SIGNALLING PATHWAYS REGULATING

BC_3H1 CELL MYOGENESIS

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By

JANICE DOREEN OAKES, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1991)
(Biology)
McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Signalling Pathways Regulating BC_3H1 Cell Myogenesis

AUTHOR: Janice D. Oakes, B.Sc. (McMaster University)

SUPERVISOR: Dr. R. W. H. Lee

NUMBER OF PAGES: xv, 130

ABSTRACT

The myogenic cell line, BC_3H1, upon cell-to-cell contact or serum starvation differentiates as monitored by the appearance of muscle-specific markers, actin, myosin light chain 1 (MLC 1) and tropomyosin (Tm) and morphological changes. The detection of MLC 1 and five Tm isoforms in this cell line is novel. To assess the role of protein kinase C (pk C)- and protein kinase A (pk A) signal transduction pathways in controlling BC_3H1 cell differentiation, activators of pk C (TPA) and pk A (cAMP analogues, dibutyryl-cAMP and 8-Br-cAMP) were used. TPA (500nM) addition caused no deviation from the normal expression patterns of actin, Tm and MLC 1. Addition of cAMP analogues (500μM) delayed the appearance of MLC 1 and muscle-specific isoforms of Tm, as well as α-actin while β- and γ-actin levels remained unchanged. However, α-actin mRNA levels were not affected by cAMP analogues yet the typical β- and γ-actin mRNA downregulation was blocked. cAMP appears to be operating at multiple levels to regulate BC_3H1 cell myogenesis such as post-transcriptional and translational. In addition, given the similarity in mechanisms through which cAMP and adenovirus early region 1A

(AdE1A) mediate gene activation, the effect of AdE1A on BC_3H1 cell differentiation was investigated. A stable transfected AdE1A clonal cell line, BC_3E7, was characterized. Together with altered morphology, BC_3E7 cells failed to show the characteristic expression of muscle-specific markers actin, Tm and MLC 1. AdE1A transfection disrupted the synchronous expression of muscle-specific proteins during BC_3H1 cell differentiation.

ACKNOWLEDGEMENTS.

I would like to thank my supervisor, Dr. R. W. H. Lee, for his never-ending support and encouragement throughout the tenure of this study. Special thanks go to all members of the lab, especially Natalie Veitch and Brenda Coopper for their assistance in cell culture, and Senthil Muthaswamy and Peter D'Amico for their advice and friendship. To my friends, Neil Fortin, Jeff Gordon, David Heaton and Tom Boyce, thank-you for your friendship and ability to help me through the rough times.

I would also like to extend my thanks to my mother and father for their encouragement and constant support.

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ABBREVIATIONS

β-TGF	type β transforming growth factor
2D-PAGE	two-dimensional PAGE
8-br-cAMP	8-bromo cAMP
AChR	acetylcholine receptor
Ad5 E1A	adenovirus 5 early region 1A
ATF	activating transcription factor
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
cAMP	cyclic adenosine 3',5'-monophosphate
CAT	chloramphenicol acetyltransferase
CRE	cAMP responsive element
CREB	CRE-binding phosphoprotein
DAG	diacylglycerol
db-cAMP	dibutyryl cAMP
DMEM	Dulbeco's modified Eagle's medium
DMF	N,N-dimethyl formamide
EGF	epidermal growth factor
FCS	fetal calf serum
FGF	fibroblast growth factor
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate

GTPase	guanosine 5'-triphosphatase
IEF	isoelectric focusing
IP_3	inositol-1,4,5-trisphosphate
MCK	muscle creatine kinase
MHC	myosin heavy chain
MLC	myosin light chain
NBT	nitro blue tetrazolium
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PIP_2	phosphatidyl inositol bisphosphate
pk C	protein kinase C
pk A	protein kinase A
PLC	phospholipase C
PS	phosphatidylserine
REF	rat embryo fibroblasts
SDS	sodium dodecyl sulfate
Tm	tropomyosin
Tn	troponin
TPA	12-O-tetradecanoyl phorbol-13-acetate

I. INTRODUCTION.

Much interest exists in identifying inducers of differentiation and how such inductions lead to the specific responses of gene activation. *In vitro* myogenesis is an attractive system for investigating the molecular mechanism of coordinate gene regulation during differentiation, having an ordered sequence of morphological and molecular events associated with it. Signalling pathways are implicated in transducing external signals from the cell membrane to the nucleus leading ultimately to altered gene expression. It was of interest in this study to investigate the role played by two signalling pathways, one protein kinase C-dependent, the other protein kinase A-dependent, on the regulation of myogenic differentiation in the mouse muscle cell line, BC_3H1. As well, the effect of the viral oncogenic transcription factor E1A on myogenesis was studied.

A. MYOGENESIS IN THE MOUSE CELL LINE, BC_3H1.

Involved in myogenic differentiation is determination, in which a multipotential mesodermal stem cell is committed to the myogenic lineage, becoming a monopotent myoblast, and the differentiation of myoblasts

into multinucleated myofibres. Accompanying the change from myoblast to myofibre are withdrawal from the cell cycle (terminal commitment), muscle-specific gene activation (leading to the development of an excitable membrane, contractile apparatus, and expression of enzymes of muscle metabolism), and cell fusion (terminal differentiation). The fact that myogenesis can be reproduced in vitro using primary myoblast cultures and established cell lines which can be induced to differentiate under controlled conditions strengthens the attractiveness of myogenesis as a system for investigating the molecular mechanisms of gene regulation during differentiation. One such cell line used extensively for studying myogenesis is the BC_3H1 cell line.

A.1. THE BC_3H1 CELL LINE.

The hypotetraploid myogenic BC_3H1 cell line was derived from a mouse brain neoplasm induced by nitrosoethyl urea in a C_3H mouse (Schubert et al., 1974). At subconfluency, BC_3H1 cells are exponentially growing (undifferentaited), adhere to the dish and exhibit a flat morphology with small processes. BC_3H1 cells can be induced to differentiate by serum depletion or the establishment of cell-to-cell contacts. At this time, they withdraw from the

cell cycle at early G_1, enter the quiescent G_0 phase (Lathrop et al., 1985) and initiate the myogenic program of muscle-specific gene expression, despite their ectodermal origin. The cells elongate and align themselves although no fusion into multinucleated myofibres occurs (Schubert et al., 1974).

When the BC_3H1 cell line was originally characterized by Schubert et al. in 1974, it was believed to be smooth muscle in type. This was based on the nonfusing nature of the cells as well as the presence of surface vesicles, lack of Z bands in sarcomeric thick filaments and electrophysiological properties (Schubert et al., 1974). The developmentally regulated synthesis of vascular smooth muscle α-actin seen in BC_3H1 cells (Strauch and Rubenstein, 1984; Strauch et al., 1986; Wang and Rubenstein, 1988) also lends support to the belief that the cells are of the smooth muscle type.

Taubman et al. (1989) further characterized the BC_3H1 cell line with respect to the contractile proteins in 1989 and provided evidence that the cell line more closely resembles skeletal muscle. Differentiated BC_3H1 cells were found to express sarcomeric isoforms of α-tropomyosin (α-Tm), myosin heavy chain (MHC), myosin light chains 2 and 3 (MLC 2, MLC 3), although no MLC 1 was detected, and troponin

T (Tn T) (Taubman et al., 1989). Tn T is important in the sarcomeric contraction apparatus but has no role in smooth muscle contraction (Adelstein and Eisenberg, 1980) nor has it been detected in smooth muscle cells (Medford et al., 1984; Marston and Smith, 1985). As well, muscle creatine kinase (MCK) (Schubert et al., 1974), nicotinic acetylcholine receptor (AChR) (Patrick et al., 1976), skeletal muscle voltage-gated Ca^{2+} channel (Caffrey et al., 1987), skeletal muscle-specific α-actin (Strauch and Reeser, 1989) and the MyoD family myogenic regulator, myogenin, which has restricted expression in skeletal muscle (Edmonson and Olson, 1989) have all been detected in BC_3H1 cells. Together these imply that BC_3H1 cells should be characterized as skeletal muscle. Taubman et al. (1989) suggest that BC_3H1 cells are merely defective for fusion.

BC_3H1 cells offer an advantage not offered by fusing myoblast cell lines. Since they do not fuse and can reversibly differentiate by re-entry into the cell cycle by the addition of serum or mitogenic factors (Lathrop et al., 1985; Wice et al., 1987; Olson et al., 1984), BC_3H1 cells can be passaged with a loss of the differentiated phenotype. This affords a wider range of experimental manipulations and an economy of time compared to earlier methods of primary cell culture development or fusing cell lines as well as

allowing the study of both induction and repression of myogenesis. A large battery of muscle-specific genes are developmentally regulated in BC_3H1 cells making this an excellent system to study coordinate gene regulation. The transition from an undifferentiated state to a differentiated one can be monitored by the contents of the contractile apparatus, the sarcomere, and thus contractile proteins can serve as excellent markers for BC_3H1 cell differentiation.

A.2. PROTEINS OF THE CONTRACTILE APPARATUS.

Perhaps the best known characteristic of muscle is its ability to contract and generate force. The contractile apparatus, or sarcomere, is composed of thick and thin filaments. The thin filament is composed of actin, tropomyosin (Tm) and the troponin (Tn) complex of Tn T, Tn I, and Tn C while the thick filament contains myosin heavy chains (MHC), two alkali myosin light chains (MLC 1 and MLC 3) and the regulatory myosin light chain (MLC 2).

A.2.1. Actin.

Actin is a major cytoskeletal protein found in both muscle and non-muscle cells. In muscle it is the most abundant by molar mass (Garrels and Gibson, 1976). In striated muscle it functions as a cable to shorten the

sarcomere during contraction. Actin exists as a multigene family in which the number varies between species. In vertebrates there are six members, encoded by separate genes. There are two non-muscle actins, termed β- and γ-cytoplasmic actins, and four muscle-specific actins, α-skeletal, α-cardiac, α-vascular smooth, and γ-enteric smooth actins (Sawtell et al., 1989). All actin isoforms are highly conserved evolutionarily (Stossel, 1978) and are distinguished by their primary amino acid sequence (Vandekerckhove and Weber, 1979). The two smooth muscle actins (both bovine and rabbit) differ by only 3 amino acid substitutions at the amino-terminus (Vandekerckhove and Weber, 1979). There exists approximately 25 amino acid substitutions between cytoplasmic and muscle-specific actins (Vandekerckhove and Weber, 1978) with the two smooth muscle actins showing the closest relationship to the non-muscle isoforms (Vandekerckhove and Weber, 1979).

Actin isoforms all have the same subunit molecular weight, 43kDa in rat muscle, however due to variations in their primary amino acid sequence, their net charges differ (Vandekerckhove and Weber, 1978). This allows for the separation on isoelectric focusing gels of three general isoforms, α (muscle-specific), and β and γ (non-muscle-specific), (Whalen et al., 1976; Rubenstein and Spudich, 1977; Garrels and Gibson, 1976) which focus at a pH of about

5.4 with α-actin being the most acidic followed by β-actin and then γ-actin (Garrels and Gibson, 1976; Whalen et al., 1976). Lee reported that the least acidic γ-actin in adrenal chromaffin cells had an isoelectric point of 5.58 and that the difference between the isoelectric points of the three actin isoforms was smaller than 0.1 pH unit, one full charge difference (Lee et al., 1979). This indicates that the differences in the isoelectric points of the actin isoforms is not due to charge modifications brought about by reactions such as phosphorylation. Muscle differentiation is accompanied by an increase in the muscle-specific isoform, α-actin, and a decrease in the non-muscle-specific isoforms, β- and γ-actins (Whalen et al., 1976; Devlin and Emerson, 1978).

A.2.2. Actin Expression in BC_3H1 cell.

The BC_3H1 cell line has proven to be an excellent system in which to study the expression of actin isoforms both at the protein and message levels. Strauch and Rubenstein (1984) identified three different actin isoforms, whose expression were developmentally regulated, by isoelectric focusing and NH_2-terminus tryptic peptide analysis. Following serum depletion at confluency, α-actin synthesis was increased and this was accompanied by decreases in both β- and γ-actin synthesis. This effect was

reversible upon re-addition of serum (Strauch and Rubenstein, 1984). The muscle-specific actin was identified as vascular smooth muscle α-actin. As well, cell-to-cell contact was implicated in having a permissive effect on differentiation (Strauch and Rubenstein, 1984).

Analysis of BC_3H1 cell mRNA indicated a 2100 nucleotide RNA, corresponding to both the β- and γ-actin transcripts, present in undifferentiated cells and whose expression decreased 3-fold following serum reduction. A 1500 nucleotide RNA corresponding to α-actin was also identified. The expression of α-actin increased 6-fold during differentiation (Strauch et al., 1986).

Upon further characterization of α-actin expression in BC_3H1 cells, it was found that skeletal muscle-specific α-actin was induced in serum-starved cells. The expression of vascular smooth muscle α-actin and skeletal α-actin was sequential. Vascular smooth muscle α-actin was induced upon confluency in high serum media, while skeletal α-actin expression occurred later in differentiation, reaching maximum induction 6 days post-confluency in reduced serum (Strauch and Reeser, 1989). A mechanism which regulates the precise temporal expression of vascular smooth and skeletal α-actin must exist. It would be of interest to determine whether these genes are activated by a common transcription

factor during differentiation in BC_3H1 cells.

A.2.3. Myosin.

Myosin is an asymmetric multimeric protein consisting of a pair of non-covalently linked large polypeptides, termed myosin heavy chains (MHC) with molecular weights in the range of 220-230 kDa, and 2 to 3 pairs of smaller polypeptides, termed myosin light chains (MLC) with molecular weights of 15 to 30 kDa, depending on cell type (Stossel, 1978; Harrington and Rodgers, 1984). In muscle, myosin functions to convert chemical energy of ATP into a force which together with actin shortens the sarcomere (reviewed in Stossel, 1984).

In skeletal muscle there are three types of MLCs: two alkaline (MLC 1 and MLC 3), and one regulatory (MLC 2). Smooth muscle, on the other hand, contains only one alkaline and one regulatory light chain. In rat and mouse, skeletal alkaline MLCs are the products of alternative splicing of primary transcripts from a single gene with two promoters (Periasamy et al., 1984; Robert et al. 1984). This gene is expressed in a developmental and tissue-specific manner (Shani et al., 1981; Periasamy et al., 1984), being regulated at the level of transcription during muscle diffentiaion in the mouse C2\7 myoblast cell line (Cox et al., 1990). The gene is not expressed in undifferentiated

BC_3H1 cells, while in differentiated cells only the transcript for MLC 3 has been detected (Taubman et al., 1989).

The regulatory subunit, MLC 2, gene is also developmentally and tissue-specifically expressed (Shani et al., 1981; Devlin and Emerson, 1978). In BC_3H1 cells, sarcomeric MLC 2 mRNA was not detected until after the cells had become post-confluent (Taubman, et al., 1989).

In vertebrates, MHC represents a multigene family, including sarcomeric and non-sarcomeric types. Sarcomeric MHC genes are clustered on a single chromosome in the genome (Czosnek et al., 1982; Leinwand et al., 1983; Weydert et al., 1985). The expression of MHCs shows developmental and tissue-specific regulation (Shani et al., 1981; Whalen et al., 1981; Mahdavi et al., 1986;). The differential expression of MHC has been shown to be regulated at a transcriptional level during myogensis (Medford et al., 1983). During BC_3H1 cell differentiation, sarcomeric MHC was induced with a corresponding reduction in non-sarcomeric MHC message (Taubman).

A.2.4. Tropomyosin.

Muscle tropomyosin (Tm) is an asymmetrical complex composed of two non-covalently linked subunits in an α-helical coiled-coil configuration which associates with

actin in a ratio of 1 Tm : 7 actin monomers (reviewed in Stossel, 1978). In skeletal muscle two Tm isoforms have been identified, each with slightly different molecular weights (in the 35kDa range) and isoelectric points (around 4.6) which some have termed α- and β-Tm (Garrels, 1979; Talbot and MacLeod, 1983). These isoforms are encoded by distinct genes, both of which are alternatively spliced in a tissue and developmentally-specific manner (MacLeod et al., 1985; Helfman et al., 1986; Wieczorak et al., 1988). Multiple isoforms of Tm have been identified in REF cells and L6 myoblasts on two dimensional gels which focus around the known pI and molecular weights for Tm (Matsumura et al., 1983). No clear demonstration of the shift from the nonmuscle-specific isoforms to the muscle-specific isoforms of Tm at the protein level has been shown in BC_3H1 cells, although it has been indicated that there was a shift from the nonmuscle isoform of α-Tm to the muscle-specific isoform at the message level during differentiation (Taubman et al., 1989).

A.2.5. Troponin.

The troponin (Tn) complex is composed of three proteins: Tn T (38kDa), Tn I (24kDa), and Tn C (18kDA). Tn T functions to attach the Tn complex to the thin filament by interacting with actin and tropomyosin. Tn I, together with

Tm blocks the interaction between actin and myosin, and Tn C binds Ca^{2+} to regulate the position of Tn I and Tm within the sarcomere and thus ultimately control contraction (reviewed in Stossel, 1978). Tn C, T, and I are encoded by distinct gene families which show developmental and tissue-specific expression (Bucher et al., 1988; Medford et al., 1984; Marston and Smith , 1985). Tn T isoform diversity is generated through an alternative splicing mechanism (Bucher et al., 1989). The existence of alternative splicing as a mechanism to generate protein diversity suggests the presence of developmental-specific splicing factors.

A.3. EFFECTS OF GROWTH FACTORS AND ONCOGENES ON BC_3H1 CELL DIFFERENTIATION

Proliferation and differentiation are mutually exclusive; exit from the cell cycle is necessary for differentiation. Owens et al. in 1986, studying cultured rat aortic smooth muscle cells, provided evidence that it is specifically growth arrest rather than cell-to-cell contact which promotes differentiation. The necessity of mitogen withdrawal for differentiation and the antagonism between growth and differentiation implicate a role for growth factors and oncogenes in regulating myogenesis.

In 1985, Lathrop et al. demonstrated that α- and β-

fibroblast growth factors (FGF) induced growth in quiescent BC_3H1 cells and a corresponding inhibition of differentiation as revealed by a decrease in MCK synthesis (Lathrop et al., 1985). Similarly, other researchers have demonstrated the ability of FGF (Wice et al., 1987; Spizz et al., 1987), type-β transforming growth factor (β-TGF) (Spizz et al., 1987) or epidermal growth factor (EGF) (Wang and Rubenstein, 1988) to inhibit the expression of muscle-specific genes in BC_3H1 cells. However, questions still remain about events which occur between the occupation of a growth factor receptor and its growth factor and the ultimate effect on differentiation.

Cellular oncogenes are potentially involved in the cascade of signal transduction. Some encode growth factor receptors, while others share homology with guanine nucleotide binding regulatory proteins or are cytoplasmic proteins which may possess kinase activity or act as transcription factors. Recent research on the oncogenes ras, myc, and fos has implicated them as mediators of external signals (Payne et al., 1987; Sejerson et al., 1985; Jamal and Ziff, 1990). Studies on BC_3H1 cells suggest the involvement of ras and myc in growth factor-mediated inhibition of myogenic differentiation (Schneider et al., 1987; Payne et al., 1987; Kelvin et al., 1989b). ras or myc

transfection could partially inhibit myogenic differentiation (Payne et al., 1987; Schneider et al., 1987). As well, as BC_3H1 cells differentiate, c-myc levels decrease (Spizz et al., 1987).

B. SIGNAL TRANSDUCTION DURING MYOGENIC DIFFERENTIATION.

That external signals such as cell-to-cell contact, serum levels, or growth factors can regulate proliferation and differentiation of BC_3H1 cells implicates a necessary role of signalling pathways in transducing signals from the cell membrane to the nucleus leading to altered gene expression. The plasma membrane provides a barrier for the inflow of information therefore extracellular signals must be converted into intracellular signals. This is accomplished by second messengers which effect a response. Protein phosphorylation appears to be an important intracellular event in response to extracellular signals.

B.1. PROTEIN KINASE C - DEPENDENT PATHWAY.

Activation of the protein kinase C (pk C) pathway elicits numerous cellular responses including the regulation of growth and differentiation, secretion, metabolism and cell surface receptors. For a review of the effects of pk C on cell proliferation and gene expression, refer to articles by Nishizuka, 1986 and Berridge, 1984.

Receptor-mediated stimulation causes the hydrolysis of phosphatidyl inositol bisphosphate (PIP_2) in the plasma membrane, yielding inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 modulates Ca^{2+} release from the endoplasmic reticulum while DAG activates pk C (reviewed in Nishizuka, 1988a, b; Bell, 1986). This is accomplished through a mechanism involving guanidine nucleotide binding protein-GTP-dependent phospholipase C (Berridge and Irvine, 1984). A diagrammatic representation of the pk C-dependent pathway is shown in Figure 1.

Pk C represents a gene family of serine/threonine kinases, each with distinct expression and localization patterns and enzymatic properties (reviewed in Nishizuka, 1988a, b). In its inactive, soluble form it is localized in the cytoplasm. Upon activation, pk C is translocated to the cell membrane (Nishizuka, 1986). The activation of pk C is regulated by DAG, phosphatidylserine (PS), and Ca^{2+}. Ca^{2+} controls the binding of pk C to the membrane while DAG affects both membrane binding and activation (Nishizuka, 1984).

The appearance of DAG is transient, being converted to inositol phospholipid via phosphatidic acid or degraded to arachidonic acid (Nishizuka, 1986). Although the period of pk C activation is short, the signal generated, through

sequential phosphorylation events, may persist for a relatively long time. Activated pk C can phosphorylate substrate proteins which may themselves be translocated to the nucleus and activate gene expression (Johnson, et al., 1987). It has been hypothesized that pk C may enter the nucleus to mediate an effect. This is based on the homology of cysteine-rich elements in the amino-terminus of pk C to some DNA-binding proteins (Parker et al., 1986) however pk C has not been found in the nucleus in appreciable amounts (Nishizuka, 1984).

B.1.1. THE PHORBOL ESTER, TPA: A PK C ACTIVATOR.

To study the role of pk C in cellular responses requires a means of activating the enzyme and examining the effects. Although DAG activates pk C, it has long fatty acid chains which limit its solubility in aqueous media making its addition to cells difficult. For this reason synthetic DAGs with short fatty acid domains have been used (Bell, 1986). One such synthetic DAG commonly used is the tumour promotor phorbol ester 12-O-tetra decanoyl-phorbol-13-acetate (TPA) which has a structure similar to DAG. TPA can substitute for DAG at low concentrations, binding to pk C at the DAG site (reviewed in Bell, 1986, Chida, 1986). This increases the affinity of pk C for Ca^{2+} which leads to full activation of pk C at physiological concentrations of Ca^{2+}

(Castagna, 1982). TPA has also been shown to induce phospholipid degradation by activating phospholipase C to generate DAG and thus indirectly activate pk C (Mufson, 1985).

B.1.2. PROTEIN KINASE C AND MYOGENESIS.

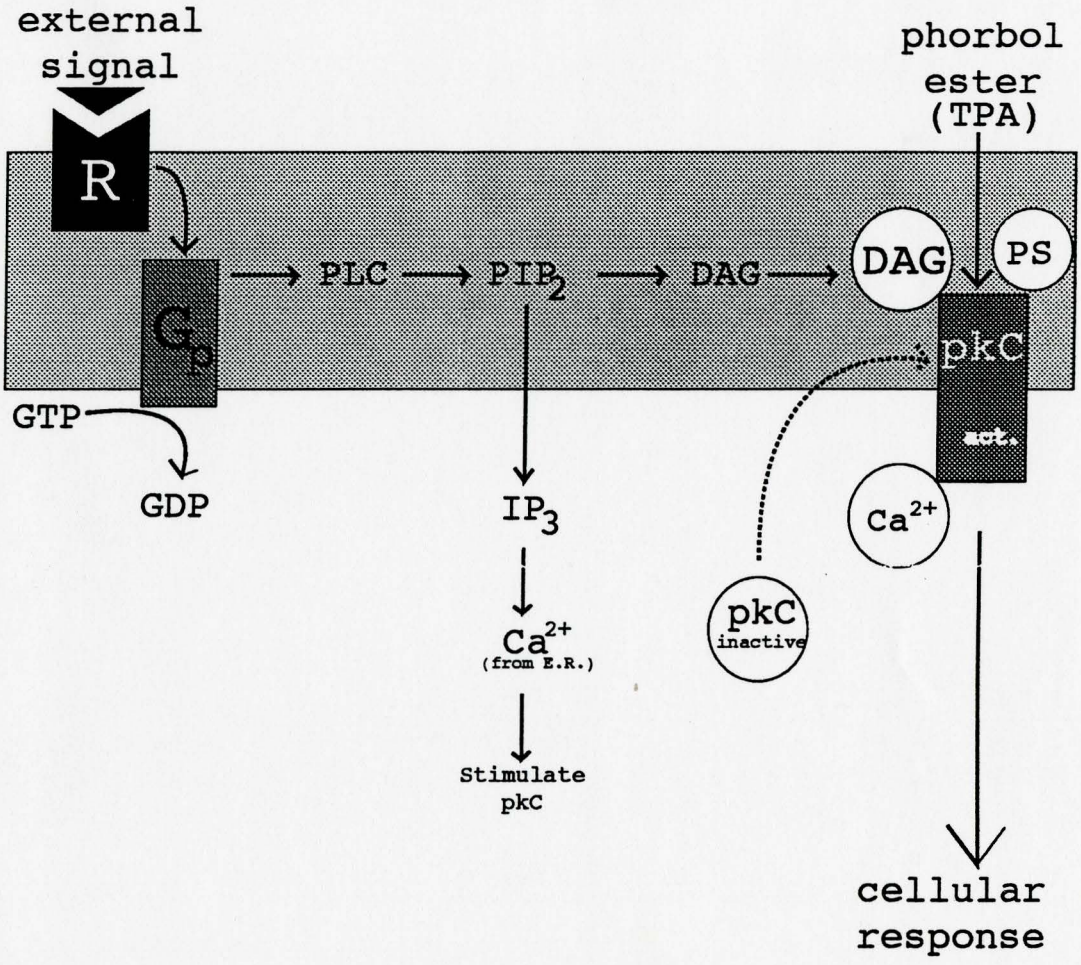
Pk C has been implicated in the regulation of myogenesis. Studying chick embryo skeletal myoblasts, Cossu and colleagues showed that TPA inhibited the expression of MCK and AChR in a manner dependent on protein synthesis (Cossu et al., 1982). This indicates that the effect was mediated by a protein synthesized in response to TPA. It has been demonstrated that membrane-bound pk C activity decreased as chick myoblasts withdrew from the cell cycle and fuse, yet pk C activity remained high when the cells had become quiescent. This suggested that pk C activity is related more to myogenic inactivation than cell cycle withdrawal (Adamo et al., 1989). It has also been shown elsewhere that there was partial translocation of pk C activity from the membrane to the cytosol after differentiation into myotubes of fetal and adult rat muscles and L6 cells (Martelly et al., 1989), also indicating a decline in pk C activity during myogenesis.

Pk C appears to play a role in myoblast fusion. Cohen and workers in 1977 demonstrated that in addition to the mitogenic effect of TPA, was its ability to specifically

block fusion of chick muscle cells. When TPA was added to primary myogenic cultures after biochemical differentiation (ie. the appearance of muscle-specific myosin), no fusion occurred but the cells still expressed muscle-specific MHC and MLCs (Cohen et al., 1977), thus indicating that biochemical and terminal differentiation could be uncoupled. A role for pk C in fusion was also revealed by David et al. (1990). High concentrations of TPA inhibited fusion of primary rat embryo skeletal myoblasts, yet at low concentrations (that which was indicated to activate pk C; 0.2nM) promoted fusion as revealed by a shift in the kinetics of fusion (David et al., 1990). TPA exerts its effect in a concentration-dependent manner.

The abiltity of TPA and hence pK C to inhibit myoblast fusion may be specific to stages of development. TPA was shown to repress myotube formation and biochemical differentiation of fetal myoblast but failed to do so in cultured satellite cells from adult skeletal muscle cells (Cossu et al., 1985; 1983). These differences were not attributed to differences in pk C but may be due to events further down the signalling pathway (Martelly et al., 1989).

Figure 1. Pk C-dependent signal transduction pathway. The external signal binding to its membrane-bound receptor (R) activates a G protein (G) which in turn activates an effector enzyme, phospholipase C (PLC). This converts phosphatidyl inositol bisphosphate (PIP_2) to the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} while DAG activates pk C, together with Ca^{2+} and phosphatidylserine (PS). Active pk C catalizes the transfer of PO_4 groups from ATP to serine/threonine residues on a substrate protein. This leads to a cellular response.


external
signal
R
phorbol
ester
(TPA)
DAG
PS
PLC
PIP2
DAG
Gp
pkC
act.
GTP
GDP
IP3
Ca2+
pkC
inactive
Ca2+
(from E.R.)
Stimulate
pkC
cellular
response

Many subspecies of pk C exist, each with distinct expression patterns and enzymatic properties. Given this, together with the variation in reported effects of TPA on myogenesis and the suggested involvement of myoblast pk C in fusion, it should prove interesting to investigate the effect of pk C activation on the non-fusing muscle cell line, BC_3H1.

B.2. PROTEIN KINASE A - DEPENDENT SIGNAL TRANSDUCTION.

The observation that glycogen phosphorylase existed in both an active and inactive state in the 1940s (Cori et al., 1945) led to the discovery of cyclic adenosine 3',5'-monophosphate (cAMP), cAMP-dependent protein kinase (pk A) and the concept that phosphorylation could act as a mechanism which regulates protein activity. The involvement of cAMP in signal transduction became apparent following the finding that in response to extracellular signals, cAMP was synthesized in cells (Robinson et al., 1971).

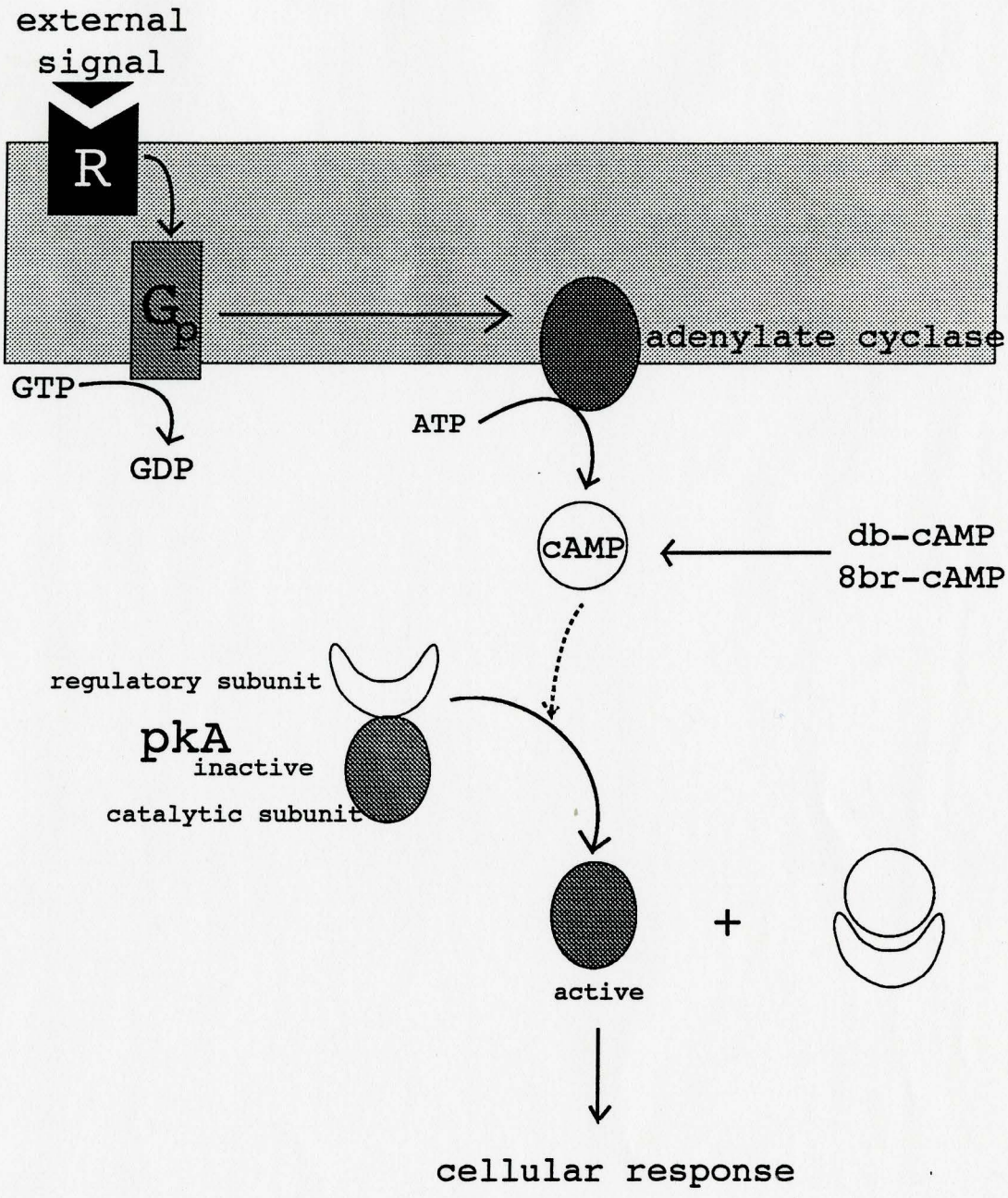
An extracellular signal, or first messenger, binds to its specific receptor, causing a conformational change in the receptor. This signal is then transduced across the membrane via the activation of a guanidine nucleotide binding protein-GTP complex which in turn activates adenylate cyclase. Adenylate cyclase catalyzes the

formation of the second messenger, cAMP, from ATP. This activation is transient and is turned off by the hydrolysis of GTP by GTPase to GDP. cAMP then activates pk A (reviewed in Gilman, 1984). The level of intracellular cAMP is also regulated, apart from its synthesis, by its degradation which is catalyzed by a phosphodiesterase.

The serine\threonine kinase, pk A, is a holoenzyme which exists as an inactive tetramer composed of two catalytic and two regulatory subunits. Each catalytic subunit consists of three major functional sites: i) a Mg^{2+}-ATP binding site, ii) a protein substrate binding site, and iii) a catalytic site for the phosphotransfer reaction. cAMP binds to the regulatory subunits of pk A and decreases its affinity for the catalytic subunit. This leads to the dissociation of the complex and the liberation of two free catalytic subunits (reviewed in Taylor et al., 1988). A diagrammatic representation of the pk A-dependent signal transduction pathway can be seen in Figure 2.

The phosphorylation of specific substrates results in many of the effects of cAMP, including enzyme activation and altered gene expression (Krebs and Beavo, 1979; Mellon et al., 1989).

Figure 2. Pk A-dependent signal transduction pathway. The external signal binds to its receptor (R) and activates a G protein (G). This in turn activates adenylate cyclase, converting ATP into cAMP. Binding of cAMP to the regulatory subunit of inactive pk A leads to its dissociation from the catalytic subunit of pk A, hence activation. The active subunit can then catalyze the transfer of PO_4 groups from ATP molecules to serine/threonine residues of substrate proteins. This leads to a cellular response.


external
signal
R
G_p
adenylate cyclase
GTP
GDP
ATP
cAMP
db-cAMP
8br-cAMP
regulatory subunit
pkA
inactive
catalytic subunit
active
+
cellular response

B.2.1. cAMP-DEPENDENT TRANSCRIPTION.

Many studies now exist which suggest that the cAMP effect is mediated at the level of the gene by cAMP responsive elements (CREs) within promoters (refer to reviews by Roesler et al., 1988 and Ziff, 1990). The CRE is the binding site for a CRE-binding phosphoprotein (CREB, MW 43 kDa) present in cells and tissues responsive to cAMP (Montminy and Bilezikjian, 1987). Necessary for CREB activation is its phosphorylation by the catalytic subunit of pk A (Gonzalez and Montminy, 1989) which gets translocated to the nucleus (Nigg et al., 1985). The DNA binding domain of CREB contains an amphipathic α helix, typical of DNA binding proteins, critical for its trans-activity. It is likely that phosphorylation modifies the structure of the transactivation region of CREB allowing interaction with the transcriptional apparatus (Lin and Green, 1989; Gonzalez and Montminy, 1989). CREs are palindromic thus CREB likely binds as a dimer, formation of which may be similarly regulated by phosphorylation (Yamamoto et al., 1988). It remains to be determined whether this be a homo- or heterodimer.

B.2.2. cAMP ANALOGUES: ACTIVATORS OF PROTEIN KINASE A.

As with pk C, activators can be used to study the role played by pk A and pk A-dependent signalling pathways in the regulation of cellular events. cAMP itself added

externally will not readily cross the plasma membrane. cAMP analogues such as dibutyryl cAMP or 8-bromo-cAMP however are commonly used to artificially elevate the level of intracellular cAMP and in turn, activate pk A.

B.2.3. cAMP AND MYOGENESIS.

A role for cAMP has been implicated in myogenesis as revealed by the effect of increased intracellular cAMP levels on myoblast fusion. Agents which elevate intracellular cAMP concentrations either directly such as cAMP analogues (db-cAMP, 8-Br-cAMP) or indirectly by activating adenylate cyclase (forskolin, cholera toxin) have been shown to have an inhibitory effect on fusion of chick myoblasts and of a rat muscle cell line (Zalin, 1973; Wahrmann et al., 1973). It was demonstrated that there is a transient increase in cAMP levels prior to fusion in primary chick myoblasts and a corresponding increase in pk A, and that this increase was preceded by an increase in adenylate cyclase activity (Zalin and Montague, 1974). Similarily, an increase in pk A activity was found to occur during myogenesis in the rat myogenic cell line, L6 (Lorimer et al., 1987). It has been indicated however, that a rapid decrease in the level of cAMP immediately prior to fusion was necessary for the event to occur (Zalin and Montague, 1974), thus correct modulation of cAMP is critical for myogenic differentiation.

Biochemical differentiation has also been demonstrated to be affected by cAMP. Olson and collegues indicated that cAMP analogues, forskolin, cholera toxin, and prostaglandin E (which also activates adanylate cyclase) could reversibly inhibit MCK and Tn T protein expression following growth arrest in BC_3H1 cells (Hu and Olson, 1988). This effect was not due to continued cell cycling caused by cAMP, suggesting that cell cycle withdrawal is independent of cAMP. These researchers also showed that FGF and β-TGF, which are known to inhibit differentiation, did not increase intracellular cAMP levels, therefore they must operate through a cAMP-independent mechanism to inhibit BC_3H1 cell myogenesis (Hu and Olson, 1988). A cAMP induced block to differentiation of BC_3H1 cells and rat vascular smooth muscle cells has also been indicated (Wice et al., 1987; Kelvin et al., 1989a; Ohara et al., 1991).

Much controversy exists as to whether cAMP negatively regulates myogenic differentiation. It has been shown that increased cAMP levels in primary chick myoblasts lead to AChR induction (Betz and Changeaux, 1979; Blosser and Appel, 1980) as well as increased cell fusion in primary chick and rat myoblasts (Curtis and Zalin, 1981; Stygall and Mirsky, 1980).

Previous studies on the effects of cAMP on myogenic

differentiation have used only a few muscle-specific markers including enzymatic (MCK), surface proteins (AChR), and cytoskeltal proteins. In light of such conflicting results on the effects cAMP has on myogenic differentiation, it should prove interesting to determine how completely cAMP affects the coordinate regulation of cytoskeletal proteins in the nonfusing cell line, BC_3H1.

C. ADENOVIRUS EARLY REGION 1A TRANSFORMING PROTEIN AND MYOGENESIS.

Oncogenes, both cellular and viral, can influence transcription factor activity at the level of signal transduction or may themselves act as transcription factors. The DNA virus, adenovirus, has been used extensively to study transformation and eukaryotic gene regulation. The adenovirus family is subdivided into five groups (A, B, C, D, and E) based on oncogenic ability and characteristics such as G-C content of DNA and sequence homology (Pin and Green, 1965; Green et al., 1979). Group C adenoviruses include type 5 adenoviruses. E1A is the first region of the viral genome to be expressed, generating two major differentially spliced mRNAs with sedimentation coefficients of 12S and 13S, coding for a 243 amino acid and a 289 amino acid nuclear phosphoprotein, respectively. These proteins

differ only in an internal sequence unique to the 289 amino acid protein. The 289 amino acid protein coordinately activates transcription of other early region genes, as well as some cellular genes (reviewed in Flint and Shenk, 1989).

C.1. ADENOVIRUS E1A-DEPENDENT TRANSCRIPTION.

Transcriptional transactivation of adenovirus early region promoters by E1A has been shown to occur via the cellular activating transcription factor (ATF, MW 45 kDa) (Lee et al., 1987). Multiple ATF binding sites confer E1A inducibility on heterologous promoters (Lee and Green, 1987). A surprising finding was that the ATF binding consensus sequence is virtually identical to CREs and ATF is capable of binding to both adenovirus early gene transcriptional control regions and CREs of cellular genes (Lin and Green, 1988; Hardy and Shenk, 1988). Studies on recombinants between ATF sites of E2A or E3 promoters and chloramphenicol acetyltransferase (CAT) gene in PC12 cells demonstrated that these ATF sites could confer cAMP inducibility on the heterologous gene in a pk A-dependent manner (Sassone-Corsi, 1988). It is speculated that ATF is equivalent to CREB based on DNA sequence specificity and molecular weight (Lin and Green, 1988). As with CREB, pk A may activate ATF by phosphorylation. Like CREB, ATF has a consensus pk A phosphorylation sequence (Gonzalez et al.,

1989; Maekana et al., 1989). E1A may converge on this pathway by similarly activating a cascade of events which leads ultimately to the phosphorylation of ATF to activate it, circumventing the need for pk A (Sassone-Corsi, 1988). E1A, however, has not been shown to affect the DNA binding activity of ATF; it may modify ATF in such a way as to allow interaction with another factor necessary for transcription (Lee et al., 1987).

Evidence also suggests that E1A and cAMP operate through different pathways. Leza and Hearing showed independent induction of the Ad E4 promoter by cAMP and E1A, uncoupling E1A transactivation from the cAMP response (Leza and Hearing, 1989). Also, Lee et al. (1989) demonstrated that the Ad E4 promoter and the human vasoactive intestinal polypeptide (VIP) promoter, which both contain an ATF consensus sequence, respond differently to E1A and cAMP: the E4 promoter was not cAMP responsive in HeLa cells, while the VIP promoter was not E1A responsive. This though may be explained in terms of different activation potentials in context of different enhancer elements rather than alternate pathways (Lee et al., 1989). Engel et al. presented results which indicated that cAMP and E1A synergize to activate E1A inducible gene transcription, suggesting alternate pathways (Engel et al., 1988). E1A may thus directly or indirectly interact with a cAMP signalling pathway or just share a

transcription factor activated by alternate pathways (Hardy and Shenk, 1988).

C.2. ADENOVIRUS E1A AND MYOGENESIS.

Adenovirus E1A has recently been used to study the molecular events associated with myogenic transcriptional activation. Webster et al., studying L8 and C2 myoblasts demonstrated that transfection of the E1A gene into these cells could block the myogenic program. Also, skeletal or cardiac α-actin promoter-directed developmentally regulated expression of a heterologous reporter gene (CAT) was inhibited in the presence of E1A (Webster et al., 1988). Similarly, Enkemann and colleagues, using a CAT expression system, demonstrated that E1A could inhibit expression of the heterologous gene driven by various muscle specific promoters and enhancers (Enkemann et al., 1990). However, in both reports, a general non-specific inhibition of transcription could not be ruled out to explain the effects. Non-muscle enhancer driven CAT expression was similarly repressed in the presence of E1A: Enkemann failed to indicate a reporter construct that was not repressed in the presence of E1A.

D. OBJECTIVES OF THE PRESENT STUDY.

1. Much variation has been reported as to the effects

of TPA and a role of pk C-dependent signal transduction on myogenesis and its suggested involvement in myoblast fusion. It was thus of interest in this study to investigate the role of pk C on myogenic regulation in the non-fusing muscle cell line, BC_3H1. TPA was used as an activator of pk C.

2. In light of the conflicting results on the effects of cAMP and the involvement of a pk A-dependent pathway of signal transduction in myogenic differentiation, it was of interest to determine how completely cAMP effects the coordinate regulation of cytoskeletal proteins in BC_3H1 cells. The cAMP analogues db-cAMP and 8-Br-cAMP were used to elevate intracellular cAMP levels and activate pk A.

3. Given the similarity by which cAMP and adenovirus E1A mediate cellular gene transcription, the effect of E1A on myogenic differentiation was examined in BC_3H1 cells.

II. MATERIALS AND METHODS

A. MATERIALS.

All biochemicals used were of the highest quality available and unless indicated, were obtained from a major supply house.

B. CELL CULTURE.

The BC_3H1 cell line, obtained from American Type Culture Collection (Rockville, MD), was maintained at logarithmic growth in Dulbeco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS)(Gibco), penicillin (100U/ml) (Sigma) and streptomycin (0.01mg/ml) (Sigma) in 5% CO_2, 95% air atmosphere at 37°C. Cells were plated at a density of 100,000 cells/60mm plate or 50,000 cells/35mm plate and media was changed every third day.

BC_3H1 cells were induced to differentiate by reducing the FCS from 20% to 0.5% at day 3 post-plating when the cells are logarithmically growing. This serves to synchronize myoblast differentiation. It was at this time that drugs were added to the cells to test their effects on this process. 12-O-tetra decanoyl-phorbol-13-acetate (TPA)(Calbiochem) was diluted in DMEM and added to the cells

to final concentrations of 0.01μM, 0.05μM, 0.1μM, 0.25μM and 0.5μM from a stock solution of 10μM TPA in 100% ethanol. Dibutyryl-cAMP (db-cAMP) (Boehringer Mannheim) or 8-bromo-cAMP (8br-cAMP) (Boehringer Mannheim) was diluted in DMEM from a 100mM stock solution in 1M HEPES and added to the cells to final concentrations of 1mM, 500μM, 250μM, 100μM, and 10μM in 1M HEPES.

The adenovirus 5 early region 1A transfected BC_3H1 cell line, BC_3E7, was developed in this lab by Dr. R.W.H. Lee (Mymryk et al., submitted) and was cultured as described above except FCS was reduced on day 4 post-plating.

C. [^{35}S]-METHIONINE LABELLING OF CULTURED CELLS.

Cells were grown on 35mm Falcon tissue culture plates as described above. At various times in culture, cells were labelled with [^{35}S]-methionine (50μCi/ml) (ICN or NEN Dupont cell labelling grade) in 1 mL methionine-free Medium 199 for 2 - 14 hours at 37°C. After labelling, cells were rinsed twice with cold phosphate buffered saline (PBS) (137mM NaCl, 2.6mM KCl, 8.3mM Na_2HPO_4, pH 7.2), collected in 200μl of lysis buffer containing 9.5 M urea and a pH range of 5-7 (O'Farrell, 1975) and stored at -20°C until all samples were collected.

D. TWO-DIMENSIONAL GEL ELECTROPHORESIS OF PROTEINS. (2D-

PAGE).

D.1. FIRST DIMENSION: ISOELECTRIC FOCUSING.

First dimension isoelectric focusing (IEF) gels were prepared essentially as described by O'Farrell (1975) using a BioRad Mini-Protein II 2-D system. The gels contained 4% acrylamide, 9M urea and 1% ampholines of pH range 5-7 (Biorad). A 30 mM NaOH cathode (-) buffer and a 10mM H_3PO_4 anode (+) buffer were used. The tube gels were prefocused at 200V for 10 minutes, 300V for 15 minutes, and 400V for 15 minutes. 20-25μl of cell lysate was loaded at the basic end of the gel using a Hamilton syringe as described in the BioRad protocol, and overlayed with 25μl of sample overlay buffer, ampholine range 5-7 (O'Farrell, 1975). Gels were electrophoresed at 500V for 10 minutes and then at 750V for 5 hours. After focusing, gels were frozen at -20°C directly in their tubes until the second dimension could be run.

D.2. SECOND DIMENSION: SDS-PAGE.

The second dimension was a discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE) system (Laemmli, 1970) with a resolution gel of 11% acrylamide, pH 8.8 and a 4.5% acrylamide stacking gel, pH 6.8. First dimension tube gels were thawed in their tubes, removed using a tube gel ejector provided by BioRad onto Parafilm, and then transferred onto the top of the second dimension

SDS PAGE gel and overlayed with SDS sample equilibration buffer (O'Farrell, 1975). Gels were equilibrated for 10 minutes. Molecular weight markers (Pharmacia) were loaded into a well created in the stacking gel by a comb provided by Pharmacia. Protein standards used as molecular weight markers were: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). A running buffer composed of 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, pH 8.8 was used. Gels were electrophoresed with no external cooling at 180V until the tracking dye had reached the bottom of the resolution gel, requiring an approximate time of 45 minutes. After electrophoresis, the gels were stained and fixed in 0.1% Coomassie blue in 50% methanol and 10% acetic acid, destained in 5% methanol and 7% acetic acid, transferred to Whatmann 3MM filter paper and dried under low vacuum at 80°C using a BioRad gel dryer for approximately 1 hour, and exposed to Kodak XAR film for autoradiography.

D.3. COMIGRATION OF COMMERCIALLY AVAILABLE PROTEINS.

Commercially available muscle-specific proteins were used to aid in the identification of muscle-specific proteins detected by two dimensional analysis of [^{35}S]-methionine labelled cellular proteins. Rabbit muscle actin,

bovine muscle tropomyosin, and bovine muscle myosin light chains (Sigma) were each brought to a concentration of 1mg/ml in water and stored at -20°C. Prior to gel electrophoresis, 5μg of each isoform (e.g. since there are 3 actin isoforms, 15μg would have been used) were then lyophilized and 20μl of lysis buffer was added. The samples were then either run alone or comigrated with cell samples on IEF gels as described above.

D.4. WESTERN BLOT ANALYSIS.

Western analysis was performed essentially as described by Towbin et al., 1979. [^{35}S]-methionine labelled cellular proteins were first separated by 2D-PAGE as described above. Gels were then soaked in equilbration buffer (25mM Tris, 192mM glycine, pH 8.3) for 45 minutes. Proteins were electrophoretically transfered to nitrocellulose filter (Schleicher and Schuell) in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, pH 8.3) overnight at 4°C at 300mA using a BioRad Trans Blot apparatus. The nitrocellulose membranes were stained for 15 minutes in Ponceau S solution (0.5% in 5% TCA) to ensure transfer of proteins, rinsed with water and destained in 1X PBS. Gels after transfer were also stained and fixed with Coomassie blue as described above to ensure transfer. The nitrocellulose membrane was pre-incubated in incubation

solution (0.9% NaCl, 10mM Tris-HCl, pH 7.4, 10% Carnation Instant Skim Milk powder) for 30 minutes at room temperature. The filter was incubated in the above solution containing the primary antibody (rabbit anti-mouse tropomyosin; ICN) at a dilution of 1:160 at room temperature for 1.5 hours followed by four 10 minute washes in 0.9% NaCl, 10mM Tris-HCl, pH 7.4, and 0.05% Tween 20. Then the filter was incubated with the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (BioRad) at a dilution of 1:3000 in incubation solution, for 30 minutes and washed as above. Fresh nitro blue tetrazolium (NBT; 0.03g) and 5-bromo-4-chloro-3-indolyl phoshate (BCIP; 0.015g) in 1ml N,N-dimethyl formamide (DMF) was added to 100ml of NBT-BCIP buffer, (100mM Tris, pH 9.5, 1mM $MgCl_2$). The colour reaction was initiated by incubating the filter in the NBT-BCIP solution at 37°C for about 15 minutes until colour had developed and then rinsed with water to terminate the reaction. Nitrocellulose filters were then exposed to Kodak XAR film for autoradiography.

E.RNA ANALYSIS.

All glassware used was autoclaved and baked at 180°C for 16-18 hours.

E.1. RNA ISOLATION.

Total cellular RNA was extracted and prepared from cells grown on 60mm Falcon tissue culture plates following the acid-guanidinium thiocyanate-phenol-chloroform method outlined by Chomczynski and Sacchi (1987).

E.2. NUCLEIC ACID QUANTITATION.

RNA was quantitated by examining the ultraviolet spectrum of samples at a 1:100 dilution in H_2O. The absorbance at 260 nm was used to calculate nucleic acid concentration, assuming that 1 O.D. unit is equivalent to 40μg/ml RNA (Maniatis et al., 1982). The ratio of $O.D._{260}/O.D._{280}$ was used to assess the purity of the sample: a pure RNA preparation has a ratio of 2.0.

E.3. PLASMID LABELLING.

DNA (50ng) to be labelled were heat denatured at 90°C for 2 minutes and immediately cooled on ice for 2 minutes. Plasmids were labelled with α-[^{32}P]-dCTP (NEN Dupont) (1μCi/μl) by using random oligonucleotide primer extension, following the protocol outlined by Pharmacia. Labelled DNA probes were heat denatured at 90°C for 2 minutes and immediately cooled on ice for 2 minutes prior to

use in hybridization.

E.4. NORTHERN ANALYSIS.

For Northern hybridization analysis, 6μg of RNA (prepared and quantitated as above) was denatured in a solution with final concentrations of 1X MOPS running buffer (20mM MOPS, pH 7; 0.8mM sodium acetate; 1mM EDTA, pH 8), 6.5% formaldehyde, and 50% formamide and incubating at 60°C for 15 minutes. 5μl of formaldehyde gel loading buffer (1mM EDTA, pH 8; 0.25% bromophenol blue; 0.25% xylene cyanol; 50% glycerol) was then added and RNA was size fractionated on a 1.2% agarose/formaldehyde gel (Ausubel et al., 1987). Electrophoresis was carried out in 1X MOPS running buffer at 140V for approximately 2 hours. To verify that equal amounts of RNA had been loaded per lane 1 μl of ethidium bromide (1mg/ml) was added to each RNA sample prior to electrophoresis. After electrophoresis, RNA was visualized using a short wave ultraviolet illuminator (Fotodyne, Inc.).

RNA was transferred by capillary blotting in 0.025M NaH_2PO_4 to Gene Screen hybridization transfer membrane (DuPont) for 18 - 20 hours and fixed to the membrane by baking at 80°C for 1.5 hours. To ensure complete transfer, the agarose gels were stained with ethidium bromide (0.5μg/ml) in H_20 and visualized under UV light.

After transfer, the membranes were prehybridized in

hybridization buffer (1% SDS, 1M NaCl and 10% dextran sulfate) at 65°C for 5 hours in heat sealable plastic bags. Hybridization was performed using fresh hybidization buffer with 1-2x10^6 cpm of α-[^{32}P]-phosphate-labelled DNA probes, added to the buffer prior to addition to the filter, at 65°C for 18-20 hours. Following hybridization, membranes were washed once at low stringency in 1X SSC (0.12M sodium chloride, 0.15M sodium citrate, pH7.0); 0.1% SDS at room temperature followed by two more high stringency washes in 0.5XSSC; 0.1%SDS for 15 minutes each at 65°C. The membranes were then wrapped in plastic and exposed to Kodak XAR film with intensification at -70°C.

E.5. PLASMID PROBES.

The plasmid probe used was pMHαA-1 (Gunning et. al, 1983) which contains the full lenghth human skeletal α-actin cDNA (obtained from L.Kedes,USC).

III. RESULTS.

A. BC_3H1 MYOGENIC DIFFERENTIATION.

Myogenic differentiation was examined morphologically and biochemically in BC_3H1 cells to elucidate the roles of protein kinase A- and C-dependent signal transduction pathways. In addition, the effect of adenovirus early region 1A on the myogenic differentiation program was also studied.

A.1. MORPHOLOGICAL DIFFERENTIATION.

Actively dividing logarithmic phase BC_3H1 cells appear fibroblast-like under phase contrast microscopy (Figure 3A, day 3). When induced to differentiate by decreasing serum from 20% to 0.5% at day 3 post-plating, the cells withdraw from the cell cycle (Patrick et. al, 1976), elongate and align themselves into streams although no fusion into myotubes occurs (Figure 3B, day 10-0.5% FCS). The alignment into parallel streams of cells upon differentiation is more visually evident if BC_3H1 cells are left in 20% FCS containing media and allowed to reach confluency and establish extensive cell-to-cell contacts

(Figure 3C, day 10-20% FCS).

A.2. BIOCHEMICAL DIFFERENTIATION.

BC_3H1 cells were induced to differentiate by reducing the serum to 0.5% from 20% at day 3 post-plating. The synthesis of muscle specific cytoskeletal proteins was examined by labelling cells with [^{35}S]-methionine and subsequently analyzing the labelled cellular proteins by two-dimensional gel electrophoresis. The protein pattern obtained from BC_3H1 cells 10 days post-plating can be seen in Figure 4. An ampholine range of 5 -7 proved sufficient to separate cytoskeletal proteins of interest. Several gels were run with highly reproducible patterns obtained. This pattern was similar to that presented by Garrels (Garrels, 1979). The areas A, B, and C outlined in Figure 4 indicate the regions where actin, tropomyosin, and myosin light chain 1 migrate, respectively. Actin was identified by its abundance and molecular weight as well as its commigration with commercially available rabbit muscle actin (Sigma) (data not shown). MLC 1 was identified by comigration with commercially available rabbit muscle MLCs (Sigma) (Figure 5A). A 12% acrylamide gel was used for the second dimension in order to detect both MLC 1 and 2. The amount of labelled

BC_3H1 cellular proteins was so low that it did not contribute to the Coomassie stained gel. It is interesting to note that while MLC 1 was detected in BC_3H1 cells, no MLC 2 was detected here. Tm was identified by comigration with bovine muscle tropomyosin (Sigma) (Figure 5B). The two spots present in the Coomassie stain represent muscle-specific α (lower) and β (upper) Tm. From this it is speculated that the Tm isoforms labelled Tm 3 and 5 in the autoradiogram represent β and α Tm, respectively. As well, Western analysis using a rabbit anti-mouse tropomyosin polyclonal antibody (ICN) identified five Tm isoforms in our gel system (Figure 5C). These have been labelled Tm 1-5. The weak cross reaction observed may be due to the species difference between the antibody and the cell line.

Having established that the contractile proteins actin, MLC 1, and Tm can be resolved on 2D-PAGEs, this technique was used to study BC_3H1 cell myogenic differentiation.

Other major muscle specific contractile proteins were not studied. Troponin T (Tn T) and I (Tn I) have basic pIs and therefore did not focus within the pH range of isoelectric focusing (Devlin and Emerson, 1978; Endo and Nadel-Ginard, 1987), Troponin C (TnC) is of a low molecular weight not resolved on these gels, and myosin heavy chain.

(MHC) can not be detected on 2D-PAGE because it stays at the point of application during focusing (Shani et.al, 1981). Due to the low molecular weights of myosin light chains 2 and 3 (MLC 2, MLC 3) these were not resolved on the gel system used.

A.2.1. ACTIN ISOFORM SWITCHING.

In logarithmic (day 3 post-plating) BC_3H1 cells the non-muscle β- and γ-actins were synthesized at high levels (Figure 6). By day 10 post-plating, α-actin became the predominant isoform while β- and γ-actin synthesis decreased.

A.2.2. MYOSIN LIGHT CHAIN 1 EXPRESSION.

It can also be seen that logarithmic, undifferentiated BC_3H1 cells did not synthesize the muscle specific marker, myosin light chain 1 (MLC 1) (indicated by the arrowhead in Figure 7). The protein spot to the right served as a point of reference. Following serum starvation MLC 1 synthesis could be detected by day 6 post-plating.

A.2.3. TROPOMYOSIN ISOFORM SWITCHING.

As seen in Figure 8, only isoforms 1 and 5 were synthesized in undifferentiated BC_3H1 cells. As the cells differentiated in response to reduced serum, Tm 1 was no longer produced by day 8 and Tm 2, 3, and 4 synthesis was detected. Tm 5 synthesis was not affected by the

differentiation state.

A.2.4. ACTIN ISOFORM MESSAGE SWITCHING.

An examination of actin message levels revealed a pattern of expression which parallels that of the protein during differentiation. Northern analysis of total cellular RNA was performed using the probe pMHαA-1 which contains a full length α-actin cDNA (Gunning et. al, 1983). Enough homology exists between the α-, β- and γ-actin messages in the coding region that pMHαA-1 will cross-hybridize to all three messages. β- and γ-actin mRNAs are both 2.1 kb (Minty et al., 1981), while α-actin mRNA is 1.5 kb (Strauch et al., 1986). This size difference allows for the separation of muscle and nonmuscle actin messages and thus isoform switching can be easily observed. As seen in Figure 9A, prior to the switch from serum containing 20% FCS to one with 0.5% FCS, confluent BC_3H1 cells at day 4 did not express significant levels of α-actin mRNA but did express β- and γ-actin mRNAs. After serum reduction at day 3, β- and γ-actin message levels decreased while α-actin message levels increased through to day 10 post-plating.

Figure 3. BC_3H1 cell morphology. Cells were plated at a density of 10^5 cells per 60mm plate. (A) Logarithmic cells in 20% FCS containing media. (B) Post-confluent cells (day 10 post-plating) in 0.5% FCS containing media. Cells were induced to differentiate by decreasing FCS to 0.5% on day 3 post-plating. (C) Post-confluent cells (day 10 post-plating) in media containing 20% FCS. Phase contrast optics were used. Magnification: 1000X.

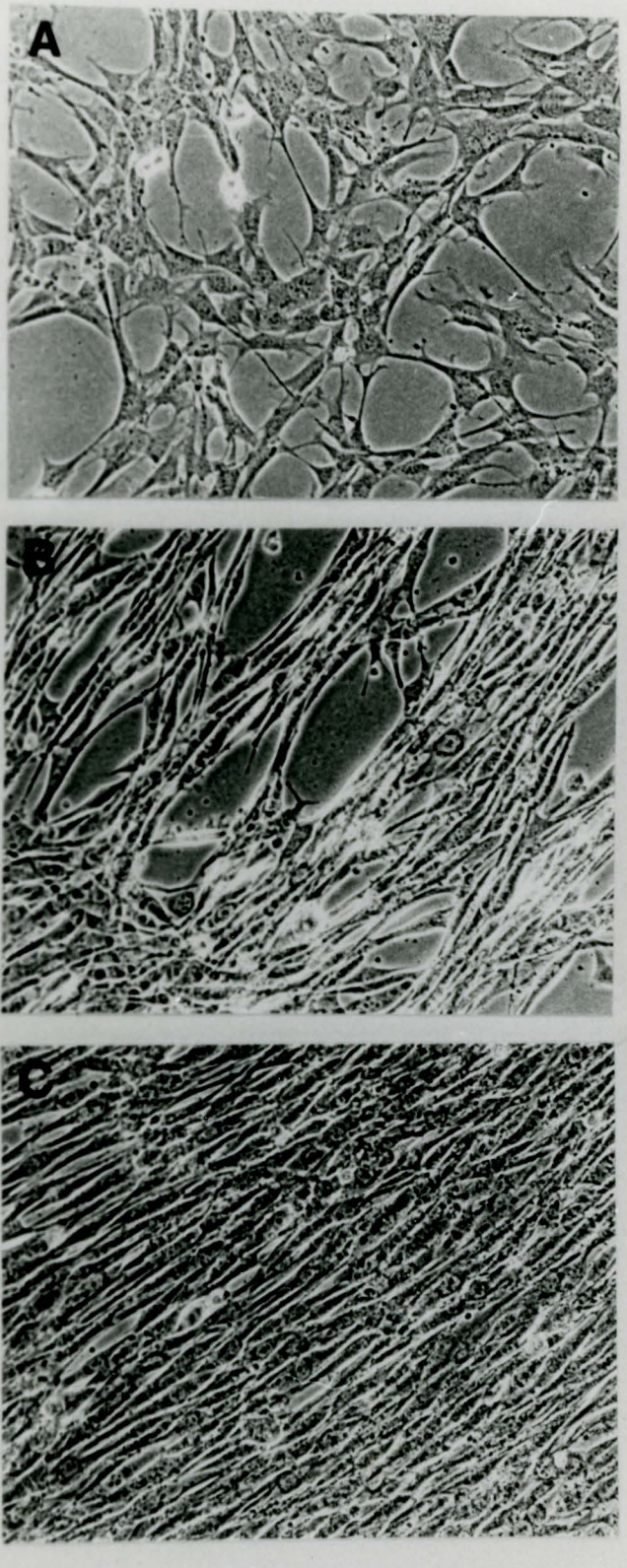

A
B
C

Figure 4. De novo protein synthesis in BC_3H1 cells. Proteins from [^{35}S]-methionine labelled cells were separated by 2D-PAGE and visualized by autoradiography. The isoelectric focusing direction (shown) is from left (basic) to right (acidic). Migration positions of molecular weight markers are indicated to the left. The regions A, B, and C outlined indicate where actin, Tm and MLC 1 migrate, respectively.

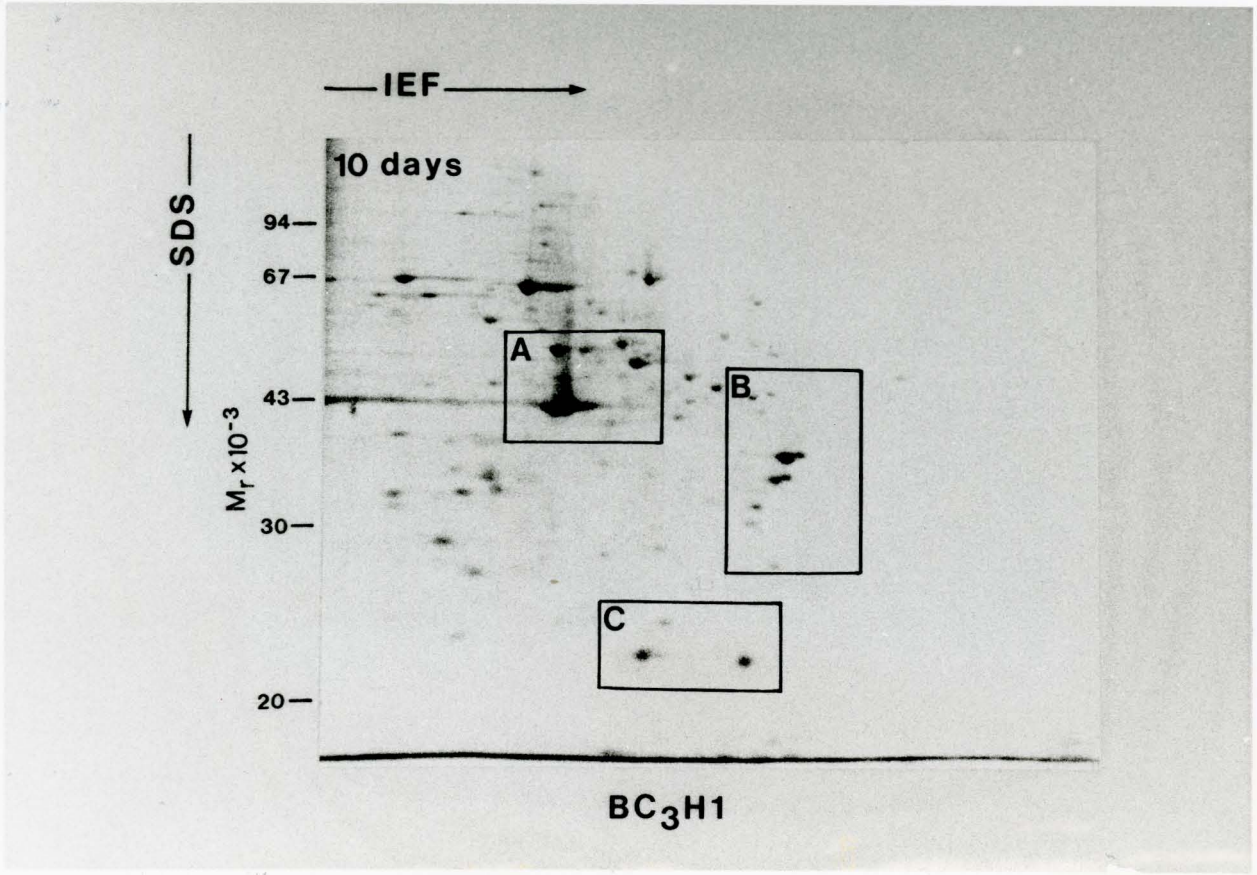
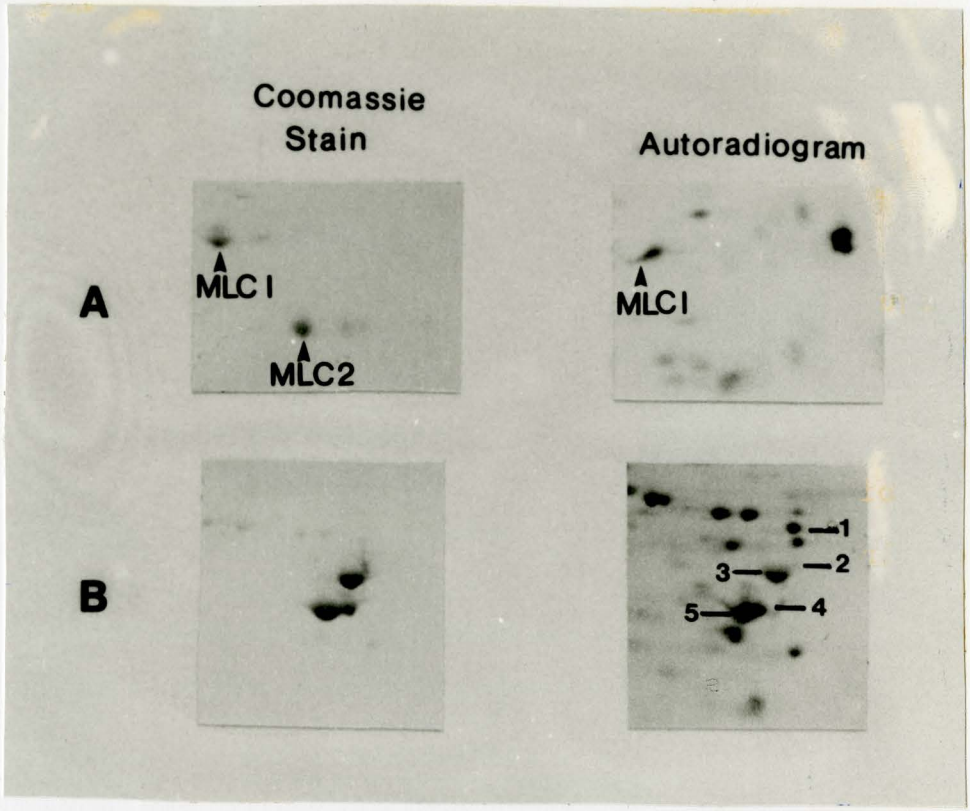

IEF
SDS
10 days
94
67
43
30
20
$M_r \times 10^{-3}$
A
B
C
BC_3H1

Figure 5. Identification of Tm and MLC 1. Commercially available muscle-specific proteins (Sigma) were comigrated with BC_3H1 [S^{35}]-methionine labelled cellular proteins. **(A)** Rabbit muscle myosin light chain standards (Coomassie staining) comigrated with the spot indicated on the autoradiogram of labelled proteins from cells day 10 post-plating. These gels were of 12% acrylamide in order to visualize MLC 2 standards. **(B)** Bovine muscle Tm standards (Coomassie staining) comigrated with spots 3 and 5 indicated on the autoradiogram of labelled proteins from cell day 6 post-plating. **(C)** Rabbit anti-mouse Tm polyclonal antibody (ICN) was used for Western analysis of Tm isoforms in BC_3H1 cells. Only the Tm region of the filter is shown here. Results for cells 10 days post-plating are shown. The focusing direction is from right to left due to the method employed in transfer. The Tm isoforms are numbered 1 to 5.


Coomassie
Stain
Autoradiogram
A
MLC I
MLC2
MLC1
B
1
2
3
4
5

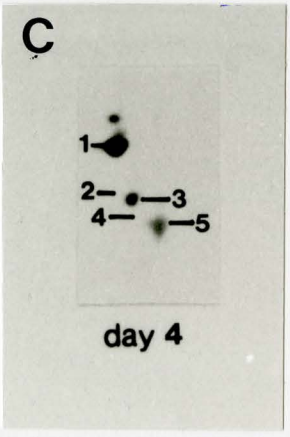

C
1
2
3
4
5
day 4

Figure 6. Actin isoform switching. BC_3H1 cells in the absence (control) and presence (TPA) of 0.1μM TPA in reduced serum at the indicated days post-plating were labelled with [^{35}S]-methionine and the proteins analyzed by 2D-PAGE followed by autoradiography. Region A from Figure 4 is shown here. The three arrowheads point to the positions of the three actin isoforms γ, β and α, from left to right, as indicated in the panel showing BC_3H1 cells day 3 post-plating.

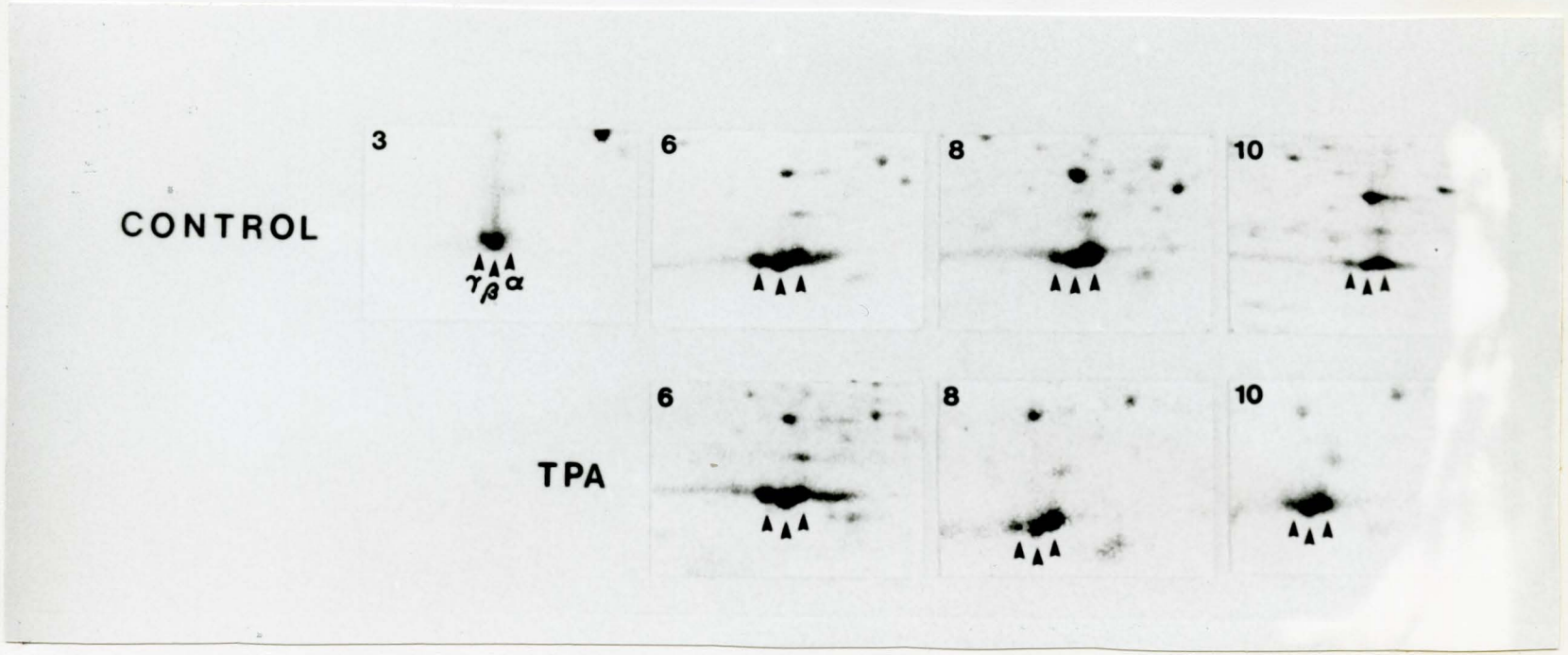

CONTROL
TPA
3
6
8
10
6
8
10
γ
β
α

Figure 7. MLC 1 expression. Region C from autoradiograms obtained as in Figure 7 are shown for BC_3H1 cells in the absence (**control**) or presence (**TPA**) of 0.1μM TPA in reduced serum. The arrowhead in each panel indicates the migration of MLC 1. An unidentified protein at the right was used as a marker and whose expression was unaffected by the differentiation state or TPA.

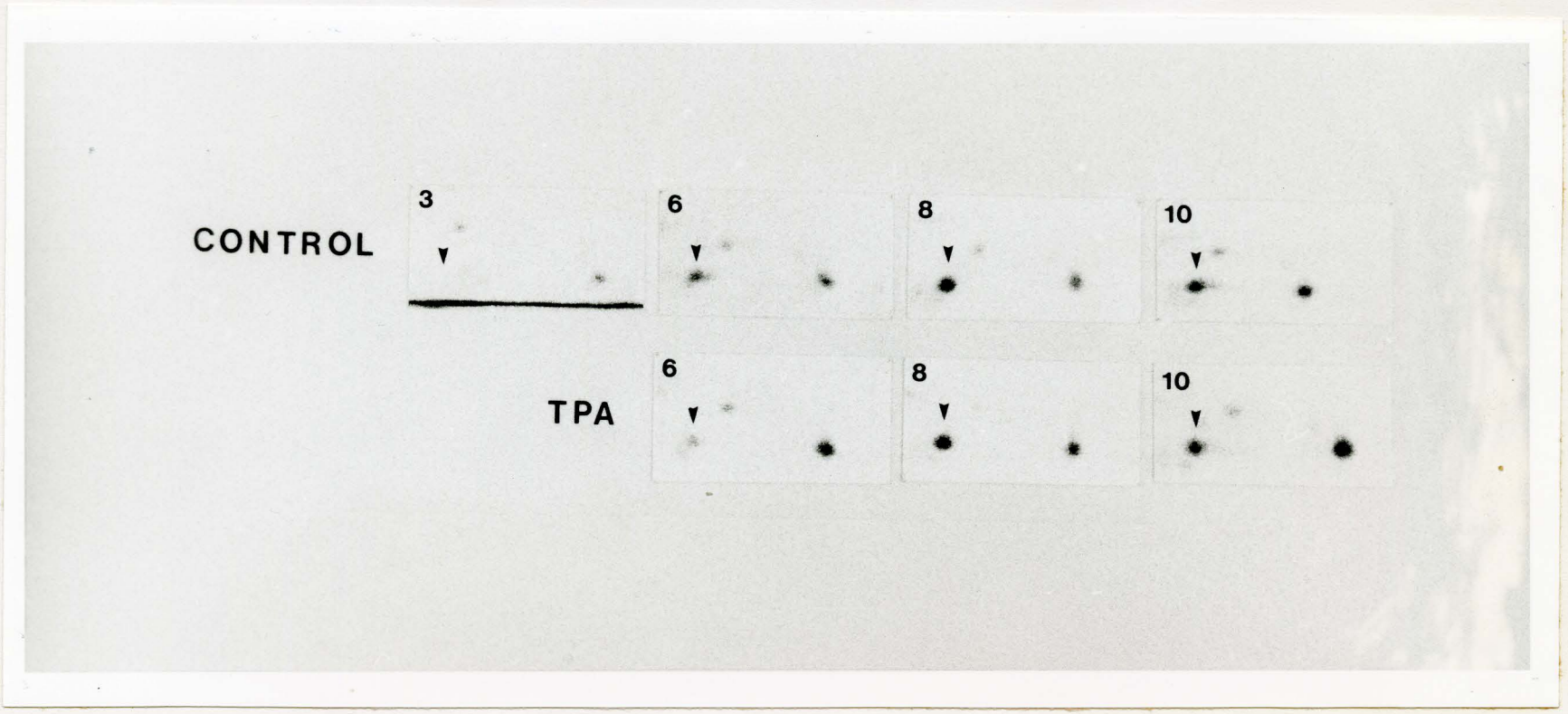

CONTROL
3
6
8
10
TPA
6
8
10

Figure 8. Tm isoform switching. Region B from autoradiograms obtained as in Figure 7 are shown. The five Tm isoforms are numbered 1 to 5. BC_3H1 cells in the absence (**control**) and presence (**TPA**) of 0.1μM TPA in media containing 0.5% FCS are shown.

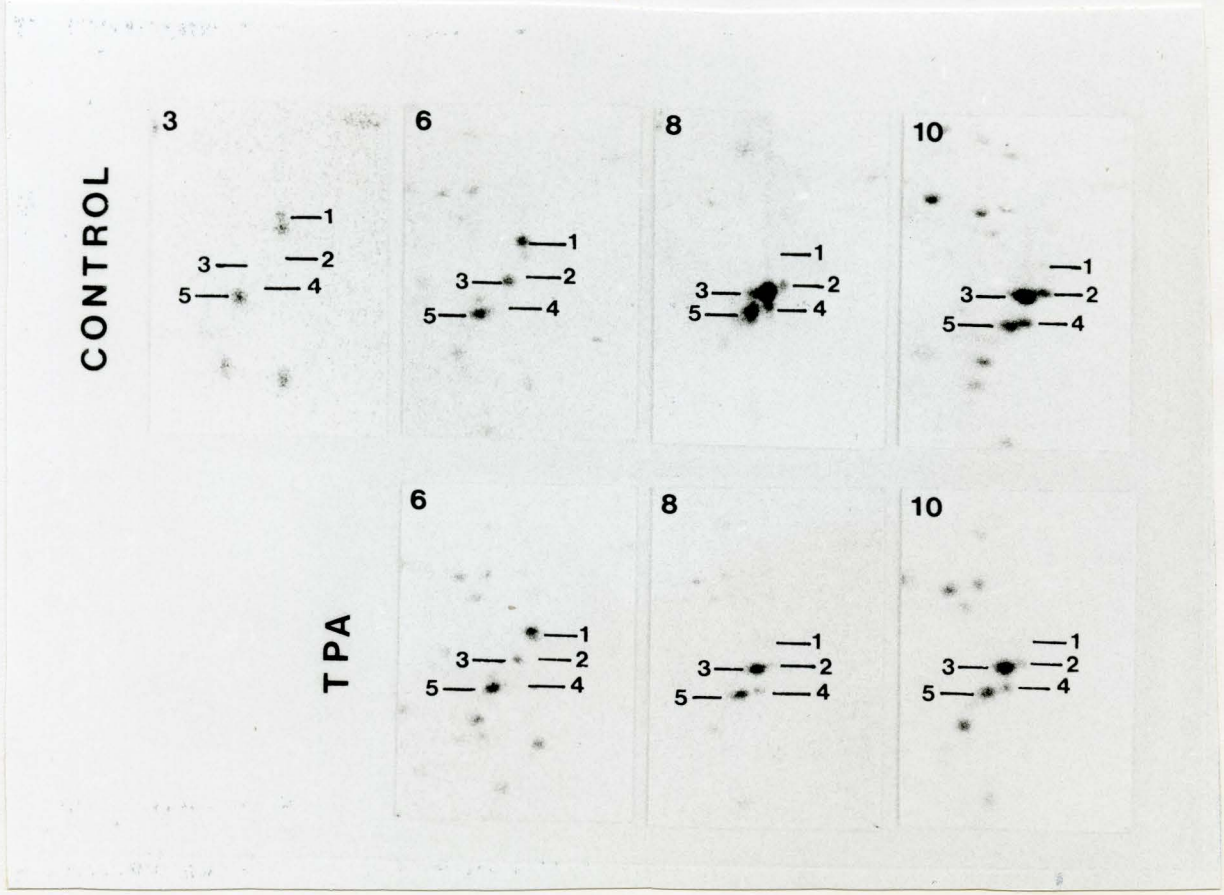
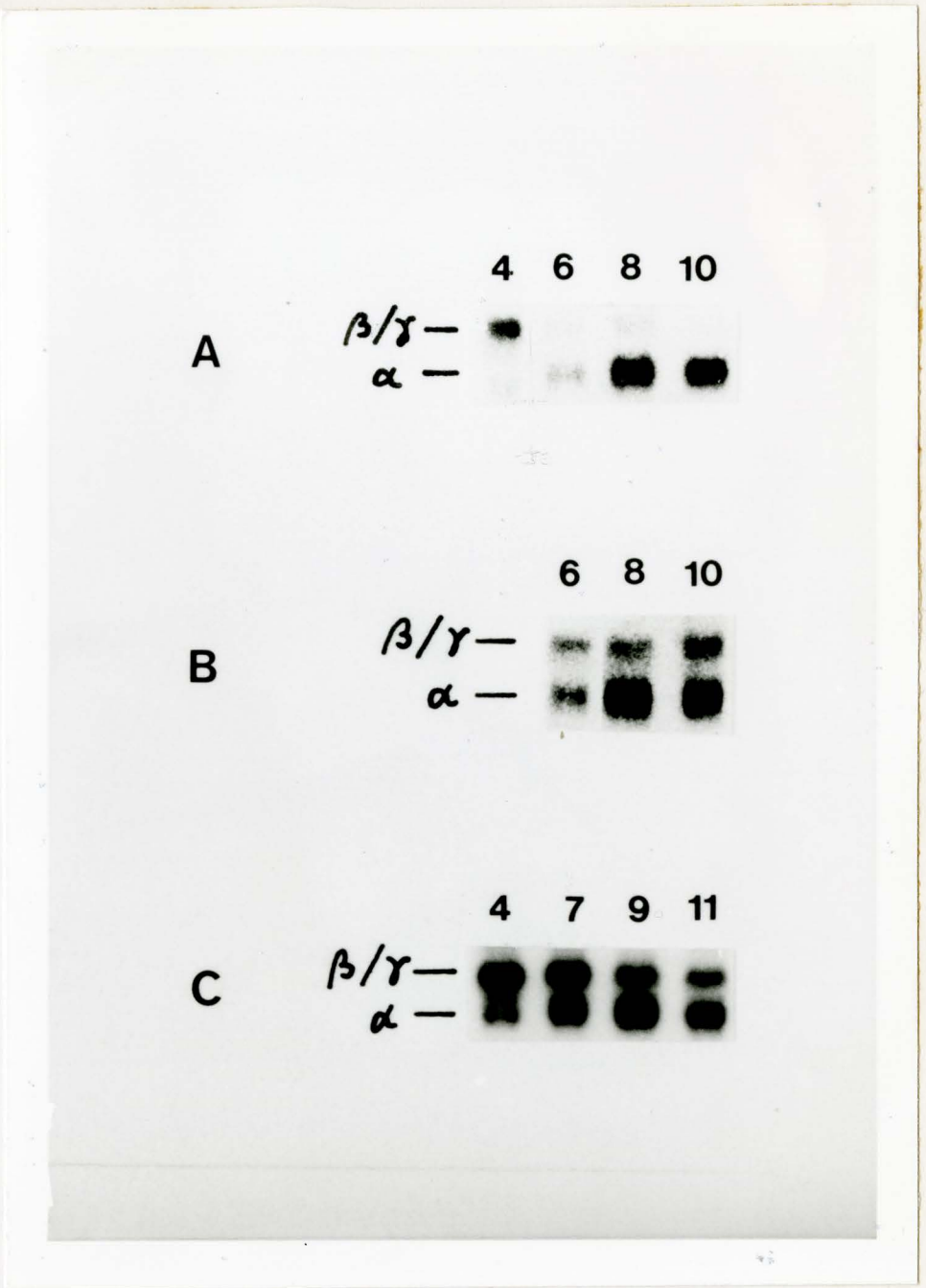

CONTROL
TPA
3
6
8
10
1
2
3
4
5

Figure 9. Actin isoform message switching. RNA was extracted from BC_3H1 cells grown in reduced serum on the indicated days post-plating and analyzed by Northern hybridization using the full length α-actin cDNA, pMHαA-1, as probe. The position of the migration of the non-muscle β- and γ-actin mRNAs which run as a single band (2100 bp) and the muscle-specific α-actin mRNA (1500 bp) are indicated to the left. (A) Actin isoform mRNA expression in BC_3H1 cells. (B) Actin isoform mRNA expression in the presence of cAMP. Cells were treated with 500μM 8-Br-cAMP in serum containing 0.5% FCS on day 3 post-plating. (C) Actin isoform mRNA expression in BC_3E7 cells.


A
4 6 8 10
β/γ —
α —
B
6 8 10
β/γ —
α —
C
4 7 9 11
β/γ —
α —

B. TPA HAS NO EFFECT ON BC_3H1 CELL DIFFERENTIATION.

The phorbol ester, TPA, was added to the cells as an activator of pk C in order to assess the role of pk C-dependent signal transduction in regulating BC_3H1 myogenic differentiation. The drug was added at day 3 post-plating at various concentrations (0.01μM, 0.05μM, 0.1μM, 0.25μM, 0.5μM). These concentrations are consistent with those used by other researchers to activate pk C (Mufson, 1985; Cossu et al., 1982; Chida et al., 1986; Adamo et al., 1989). The results presented here are for a TPA concentration of 0.1μM, although similar results were obtained for all concentrations tested.

B.1. NO EFFECT OF TPA ON MORPHOLOGICAL DIFFERENTIATION.

As can be seen in Figure 10, BC_3H1 cell cultures treated with TPA in media containing 0.5% FCS appeared no different under phase contrast optics morphologically than the untreated cells. TPA treated cells (day 10) exhibited the characteristic elongation and alignment into parallel streams of differentiated BC_3H1 cells (day 10).

B.2. NO EFFECT OF TPA ON BIOCHEMICAL DIFFERENTIATION.

TPA treated BC_3H1 cells in low serum media were [^{35}S]-methionine labelled and the labelled proteins extracted and analyzed by 2-dimensional gel electrophoresis.

B.2.1. ACTIN ISOFORM SWITCHING.

By day 10 post-plating in starvation media in the presence of TPA, muscle-specific α-actin was produced while β- and γ-actin isoforms were down-regulated, typical of untreated cells (Figure 6).

B.2.2. MYOSIN LIGHT CHAIN 1 PROTEIN PROFILE.

MLC 1 expression could be detected by day 6 in TPA treated BC_3H1 cells with kinetics similar to untreated cells (Figure 7).

B.2.3. TROPOMYOSIN ISOFORM SWITCHING.

Tropomyosin expression was also unaltered by TPA treatment (Figure 8). By day 10 post-plating, TPA treated cells in 0.5% FCS containing media exhibited the characteristic Tm shift as revealed by 2D gel electrophoresis of [^{35}S]-methionine labelled proteins: Tm1 expression decreased while Tm 2, 3, and 4 increased.

B.2.4. ACTIN ISOFORM mRNA SWITCHING.

Northern analysis, using as probe the plasmid pMHαA-1, of actin messages from TPA treated BC_3H1 cells revealed

no deviation from the characteristic shift in actin isoform expression: α-actin mRNA levels increased with a concomitant decrease in β- and γ-actin mRNA levels by day 10 post-plating (Figure 11).

Figure 10. Effect of TPA on the morphology of BC_3H1 cells. BC_3H1 cells were switched to low serum media and treated with 0.1μM TPA 3 days post-plating and maintained in the presence of TPA up to day 10 post-plating. **(A)** untreated BC_3H1 cells, day 10 post-plating. **(B)** BC_3H1 cells plus TPA, day 10 post-plating. Cells were plated at a density of 10^5 cells per plate. The micrographs were taken using phase contrast optics. Magnification: 1000X.

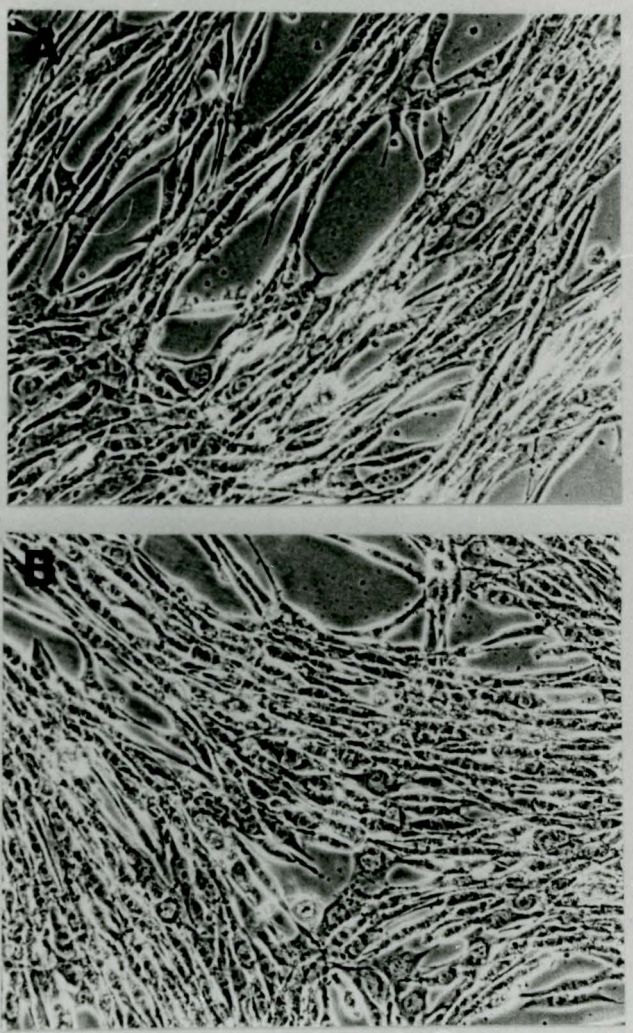
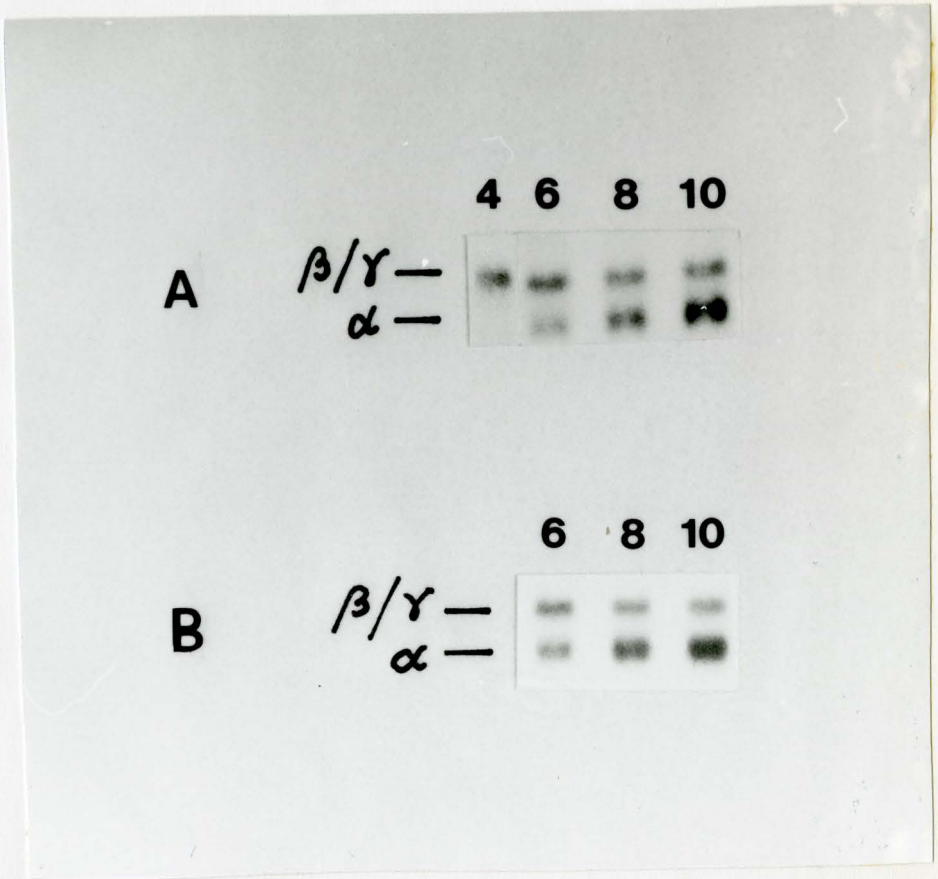

A
B

Figure 11. Effect of TPA on actin isoform mRNA switching. RNA from BC_3H1 cells treated at day 3 post-plating with 0.1μM TPA in low serum media was analyzed as outlined in Figure 9. **(A) Control no drug. (B) TPA addition.**


4 6 8 10
A
β/γ —
α —
6 8 10
B
β/γ —
α —

C. cAMP ANALOGUES DISRUPTED BC_3H1 CELL DIFFERENTIATION.

The cAMP analogues dibutyryl cAMP and 8-bromo-cAMP were added separately to BC_3H1 cells at day 3 post-plating at various concentrations (1mM, 500μM, 250μM, 100μM, 10μM) in the presence of 0.5% FCS containing media as activators of pk A, in order to assess the role of the pk A-dependent signal transduction pathway on BC_3H1 cell myogenic differentiation. The 500μM concentration is consistent with that used in other labs to study pk A activation and cAMP effects (Hu and Olson, 1988; Narindrasorasak et. al., 1986; Blosser and Appel, 1980). Both db-cAMP and 8-br-cAMP addition resulted in similar effects, however only 8-br-cAMP results at a 500μM concentration are presented here.

C.1. cAMP ADDITION ALTERED MORPHOLOGICAL DIFFERENTIATION.

cAMP treatment resulted in a drastic alteration in the morphology of BC_3H1 cells 10 days post-plating as revealed by phase contrast optics (Figure 12). Treated cells (12 C,D) did not show the characteristic alignment into parallel streams of differentiated cells (12A,B) as seen at day 10. Instead, cAMP treated cells were less refractile and had thin processes extending between cells, somewhat resembling neurite-like processes. In order to fully visualize the effect of cAMP on impairing the

alignment into parallel streams of cells, cells were left in media containing 20% FCS and allowed to reach confluency and establish extensive cell to cell contacts in the absence/presence of cAMP (12B, D).

C.2. cAMP ADDITION DISRUPTS BIOCHEMICAL DIFFERENTIATION.

C.2.1. ACTIN ISOFORM SWITCHING.

Two dimensional gel electrophoresis revealed that cAMP treated BC_3H1 cells were unable to up-regulate α-actin expression at the protein level, as can be seen in Figure 13. A delay in the synthesis of α-actin could be seen in treated cells at day 6 post-plating. Also apparent was a decreased down-regulation of β- and γ-actin protein synthesis in cAMP treated cells as compared to untreated. Both β- and γ-actin remain as prominent isoforms even at day 10 post-plating in treated cells.

C.2.2. MYOSIN LIGHT CHAIN 1 EXPRESSION.

An inhibition of MLC1 protein expression in cAMP-treated cells was also indicated by two dimensional (2D) gel electrophoresis (Figure 14). MLC 1 which was normally present by day 6 post-plating in untreated cells was not detectable and only faintly seen at day 10.

C.2.3. TROPOMYOSIN ISOFORM SWITCHING.

Tropomyosin expression was also disrupted by cAMP addition in BC_3H1 cells (Figure 15). During the period in which untreated cells differentiated and down-regulated Tm1 and up-regulated Tm2, 3, and 4, 2D analysis indicated an inhibition in the upregulation of Tm2, 3, and 4 in cAMP treated cells.

Various [^{35}S]-methionine labelling times were used (2 -14 hours). All labelling periods resulted in similar 2D profiles of the labelled proteins (data not shown).

C.2.4. ACTIN ISOFORM mRNA SWITCHING.

Northern analysis of actin messages revealed results which proved unexpected, as seen in Figure 9B. Although α-actin expression was altered at the protein level, no apparent effect was observed at the message level; α-actin mRNA levels increased in cAMP treated cells, typical of untreated cells. However, in accordance with the effect observed at the protein level for β- and γ-actins in cAMP treated cells, their message levels also showed no down-regulation through to day 10.

Figure 12. Effect of cAMP on the morphology of BC_3H1 cells. Cells were serum starved on day 3 post-plating and maintained in culture up to 10 days post-plating in the absence (A) or presence (C) of 500μM 8-Br-cAMP. As well, BC_3H1 cells were maintained in culture up to 10 days post-plating in media containing 20% FCS in the absence (B) or presence (D) of 500μM 8-Br-cAMP added on day 3 post-plating. Cells were plated at a density of 10^5 cells per 60mm dish. Phase contrast optics were used. Magnification: 1000X.

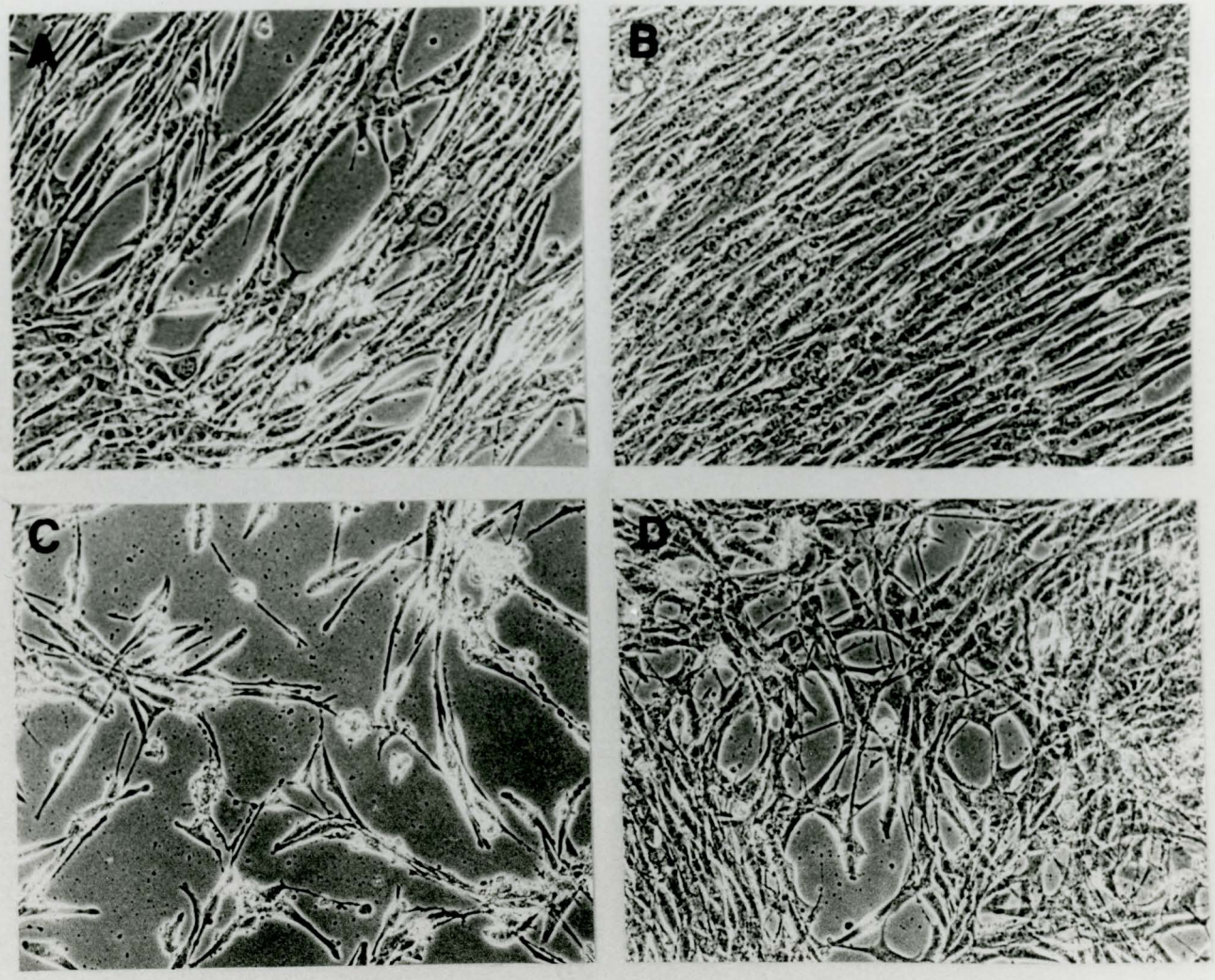

A
B
C
D

Figure 13. Effect of cAMP on actin isoform switching. Cells were treated with 500μM 8-Br-cAMP on day 3 post-plating in serum containing 0.5% FCS. Region A from autoradiograms obtained as described in Figure 6 is shown. Autoradiograms from untreated BC_3H1 cells are shown as control.

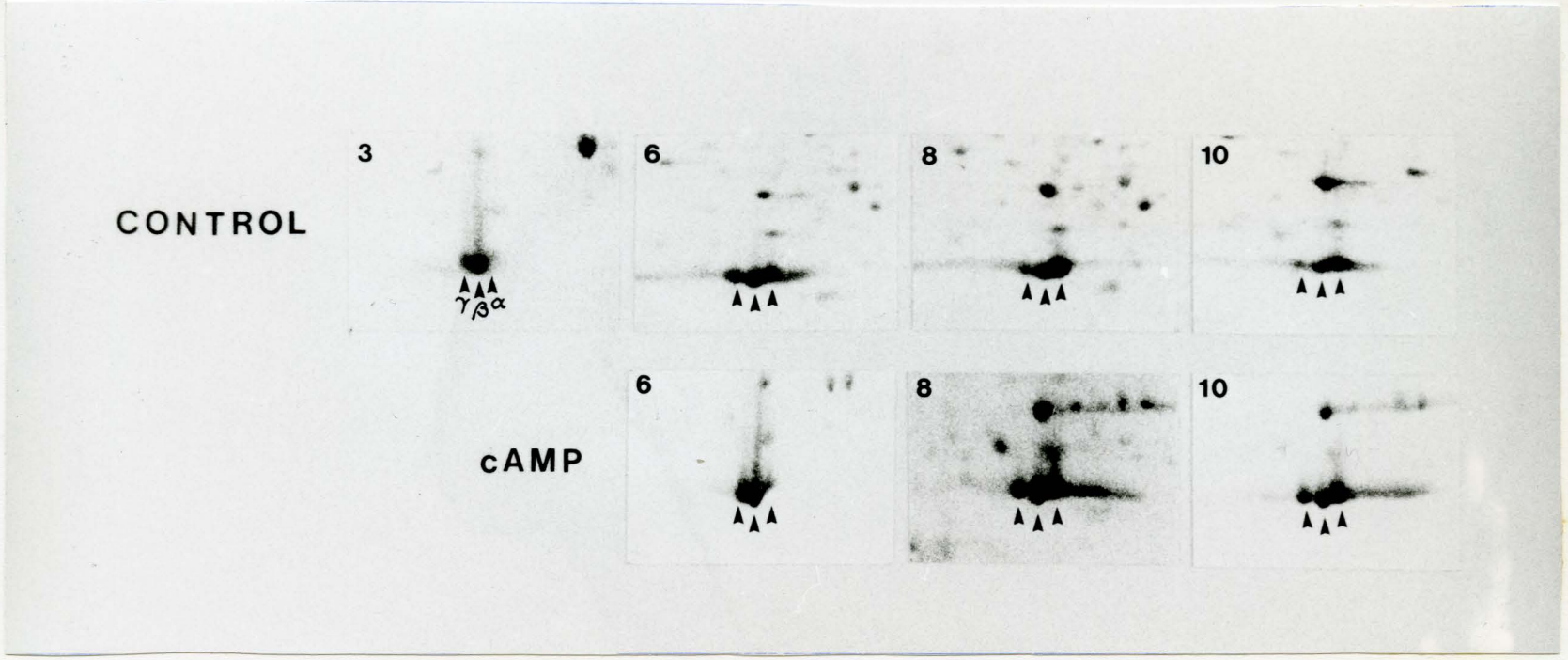

CONTROL
3
6
8
10
γβα
cAMP
6
8
10

Figure 14. MLC 1 expression: effect of cAMP. Region C from autoradiograms obtained as described in Figure 6 is shown. MLC 1 expression in BC_3H1 cells was examined in the absence (**control**) and presence (**cAMP**) of 500μM 8-Br-cAMP in low serum media.

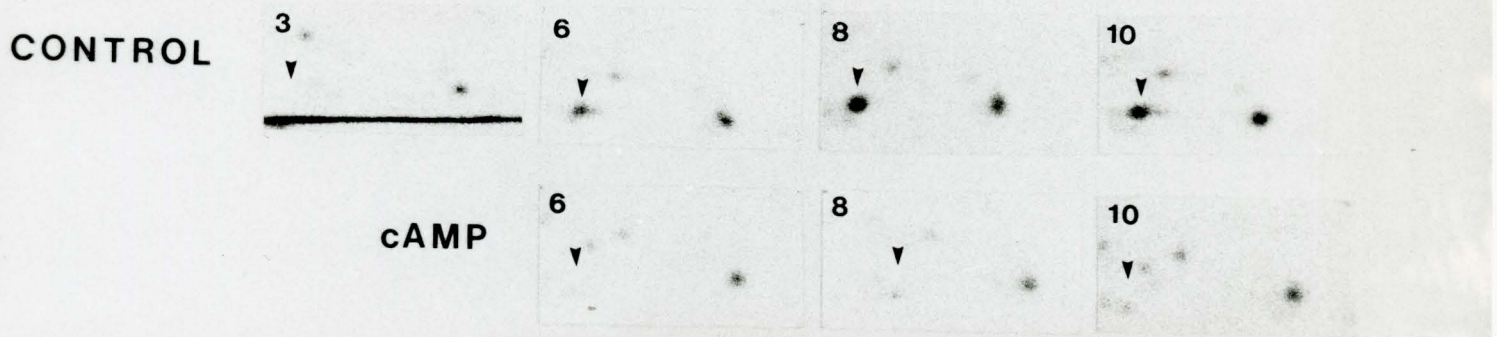
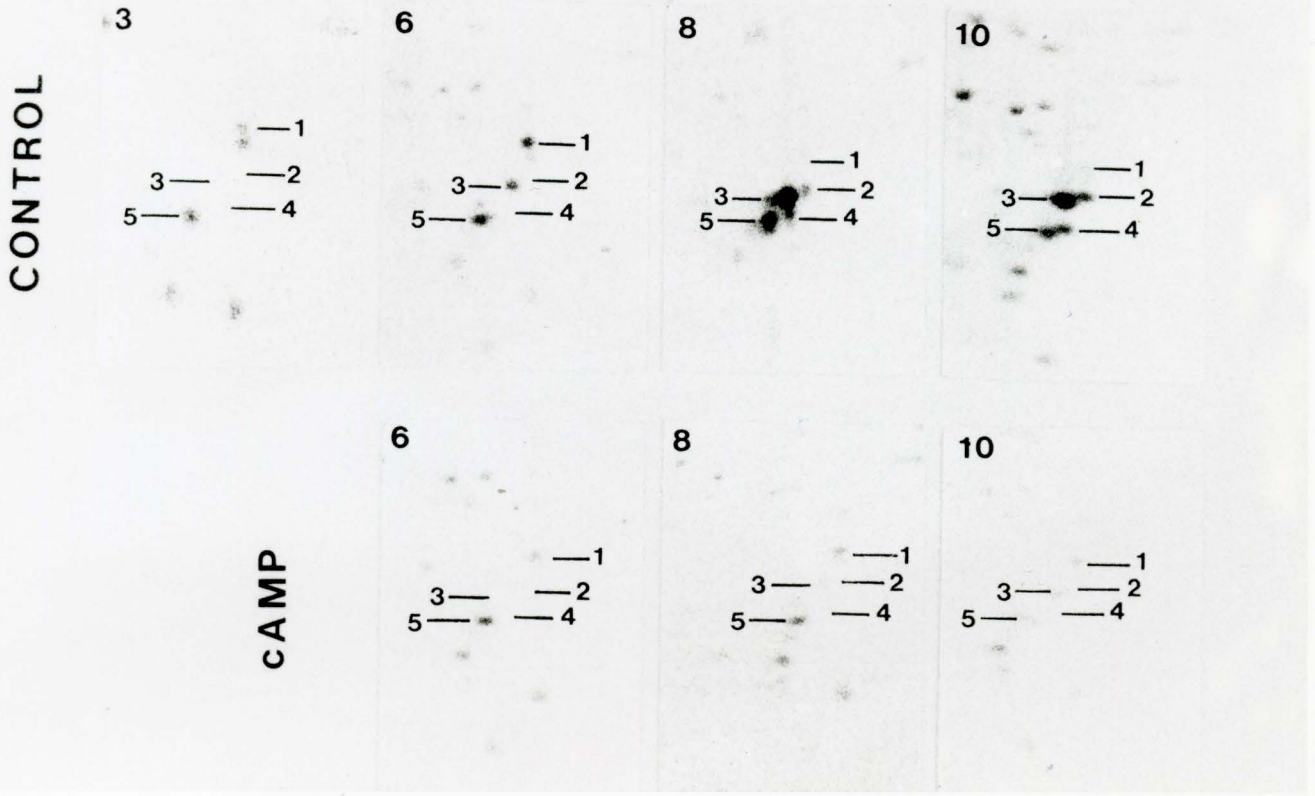

CONTROL
3
6
8
10
cAMP
6
8
10

Figure 15. Effect of cAMP on Tm isoform switching. BC_3H1 cells were treated with 500μM 8-Br-cAMP in low serum media on day 3 post-plating. Region B from autoradiograms obtained as described in Figure 6 is shown. Untreated BC_3H1 cells served as control.


CONTROL
cAMP
3
6
8
10
1
2
3
4
5

D. EFFECTS OF ADENOVIRUS 5 EARLY REGION 1A GENE TRANSFECTION ON BC_3H1 CELL DIFFERENTIATION.

The BC_3E7 cell line represents a clone of adenovirus 5 early region 1A (Ad5 E1A) transfected BC_3H1 cells developed in this lab by Dr.R.W.H. Lee (Mymryk et al., submitted).

D.1. BC_3E7 CELLS DO NOT MORPHOLOGICALLY DIFFERENTIATE.

At subconfluency, day 4, (Figure 16D), BC_3E7 cells were indistinguishable from subconfluent BC_3H1 cells, day 3 (Figure 18A). The differences in days serve to compensate for the decreased growth rate of BC_3E7 cells (Mymryk et al., submitted). BC_3E7 cells upon reaching confluency became contact inhibited and did not elongate as did BC_3H1 cells (Figure 16C) but rather appeared flattened (Figure 16F) when viewed by phase contrast microscopy. In an attempt to induce differentiation, BC_3E7 cells were serum starved in media containing 0.5% FCS at day 4 post-plating. The cells similarly failed to show the characteristic alignment and elongation at day 11 post-plating (Figure 16E).

D.2. BC_3E7 CELLS EXHIBIT ALTERED BIOCHEMICAL DIFFERENTIATION.

D.2.1. De Novo PROTEIN SYNTHESIS IN BC_3E7 CELLS.

De novo protein synthesis in BC_3E7 was examined cells by 2D gel electrophoresis of [^{35}S]-methionine labelled proteins as seen in Figure 17. The protein pattern obtained from BC_3E7 cells 11 days post-plating was compared with that of BC_3H1 cells 10 days post-plating (Figure 4). Both induction and repression of cellular proteins could be observed but were not studied further. Again, the areas outlined A, B, and C indicate regions in which the cytoskeletal proteins actin, tropomyosin and myosin light chain 1 migrate respectively, and were further investigated.

D.2.2. ACTIN ISOFORM SWITCHING IN BC_3E7 CELLS.

Two dimensional analysis of [^{35}S]-methionine labelled BC_3E7 proteins indicated that α-actin was expressed in proliferating (day 4) cells (Figure 18) and persisted through all days examined. Also, there was no down-regulation of the non-muscle β- and γ- actin isoforms once the cells reached confluency, through to day 11 post-plating.

D.2.3. MYOSIN LIGHT CHAIN 1 EXPRESSION IN BC_3E7 CELLS.

Examination of the MLC 1 regions of 2D gels of [^{35}S]-methionine labelled proteins from BC_3E7 cells revealed

that MLC 1 was induced in these cells upon confluency, however its appearance was delayed until day 9 at which point synthesis was detected but at lower levels (Figure 19).

D.2.4. TROPOMYOSIN ISOFORM SWITCHING IN BC_3E7 CELLS.

As seen in Figure 20, unlike Tm regulation in BC_3H1 cells, Tm 1 down-regulation was inhibited in BC_3E7 cells, its presence detected even at day 11. No effect however was observed on the regulation of the other Tm isoforms in BC_3E7 cells, which followed the shift in pattern characteristic of differentiated BC_3H1 cells.

D.2.5. ACTIN ISOFORM mRNA SWITCHING IN BC_3E7 CELLS.

Northern analysis was performed as decribed to examine actin transcript levels in subconfluent and post-confluent BC_3E7 cells (Figure 9C). In accordance with the protein results, α-actin message was present at high levels even in proliferating cells (day 4) and persisted through all days in culture examined (days 4-11). In addition, no down-regulation of the non-muscle β- and γ-actin isform messages was observed following confluency through to day 11.

Figure 16. BC_3E7 cell morphology. BC_3H1 cells (A, B, C) and BC_3E7 cells (D, E, F) were plated at a density of 10^5 cells per 60mm dish. Subconfluent proliferating cells were similar in appearance (A, D). Post-confluent, day 10 BC_3E7 cells in serum containing 20% FCS (F) did not elongate typical of day 10 BC_3H1 cells (C). In reduced serum, BC_3E7 cells at day 10 (E) also failed to show the characteristic elongation observed in BC_3H1 cells in low serum media at day 10 (B). The micrographs were taken using phase contrast optics. Magnification: 1000X.

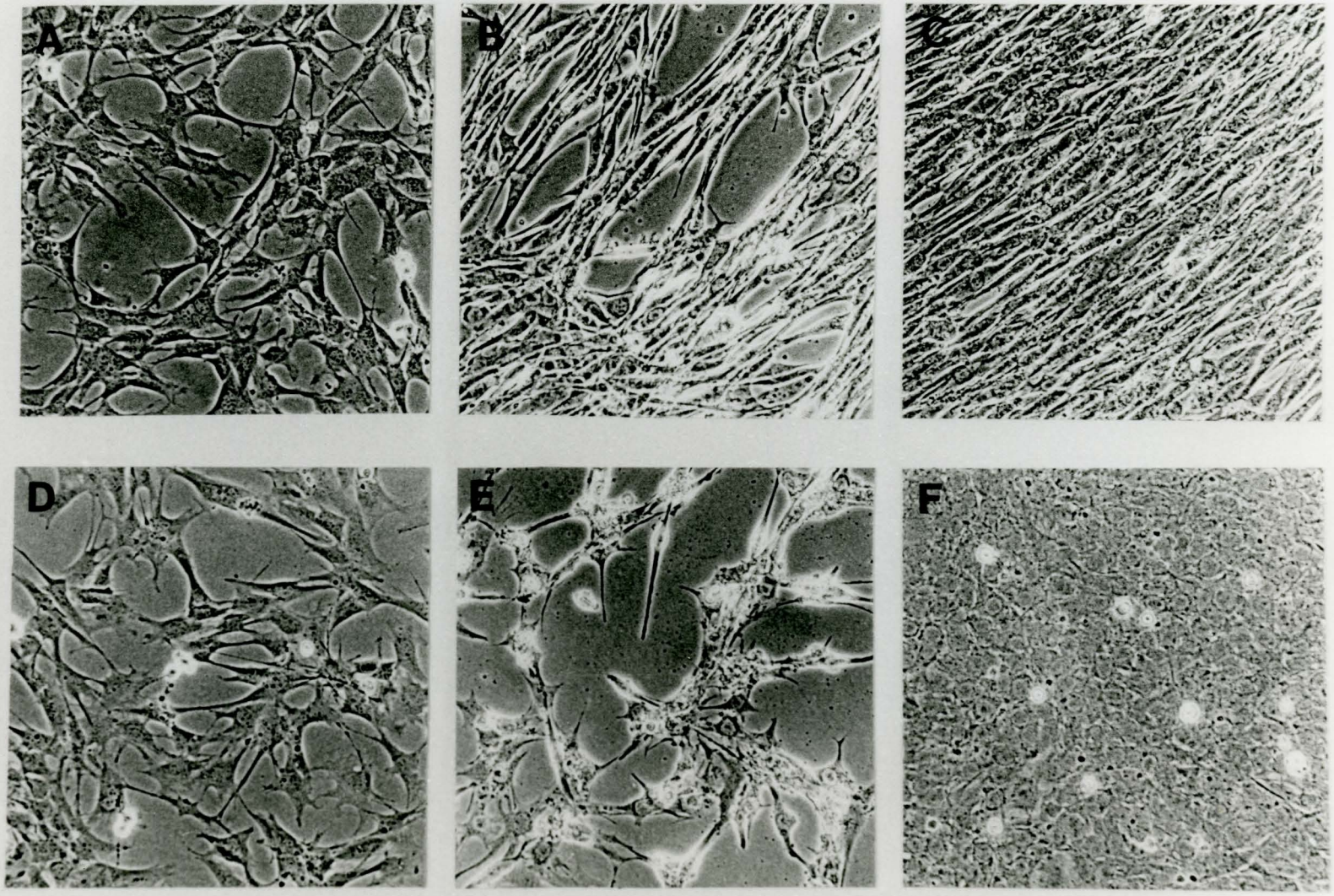

A
B
C
D
E
F

Figure 17. <u>De novo</u> **protein synthesis in BC_3E7 cells.** Proteins from BC_3E7 cells were analyzed as outlined in Figure 4. Again, the regions A, B and C indicate the areas to which actin, Tm and MLC1 migrate, respectively.

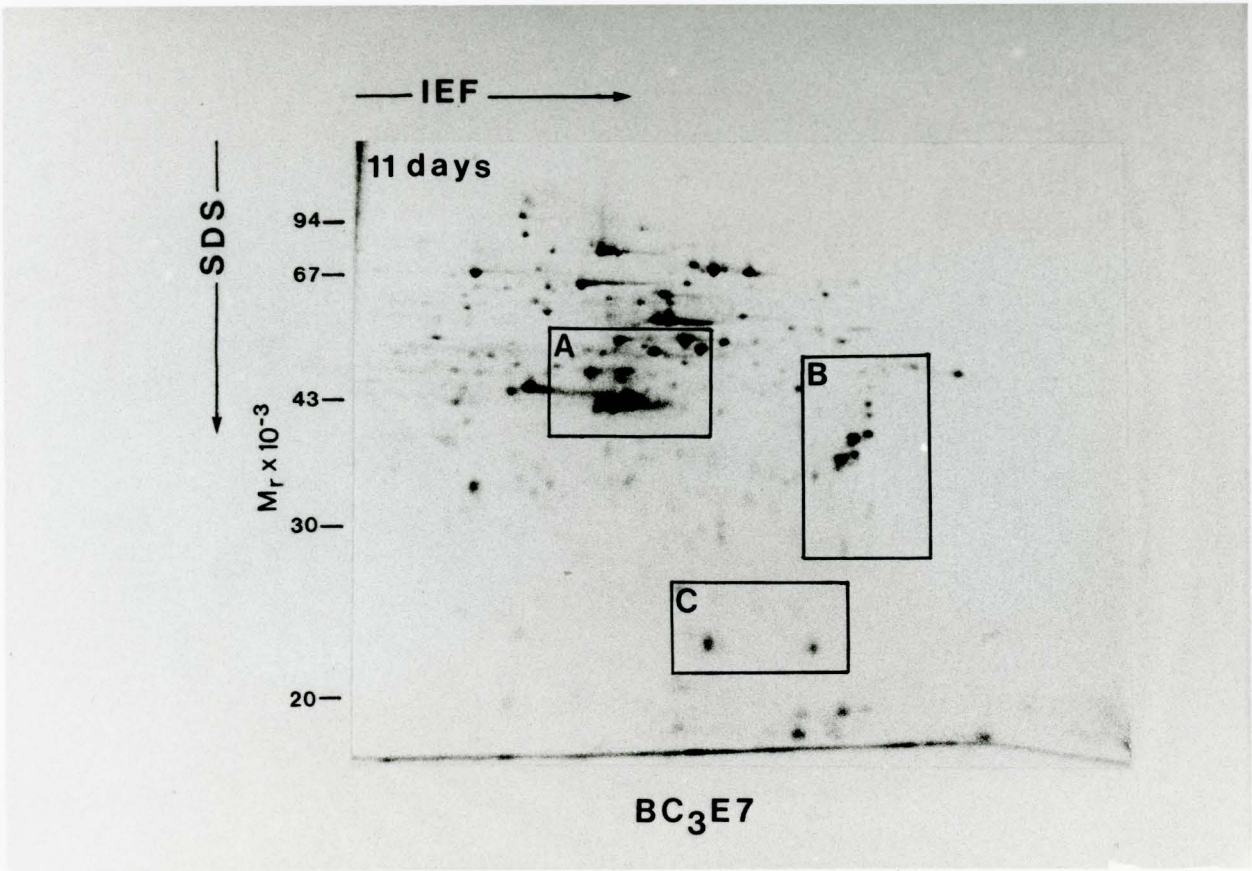

IEF
SDS
11 days
94
67
43
30
20
$M_r \times 10^{-3}$
A
B
C
BC_3E7

Figure 18. Actin expression in BC_3E7 cells. Region A from autoradiograms obtained as described in Figure 6 is shown here for both BC_3H1 and BC_3E7 cells. The difference in days between BC_3H1 and BC_3E7 cells serves to compensate for the decreased growth rate of BC_3E7 cells.

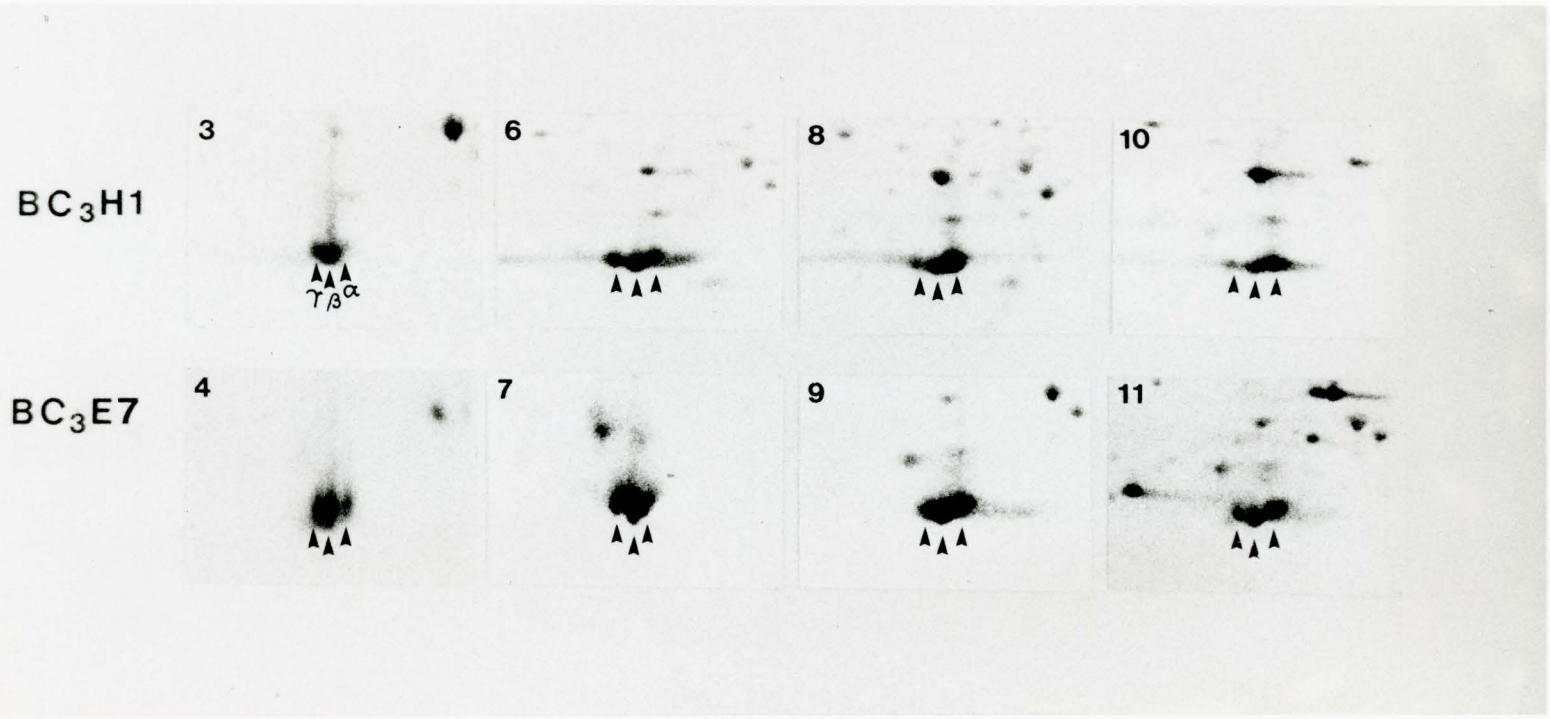

BC3H1
BC3E7
3
4
6
7
8
9
10
11
γ
β
α

Figure 19. MLC 1 expression in BC_3E7 cells. Proteins from both BC_3H1 cells and BC_3E7 cells were analyzed as outlined in Figure 6 and region C from the autoradiograms is shown.

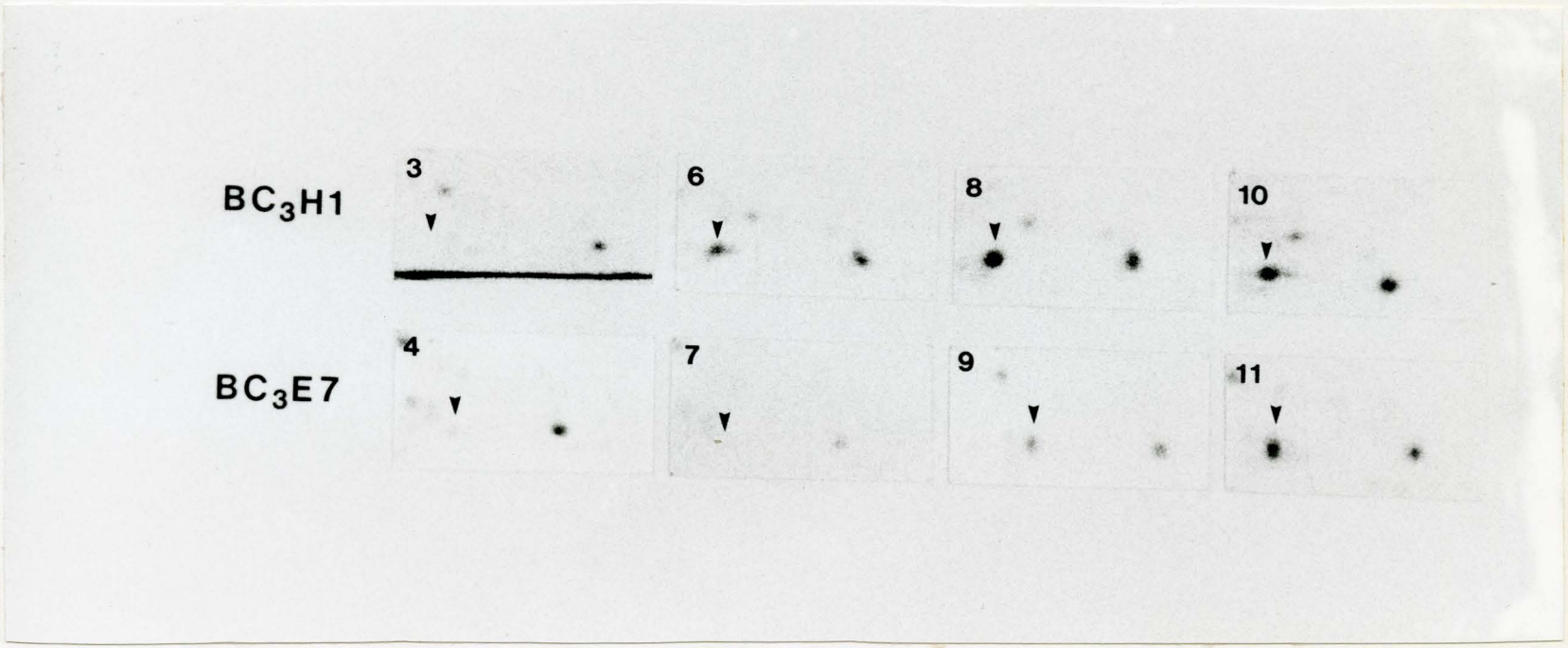
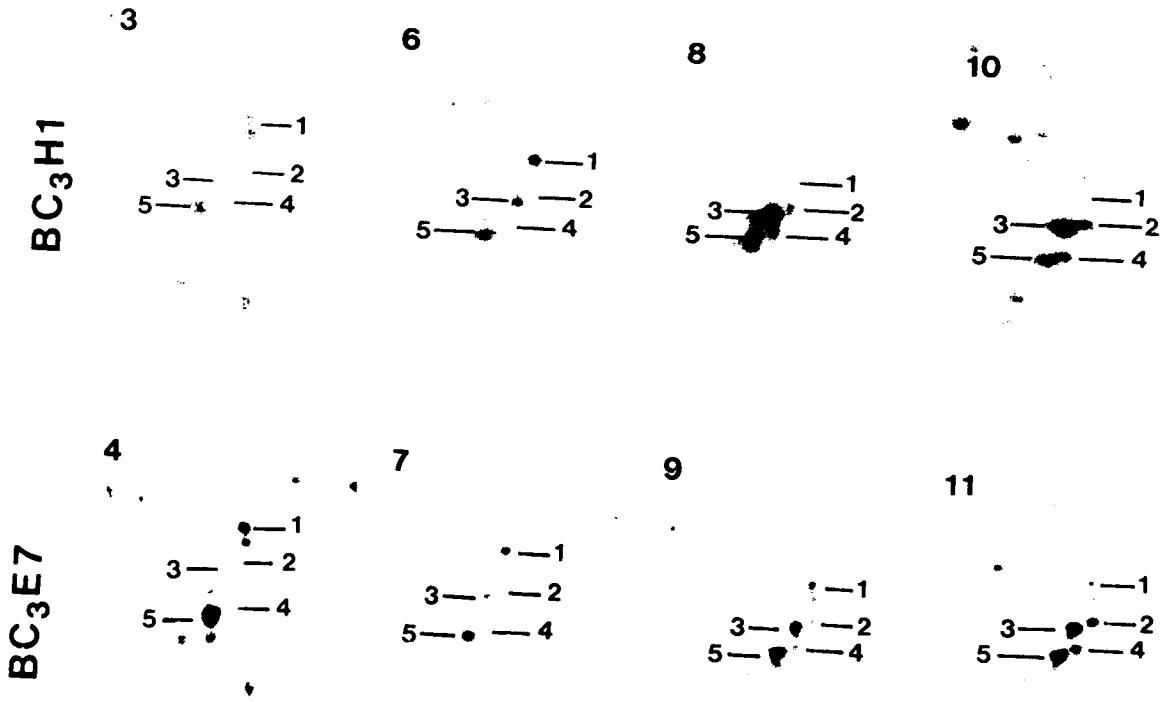

BC3H1
3
6
8
10
BC3E7
4
7
9
11

Figure 20. Tm expression in BC_3E7 cells. Region B from autoradiograms obtained as described in Figure 6 is shown here for both BC_3H1 cells and BC_3E7 cells.


3
6
8
10
BC3H1
1
2
3
4
5
4
7
9
11
BC3E7

IV. DISCUSSION.

The intent of this study was to elucidate the molecular links between signal transduction and protein synthesis leading to the transition to the differentiated state in the mouse muscle cell line, BC_3H1. The involvement of both pk C- and pk A-dependent signal transduction pathways was examined. As well, given the similarity between adenovirus E1A and cAMP-dependent transcription involving CREBs and ATFs, the effect of Ad5 E1A transfection on BC_3H1 cell myogenic differentiation was also examined.

A. EXPRESSION OF MYOSIN LIGHT CHAIN 1 IN BC_3H1 CELLS: A NOVEL FINDING.

The end-point of myogenic differentiation in BC_3H1 cells has been indicated to be 6 days after the initial exposure to serum-free medium. At this point changes in cell morphology were visually complete and α-actin protein and mRNA levels had reached final steady state levels of expression (Strauch et al., 1986). It was for this reason that differentiation in this study was taken to day 10 post-plating which represents 6 days after serum starvation. Although there has been extensive work on actin expression in BC_3H1 cells, little is known about Tm isoform expression

patterns. The identification of five Tm isoforms by two-dimensional IEF analysis (Figures 5 and 8) as well as their developmentally regulated expression in BC_3H1 cells presented in this report is novel. The identity of these five proteins as Tm should be further verified by a lack of [^{35}S]-proline or tryptophan incorporation, as all tropomyosins lack these amino acids (Garrels, 1979 and references within).

Taubman et al. (1989) failed to detect MLC 1 expression at the level of the message as revealed by Northern analysis using a rat skeletal muscle MLC 1 cDNA probe even by day 11 post-plating. Similarly, Kelvin et al. (1989a) did not detect MLC 1 expression using immunoblot techniques with a monoclonal antibody directed against skeletal muscle myosin. In contrast, results from two dimensional analysis of [^{35}S]-methionine labelled cellular proteins presented here clearly indicate the presence of a protein with molecular weight and pI values similar to MLC 1 which is developmentally regulated (Figure 7) and which comigrates with commercially available rabbit muscle MLCs (Figure 5). It was concluded from these results that this protein was MLC 1. The identification of such in BC_3H1 cells is novel. It is unlikely that this protein is an artefact due to the high reproducibility of these results.

Western analysis using a monoclonal antibody directed towards MLC 1 is required to further identify this as MLC 1. Such a discrepancy between this report and the previous ones (Taubman et al., 1989; Kelvin et al., 1989) in the finding of MLC 1 may simply be explained by the use of different clonal BC3H1 cell lines. In the clone studied by Kelvin et al. (1989a) the MLC 1 gene is present and can be activated by treatment with pertussis toxin, an inducer of differentiation. Since the BC_3H1 cell line does not fuse, representing incomplete myogenic differentiation, it is possible that the clone used here is one which can progress further to the differentiated state than those used elsewhere without the need for extra inducers of differentiation.

B. TPA HAD NO EFFECT ON BC_3H1 CELL MYOGENIC DIFFERENTIATION.

In this study, the tumour promoter phorbol ester, TPA, was used as an activator of pk C in order to assess the role played by pk C-dependent signalling pathways in the regulation of BC_3H1 cell myogenesis. It was observed that treatment of BC_3H1 cells at 3 days post-plating in the presence of reduced serum (0.5%) with TPA, in the concentration range of 0.01-0.5μM, had no inhibitory effect on myogenic differentiation. A summary of the effects of TPA can be found in Table 1. TPA-treated cells were

Table 1. Summary of the effects of TPA and cAMP analogue addition to BC_3H1 cells and BC_3E7 cell characteristics.

* Day 10 post-plating in BC_3H1 cells corresponds to day 11 post-plating in BC_3E7 cells.

	ACTIN mRNA		ACTIN		MLC 1		Tm		MORPHOLOGY	
	log	d10*	log	d10	log	d10	log	d10	log	d10
Control	high levels β/γ minimal α	↓ β/γ ↑ α	high levels β/γ minimal α	↓ β/γ ↑ α	-	+	Tm 1,5	Tm 2,3,4,5	fibroblastic	elongated aligned
TPA	- as control -									
cAMP	high levels β/γ minimal α	delayed ↓ β/γ delayed ↑ α	high levels β/γ minimal α	high levels β/γ delayed ↑ α	-	minimal	Tm 1,5	Tm 1	fibroblastic	no alignment neuron-like processes
BC3E7	high levels β/γ high levels α	high levels β/γ high levels α	high levels β/γ α-actin present	high levels β/γ high levels α	-	minimal	Tm 1,5	Tm 1,2,3,4,5	fibroblastic	no elongation no alignment

morphologically indistinguishable from untreated cells(Figure 10). Biochemical differentiation as indicated by the cytoskeletal proteins studied, was also unaffected by TPA addition. Hu and Olson (1988) indicated that TPA addition to BC_3H1 cells up-regulated the expression of growth factor induced early genes (c-fos, c-myc and ornithine decarboxylase). This, together with unpublished results from this lab which indicate that addition of TPA inhibits the expression of surface acetylcholine receptors, suggests that it is unlikely that the lack of an effect of TPA addition seen in the results presented here was due to an unresponsiveness to TPA. To further verify that pk C is responsive to TPA addition, it would be beneficial to assess pK C activation in the presence of TPA.

If one considers the fact that TPA activates pk C (Nishizuka, 1984), it could be concluded that pk C activation failed to have an inhibitory effect on myogenic differentiation in BC_3H1 cells and thus signal transduction via the pk C-dependent pathway is not directly involved in negative regulation of muscle-specific gene expression. Results presented here are in accordance with those of Hu and Olson (1988) which demonstrated that TPA at a concentration of 3.2μM had no effect on BC_3H1 cell differentiation as revealed by MCK activity and AChR levels.

However, Cossu et al. in 1980 found that TPA (0.16μM) had an inhibitory effect on surface AChRs and MCK activity in chick embryo skeletal muscle cells. Also, as indicated previously, similar results have been obtained in this lab which have indicated that TPA inhibits the expression of surface AChR. We believe that TPA exerts its negative effect on surface AChRs at a level other than muscle-specific transcription or translation since no inhibitory effect was seen on proteins of the contractile apparatus. One such level of regulation currently under investigation is cell surface transport.

Other researchers have indicated that while TPA specifically blocked myoblast fusion, it had no effect on the expression of muscle-specific MHC and MLCs (Cohen et al., 1977). These results are not inconsistent with those presented in this study and lend support to the suggestion that TPA, and hence pk C, operates distal to muscle-specific gene transcription in the myogenic regulatory pathway to prevent complete myogenesis. The developmental regulation of the contractile proteins examined here likely lies external to a pk C-dependent pathway. However, one must be careful in making conclusions such as this. If pk C was invovled in initiating differentiation, it would be difficult to detect an induction in the presence of TPA since pk C may be active prior to addition.

Important in evaluating an effect of a drug is the concentration used, TPA being no exception. Although the concentrations used in this study were within the range used by others to activate pk C (Mufson, 1985; Cossu et al., 1982; Chida et al., 1986; Adamo et al., 1989) a problem which must be considered is that TPA is not degraded as rapidly as DAG (Nishizuka, 1986). Persistent exposure to high concentrations of TPA can prolong the association of pk C with the membrane, leading to the degradation of pk C and hence to its decrease in activity (Nishizuka, 1988). It has been demonstrated that TPA concentrations of 0.2μM and greater partially decreased both cytosolic and membrane-bound pk C activities in rat skeletal myoblasts (Narindrasorak et al., 1987). Hu and Olson (1988), using 3.2μM TPA may have in fact inhibited pk C activity rather than activate it. This could lead to false conclusions regarding the role of pk C-dependent signalling pathways. Since the concentrations used in this study were within a range known to activate pk C, it is presently suggested that a pk C-dependent signal transduction pathway is not directly involved in regulation of muscle-specific gene expression. In light of what is known regarding sensitivity towards TPA, it remains to be determined whether the duration of exposure and the associated pk C activity is important in evaluating the effect of TPA on myogenic differentiation.

C. cAMP ANALOGUES DISRUPT BC_3H1 CELL MYOGENIC DIFFERENTIATION.

In an attempt to determine whether BC_3H1 cell differentiation was subject to regulation by pk A-dependent signalling pathways, cAMP analogues were added to the cells in culture as activators of pk A. Although only results of 8-br-cAMP addition were presented, both 8-br-cAMP and db-cAMP produced similar effects of disrupting myogenic differentiation. When 500μM 8-br-cAMP was added to BC_3H1 cells in the presence of reduced serum at day 3 post-plating, the characteristic morphological differentiation was disrupted, with the cells showing neuron-like processes (Figure 12). As well, two dimensional analysis of cytoskeletal proteins indicated that biochemical differentiation was disrupted (Figures 14, 15). The effects of cAMP analogues are summarized in Table 1. Unpublished results from this lab also indicated that surface AChR expression was inhibited by cAMP analogues. The appearance of neuronal-like processes upon cAMP addition has been previously documented. Smith (1984) found that agents which increased intracellular cAMP induced cytoplasmic arboration in cultured arterial muscle cells, with the cytoplasm becoming concentrated into the perinuclear area leaving thin dendrite-like processes (Smith, 1984). The question remains

here as to whether this altered morphology is a result of a disturbed cytoskeleton, given the fact that biochemical differentiation was also disrupted. The precise functional significance of the various cyotskeletal protein isoforms remains vague at present and thus cAMP-treated BC_3H1 cells may aid in this pursuit.

It is interesting to speculate, given the fact that BC_3H1 cells originated from a brain tumour (Schubert et al., 1974), cAMP may divert these cells into a neuronal pathway of differentiation. Dibutyryl cAMP has been reported to cause embryonal carcinoma cells to express neuron-specific markers such as neuron-specific enolase and the medium neurofilament (MW 150 kDa) as well as take on a neuron-like ultrastructure (Sharma et al., 1990). Preliminary results from this lab of Western analysis to detect the presence of neurofilaments in cAMP-treated BC_3H1 cells have been inconclusive.

Hu and Olson (1988) presented results which demonstrated that cAMP inhibited the expression of the genes encoding muscle specific MCK and TnT. The results of Hu and Olson (1988) also suggested that cAMP inhibited the expression of α-actin at the level of transcription. However, the results of this study suggest otherwise. Although cAMP addition lead to an inhibition of α-actin

expression at the level of protein synthesis (Figure 13), it did not prevent expression of the gene at the message level (Figure 9B). In the presence of cAMP analogues, α-actin message was detected at a level similar to untreated cells by day 6 post-plating. This suggests that cAMP is exerting its effect on α-actin expression at a level other than transcription. The delay in the appearance of α-actin protein (Figure 13) in the presence of cAMP seen at day 6 post-plating may indicate that cAMP affects translational efficiency of α-actin messages.

Recent results from this lab indicated that cAMP does affect α-actin expression. Cells treated with cAMP at an early stage prior to the establishment of cell-to-cell contacts showed complete inhibition of α-actin expression. Those treated on days 3 or 4 post-plating showed only a delay in the onset of α-actin expression. It has previously been demonstrated that α-skeletal actin requires both cell-to-cell contact and serum withdrawal for maximum induction (Strauch and Reeser, 1989). These findings suggest that the two differentiation signals may be independently regulated by cAMP with only serum starvation being sensitive to increased cAMP levels.

It has been shown that FCS- or FGF-induced repression of α-actin synthesis occured at the level of translational efficiency of α-actin mRNA rather than at the

level of transcription (Wice et al., 1987). Hu and Olson (1988) indicated that neither FGF nor β-TGF increased cAMP levels in BC_3H1 cells and thus it was concluded that they affected differentiation through a cAMP-independent mechanism. Both these growth factors are speculated to operate through tyrosine-specific kinase activation; addition of an inhibitor of tyrosine-specific phosphatase, vanadate, resulted in similar effects on BC_3H1 cell actin expression as FGF and β-TGF (Wice et al., 1987). However, vanadate has been shown to increase intracellular cAMP levels (Schwabe, et al., 1979). This result is consistent with those presented here in which cAMP addition appeared to be affecting the translational efficiency of α-actin mRNA. Either vanadate operates through a cAMP-dependent pathway to affect differentiation or the increase in cAMP is only secondary to a direct effect of tyrosine phosphorylation on differentiation. Apparently a cAMP-dependent and independent pathway converge to regulate α-actin mRNA translation.

Cyclic AMP treatment also interfered with the down-regulation of β- and γ-actin expression, both at the protein and message level (Figures 13 and 9B). This cAMP effect may operate at either a transcriptional or post-transcriptional level, such as message stability. Results from this lab

suggest the latter possibility is operational: cAMP analogues appeared to increase the half-lives of both β- and γ-actin messages.

Differentiation and proliferation are mutually exclusive thus one could inhibit differentiation by preventing cell cycle withdrawal. The inhibitory effects of cAMP analogues on myogenic differentiation observed should not be attributed to continued cell proliferation and DNA synthesis, since Hu and Olson (1988) indicated that neither the doubling time nor the rate of cell cycle withdrawal were affected by cAMP in BC_3H1 cells. Nor should the cAMP effect observed be attributed to a cytotoxic effect of the cAMP analogues used since Hu and Olson also indicated that the rate of total protein synthesis and degradation were unaffected by cAMP (Hu and Olson, 1988). In this study, cell numbers in cAMP-treated and untreated cells were comparable therefore it is unlikely that cAMP had a cytotoxic effect. Since both 8-br-cAMP and db-cAMP addition to BC_3H1 cells produced similar effects of disrupting myogenic differentiation, it is unlikely that the effects observed were due to the 8-bromo or dibutyryl groups rather than cAMP itself. Thus, given that cAMP activates pk A, BC_3H1 myogenic differentiation is subject to a pk A-dependent signalling pathway which negatively regulates

muscle-specific gene expression at multiple levels including transcriptional, post-transcriptional and translational.

D. INTERACTION BETWEEN PK C- AND PK A-DEPENDENT PATHWAYS?

In BC_3H1 cells, it has recently been demonstrated that insulin addition (an inducer of differentiation) led to a decrease in cytosolic pk C activity and a corresponding increase in membrane-bound pk C activity (Cooper et al., 1990). If pk C was normally active in BC_3H1 cells during differentiation, further activation by TPA would likely fail to show an effect on differentiation. With respect to this, it remains to be determined whether cAMP affects pk C activity. It has been shown that cAMP had an inhibitory effect on phosphatidyl inositol turnover. Pk C activity decreased when rat skeletal muscle cells or a neurotumor cell line, NCB-20, were exposed to agents that increase cAMP (Narindrasorasak et al., 1987; McAtee and Dawson, 1989). This decrease was dependent on pk A activation and required ATP, thus phosphorylation is likely involved (Narindrasorasak et al., 1987). Second messenger generation was inhibited through a cAMP-mediated inhibition of DAG release. This was due to an inhibition of phospholipase C (McAtee and Dawson, 1989). Perhaps pk A-dependent phosphorylation regulates phospholipase C. Pk C activity in the presence of cAMP must be determined to assess the

possibility that pk C inactivation may be ultimately inhibiting differentiation. As well, it should be interesting to determine if TPA in the presence of cAMP could bypass the cAMP-induced block to BC_3H1 cell differentiation. In evaluating a role of pk A-dependent signal transduction, one may indirectly be evaluating pk C-dependent signal transduction.

E. ADENOVIRUS 5 EARLY REGION 1A DISRUPTS BC_3H1 CELL DIFFERENTIATION.

It is generally accepted that transformation and differentiation are mutually exclusive. Oncogenes are known to operate within signal transduction pathways, affecting transcription factor activity or acting as transcription factors themselves. Although the cellular oncogenes c-*ras* and c-*myc* have been implicated in growth factor-mediated inhibition of myogenesis (Schneider et al., 1987; Payne et al., 1987; Kelvin et al., 1989b), these oncogenes are closely linked to cell cycle withdrawal and thus may operate at the level of terminal commitment rather than further down the differentiation pathway such as muscle-specific gene activation which results in terminal differentiation. Results of cAMP addition to BC_3H1 cells presented here suggest that the block to differentiation observed operates at the level of muscle-specific gene expression rather than

cell cycle withdrawal. It was thus of interest to investigate the role of potential transcription factors.

Given the fact that adenovirus E1A mediated transactivation of viral and cellular genes is believed to occur through a mechanism which shares similarity to the mechanism by which cAMP mediates cellular gene activation, we wanted to examine the effect of E1A on BC_3H1 cell differentiation. It was necessary to examine E1A transactivation in the absence of other consequences of viral infection and thus stable Ad5 E1A transfected BC_3H1 cell lines were developed.

One such Ad5 E1A-transfected BC_3H1 cell line which was further characterized was called BC_3E7. Results from this lab suggested that is was unlikely that this cell line resulted from spontaneous mutation and that the disruption of differentiation observed was due to growth stimulation by E1A (Mymryk et al., submitted). It is likely that the effects observed were due to E1A since the BC_3P7 cell line which was transfected with a plasmid containing only the neomycin resistance gene was morphologically and biochemically similar to BC_3H1 cells (data not shown). The presence of E1A was verified at the levels of message, protein, and biological activity (Mymryk et al., submitted).

Ad5 E1A transfection resulted in the disruption of

the coordinate regulation of muscle-specific gene activation in BC_3H1 cells. Multiple effects on muscle-specific markers were observed. Refer to Table 1 for a summary of these effects. Earlier reports demonstrated a block to myogenesis in L6, C2, and 23A2 cells transfected with the Ad 5 E1A gene (Webster et al., 1988; Enkemann et al., 1990). Presented here are results which indicate a lack of down-regulation of non-muscle markers β- and γ-actins upon confluency, both at the protein and message levels (Figures 18, 9C) and the failure to morphologically differentiate (Figure 16). This E1A-induced block to myogenesis is in accordance with the earlier studies. Results from this lab also revealed a repression of muscle-specific expression of MCK activity and surface AChR (Mymryk et al., submitted).

But, in contrast to those earlier reports by Webster et al. (1988) and Enkemann et al. (1990) in which E1A transfection led to inhibition of all muscle-specific genes studied, not all muscle-specific markers were repressed in BC_3E7 cells. E1A transfection had no effect on the expression of Tm isoforms 2-5 (Figure 20) nor MLC 1 (Figure 19). As well, unexpectedly, E1A transfection of BC_3H1 cells led to an activation of muscle-specific α-actin in proliferating and presumably undifferentiated BC_3E7 cells at both message and protein levels (Figures 18, 9C).

These results, as well as the fact that both induction and repression of cellular proteins were observed, suggests that E1A did not cause a general non-specific inhibition of transcription. A general non-specific repression of transcription by Ad E1A could not be ruled out, however, for the results presented by Webster et al. (1988) and Enkemann et al. (1990). The results presented in this thesis thus suggest that E1A is exerting an effect specific to myogenesis in BC_3H1 cells.

F. COORDINATE REGULATION OF MUSCLE-SPECIFIC GENES: AN INCREASINGLY COMPLICATED STORY.

The coordinate regulation of such a vast array of proteins encoded by unlinked genes raises the question of whether there is a common underlying regulatory mechanism. It has been suggested that trans-acting factor(s), both positive and negative, interact with *cis*-acting elements associated with each gene. Common *cis*-acting sequences have been identified in several muscle-specific genes including MCK (Buskin, and Hauschka, 1989; Jaynes et al., 1988; Sternberg et al., 1988), MLC 1/3 (Donoghue et al., 1988), cardiac actin (Minty and Kedes, 1986), cardiac Tn T (Mar et al., 1988) and δ subunit of AChR (Baldwin and Burden, 1988), however no universal mechanism for gene activation has been

identified. It is becoming increasingly difficult to explain the coordinate regulation of muscle-specific genes. As has been suggested in this study and by other researchers, levels of control may vary. When BC_3H1 cells were induced to de-differentiate and re-enter the cell cycle, MCK mRNA synthesis decreased and messages decayed, producing a corresponding decrease in the level of protein (Glaser and Wice, 1989). However, the decrease in α-actin seen at the protein level did not reflect the message level which remained high (Glaser and Wice, 1989). Similar results which suggest multiple levels of regulation were obtained here in the presence of cAMP analogues. Here, cAMP appears to operate at a translational level to regulate α-actin expression while β- and γ-actin expression appears to be regulated by cAMP at a post-transcriptional level. Also, distinct regulatory mechanisms are speculated to exist for muscle-specific gene expression given the fact that human cardiac and skeletal α-actin genes were expressed constitutively when introduced into C2C12 cells (Minty et al., 1986; Muscat and Kedes, 1987) while the expression of a MCK-CAT fusion gene was growth factor mediated, only detected after serum withdrawal (Johnson et al., 1989). Human cardiac and skeletal α-actin genes are thus regulated by a common transcription factor present in undifferentiated

C2C12 cells (Muscat et al., 1988). However, the MCK gene must be regulated by a different transcription factor not present or active until after mitogen withdrawal. The precise tissue and developmental-specific regulation of cardiac and skeletal α-actin likely involves cell-specific accessory factors and/or chromosomal mechanisms which govern promoter accessibility (Muscat et al., 1988). In BC_3H1 cells, the expression of cardiac α-actin precedes that of skeletal α-actin (Strauch and Reeser, 1989). As well, the expression of cardiac α-actin mRNA precedes that of MHC and MLC 2 by four hours in cultured chick myoblasts (Lawrence et al., 1989). This sequential expression of muscle-specific genes does not lend support to a "master" gene which directly activates all muscle-specific genes. It is possible though that one may indirectly act on muscle-specific genes via a cascade mechanism.

While investigating myogenic regulation, researchers identified several "master" myogenic regulators , including MyoD1, myd, and myogenin (Davis et al., 1987; Pinney et al., 1988; Wright et al., 1989), which had the ability to convert nonmyogenic cell lines into myogenic ones. It was speculated that these proteins function as transcriptional activators of muscle-specific genes. It appears that myd fuctions distal to both MyoD1 and myogenin (Pinney et al.,

1988), yet it remains unclear where MyoD1 and myogenin function with respect to each other in the temporal sequence of myogenic regulation. Myogenin is expressed embryologically prior to MyoD1. Yet, although myogenin is expressed in the fusing myogenic cell line, L6, and in BC_3H1 cells, MyoD1 is not (Wright et al., 1989).

Dispite these incongruencies, it is generally believed that the expression of a "master" regulatory molecule is necessary for the subsequent expression of muscle-specific genes and the attainment of the differentiated phenotype. In support of this, Enkemann et al. indicated that E1A transfection led to an inhibition of myogenin expression. Accordingly, if E1A, operating directly on myogenin and not on muscle-specific genes, inhibited the expression of myogenin, then this would lead necessarily to the repression of other muscle-specific genes, provided myogenin was "master" over all muscle-specific genes. However, in light of results presented here in which E1A prevented morphological differentiation yet did not completely block biochemical differentiation, the myogenic regulatory pathway no longer seems so linear and myogenin no longer the "master" regulatory molecule. Several branches may exist leading to distinct parameters of differentiation. These may have unique members of the MyoD

family of regulators operating on the individual branches. Since cAMP addition to BC_3H1 cells resulted in a more synchronous inhibition of differentiation than E1A, it is suggested that cAMP operates further upstream in the pathway of myogenesis than E1A.

G. FUTURE RESEARCH.

It is now necessary to investigate the expression of myogenin in BC_3E7 cells. Its presence or absence in this cell line should prove equally intriguing. If, in accordance with the previous studies, myogenin expression is inhibited in BC_3H1 cells by E1A transfection, then the fact that morphological differentiation was prevented even in the presence of some biochemical differentiation weakens the theory of myogenin as a "master" regulatory molecule. It may, rather, be operating further down the regulatory pathway or only on one branch, many of which together allow for stable and complete myogenesis. If present in BC_3E7 cells, E1A should be functioning more distal to myogenin than Enkemann et al. suggest, perhaps either directly on some muscle-specific genes or on a lower level regulatory protein(s). It will also be interesting to determine how E1A mediated an up-regulation of α-actin. Also to be addressed is whether cAMP affects the level of myogenin expression or its activation, perhaps through a pK A-

dependent phosphorylation. The BC_3H1 cell line in the presence of cAMP, as well as the BC_3E7 cell line, should prove useful in the identification of a novel set of myogenic regulators involved in the increasingly complicated myogenic pathway. cAMP-treated BC_3H1 cells should also prove to useful in determining the functional significance of cytoskeletal proteins as well as in elucidating the regulatory mechanisms governing α-, β- and γ-actin expression. Further work needs to be conducted in order to confidently determine the role of pk A- and pk C-dependent pathways in BC_3H1 cell myogenesis. The effect of cAMP on pk c activity needs to be addressed before any conclusion is drawn regarding the ultimate involvement of these two pathways.

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