of muscle-specific proteins in BrdUrd- and phloretin-grown rat L6 myoblasts.

Northern blot analysis and quantitation of the total RNA extracts from control and myogenesis-inhibitor treated cultures were carried out as described in Fig. 4.2. Labelled MHC, MLC, TnT, ß2-microglobulin cDNAs were used to probe the respective mRNAs. Panels A, B, and C show the levels of MHC, MLC and TnT in untreated (●), BrdUrd-grown (▲), and phloretin-grown (△) rat L6 cells on different days after subculturing. The MHC, MLC and TnT mRNAs in each sample were normalized according to the amount of ß2-microglobulin mRNA present in the same sample. Transcript levels present in day-2 culture of untreated L6 myoblast were used as 100%.


A
ALTERATION IN MHC mRNA (%)
3000
2700
2400
2100
1800
1500
1200
900
600
300
0
1
2
3
4
5
6
7
DAYS
B
C

(Chen & Lo, 1989). Thus this mutant was used to examine the relationship between p112 and the transcript levels of myogenesis-associated genes.

Regardless of the horse serum concentrations used, a similar rate of decline in the Myf5 mRNA was observed in mutant D1 and its parental L6 cells (Fig. 4.5). While the Myf4 and NCAM mRNAs were present in comparable levels in L6 myoblasts grown in 1% or 10% horse serum (Figs. 4.5A and 4.5B), and in the $p112^+$ D1 cells (those grown in 1% horse serum) (Fig. 4.5C), these mRNAs were significantly reduced in the $p112^-$ D1 cells (those grown in 10% serum) (Fig. 4.5D). Dramatic reduction in the MLC, MHC, TnT transcripts was also observed with the $p112^-$ D1 cells (Fig. 4.6). Thus these studies showed that the drop in p112 in mutant D1 was accompanied by decreases in the Myf4, NCAM, MHC, MLC and TnT mRNAs. However, growth of this mutant in 1% horse serum restored not only the p112 level, but also the levels of the above mentioned transcripts.

4.3.4 Expression of myogenesis-associated genes in mutants defective either in the cell surface 112 kDa protein or in the ecto-protein kinase

The possibility exists that the phosphorylation of the 112 kDa protein may not be essential for myogenesis; the BrdUrd/phloretin-grown L6 cells and mutant D1 (when grown in 10% horse serum) may be impaired in a regulatory element, which controls the expression of Myf4 and the p112 level. If this were the case, then mutants defective in either the ecto-protein kinase or the

Figure 4.5 Transcript levels of Myf4, Myf5 and NCAM in a conditional myogenesis-defective mutant (D1) and its parental L6 myoblast.

Both mutant D1 and its parental L6 cells were initially grown in 10% horse serum for 1 day, and then subcultured in 1% or 10% horse serum for a further 2 to 6 days. Total cellular RNA was then extracted from these cells. Detection and quantitation of various mRNAs were carried out as described in Figs. 4.2 and 4.3. Panels A, B, C, and D indicate transcript levels in L6 cells grown in 1% horse serum; L6 cells grown in 10% horse serum; mutant D1 grown in 1% horse serum; and mutant D1 grown in 10% horse serum, respectively. Days refer to the number of days after switching to the final horse serum concentration. Transcript levels present in day-2 culture of L6 myoblast grown in 10% horse serum were used as 100%. ●, ▵, and ▽ denote mRNA levels of Myf4, Myf5 and NCAM, respectively.

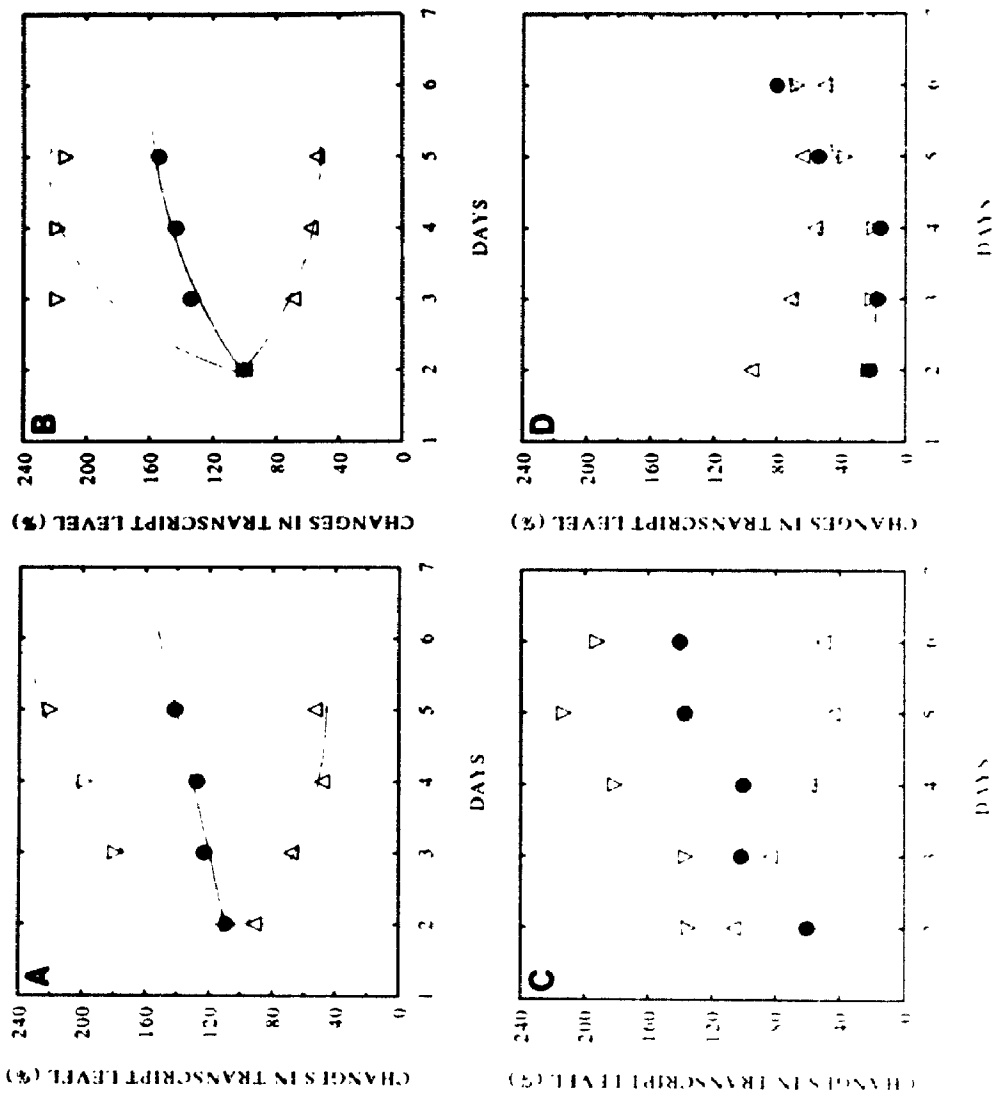
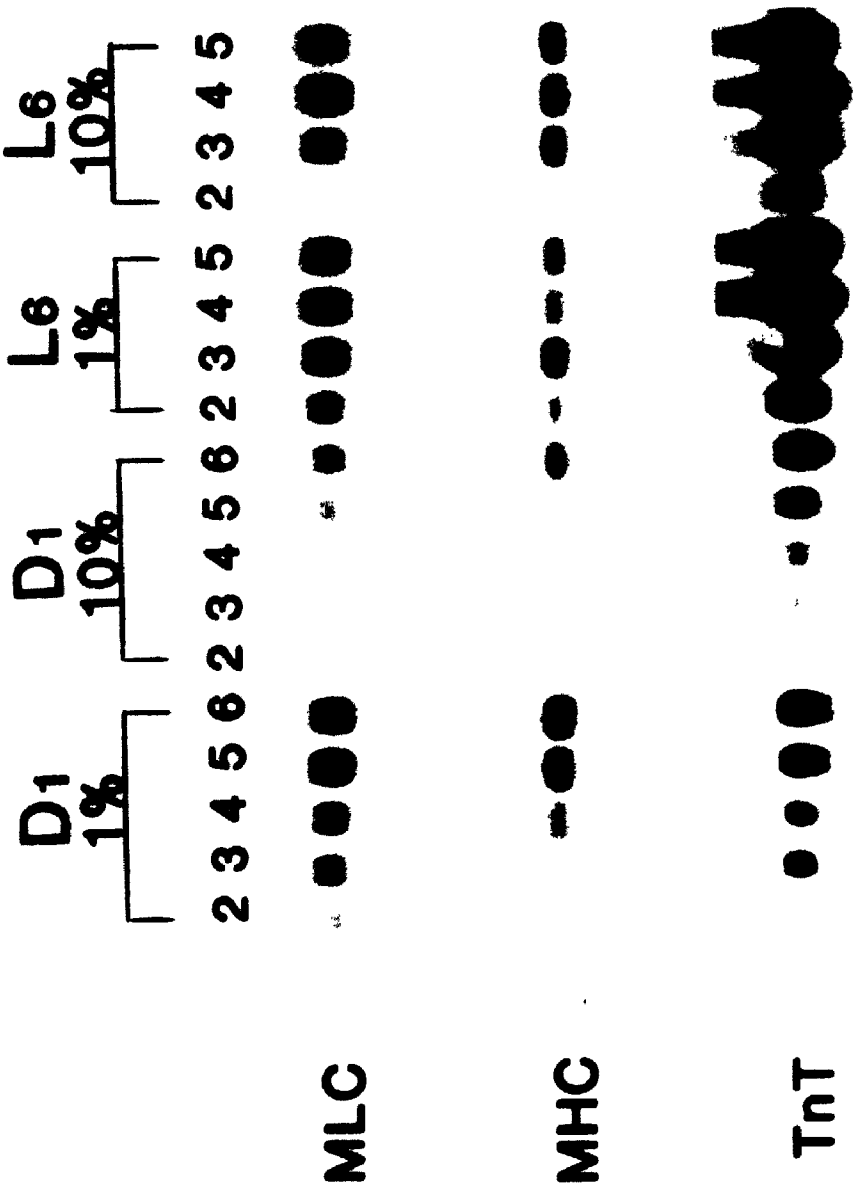

A
B
C
D
CHANGES IN TRANSCRIPT LEVEL (%)
DAYS
240
200
160
120
80
40
0
1
2
3
4
5
6
7

Figure 4.6 Transcript levels of MLC, MHC, and TnT in mutant D1 and its parental L6 myoblast.

Extraction of total cellular RNA and northern blot analysis were carried out as described in Fig. 4.5. Labelled MLC, MHC, and TnT cDNAs were used to probe for the respective mRNAs. Each lane was loaded with 20μg RNA. 1% and 10% refer to the percentage of horse serum used in culturing the cells. The numbers on top of each lane indicate the days after switching to the final horse serum concentration.


D1 1%
D1 10%
L6 1%
L6 10%
2 3 4 5 6 2 3 4 5 6 2 3 4 5 2 3 4 5
MLC
MHC
TnT

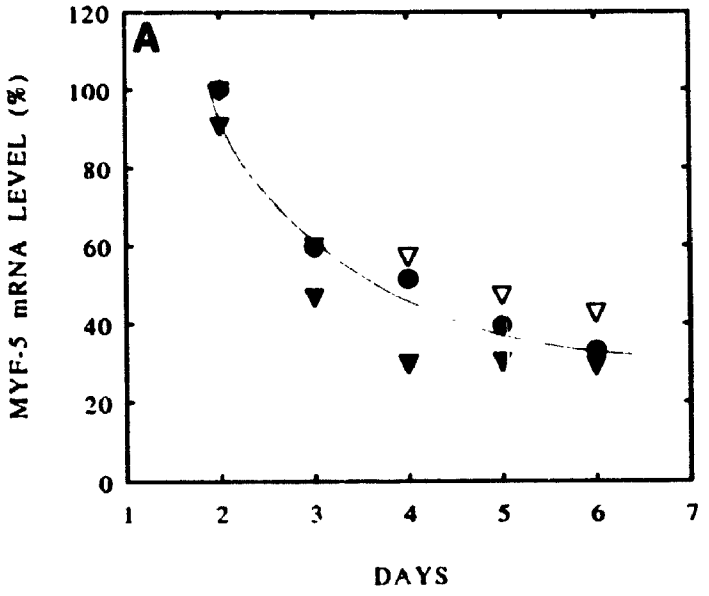
112 kDa protein should possess normal levels of the Myf4, MLC, MHC, and TnT transcripts. Mutants F72 and D1/S4 were previously shown to possess around 37% of the normal p112 (Chapter 3). Mutant F72 was defective only in the ecto-protein kinase (38% of the L6 level), and its level of the 112 kDa protein remained similar to L6 cells. On the other hand, mutant D1/S4 contained normal level of the ecto-protein kinase activity, while retaining only 35% of the 112 kDa protein present in L6 cells. These two mutants were therefore invaluable in assessing the relationship between p112 and the expression of various myogenesis-associated genes.

The rate of decline of the Myf5 mRNA in these two mutants was similar to that of their parental L6 cells (Fig. 4.7A). This showed that the expression of Myf5 was not affected by the ecto-protein kinase or by the 112 kDa protein. In contrast, day-2 cultures of these two mutants contained less than 20% of the Myf4 mRNA present in L6 cells (Fig. 4.7B). Since the Myf4 transcript was dramatically reduced in mutants defective in either the ecto-protein kinase or in the 112 kDa protein, these two proteins must play important roles in regulating the level of the Myf4 transcript.

The defect in the ecto-protein kinase or in the 112 kDa protein also reduced the levels of the NCAM and the muscle-specific transcripts. The 3 kb NCAM mRNA in both mutants was about 46% of that present in day-2 L6 culture. Similar to the BrdUrd- and phloretin-grown cells, the NCAM mRNA

Figure 4.7 Myf5 and Myf4 transcript levels in mutants defective in the ecto-protein kinase or in the cell surface 112 kDa protein.

Total cellular RNA extracts were prepared from mutants F72, D1/S4 and their parental L6 myoblast on different days after subculturing. Northern blot analysis and quantitation of the Myf5 and Myf4 mRNAs were carried out as described in Fig. 4.2. The Myf5 and Myf4 mRNAs in each sample was normalized according to the amount of ß2-microglobulin mRNA present in the same sample. Panels A and B show the levels of the Myf5 and Myf4 mRNAs, respectively. The transcript level present in day-2 culture of L6 myoblast was used as 100%. ●, ▼, and ▽ denote mRNA levels in L6, F72 and D1/S4 cells, respectively.


A
MYF-5 mRNA LEVEL (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
DAYS

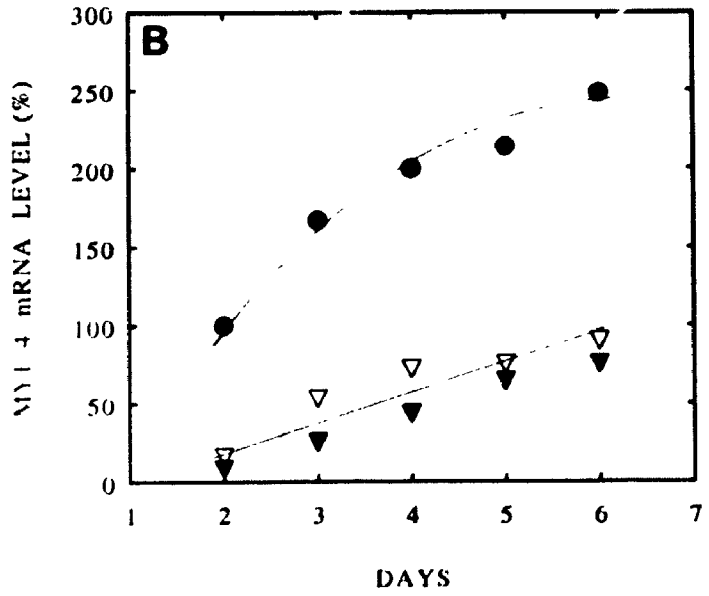
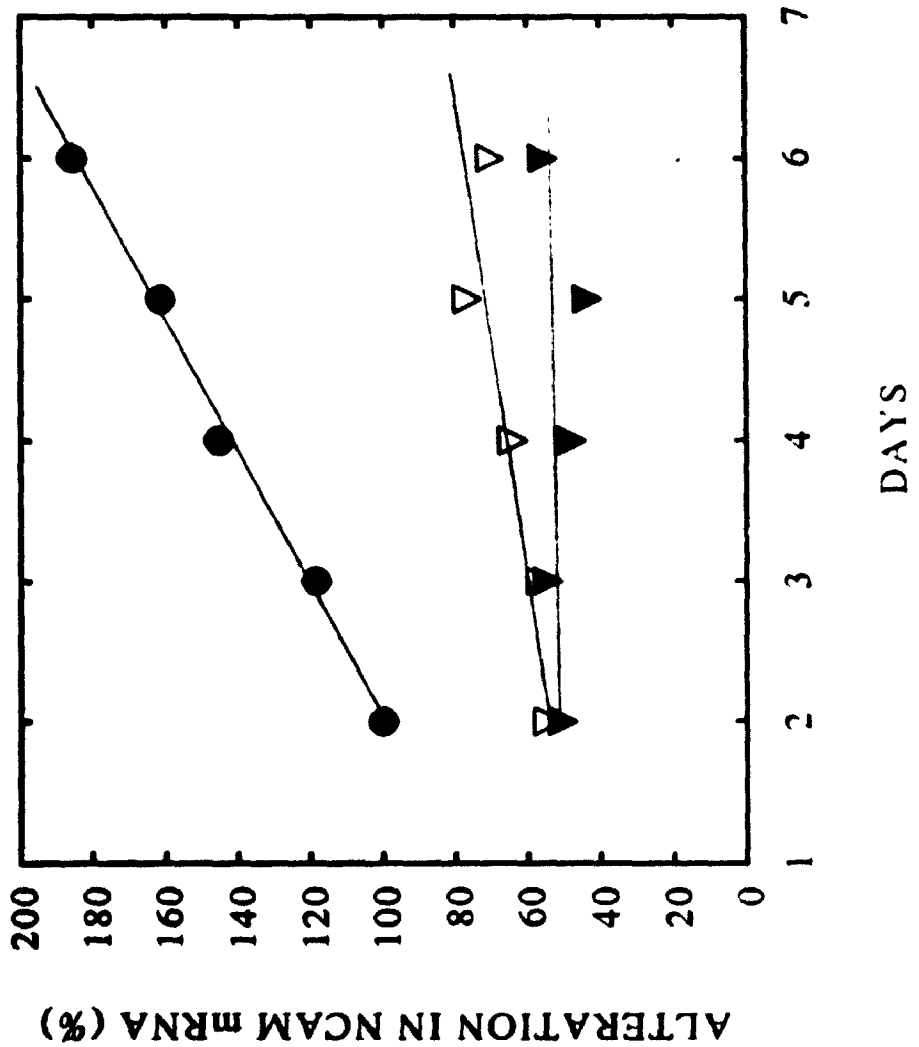

B
MYF-4 mRNA LEVEL (%)
300
250
200
150
100
50
0
1
2
3
4
5
6
7
DAYS

Figure 4.8 NCAM mRNA levels in mutants defective in the ecto-protein kinase or in the cell surface 112 kDa protein.

Experiments were carried out as described in Fig. 4.7, except that ^{32}P-labelled NCAM cDNA was used to probe the mRNAs. The 3.0 kb NCAM mRNA in each sample was normalized according to the amount of ß2-microglobulin mRNA present in the same sample. The transcript level present in day-2 culture of L6 myoblasts was used as 100%. ●, ▼, and ▽ denote NCAM mRNA levels in L6, F72 and D1/S4 cells, respectively.


ALTERATION IN NCAM mRNA (%)
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
DAYS

levels in both mutants remained almost unchanged during growth (Fig. 4.8). While the MLC, MHC, and TnT mRNAs were dramatically reduced in mutant D1/S4, these mRNAs were hardly detectable in mutant F72 (Fig. 4.9). Thus defects in either the ecto-protein kinase or the 112 kDa protein were also accompanied by the inability to activate transcription of the myogenesis-associated genes. This may explain why both mutants F72 and D1/S4 were impaired in myogenesis (D'Amore & Lo, 1988; Chapter 3).

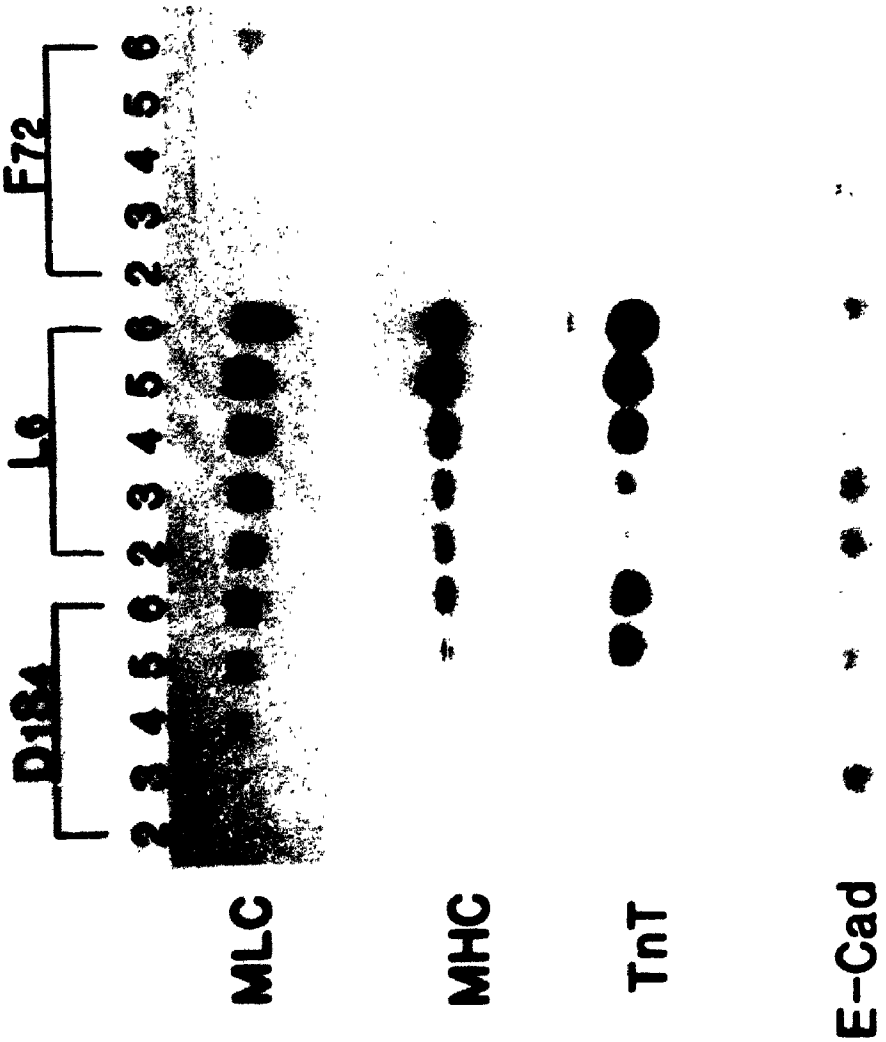
4.4 DISCUSSION

Studies using chemical reagents suggest that myogenesis is comprised of a sequential cascade of multiple steps leading to terminal differentiation and formation of multinucleated myotubes. Components such as the myogenic factors and the neural cell adhesion molecule (NCAM) have recently been demonstrated to play vital roles in myogenesis (Moore et al., 1987; Dickson, et al., 1990; Emerson, 1990; Knudsen, 1990). While exciting new information is emerging on the mode of action of the myogenic factors, not much is known on the temporal order of expression of myogenic components (Weintraub et al., 1991).

The present investigation examines the possible site of involvement of cell surface phosphorylation in regulating the expression of myogenesis-associated genes. The ecto-protein kinase involved is a Ca^{++}-dependent phosphoserine kinase, and its substrate protein is a 112 kDa cell surface protein (Chapter 2). More than sufficient extracellular ATP was present on the cell

Figure 4.9 Transcript levels of muscle-specific proteins in mutants defective in the ecto-protein kinase, or in the cell surface 112 kDa protein.

Experiments were carried out as described in Fig. 4.7, except that ^{32}P-labelled MLC, MHC, TnT and E-cadherin cDNAs were used in northern blot studies. The numbers on top of each lane indicate the days after subculturing of each cell type.


D18
L9
F72
2 3 4 5 0 2 3 4 5 0 2 3 4 5 6
MLC
MHC
TnT
E-Cad

surface for the optimal phosphorylation of the 112 kDa protein (Chapter 2). Studies with myogenesis inhibitors and with a conditional myogenesis-defective mutant (D1) suggested that the phosphorylated 112 kDa protein (p112) might play a role in myogenesis (Chapter 3). This finding was corroborated by the observation that mutants defective in either the ecto-protein kinase or the 112 kDa protein were also impaired in myogenesis.

Five different types of $p112^-$ myoblasts were used to examine the temporal order of expression of myogenic components. The first two types were L6 cells grown in the presence of 7.5 μM BrdUrd or 50 μM phloretin. By exerting their effects on both the ecto-protein kinase and the 112 kDa protein, these chemicals reduced the p112 by 64% (Chapter 3). The third type was mutant D1. This mutant possessed normal p112 level when grown in 1% horse serum; however, a 74% reduction of p112 was observed when this mutant was grown in 10% horse serum (Chapter 3). The fourth type was a mutant (F72) defective only in the ecto-protein kinase, and the fifth type was a mutant (D1/S4) deficient only in the 112 kDa protein. Both mutants had much reduced p112 level (Chapter 3). It should be noted that all three mutants (D1, F72 and D1/S4) were isolated from L6 myoblasts.

An examination of the transcript levels for the myogenic factors revealed several interesting observations. While Myf3 (human MyoD1) and Myf6 (human MRF4) cDNAs could not detect any transcripts in northern blot studies,

both Myf5 and Myf4 (human myogenin) cDNAs were able to recognize specific transcripts in total mRNAs prepared from rat L6 myoblasts (Fig. 4.1; Braun et al., 1989 a,b; Emerson, 1990). The Myf5 mRNA level was found to decline with myogenesis (Fig. 4.2A; Hinterberger et al., 1991), the largest drop occurred between day-2 and day-3 after subculturing (Fig. 4.2A). This suggested that the Myf5 gene was involved in myogenic determination, and was not activated by myogenic differentiation (Braun et al., 1989 a,b; Peterson et al., 1990). Since the rate of decline of Myf5 mRNA was similar in all myoblasts examined, regardless of their p112 content (Figs. 4.2A, 4.5 and 4.7A), both the ecto-protein kinase and the 112 kDa protein were not likely involved in regulating the Myf5 expression. Thus the p112 site is probably located downstream from the Myf5 site, if they are involved in the same myogenic pathway. Alternatively, they may participate in paralle. pathways leading to myotube formation.

Unlike the Myf5 mRNA, the Myf4 mRNA level was elevated during myogenesis (Figs. 4.2B, 4.5 and 4.7B; Hinterberger et al., 1991). This increase was initiated before the alignment of myoblasts, and the rate of increase was slower after the appearance of myotubes (day-4) (Fig. 4.2B). Its level in the myotubes was about twice that present in myoblasts. More importantly, all $p112^{-}$ myoblasts possessed much reduced levels of this transcript. The 64% reduction of p112 in L6 myoblasts by growth in BrdUrd/phloretin was accompanied by a 60-70% decrease of the Myf4 mRNA (Fig. 4.2B). Growth of mutant D1 in 10% horse serum resulted in a 74% and a 85% decrease in p112 and Myf4 mRNA,

respectively; whereas these two components were not altered when this mutant was grown in 1% horse serum (Fig. 4.5). Mutant F72 contained around 40% and 15% of the p112 and Myf4 mRNA present in L6 cells, respectively; whereas mutant D1/S4 possessed around 35% and 31% of the normal p112 and Myf4 transcript, respectively (Fig. 4.7).

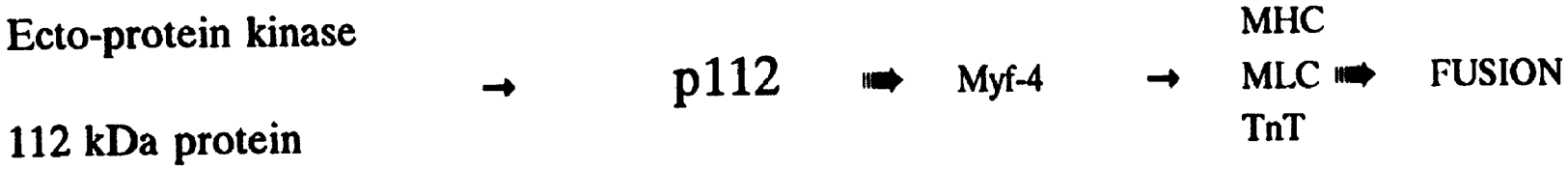
A comparison of the transcript levels of Myf4 and the muscle-specific genes revealed several interesting features. First, the major increase in the Myf4 mRNA occurred between days-2 and -4 (Fig. 4.2B); this preceded the major increase of the muscle-specific transcripts, which occurred after day-4 (Figs. 4.4A and 4.6). Second, while a two fold increase in the Myf4 mRNA was observed in myotubes, 7 to 28 fold increases in the muscle-specific transcripts were found in the same myotubes. Third, studies with the p112$^-$ myoblasts revealed that decreases in the Myf4 mRNA were always accompanied by reductions in the muscle-specific mRNAs (Figs. 4.4, 4.6, and 4.9). These findings therefore suggested that the expression of MHC, MLC, and TnT was subsequent to and controlled by Myf4 (Figs. 4.2 and 4.4); and that a relatively small increase in Myf4 could trigger a much larger increase in the muscle-specific mRNAs. This is in agreement with the hypothesis that transcription of the muscle-specific genes is activated by binding of Myf4 to their enhancer regions (Lin & Konieczny, 1992). The much reduced levels of these myogenesis-associated transcripts may explain why all these p112$^-$ myoblasts are impaired in myogenesis.

The major question concerns the temporal order of involvement of Myf4 and p112 in the myogenic pathway. Based on currently available data, a tentative working model can be proposed. This model postulates that the p112 site is situated upstream of the Myf4 site in the myogenic pathway (Fig. 4.10), and thus the expression of Myf4, MLC, MHC, and TnT, and morphological differentiation are dependent on p112. Studies with mutants F72 and D1/S4 showed that both the ecto-protein kinase and the 112 kDa protein might be essential for the elevated Myf4 mRNA level observed in myogenesis-competent cells. Moreover, L6 cells grown in phloretin/BrdUrd, and mutant D1 grown in 10% horse serum were deficient not only in p112, but also in the Myf4 transcript (Figs. 4.2B, 4.5, and 4.7). In other words, all the $p112^-$ cells examined were also deficient in the Myf4 mRNA. As discussed earlier, our findings indicated that the expression of MHC, MLC, and TnT was subsequent to and controlled by Myf4 (Figs. 4.2 and 4.4). The much reduced level of Myf4 in the $p112^-$ cells may therefore explain why these cells are also deficient in transcripts for various muscle-specific genes and why they are impaired in myogenesis (Figs. 3.4, 3.6 and 3.9 of chapter 3). Another way to test this working model is to determine the levels of various transcripts in $p112^-$ mutants transfected with the Myf4 cDNA.

We have also shown that the levels of the Myf4 mRNA and p112 in the myogenesis-competent L6 cells were regulated in an opposite manner. While an 80% drop in the p112 level was observed between day-2 and day-4 L6 cultures (Chapter 3), an 80% increase in the Myf4 mRNA was observed in these cultures (Fig. 4.2B). These findings suggest that p112 is only involved in the initiation of

Figure 4.10 A tentative working model of the myogenic pathway.

The tentative working model presented here depicts the temporal order of the expression of components involved in the myogenic pathway. Attempts are made to account for the changes in mRNA levels observed the myogenic L6 cells, and in the five p112$^-$ cell lines. Studies with mutants F72 and D1/S4 indicate that both the ecto-protein kinase and the 112 kDa protein are essential for the elevated level of the Myf4 transcript in myogenesis-competent cells. The increase in Myf4 mRNA was found to occur before the major increases in the MLC, MHC, and TnT mRNAs. The large broken arrows indicate the probable involvement of more than one steps. Not included in this model are the Myf5 and NCAM sites. Since changes in p112 has no effect on the rate of decline of the Myf5 mRNA, the p112 site is probably located downstream of the Myf5 site, if they are involved in the same myogenic pathway. As discussed in the text, the NCAM site is probably situated between the p112 and the Myf4 sites.


Ecto-protein kinase
112 kDa protein
p112
Myf-4
MHC
MLC
TnT
FUSION

myogenesis, after which its presence is no longer required.

Aside from p112, similar myogenesis-associated changes have also been observed with other cell surface components. The pattern of NCAM expression was found to be tightly regulated during myogenesis of mouse myoblasts (Moore et al., 1987). The observation that mouse myoblasts transfected with NCAM cDNA exhibited a much elevated level of muscle specific enzyme and a faster rate of fusion suggested NCAM might play a role in activating expression of the muscle-specific genes (Dickson et al., 1990). A similar increase in the NCAM mRNA during myogenesis was also observed with the $p112^{+}$ rat L6 myoblasts (Figs. 4.3 and 4.5). Since increases in this transcript could not be observed in all five types of $p112^{-}$ myoblasts, it is likely that p112 may play a role in regulating the expression of NCAM.

In summary, studies using five different p112-deficient cell lines suggest that both ecto-protein kinase and the cell surface 112 kDa protein may be involved at sites upstream from the Myf4, MLC, MHC, TnT, and NCAM sites in the myogenic pathway. If this p112 site is in the same pathway as Myf5, then it would be located downstream from this myogenesis-determination gene. Not much is presently known about the mechanism(s) by which cell surface components, such as NCAM or p112, can modulate myogenic activities. Our present studies and those of others (Dickson et al., 1990) suggest that these cell surface components may activate, directly or indirectly, the transcription of various

myogenesis-associated genes, presumably by mechanisms involving signal transduction.

CHAPTER 5

USE OF TRANSFECTANTS TO DETERMINE THE TEMPORAL ORDER OF EXPRESSION OF MYOGENIC COMPONENTS

5.1 INTRODUCTION

Myogenic differentiation involves proliferation of myoblasts, withdrawal of cells from the cell cycle, cell-cell recognition, synthesis of muscle-specific proteins and membrane fusion. However, little is known about the molecular events involved in the regulation and initiation of myogenesis.

Recently, a family of related myogenic regulatory factors was discovered. The first factor identified was MyoD (Davis et al., 1987). Subsequent studies revealed other members of this family. They are Myf5 (Braun et al., 1989b), Myf4/myogenin (Braun et al., 1989a; Wright et al., 1989; Edmondson and Olson, 1989), Myf6/MRF4/herculin (Braun et al., 1990; Miner and Wold, 1990; Rhodes and Konieczny, 1989). Myf3 is the human homologue to the mouse MyoD (Braun et al., 1989a). These myogenic factors are expressed exclusively in muscle cells.

The predicted amino acid sequences of these myogenic factors share a region of homology with a segment of MyoD. This region has the potential to form two amphipathic alpha-helices separated by a non-helical loop. This region is referred to as the helix-loop-helix (HLH) motif (Murre et al., 1989a).

These myogenic factors also have a region rich in basic amino acids located adjacent to the amino-terminal of the HLH domain. This basic domain confers DNA binding activity and specificity (Davis et al., 1990). The bHLH region is both necessary and sufficient for biological activity and for sequence-specific DNA binding (Tapacott et al., 1988). Myogenic proteins are therefore thought to be DNA binding basic HLH (bHLH) proteins with transcriptional regulatory functions.

When transfected into non-muscle cell lines, each myogenic factor is able to induce synthesis of muscle-specific proteins and subsequently myogenesis (Weintraub et al., 1991). Although not much is known about the mechanism involved, the expression of any member of the MyoD family was found to activate expression of other members of the same family (Thayer et al., 1989; Edmondson and Olson, 1989; Braun et al., 1989a). It has also been shown that members of the MyoD family can positively regulate their own expression (Edmondson et al., 1992). Although quantitative differences in activity and time of embryonic expression are detectable, it is not clear whether these myogenic factors have biologically distinct functions.

Although the neural cell adhesion molecule (NCAM) is a membrane glycoprotein originally characterized in nervous tissue, it is also expressed in other cell types (Knudsen et al., 1990a). Recent studies suggested that NCAM was involved in myogenesis (Knudsen et al., 1989 & 1990b). When mouse myoblasts

were transfected with the human muscle-specific NCAM cDNA, their rate of myogenesis was faster than that of control cells. This increased rate of myogenesis could be completely reversed when the transfected cells were treated with specific anti-human NCAM monoclonal antibody. Besides myotube formation, the creatine phosphokinase (CPK) activity was also elevated in the NCAM-transfected myoblasts (Dickson et al., 1990). These results suggested that NCAM might play a role in regulating myogenesis. In spite of their ability to induce myogenesis, not much is known about the relationship of NCAM and myogenic regulatory factors.

We have demonstrated in previous chapters that an ecto-PK and a cell surface 112 kDa protein might be involved in myogenesis. Northern blot analyses showed that mutants defective in either ecto-PK or the 112 kDa protein have much reduced Myf4, NCAM, MHC, MLC, and TnT transcript levels. Similar results were obtained with cells in which the phosphorylation of cell surface 112 kDa protein was inhibited by the myogenesis inhibitors, 5-bromo-2-deoxyuridine (BrdUrd) or phloretin. This suggested that phosphorylation of cell surface 112 kDa protein by the ecto-PK might occur before the expression of Myf4, NCAM, and the muscle-specific proteins MHC, MLC, TnT (Chapter 4). To further determine the temporal order of expression of myogenesis-associated components, transfection studies were carried out in the present chapter. The cDNAs of *myf4* and *myf5* were transfected into p112- defective mutants D1/S4 and F72. The transcript levels of Myf4, Myf5, NCAM, MLC, MHC, and TnT

were then determined in those transfectants.

5.2 METHODS AND MATERIALS

5.2.1 Cell lines and culture media

Yaffe's L6 rat skeletal myoblast line (Yaffe, 1968), mutant D1/S4, and mutant F72 were grown as previously described in Section 4.2.1.

5.2.2 Preparation of cDNAs for transfection

The cDNAs for Myf4 and Myf5 were originally inserted in PBS$^+$ a non-eukaryotic expression vector, when purchased from the Repository of Human DNA Probes and Libraries, American Type Culture Collection, Rockville, MD.. Plasmid DNA was isolated by the QIAGEN plasmid kit (QIAGEN, Germany) using the protocol recommended by the supplier. The cDNA inserts for Myf4 and Myf5 were isolated and purified as previously described in Section 4.2.3. Both the cDNA inserts, Myf4 and Myf5, were then re-cloned into an eukaryotic expression vector, pRc/RSV, using methods as described by Sambrook et al. (1989). Both PBS$^+$ vector containing the *myf4* insert (PBS-M4) and pRc/RSV vector containing the *myf4* (RSV-M4) or *myf5* (RSV-M5) were used in transfection studies.

5.2.3 Transfection study

The PBS-M4, RSV-M4 and RSV-M5 plasmids were transfected into mutants F72 and D1/S4 by the calcium phosphate-DNA co-precipitation method

(Sambrook et al., 1989). Mutants F72 and D1/S4 were seeded at a density of $5x10^5$ cells/100mm plate. After 24 hr, DNA and calcium phosphate precipitates were added to cells. In transfection studies with RSV-M4 and RSV-M5, 10μg DNA per plate were used. In transfection studies using the PBS vector, co-transfection with the pSV-neo plasmid was carried out. This is because PBS-M4 does not contain the *neo* gene or any other usable selection marker. In these studies 40μg PBS-M4 DNA were co-precipitated with 1μg of the eukaryotic expression vector pSV2-neo (Southern and Berg, 1982) in calcium phosphate buffer. Transfectants were then selected in alpha medium containing 400μg/ml G418 (Geneticin, from GIBCO). After cloning, transfectants were analyzed for the expression of Myf4, Myf5, NCAM, MHC, MLC, and TnT.

5.2.4 Isolation of DNA from transfectants

DNAs were extracted from day-3 cultures of transfectants using the following procedure. After washing with ice-cold, sterile phosphate-buffered saline (150mM NaCl, 30mM KCl, 80mM Na_2HPO_4, 15mM KH_2PO_4, pH 7.4), 2.5ml of the lysis buffer (10mM Tris-HCl, pH 7.9, 10mM EDTA, 10mM NaCl, 0.1% SDS, and 200μg/ml proteinase K) was added to each plate. After incubating for 3 hrs. at 37°C, cell lysate was transferred to a sterile tube and incubated at 55°C overnight. DNA lysate was extracted with phenol/chloroform (24:1) saturated with TE buffer (100mM Tris, pH 8.0, 10mM EDTA, 10mM NaCl). Samples were then centrifuged at 2500 xg for 20 min. at room temperature in a table-top centrifuge. The aqueous phase was transferred to a new tube, and precipitated

by adding absolute ethanol to a concentration of 70%. The DNA was spooled out with a Pasteur pipette, washed once in 70% ethanol, and then once in absolute ethanol. After drying briefly in air, the DNA was dissolved in TE buffer.

5.2.5 Isolation of total RNA from transfectants

Total cellular RNA was extracted from transfectants with day-2, day-4, day-5 and day-6 cultures using the method as previously described in Section 4.2.2.

5.2.6 Preparation of cDNA probes

The cDNA inserts for Myf4, Myf5, N-CAM, MLC, MHC, TnT, and β2-microglobulin were isolated, purified, and labelled with ^{32}P using the method as described in Section 4.2.3.

5.2.7 Southern blot analysis

For Southern blotting, DNAs were digested with the appropriate enzymes: EcoRI for DNA extracted from the PBS-M4 transfectants; HindIII for DNA extracted from the RSV-M4 transfectants; HindIII and XbaI for DNA extracted from RSV-M5 transfectants. After incubating at 37°C for 4 hr., the DNAs (10μg/lane) were separated by electrophoresis through a 0.7% agarose gel, then transferred to a Biotrans nylon membrane (ICN Biochemicals) by vacuum blotting (VacuGene XL, Pharmacia) using the alkaline vacuum transfer protocol

recommended by the supplier. The DNA gel was transferred first in 0.2N HCl until the bromphenol blue turned yellow (about 40 min.); the transfer was continued in 1N NaOH for another 1.5 hrs. After transfer, the membrane was washed in 2X SSC for 10 min. and air dried for 30 min.

Hybridization was then carried out with the appropriate labelled cDNA probes (Myf4 or Myf5). The membrane was prehybridized at 37°C for at least 2 hrs in a solution containing 45% formamide, 5X SSC, 2X Denhardt's solution, 20 mM sodium phosphate buffer, pH6.5, 0.1% sodium dodecyl sulfate (SDS), and 100μg/ml denatured herring sperm DNA. Hybridization was carried out at 37°C overnight in the prehybridization solution with the labelled cDNA probe ($1x10^6$cpm/ml of ^{32}P labelled insert). After washing in 2X SSC and 0.1% SDS two times at room temperature for 15 min. and then at 42°C for 1 hr, the blot was exposed to Kodak XAR-5 film at -80°C.

5.2.8 Northern gel blotting studies

RNA blotting was carried out as described in Section **4.2.4.**

5.2.9 Phosphorylation of cell surface 112 kDa protein

The phosphorylation of cell surface proteins was carried out as described in Section **2.2.2.**

5.2.10 Materials

Various restriction enzymes, Prime-a-Gene kit and molecular markers were purchased from Promega. α-^{32}P-dCTP was purchased from Amersham Corp.. All other chemicals were obtained from commercial sources and were of the highest available purity.

5.3 RESULTS

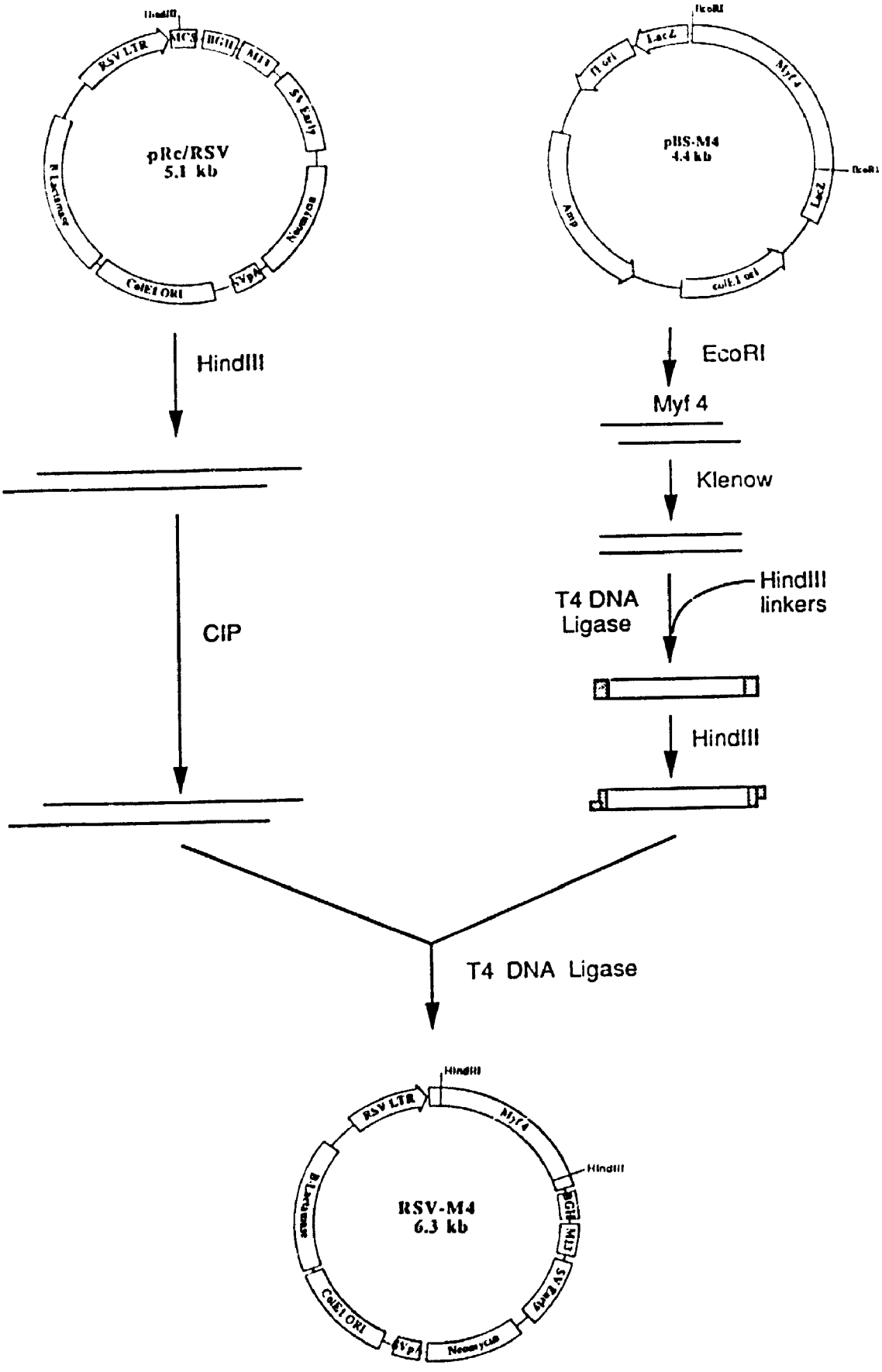
5.3.1 Insertion of *myf4* and *myf5* cDNAs into the pRc/RSV vector

The cDNA inserts for Myf4 and Myf5 were excised and purified from PBS$^+$ vectors with EcoRI as described in Section **4.2.3.** The *myf4* and *myf5* inserts from PBS$^+$ vector were recloned into the pRc/RSV vector (from Invitrogen corp., Appendix I) according to the methods described in the Maniatis' Laboratory Manual (Maniatis et al., 1982). The strategy is shown in Figures 5.1 and 5.2. After excision and purification, the EcoRI-digested recessed ends of *myf4* and *myf5* cDNAs were enzymatically filled by Klenow.

For cloning the *myf4* cDNA, a phosphorylated HindIII linker (from Promega) was ligated to the *myf4* cDNA by blunt-ended ligation in the presence of T4 DNA ligase (Promega) (Fig. 5.1). 0.5μg blunt-ended *myf4* cDNA, 2μg phosphorylated HindIII linker, and 2 Weiss units T4 ligase were used. After ligation, the HindIII-recessed ends of *myf4* cDNA were then produced by digestion with HindIII. The pRc/RSV vector was linearized with HindIII and dephosphorylated with CIP (Calf intestinal alkaline phosphatase, from Boehringer Mannheim). The *myf4* cDNA with HindIII-recessed ends was then ligated to the

Figure 5.1 The strategies for recloning the *myf4* cDNA into pRc/RSV vector

Figure 5.1 shows the strategy for cloning the *myf4* cDNA into the pRc/RSV vector through HindIII linker.


pRc/RSV
5.1 kb
RSV LTR
MCS
SV Early
Neomycin
SVPA
ColE1 ORI
R Lactamase
HindIII
CIP
pBS-M4
4.4 kb
f1 ori
LacZ
Myf 4
Amp
ColE1 ori
EcoRI
Myf 4
Klenow
T4 DNA
Ligase
HindIII
linkers
HindIII
T4 DNA Ligase
RSV-M4
6.3 kb
HindIII
RSV LTR
Myf 4
HindIII
SV Early
Neomycin
SVPA
ColE1 ORI
B Lactamase

HindIII-linearized and dephosphorylated pRc/RSV vector by T4 ligase (1μg vector DNA/Weiss unit).

For cloning the *myf5* cDNA, the pRc/RSV vector was linearized with SpeI and the recessed ends were enzymatically filled by Klenow (Fig. 5.2). The linearized and blunt-ended pRc/RSV vector was then dephosphorylated by CIP. The blunt-ended *myf5* cDNA was directly ligated to the linearized, blunt-ended, and dephosphorylated pRc/RSV vector by blunt-ended ligation in the presence of T4 ligase.

The pRc/RSV vector containing *myf4* or *myf5* cDNA were then transformed into E. coli HB101 using the calcium chloride procedure (Sambrook et al., 1989). Plasmid DNA was extracted from the positive colonies using the alkali lysis method (Sambrook et al., 1989). The plasmid DNA was digested with HindIII (for Myf4) or XbaI and HindIII (for Myf5) to determine the presence of *myf4* or *myf5* cDNA in the pRc/RSV vector.

The orientation of *myf4* cDNA in pRc/RSV vector was determined by digesting the plasmid DNA with PstI. According to the sequences of pRc/RSV and *myf4*, the pRc/RSV vector with *myf4* cDNA in sense orientation (RSV-M4) should be cleaved by PstI into 3 bands, 0.9, 1.3, 4.2 kb. This was indeed found to be the case (Figure 5.3A, lane 2). This demonstrated that the *myf4* insert in RSV-M4 was in the sense orientation.

Figure 5.2 The strategies for recloning the *myf5* cDNA into pRc/RSV vector

Figure 5.2 shows the strategy for cloning the *myf5* cDNA into the pRc/RSV vector by blunt-ended ligation.

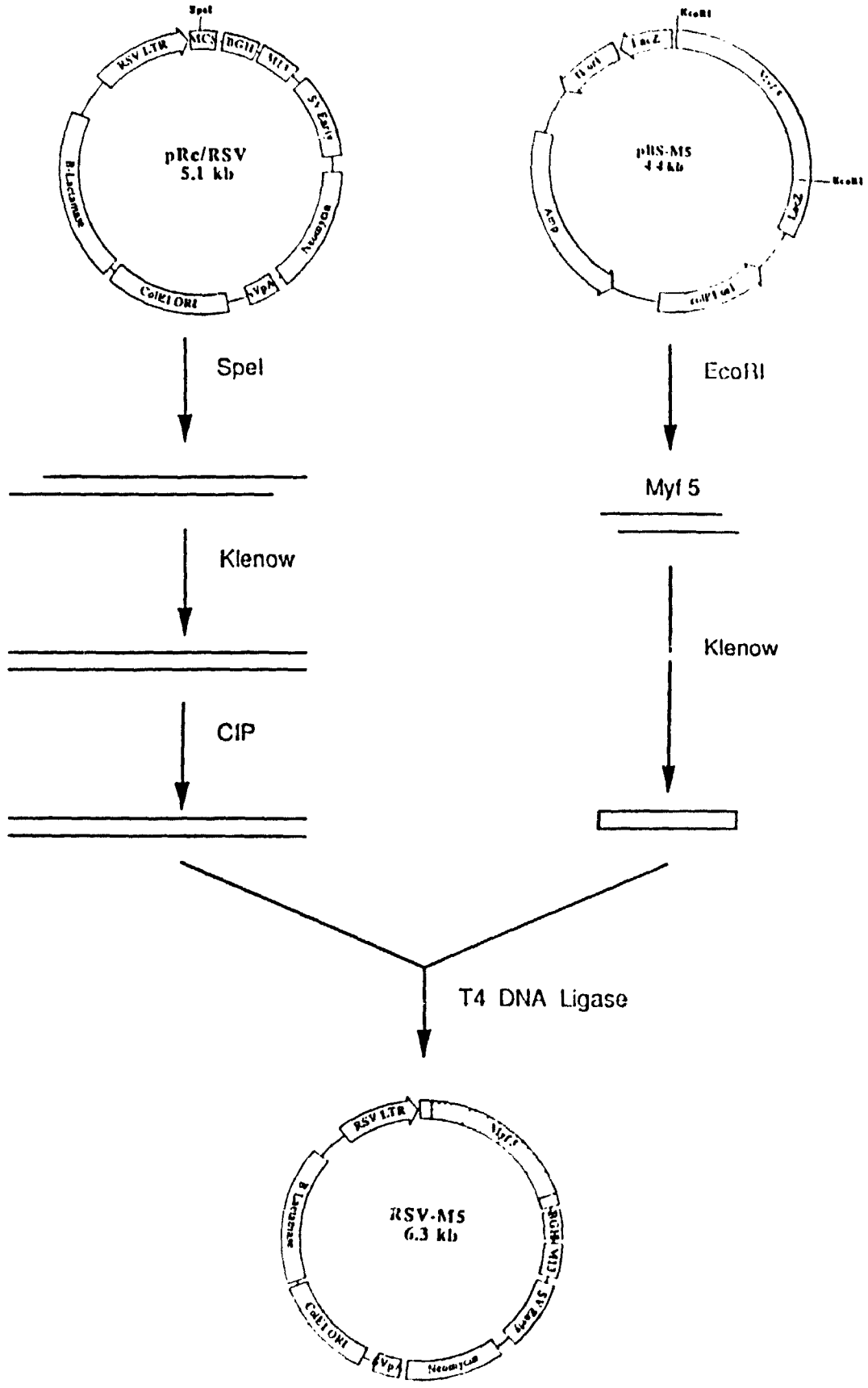
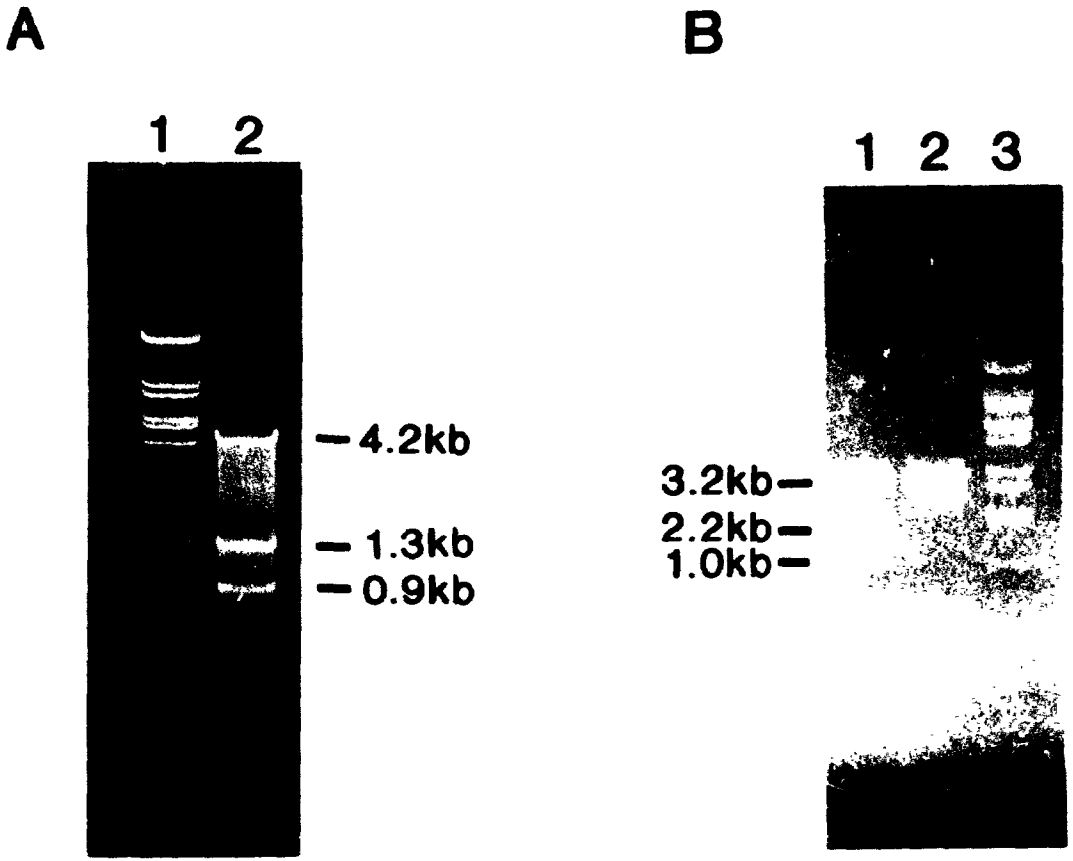

pRc/RSV
5.1 kb
RSV LTR
MCS
BGH
SV Early
Neomycin
SVPA
ColE1 ORI
β-Lactamase
pBS-M5
4.4 kb
LacZ
Myf 5
SpeI
EcoRI
Klenow
CIP
T4 DNA Ligase
RSV-M5
6.3 kb

Figure 5.3 Gene mapping of RSV-M4 and RSV-M5

Plasmid DNA RSV-M4 and RSV-M5 were cut by the appropriate enzymes and run on a 1% agarose gel. Panel A shows the map of RSV-M4 cut by PstI. Lane 1 is BstE II digested λ DNA marker. Lane 2 is PstI digested RSV-M4 DNA, and three DNA bands (4.2, 1.3, and 0.9 kb) were observed. Panel B shows the map of RSV-M4 cut by HincII and XbaI. Lane 3 is BstE II digested λ DNA marker. Lanes 1 and 2 are RSV-M5 DNA digested with HincII and XbaI. Three DNA bands (3.2, 2.2, 1.0 kb) were seen in lane 1. This indicated that the insert was in the sense orientation. Only two DNA bands were present in lane 2. This indicated that the insert was in the antisense orientation.


A
1 2
4.2kb
1.3kb
0.9kb
B
1 2 3
3.2kb
2.2kb
1.0kb

The orientation of *myf5* cDNA in pRc/RSV vector was determined by digesting the plasmid DNA with HindII and XbaI. According to the sequences of pRc/RSV and *myf5*, the pRc/RSV vector with *myf5* cDNA in the sense orientation (RSV-M5) should be cleaved into 3 bands, 1.0, 2.2, 3.2 kb. The result presented in lane 1 of Figure 5.3B therefore indicated that the Myf5 insert in RSV-M5 was in the sense orientation. Both RSV-M4 and RSV-M5 in the sense orientation were then used in transfection studies.

5.3.2 Southern blot analysis of transfectants

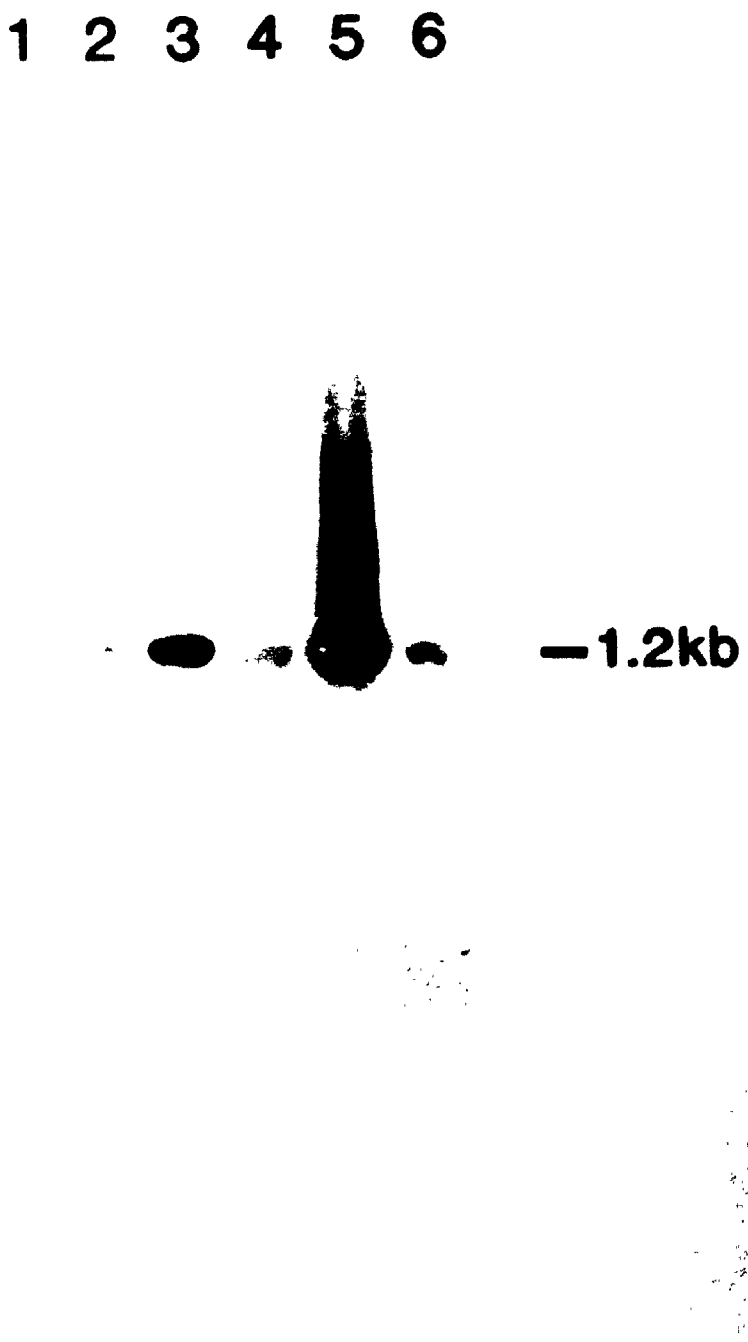
Southern blot analyses were carried out to determine whether these transfectants contained the full length *myf4* or *myf5* cDNA. DNAs were extracted from these transfectants, digested, ran on an agarose gel, transferred onto nylon membrane, and hybridized with ^{32}P-labelled Myf4 or Myf5 cDNA probes using methods described in Sections **5.2.4** and **5.2.7**. It should be noted that the DNAs were digested with the same enzymes used for excising the corresponding cDNA from the vectors.

5.3.2.1 Analysis of D1/S4 cells transfected with PBS-M4

Figure 5.4 shows the results of Southern blot analysis of D1/S4 transfected with PBS-M4 (DS-T4). One positive DNA band (1.2 kb) could be detected in the transfectants. More importantly this 1.2 kb band was absent in D1/S4 transfected with PBS vector alone (DS-Tc) (Fig. 5.4, lane 1). This together with the fact that this band has the same size as the original *myf4*

Figure 5.4 Examination of transfected *myf4* cDNA in transfectant DS-T4

DNA was extracted from 5 positive clones of D1/S4 transfected with *myf4* and subjected to Southern blot analysis. 10μg DNA/lane of each sample was loaded onto the agarose gel. The labelled *myf4* cDNA was used to probe the DNA. Lane 1 is DNA from control transfectant DS-Tc. Lanes 2, 3, 4, 5, and 6 are DNA from the 5 different clones of DS-T4. A 1.2 kb DNA band was detected in all transfectants, except the control transfectant, thus indicating the presence of the transfected *myf4* cDNA.


1 2 3 4 5 6
—1.2kb

cDNA suggested that the observed 1.2 kb band might indeed be the transfected *myf4* cDNA. Figure 5.4 also showed that some transfectants have a high level of the transfected *myf4* cDNA (Fig.5.4, lanes 3 and 5), whereas others have a lower level of transfected *myf4* cDNA (Fig.5.4, lanes 2, 4, and 6). In addition to the 1.2 kb band, a higher molecular weight band could be also seen in all transfectants after exposing the blot for an extended period of time. This band was probably the endogenous *myf4* gene. The transfectant from lane 3 was further subjected to northern blot analyses.

5.3.2.2 Analysis of F72 cells transfected with RSV-M4

Figure 5.5 shows the results of Southern blot analysis of F72 transfected with RSV-M4 (F-T4). A 11 kb DNA band was observed in the control transfectant (F-Tc) and in all F-T4 transfectants. Two of the transfectants (lanes 1 and 4) also had an additional 1.2 kb positive band. Since this band has the same size as the full length *myf4* cDNA in RSV-M4, these transfectants probably possessed the full length *myf4* cDNA, as the enzymes used to digest the transfectant DNA were the same as those used to excise the *myf4* cDNA from RSV-M4. The transfectant shown on lane 3 has an additional 1 kb DNA band besides the 11 kb DNA band. Since this band is smaller than the full length Myf4, this transfectant may contain only a part of the *myf4* cDNA. The transfectant from lane 1 was further subjected to northern blot analyses.

5.3.2.3 Analysis of F72 cells transfected with RSV-M5

Figure 5.5 Examination of transfected *myf4* cDNA in transfectants F-T4

DNA was extracted from 4 positive clones of F72 transfected with *myf4* and then subjected to Southern blot analysis. 10μg DNA/lane of each sample were loaded onto the agarose gel. Labelled *myf4* cDNA was then used to probe the DNA. Lane 5 is the DNA from control transfectant F-Tc. Lanes 1, 2, 3, and 4 are DNAs from 4 different clones of F-T4. The 1.2 kb band represents the transfected *myf4* cDNA.

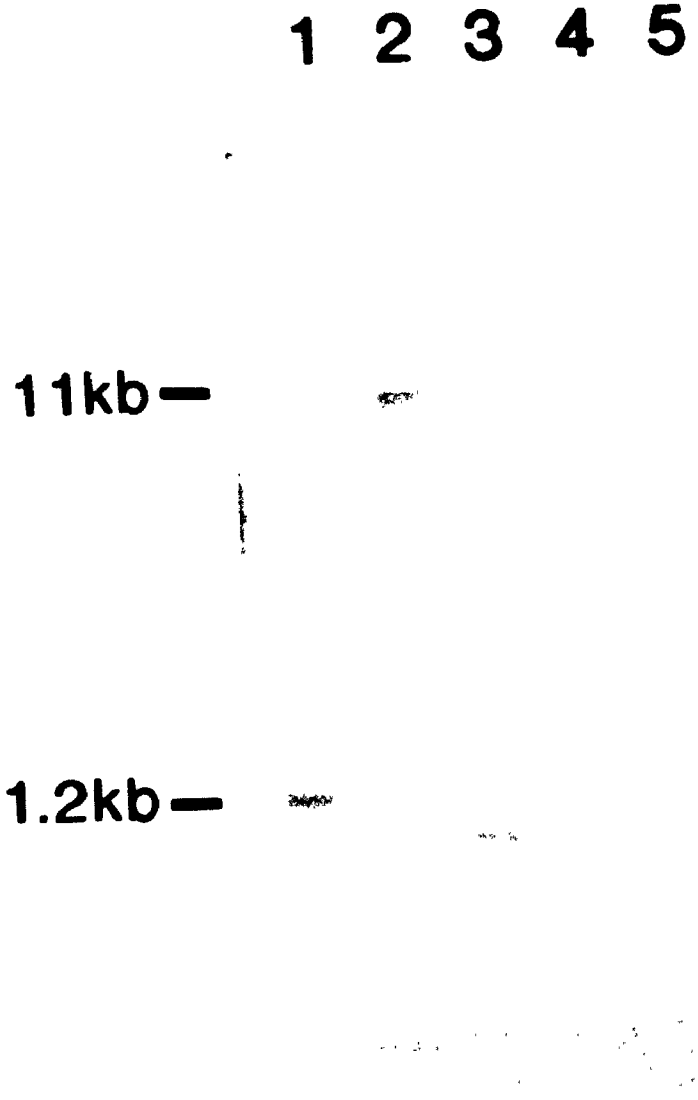

1 2 3 4 5
11kb
1.2kb

Figure 5.6 shows the results of Southern blot analysis of F72 transfected with RSV-M5 (F-T5). Two positive DNA bands (2.9 and 1.9 kb) were detected in the control transfectant F-Tc (lane 5) and in all the transfectants (lanes 1-4). Two transfectants had an additional 1.2 kb DNA band (lanes 1 and 2). The 2.9 and 1.9 kb bands were probably the endogenous *myf5* gene since they were present in control transfectant F-Tc. Since the size of the *myf5* cDNA in RSV-M5 was around 1.2 kb, and this DNA band was only present in the transfectants, this band was probably the transfected *myf5* cDNA. The transfectants from lanes 1 and 2 were further subjected to northern blot analyses.

5.3.3 Expression of the transfected cDNAs in the transfectants

Transfectants containing full length *myf4* or *myf5* cDNA (DS-T4, F-T4, and F-T5) were further examined for the expression of the *myf4* or *myf5*. It was previously shown that during myogenesis the transcript level of Myf4 was increased, whereas the Myf5 transcript level was reduced (Chapter 4). Thus, the transcript levels of Myf4 or Myf5 in transfectants were examined. Transcript levels in day-2, day-4 and day-6 cultures of DS-T4 and day-2 and day-5 cultures of F-T4 and F-T5 were determined.

Figure 5.7A shows the results of northern blot analysis of DS-T4. Compared with the control transfectant DS-Tc (lanes 1, 2, and 3) the Myf4 transcript levels were much higher in transfectant DS-T4 (lanes 4, 5, and 6). In myogenesis-competent cells, the Myf4 transcript was elevated upon prolonged

Figure 5.6 Examination of transfected *myf5* cDNA in transfectant F-T5

DNA was extracted from 4 positive clones of F72 transfected with *myf5* and then subjected to Southern blot analysis. 10μg DNA/lane of each sample were loaded onto the agarose gel. Labelled *myf5* cDNA was used to probe the DNA. Lane 5 is the DNA from control transfectant F-Tc. Lanes 1, 2, 3, and 4 are DNAs from 4 different clones of F-T5. The 1.2 kb band denotes the transfected *myf5* cDNA.

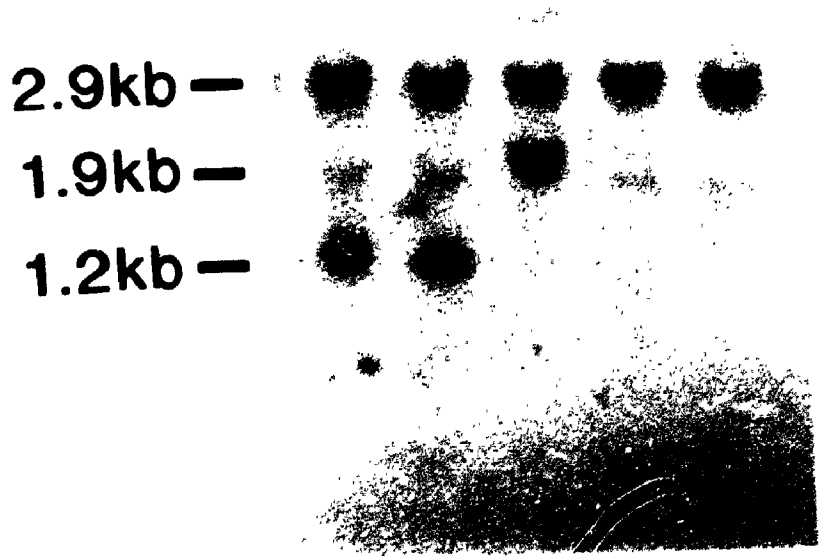
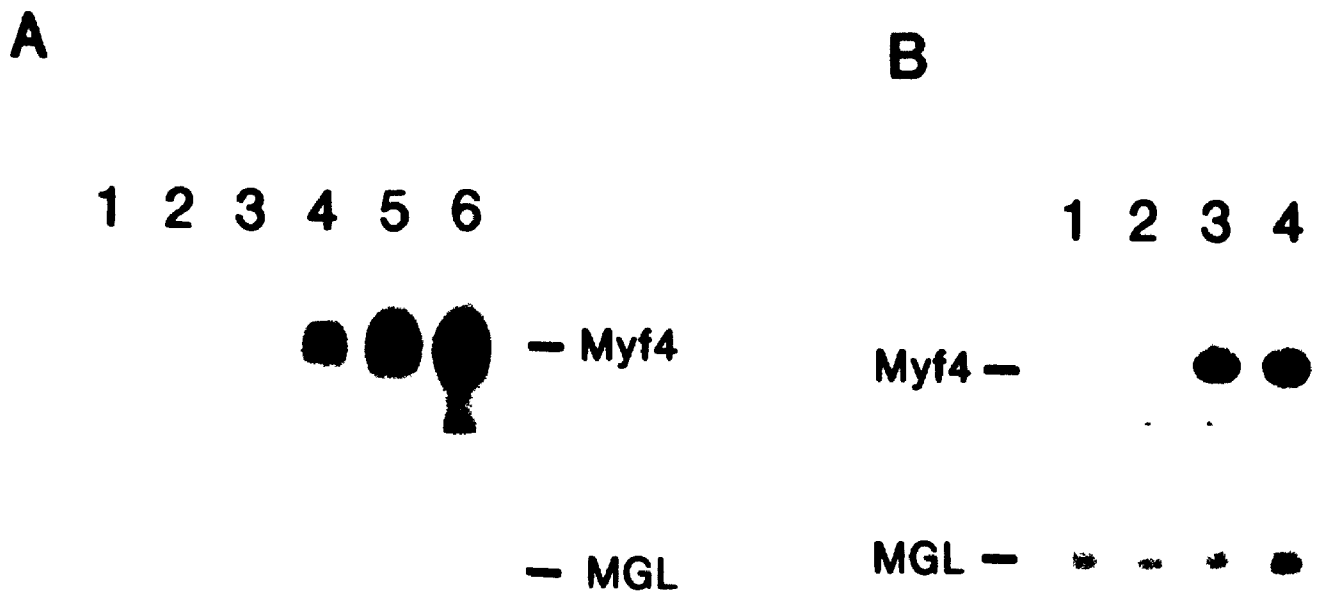

1 2 3 4 5
2.9kb
1.9kb
1.2kb

Figure 5.7 Expression of transfected *myf4* in transfectant DS-T4 and F-T4

Total RNA was extracted from a clone of transfectant DS-T4 (Fig. 5.4, lane 3) and a colony of F-T4 (Fig. 5.5, lane 1), and subject to northern blot analysis. 20μg RNA/lane of each sample were loaded onto an agarose gel. Labelled *myf4* cDNA was then used to probe the Myf4 transcript level. In order to compare the amount of RNA loaded in each lane, labelled β2-microglobulin (MGL) cDNA was used to probe all the samples. Panel A shows the Myf4 transcript levels in DS-T4. Lanes 1, 2, and 3 are RNAs from day-2, day-4 and day-6 cultures of control transfectant DS-Tc. Lanes 4, 5 and 6 are RNAs from day-2, day-4 and day-6 cultures of transfectant DS-T4. Panel B shows the Myf4 transcript levels in F-T4. Lanes 1 and 2 are RNAs from day-2 and day-5 cultures of the control transfectant F-Tc. Lanes 3 and 4 are RNAs from day-2 and day-5 cultures of F-T4. A 1.9 kb RNA band was detected with the labelled *myf4* cDNA in both DS-T4 and F-T4.


A
1 2 3 4 5 6
– Myf4
– MGL
B
1 2 3 4
Myf4 –
MGL –

subculturing. This increase was also observed in transfectant DS-T4. We noticed that the size of the transcript was around 1.9 kb, which was larger than that of the cDNA (1.2 kb). This size difference was also observed by Braun (1989a). This may be due to that the expression of the endogenous *myf4* gene is activated by the transfected *myf4* cDNA.

Figure 5.7B shows the results of northern blot analysis of F-T4. Similarly, the transfectant F-T4 has much higher Myf4 transcript levels (lanes 3 and 4) than the control transfectant F-Tc (lanes 1 and 2). Again this showed that the Myf4 transcript level was elevated in transfectant F-T4.

Figure 5.8 shows the Myf5 transcript levels in the two different clones of F-T5 (lanes 3, 4, 5, and 6). The Myf5 transcript levels in these two Myf5 transfectants were similar to the control transfectant F-Tc (lanes 1 and 2). Overexpression of *myf5* was not observed in these Myf5 transfectants.

5.3.4 Expression of other myogenic factors in transfectants

The expression of *myf4* was also examined in these two Myf5 transfectants. As expected, the Myf4 transcript level in F-T5 remained similar to that in control transfectant F-Tc (Figure 5.9). This suggested that the transfected *myf5* cDNA could not activate the expression of the endogenous *myf4* gene.

Figure 5.8 Expression of the transfected *myf5* in transfectant F-T5

Total RNA was extracted from two clones of F-T5 (Fig. 5.6, lanes 1 and 2), and subjected to northern blot analysis. 20μg RNA/lane of each sample were loaded onto the agarose gel. Labelled *myf5* cDNA was used to probe the Myf5 transcript level in the transfectants. In order to compare the amount of RNA loaded in each lane, labelled β2-microglobulin (MGL) cDNA was used to probe all samples. Lanes 1 and 2 are RNAs from day-2 and day-5 cultures of the control transfectant F-Tc. Lanes 3 and 4 are RNAs from day-2 and day-5 cultures of one F-T5 transfectant. Lane 5 and 6 are RNAs from day-2 and day-5 cultures of another F-T5 transfectant. A 1.9 kb RNA band was detected with the labelled *myf5* cDNA.

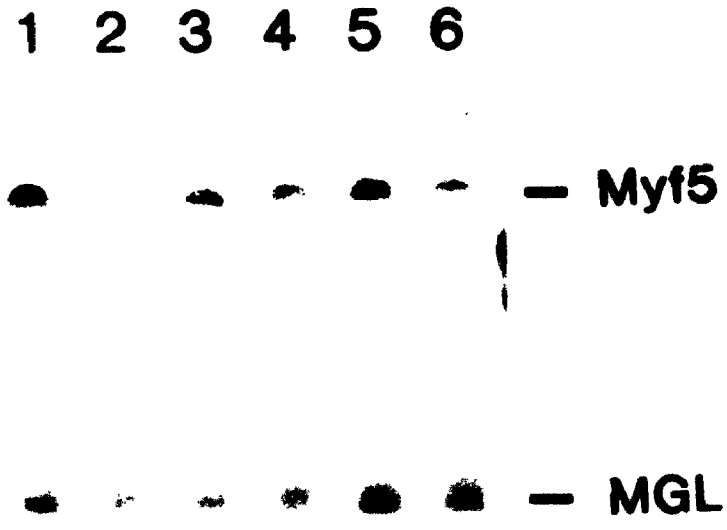
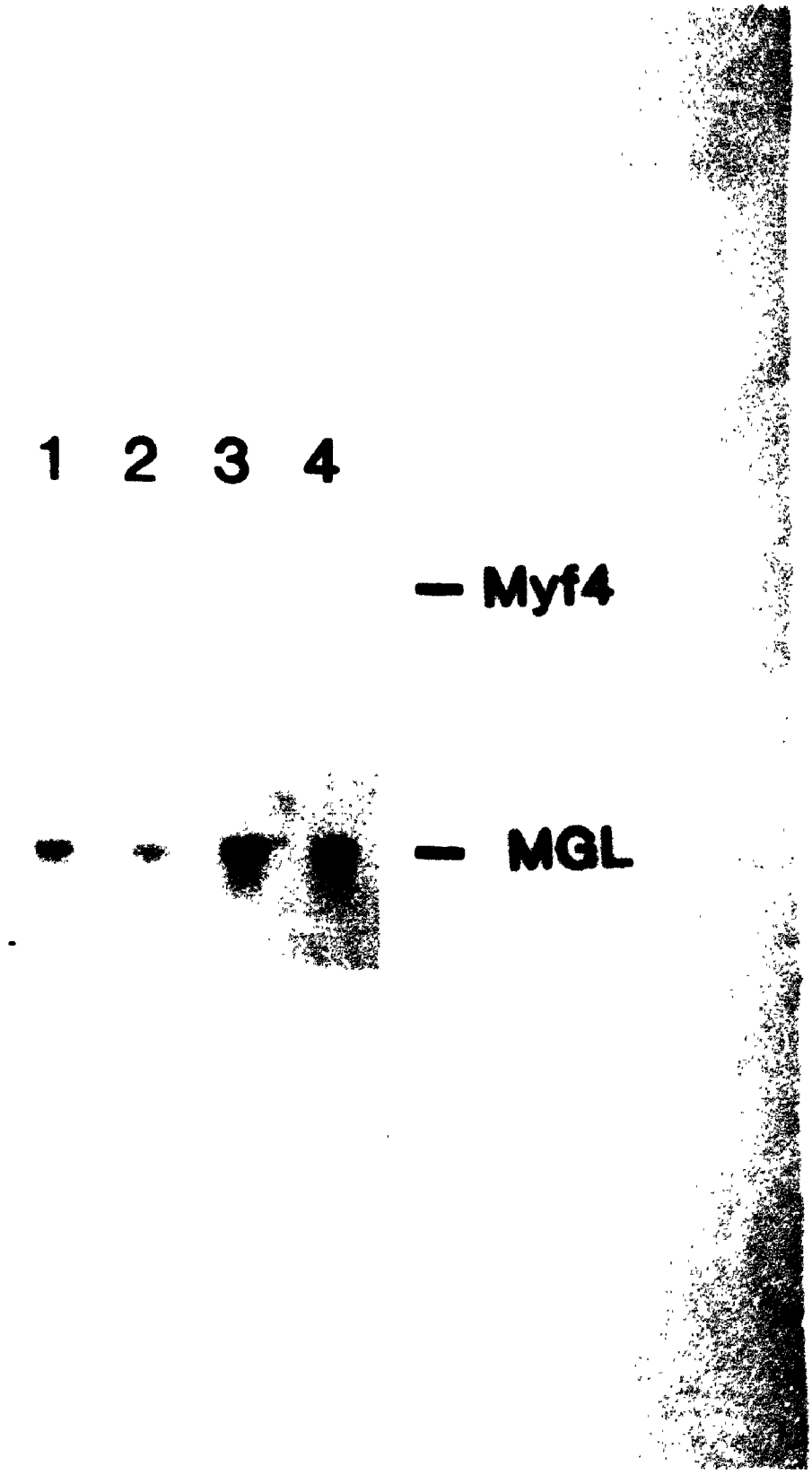

1 2 3 4 5 6
Myf5
MGL

Figure 5.9 Expression of *myf4* in transfectant F-T5

Northern blot analysis with total RNA was carried out to examine the expression of *myf4* in F-T5. 20μg RNA/lane of each sample were loaded onto the agarose gel. Labelled *myf4* cDNA was then used to probe the Myf4 transcript levels. In order to compare the amount of RNA loaded in each lane, labelled β2-microglobulin (MGL) cDNA was used to probe all samples. Lanes 1 and 2 are RNAs from day-2 and day-5 cultures of control transfectant F-Tc. Lanes 3 and 4 are RNAs from day-2 and day-5 cultures of F-T5. A 1.9 kb RNA band was detected with the labelled *myf4* cDNA in the transfectants.


1 2 3 4
— Myf4
— MGL

The expression of *myf5* was also examined in transfectants DS-T4 and F-T4. Northern blot analysis showed that although DS-T4 and the F-T4 had much higher Myf4 transcript levels, the Myf5 transcript level in these two transfectants remained similar to that of the control transfectants (Fig. 5.10A and Fig. 5.10B). In other words, the increase of *myf4* expression in DS-T4 and F-T4 did not affect the expression of *myf5*.

5.3.5 Expression of NCAM in transfectants DS-T4, F-T4 and F-T5

NCAMs are thought to be involved in myogenesis (Moore et al., 1987; Dickson et al., 1990; and Chapter 4). When compared with their parental L6 cells, both myogenesis-defective mutants D1/S4 and F72 were found to have very low NCAM and Myf4 transcript levels (Chapter 4). However, the relationship between Myf4 and NCAM was not known. In order to examine the temporal order of expression of *myf4* and NCAM, the expression of NCAM was determined in transfectants DS-T4, F-T4 and F-T5. Three transcripts, 6.7 kb, 4.8 kb, 3.0 kb, were recognized by the NCAM cDNA in L6 cells; of these, the 3.0 kb NCAM mRNA was the most prominent one. Northern blot analyses showed that the NCAM transcript levels remained unaltered in DS-T4 and F-T4 (Fig. 5.11 A and B). This demonstrated that the expression of NCAM was not regulated by Myf4. This figure also showed that the NCAM transcript level was not elevated in F-T5. This suggested that the transfected *myf5* cDNA in mutant F72 has no effect on the expression of NCAM.

Figure 5.10 Expression of *myf5* in transfectants DS-T4 and F-T4

Northern blot analysis with total RNA was carried out to examine the expression of *myf5* in DS-T4 and F-T4. 20μg RNA/lane of each sample were loaded onto the agarose gel. Labelled *myf5* cDNA was used to probe the Myf5 transcript levels. In order to compare the amount of RNA loaded in each lane, labelled β2-microglobulin (MGL) cDNA was used to probe all samples. Panel A shows the Myf5 transcript levels in DS-T4. Lanes 1, 2 and 3 show RNAs from day-2, -4 and -6 cultures of control transfectant DS-Tc. Lanes 4, 5 and 6 are RNAs from day-2, -4 and -6 cultures of transfectant DS-T4. Panel B shows the Myf5 transcript levels in F-T4. Lanes 1 and 2 show RNAs from day-2 and day-5 cultures of the control transfectant F-Tc. Lanes 3 and 4 are RNAs from day-2 and day-5 cultures of transfectant F-T4. A 1.9 kb RNA band was detected with the labelled *myf5* cDNA in both DS-T4 and F-T4.

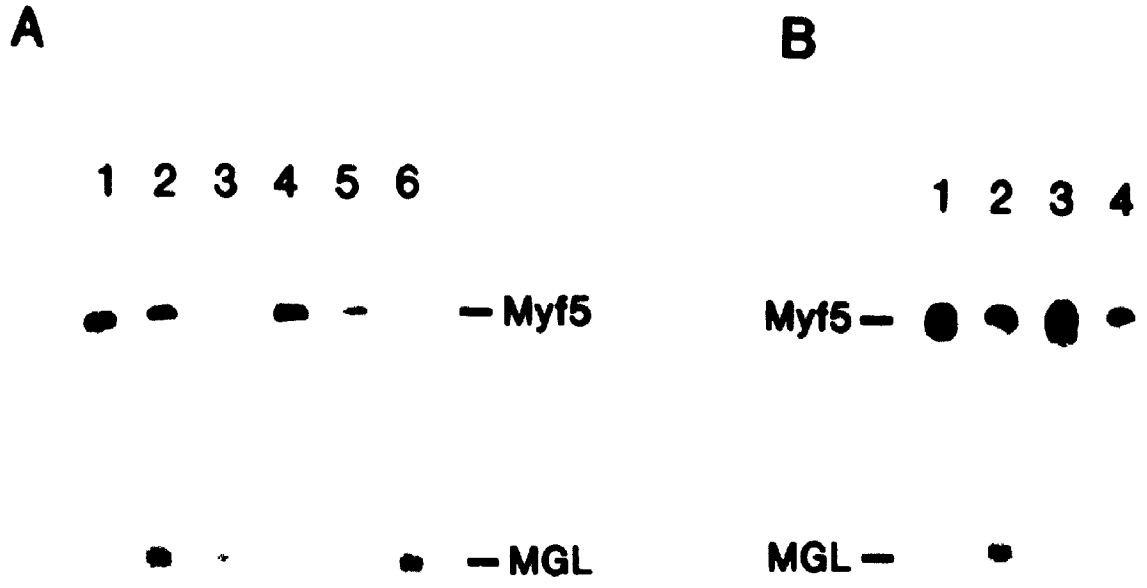
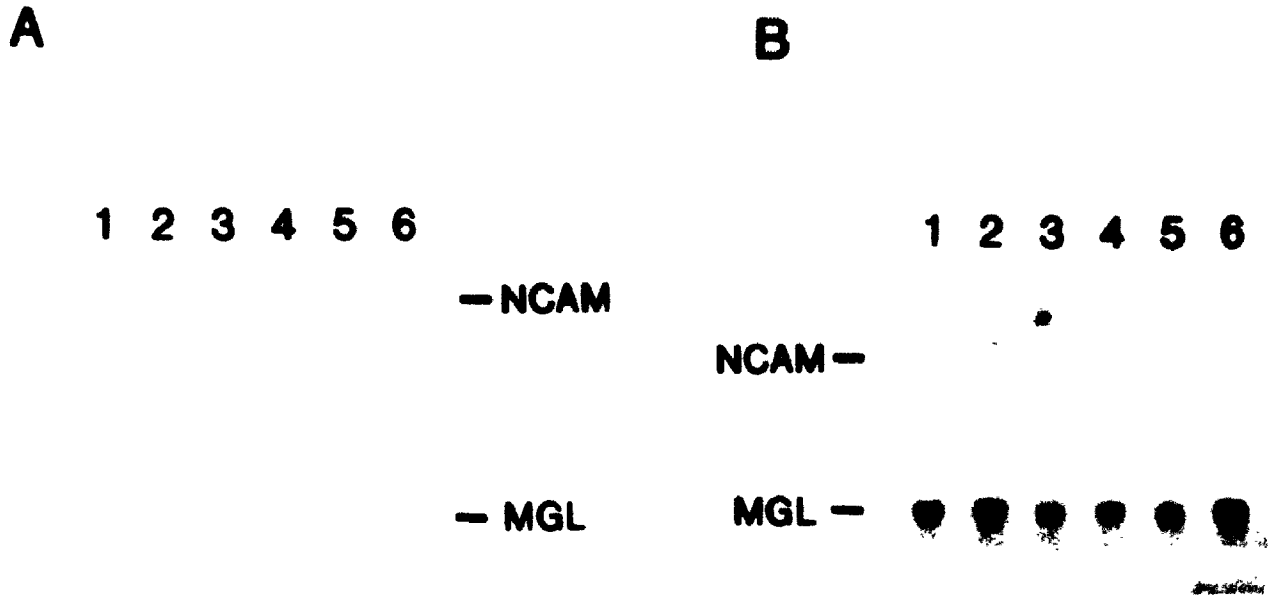

A
1 2 3 4 5 6
Myf5
MGL
B
1 2 3 4
Myf5
MGL

Figure 5.11 Expression of NCAM in transfectants DS-T4, F-T4 and F-T5

Northern blot analysis using total RNA was carried out for examining the expression of NCAM in DS-T4, F-T4 and F-T5. 20μg RNA/lane of each sample were loaded onto agarose gel. Labelled NCAM cDNA was then used to probe the NCAM transcript levels. In order to compare the amount of RNA loaded in each lane, labelled β2-microglobulin (MGL) cDNA was used to probe all samples. Panel A shows the NCAM transcript levels in DS-T4. Lanes 1, 2 and 3 show RNAs from day-2, day-4 and day-6 cultures of the control transfectant DS-Tc. Lanes 4, 5 and 6 are RNAs from day-2, day-4 and day-6 cultures of transfectant DS-T4. A 3.0 kb RNA band (most prominent one in 3 bands) was detected with the labelled NCAM cDNA. Panel B shows the expression of NCAM transcript levels in F-T4 and F-T5. Lanes 1 and 2 show RNAs from day-2 and day-5 cultures of the control transfectant F-Tc. Lanes 3 and 4 show RNAs from day-2 and day-5 cultures of F-T4. Lanes 5 and 6 show RNAs from day-2 and day-5 cultures of F-T5. A 3.0 kb RNA band (most prominent one in 3 bands) was detected with the labelled NCAM cDNA.


A
1 2 3 4 5 6
— NCAM
— MGL
B
1 2 3 4 5 6
NCAM —
MGL —

5.3.6 Expression of MLC, MHC, and TnT in transfectants DS-T4, F-T4 and F-T5

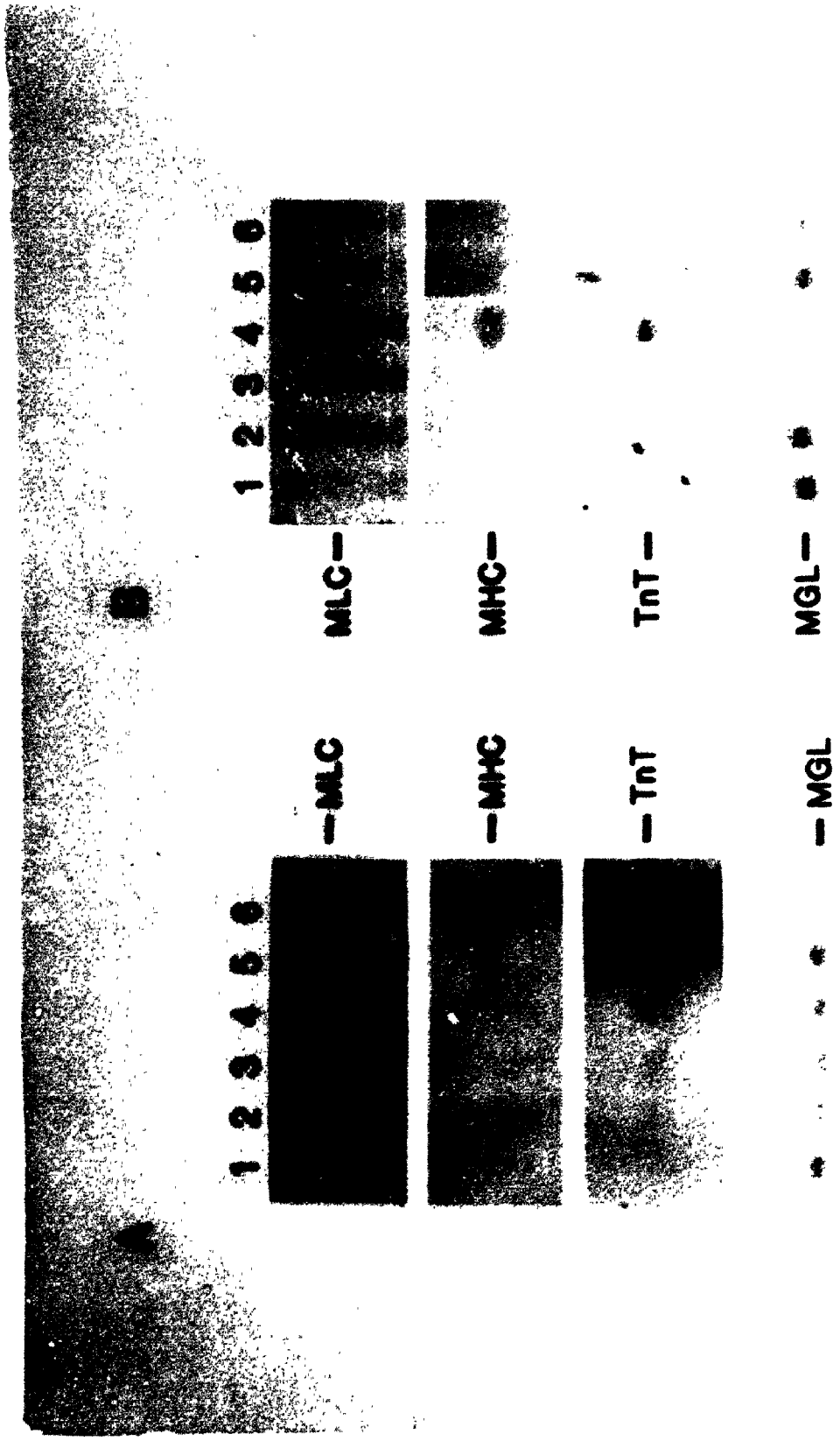
Synthesis of muscle-specific proteins is induced during myogenesis. The expression of these muscle-specific proteins is regulated by myogenic factors. We have previously shown that the myogenesis-defective mutants, D1/S4 and F72, have much reduced levels of the Myf4, MLC, MHC, and TnT transcripts. Therefore, we were interested in seeing if the transfected myogenic factors had any effect on the expression of MLC, MHC, and TnT. As shown in Fig. 5.12A, transfectant DS-T4 exhibited much higher MLC, MHC, and TnT transcript levels than the control transfectant DS-Tc. Similarly, transfectant F-T4 also had higher MLC, MHC, and TnT transcript levels than the control transfectant F-Tc (Fig. 5.12B, lanes 3 and 4). However, the MLC, MHC, and TnT transcripts could hardly be detected in F-T5. These results showed that the increased expression of *myf4* resulted in elevated levels of the MLC, MHC, and TnT transcripts in DS-T4 and F-T4. In other words the expression of muscle-specific proteins MLC, MHC, and TnT were regulated by Myf4 in DS-T4 and F-T4. As expected, the transcript levels of these muscle-specific proteins were not increased in F-T5 (Fig. 5.12B, lanes 5 and 6).

5.3.7 Phosphorylation of cell surface 112 kDa protein in transfectants DS-T4, F-T4 and F-T5

We have previously shown that mutants D1/S4 and F72 were defective in the phosphorylation of cell surface 112 kDa protein (Chapter 3) and

Figure 5.12 Expression of MHC, MLC, and TnT in transfectants DS-T4, F-T4 and F-T5

Northern blot analysis using total RNA was carried out to examine the expression of MLC, MHC and TnT in DS-T4, F-T4 and F-T5. 20μg RNA/lane of each sample were loaded onto the agarose gel. Labelled MHC, MLC and TnT cDNAs were then used to probe for the respective transcripts. In order to compare the amount of RNA loaded in each lane, labelled β2-microglobulin (MGL) cDNA was used to probe all samples. Panel A shows the MHC, MLC and TnT transcript levels in DS-T4. Lanes 1, 2 and 3 are RNAs from day-2, day-4 and day-6 cultures of the control transfectant DS-Tc. Lanes 4, 5 and 6 are RNAs from day-2, day-4 and day-6 cultures of transfectant DS-T4. Panel B shows the MHC, MLC and TnT transcript levels in F-T4 and F-T5. Lanes 1 and 2 are RNAs from day-2 and day-5 cultures of the control transfectant F-Tc. Lanes 3 and 4 are RNAs from day-2 and day-5 cultures of F-T4. Lanes 5 and 6 are RNAs from day-2 and day-5 cultures of F-T5.


1 2 3 4 5 6
MLC
MHC
TnT
MGL
1 2 3 4 5 6
MLC
MHC
TnT
MGL

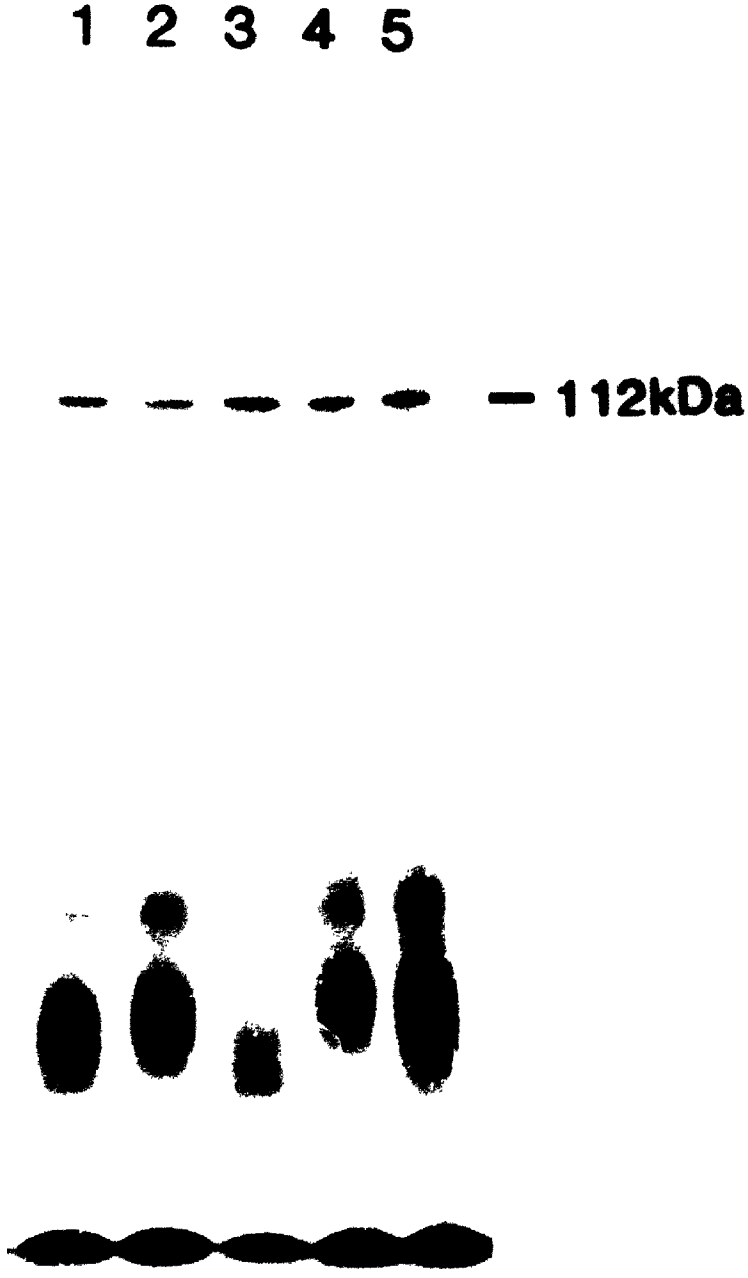
in the expression of *myf4* (Chapter 4). Both myogenesis inhibitors phloretin and BrdUrd inhibited the phosphorylation of cell surface 112 kDa protein and the expression of *myf4*. In order to understand the relationship between these two events, the phosphorylation of the cell surface 112 kDa protein in DS-T4, F-T4 and F-T5 was examined. These studies revealed that the p112 (phosphorylated 112 kDa protein) levels were similar in transfectants DS-T4, F-T4, F-T5 and their corresponding control transfectants (DS-Tc and F-Tc) (Fig. 5.13). It may be surmised from these studies that the site of action of p112 occurs upstream from the Myf4 site in the myogenic pathway.

5.3.8 Morphological fusion of transfectants

Since the transcripts of MLC, MHC and TnT were much higher in transfectants DS-T4 and F-T4 than those in the control transfectants, the morphological fusion of these transfectants was examined. Day-6 culture of transfectant DS-T4 was found to have a fusion index of 32%; whereas the fusion index of the control transfectant DS-Tc was almost 0%. Multinucleated myotubes could not be observed in the transfectant F-T4 and in the control transfectant F-Tc. This was probably due to the amount of the *myf4* mRNA present in the transfectants. Transfectant DS-T4 had very high levels of transfected *myf4* cDNA and *myf4* mRNA, whereas the levels of *myf4* cDNA and *myf4* mRNA were relatively low in F-T4. This suggests that a critical level of the *myf4* mRNA was required for morphological differentiation.

Figure 5.13 Phosphorylation of cell surface 112 kDa protein in transfectants DS-T4, F-T4 and F-T5

The phosphorylation assay (section **5.2.9**) was carried out to examine the level of the phosphorylated 112 kDa protein in transfectants DS-T4, F-T4 and F-T5. Lanes 1, 2, 3, 4 and 5 denote the p112 levels in DS-Tc, DS-T4, F-Tc, F-T4 and F-T5, respectively.


1 2 3 4 5
112kDa

5.4 DISCUSSION

Myogenic differentiation includes the synthesis of muscle-specific proteins and the formation of myotubes from myoblasts. The synthesis of muscle-specific proteins is regulated by myogenic factors. Transfection of myogenic factors into non-muscle cells was found to induce myogenic differentiation (Thayer et al., 1989; Edmondson and Olson, 1989; Braun et al., 1989a). NCAM was also found to play an important role in myogenic differentiation. Overexpression of NCAM by transfection increased not only the CPK activity but also the rate of fusion (Dickson et al., 1990). As indicated in Chapters 3 and 4, myogenesis may be regulated by an ecto-PK mediated phosphorylation of a cell surface 112 kDa protein. Myoblasts impaired in myogenesis (L6 cells grown in phloretin or BrdUrd; mutants D1/S4 and F72) were all defective in the phosphorylation of cell surface 112 kDa protein and the expression of the Myf4, MLC, MHC, TnT. However, it should be noted that the expression of *myf5* was not altered in these cells. This correlation was confirmed by studies using a conditional myogenesis-defective mutant D1 (Chapter 4). When mutant D1 was grown in the permissive condition (1% horse serum) the ability to phosphorylate the cell surface 112 kDa protein and the expression of Myf4, NCAM, MLC, MHC, TnT were restored back to normal. In the myogenic pathway, it is known that the myogenic factors are involved in activating the expression of muscle-specific proteins (Thayer et al., 1989; Edmondson and Olson, 1989; Braun et al., 1989a). However, the relationship between the phosphorylation of the cell surface 112 kDa protein and the expression of Myf4, Myf5, and NCAM was not clear.

In order to understand the temporal order of expression of these factors in the myogenic pathway, this investigation examined the expression of Myf5, NCAM, MLC, MHC, and TnT, and the phosphorylation of the cell surface 112 kDa protein in mutants D1/S4 and F72 transfected with the *myf4* or *myf5* cDNA.

Studies with transfectants harbouring higher levels of the transfected *myf5* cDNA revealed that there was no change in the Myf5, Myf4, NCAM, MLC, MHC, and TnT transcript levels (Figs. 5.8, 5.9, 5.11B, and 5.12B). This indicated that the exogenously added *myf5* cDNA was unable to activate the expression of the above-mentioned genes in mutant F72. There are at least two possible explanations. First, transcription of the transfected *myf5* cDNA did not occur in the transfectants. Since these transfectants expressed the neomycin-resistant phenotype, this could only happen if the *neo* gene in transfected plasmid was integrated into the genomic DNA. Second, the Myf5 transcript formed might be very unstable. As indicated in Chapter 4, the Myf5 transcript level decreased dramatically during the early stage of growth. Thus the inability to observe a higher level of Myf5 transcript in F-T5 might be due to the rapid degradation of this transcript. Since antibodies directed against the Myf5 protein are not available, we are unable to determine the level of this protein in these transfectants. If increased levels of the Myf5 protein were present in these transfectants, then the absence of the Myf4, NCAM, MLC, MHC, and TnT transcripts in these transfectants could be due to the inability of Myf5 to activate

the transcription of *myf4* and/or NCAM. In other words, some other factors must be impaired in these transfectants, and these factors are essential for the proper functioning of Myf5. The observation that the expression of *myf5* was dramatically reduced during the very early stage of growth (Section **4.2.1**) also suggests that Myf5 may play a role at a very early stage of myogenesis. It was recently demonstrated that Myf5 might play a role in the determination of myogenic progenitor cells (Braun et al., 1992). Since the expression of *myf5* was not altered in mutants D1/S4 and F72, it is possible that Myf5 is involved at a site upstream of Myf4, NCAM and the phosphorylation of cell surface 112 kDa protein in the myogenic pathway, or it may participate in a parallel pathway.

It has been shown that NCAM could increase the activity of a muscle-specific enzyme, MCK (muscle creatine kinase) (Dickson et al., 1990). This suggests that NCAM is located upstream of the muscle-specific proteins in the myogenic pathway. Studies with both DS-T4 and F-T4 transfectants revealed that the increase of *myf4* expression did not affect the NCAM transcript level (Fig. 5.11). This observation suggested two possibilities: (i) If NCAM and Myf4 are involved in the same pathway, NCAM may be acting upstream of Myf4. (ii) NCAM and Myf4 may not be involved in the same pathway; they may participate in parallel pathways. They may be responsible for regulating different sets of muscle-specific proteins and enzymes.

Studies with transfectants DS-T4 and F-T4 also showed that the

increase of *myf4* expression resulted in elevated levels of the MLC, MHC, and TnT transcripts (Fig. 5.12). This suggested that Myf4 might be involved at sites upstream of the muscle-specific proteins in the myogenic pathway. More importantly, this showed that Myf4 alone was sufficient to trigger the expression of the downstream factors.

We have previously shown that the p112$^-$ (impaired in the phosphorylation of cell surface 112 kDa protein) cell lines (D1/S4, F72, D1, and L6 treated with phloretin or BrdUrd) were defective in the expression of NCAM and Myf4 (Chapter 4). This suggests that Myf4 and p112 may be involved in the same myogenic pathway. An examination of various transfectants revealed that there was no increase in p112 in these cells (Fig. 5.13). It may be surmised that the p112 site is located upstream of Myf4 in the myogenic pathway.

It has been shown that the expression of any one of the myogenic factors can activate the expression of other myogenic factors (Thayer et al., 1989; Edmondson and Olson, 1989; Braun et al., 1989a). However, studies with the p112$^-$ cells showed that the presence of Myf5 could not activate the *myf4* expression. This observation suggests two possibilities: (i) the Myf4 and Myf5 proteins cannot positively activate each other in rat myoblasts; (ii) the activation of Myf4 by Myf5 may involve other factor(s). If the latter is the case, the p112$^-$ cells are defective in the factor(s) responsible for the Myf5-mediated activation of Myf4. Our data suggested that the factor(s) could be the p112 or NCAM.

In agreement with this observation, there was no correlation between the Myf4 and Myf5 levels in the various transfectants. The Myf5 transcript level was found to be unaltered in transfectants expressing high Myf4 transcript levels (Fig. 5.10).

In summary, studies using mutants D1/S4 and F72 transfected with the *myf4* cDNA suggest that the Myf5 site may be upstream of the p112 site in the myogenic pathway, if they are involved in the same pathway. If NCAM is in the same pathway as Myf4, then the NCAM site will be upstream of the Myf4 site; both sites are downstream of the p112 site. The muscle-specific proteins and enzymes are located downstream of the Myf4 site.

CHAPTER 6

SUMMARY

Although myogenesis has been studied intensively, little is known about the initiation and the temporal order of expression of myogenic components. This thesis examined the involvement of an ecto-PK and its cell surface substrate proteins in the initiation of myogenesis. The possible site of action of this cell surface phosphorylation in the myogenic pathway was also investigated.

We have shown that a cell surface 112 kDa protein can be phosphorylated by an ecto-PK (Chapter 2). This phosphorylation reaction was dependent on the presence of Ca^{++}, Mg^{++}, and F^-. Phospho-amino acid analysis revealed that this is a phosphoserine protein kinase. It was interesting to note that the level of phosphorylated 112 kDa protein (p112) was reduced during myogenesis. This suggested that the phosphorylation of this 112 kDa protein might be regulated by components associated with myogenesis (Chapter 3).

Studies with myogenesis inhibitors and myogenesis-defective mutants provided evidence for the role of p112 in the initiation of myogenesis (Chapter 3). Phosphorylation of the 112 kDa protein was inhibited by myogenesis inhibitors, 5-bromo-2-deoxyuridine (BrdUrd) and phloretin. More importantly,

mutants defective in either the ecto-PK or the 112 kDa protein were also impaired in myogenesis. Studies with a conditional myogenesis-defective mutant, D1, showed that this mutant exhibited a normal p112 level and the ability to form myotubes, when grown in myogenesis-permisive conditions (1% horse serum). However, growth of this mutant in the non-permissive conditions (10% horse serum) abolished both the fusion ability and the phosphorylation of the 112 kDa protein. Since a normal p112 level could be observed in another myogenesis-impaired mutant D23, this suggests that the phosphorylation of the cell surface 112 kDa protein is not one of the end results of myogenesis. The above findings suggest that the functioning of the ecto-PK and the 112 kDa protein may be required for the initiation of myogenesis.

The next question concerns the site of action of the cell surface phosphorylation in the myogenic pathway. It is known that myogenic factors can regulate the expression of muscle-specific proteins and morphological differentiation. We have shown in Chapter 4 that mutants defective in either the ecto-PK or the 112 kDa protein are also impaired in forming multinucleated myotubes. Northern blot analyses revealed that these two mutants possessed much reduced transcript levels of MHC, MLC, and TnT. Inhibition of cell surface phosphorylation by BrdUrd or phloretin also resulted in very low transcript levels of MHC, MLC, and TnT. These results illustrate the close relationship between the phosphorylation of cell surface 112 kDa protein by ecto-PK and the expression of muscle-specific proteins.

Our next effort was to examine the relationship between the phosphorylation of cell surface 112 kDa protein and the expression of myogenic factors (Chapter 4). We examined the expression of four myogenic factors: Myf3, Myf4, Myf5 and Myf6 in various myogenesis-impaired cells. The relationships of these myogenic factors in the rat myoblast myogenic pathway are not clear. The Myf5 transcript level was found to decline upon prolonged growth of the culture, whereas the Myf4 transcript was found to be elevated during myogenesis. It should be noted that Myf3 and Myf6 transcripts could not be detected. Northern blot analyses showed that mutants defective in ecto-PK or 112 kDa protein exhibited the normal pattern of decline of the Myf5 transcript; however the expected increase in the Myf4 transcript was not observed in these myogenesis-impaired cells. Similar observations were also made in the cells in which the phosphorylation of the cell surface 112 kDa protein was inhibited by BrdUrd or phloretin. These findings therefore suggest that the p112 site may occur before the Myf4 site, but after the Myf5 site in the myogenic pathway.

The fact that NCAM could increase the activity of the muscle-specific enzyme CPK and enhance myoblast fusion indicated that the expression of NCAM may occur upstream of the muscle-specific proteins and enzymes in the myogenic pathway. The relationship between the p112 site and the expression of NCAM was therefore examined. These studies revealed that $p112^-$ cells (mutants defective in the ecto-PK or the 112 kDa protein, and L6 myoblast grown in the presence of BrdUrd or phloretin) had very low levels of the NCAM

transcript. These results indicated that the p112 site might be upstream of the NCAM site in the myogenic pathway.

Myogenic factors have been shown to cross-activate each other's expression. We have shown in the above studies that mutants D1/S4 and F72 were defective in the expression of Myf4, NCAM and muscle-specific proteins. This suggested that the deficiency in p112 may block the message flow from Myf5 to its downstream elements. If all the components examined in this study were involved in the same myogenic pathway, then it seemed possible that the p112 site might be located downstream from the Myf5 site but upstream from the Myf4 and NCAM sites in the myogenic pathway.

It has been demonstrated that the expression of any one of the myogenic factor genes in fibroblasts can convert fibroblasts to cells exhibiting the ability to form multinucleated myotubes. In order to determine the possible sequence of the events during myogenesis, transfection studies were carried out (Chapter 5). The *myf4* cDNA was transfected into the mutants D1/S4 and F72. When the transfected *myf4* gene was expressed in mutants D1/S4 and F72 the expression of the muscle-specific proteins MHC, MLC, TnT was elevated, whereas the level of the phosphorylated 112 kDa protein and the NCAM transcript remained low. The fact that the transfected *myf4* cDNA can restore the ability of the transfectants to express high levels of the muscle-specific transcripts suggested that as long as the Myf4 level was maintained at a high level, the

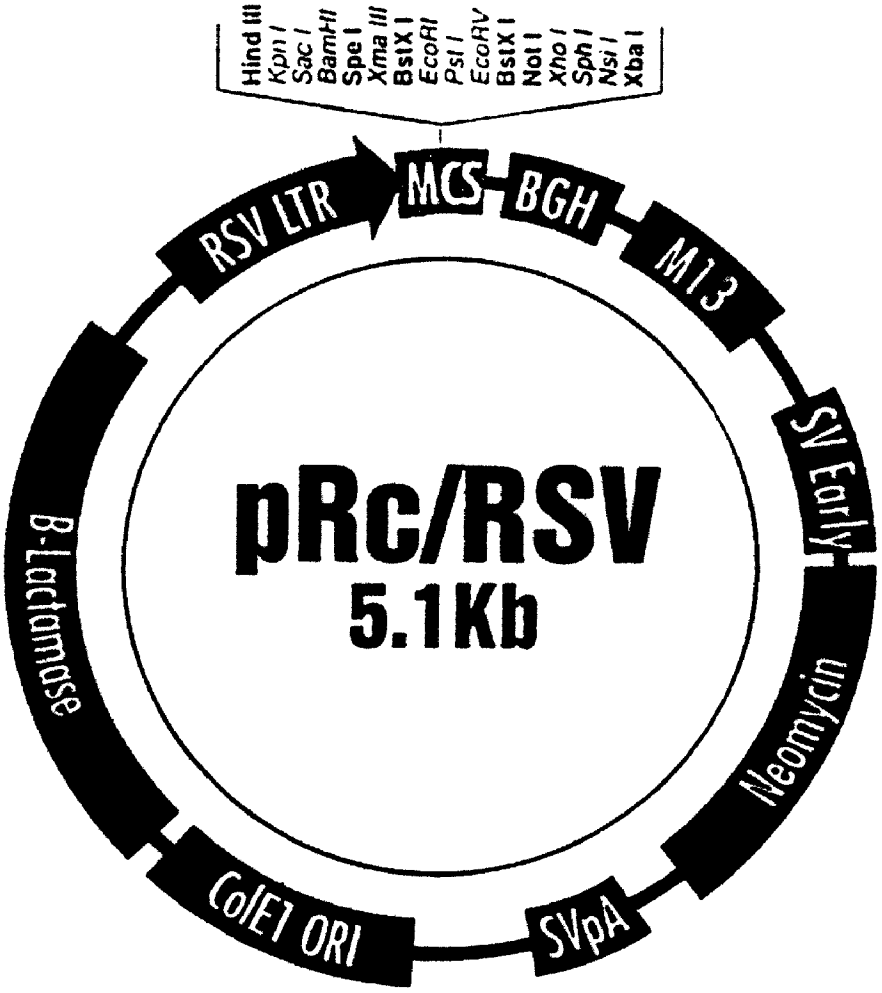
Myf5, ecto-PK and the cell surface 112 kDa protein were not required for myogenesis. However, these studies do demonstrate the importance of these components in activating the expression of Myf4.

In conclusion, an ecto-PK and its substrate cell surface 112 kDa protein were detected in the rat myoblast L6 cell line. The phosphorylation of the cell surface 112 kDa protein by ecto-PK may play an important role in the initiation of myogenesis. Our findings suggested that the phosphorylated 112 kDa protein probably participated at a site between Myf5 and Myf4 in the myogenic pathway. One possibility is that the components of the rat myoblast myogenic pathway may participate in the following sequence: Myf5, p112, NCAM, Myf4. The expression of Myf4 is essential for the activation of muscle-specific proteins and morphological differentiation. If the NCAM and Myf4 are in the same pathway, then NCAM may act upstream of Myf4. Another possibility is that NCAM and Myf4 are functionally parallel in the myogenic pathway. Both can regulate the expression of muscle-specific proteins and enzymes.

In future studies the relationships of Myf4, Myf5 and NCAM can be examined by transfecting the respective cDNAs into L6 myoblasts. The expression of Myf4, Myf5, and NCAM and the level of p112 in these transfectants should provide valuable information about the relationships of Myf4, Myf5, NCAM and p112.

Further studies can be carried out to determine the properties of the 112 kDa protein and the ecto-PK. The 112 kDa protein defective mutant D1/S4 and ecto-PK defective mutant F72 can be fused. This may produce cells with normal ecto-Pk activity and normal cell surface 112 kDa protein. Then the fused cells will be examined for the phosphorylation of cell surface 112 kDa protein and the myogenic ability. Besides, the cDNA of the 112 kDa protein should be cloned. One can isolate the mRNA for the 112 kDa protein and clone it by using the subtractive hybridization technique, which was used successfully in isolating and cloning the myogenic factor cDNAs. Mutant D1/S4 and its parental wild type L6 cells can be used in these studies. The alternative approach is to isolate the 112 kDa protein and to determine the terminal sequences of the protein. We have already in our possession an antibody directed against the 112 kDa protein. It is therefore possible that this protein can be isolated by an immunoadsorbant column. The DNA primers for the 112 kDa protein can be synthesized according to the protein sequence. The primers then can be used in a PCR (polymerase chain reaction) study to select for the cDNA for the 112 kDa protein. Once full-length cDNA for the 112 kDa protein has been cloned, it can be transfected into D1/S4 to examine the expression of NCAM, Myf4 and muscle-specific proteins. The anti-sense RNA of the 112 kDa protein can also be used to block the expression of the 112 kDa protein in L6 cells, and then the expression of Myf5, Myf4, NCAM and muscle-specific proteins can be examined. These studies should yield important information about the myogenic pathway in rat myoblasts.

APPENDIX I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Sac I
BamHI
Spe I
Xma III
BstX I
EcoRI
Psi I
EcoRV
BstX I
Not I
Xho I
Sph I
Nsi I
Xba I
RSV LTR
MCS
BGH
M13
SV Early
Neomycin
SVpA
ColE1 ORI
B-Lactamase
pRc/RSV
5.1Kb

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