ACTIN GENE EXPRESSION IN BC_3H1 CELLS

ACTIN GENE EXPRESSION DURING MYOGENIC DIFFERENTIATION OF BC_3H1 CELLS

BY

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ABSTRACT

Myogenic differentiation of muscle cells in culture is characterized by changes in morphology and in pattern of gene expression. When the myoblasts in culture are induced to differentiate either by cell to cell contact or by serum-starvation, a vast array of muscle tissue-specific genes including α-actin, are activated and is accompanied by a concomitant down-regulation of non-muscle genes such as, β-and γ-actins, tubulins etc.. The coordinate activation of muscle-specific genes is suggested to be mediated by *cis*-acting regulatory sequences in the muscle gene-promoters and muscle-tissue-specific DNA-binding proteins belonging to the MyoD class of regulators. But the mechanism behind the repression of non-muscle gene expression during differentiation has not yet been well understood. To date, no consensus has been achieved on the mechanism governing the down-regulation of β-actin gene, and no information is available on the regulation of γ-actin gene during muscle differentiation. The results from the present study showed that during differentiation of BC_3H1 cells the β-and γ-actin genes were down-regulated to $\approx 25\%$ of their initial levels in undifferentiated cells. Measurement of half-life during differentiation indicated that the half-lives of both β-and γ-actins decreased to $\approx 25\%$ of their original levels in myoblasts. These results suggest that changes in mRNA stability play an important role in the down-regulation of non-muscle actin genes. Second messengers and oncogenes are known to block the differentiation program of muscle cells. In the present study cAMP and E1A were observed to inhibit the down-regulation of β-and γ-actin genes in BC_3H1 cells. In both cAMP- and E1A-

treated cells the β-and γ-actin mRNAs were found to have a higher half-life than the untreated differentiated BC_3H1 cells. This observation also suggests that mRNA stability might play an important role in the regulation of β-and γ-actin gene expression.

The muscle-specific α-actin is activated by cell-cell contact and serum-starvation. Results in the present study suggested that cAMP was able to inhibit the activation of α-actin expression mediated by serum-starvation while it had no significant effect on the signal mediated by cell-cell contact. It is hypothezised that the two signals mediating α-actin activation might follow different intracellular signalling pathways. The effects of cAMP and E1A on the expression of muscle-specific and non-muscle actins could be a direct primary event or might be an indirect secondary event, mediated by other intracellular factors such as myogenin. The results showed that cAMP did not block the transcription of the myogenin while secondary evidences suggested that cAMP might negatively-regulate myogenin at a point downstream of transcription. E1A was observed to block the expression of myogenin gene suggesting that E1A might be mediating its effect through myogenin. Because the muscle-specific(α) and non-muscle(β and γ) isoforms were expressed both in the presence and in the absence of myogenin, myogenin's role in the regulation of actin genes is unclear.

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Muscle cells in culture pass from the undifferentiated state called myoblasts to a differentiated state known as myocytes. This process of differentiation involves activation of an array of muscle specific genes eg., α-actin and is accompanied by a decrease in the level of expression of non-muscle genes such as β- and γ-actins. The primary objective of the present project was to understand the mechanism by which the β-and γ-actin gene expression was down-regulated during differentiation of the muscle cell line BC_3H1. Differentiation blocking agents such as cAMP, a second messenger, and an oncogene E1A were used as tools to understand this process. The second objective was to investigate the role played by cAMP in the expression of muscle-specific α-actin and a muscle differentiation regulatory gene, myogenin.

A.1 MUSCLE DIFFERENTIATION: A HISTORICAL OVERVIEW

Early studies on muscle development were focused on understanding the development of the somites, the contractile apparatus, and the relationship between them (Herrmann, 1952). It was believed that the somite mesoderm was responsible for the musculature of the dorsal one-third of the trunk, and the lateral plate mesoderm gave rise to both, the remaining two-thirds and the musculature of the chicken limb (Straus and Rawles, 1953; Boyd, 1960; Konigsberg, 1965). The

skeletal muscle elements in the chick embryo were first recognised in the somites on the third day of incubation (Holtzer *et al.*, 1957), and later by the fifth day they were observed in the limb (Kitiyakara, 1959). Muscle element formation continues at least for two or three weeks of development (Coleman and Coleman, 1968). Detailed analysis of the changes during the muscle differentiation was challenging to the scientists, because the muscle differentiation *in vivo* was a highly asynchronous process.

Myogenesis in cell-culture system, on the other hand, can be a highly synchronous process permitting frequent observations, and a wide range of experimental manipulations. In the early experiments, the cells for culture were obtained from the thigh muscle tissues of a 12 day old chick embryo. The tissues were dissociated into individual cells by treating with trypsin, and the cells were grown in a medium containing 15% horse serum and 2.5% chick embryo extract. During the proliferative-phase, the culture was composed of fibroblast-like cells but upon confluency, they formed long multinuclear structures by the process of successive cell-fusion. Vigorous spontaneous contractions were observed in the elongated muscle cells, and these structures were observed to resemble the muscle fibres *in vivo* (Konigsberg, 1963; Coleman and Coleman, 1968). The fusion of myoblasts to form myofibers was accompanied by the synthesis of a variety of muscle-specific proteins, such as, the energy metabolism enzyme creatine phosphokinase (Konigsberg, 1963), the receptor for the neurotransmitter

acetylcholine(AChR) (Devreotes and Fambrough, 1975), and the proteins of the muscle contractile apparatus: the sarcomere (Devlin and Emerson, 1978; Garrells, 1979; Affara *et al.*, 1980). The primary components of the muscle sarcomere can be divided into thick and thin filament proteins. The thick filament is composed of myosin heavy chain (MHC), alkali myosin light chains (MLC1, MLC3), and regulatory myosin light chains (MLC2). The thin filament is composed of actin, α-tropomyosin, and the troponin complex: troponin T (TnT), troponin C (TnC), and troponin I (TnI).

The simultaneous activation of a group of genes in one cell type raises the question, how these genes are coordinately activated as a functional set in these cells. The muscle cell culture system provides an opportunity to study the coordinate activation of a set of genes encoding a structurally diverse group of proteins that is responsible for the major activity of the muscle cell type ie., contraction.

A.2 MYOGENIC REGULATORS

Evidence for the presence of myogenic-regulatory factors came from studies where expression of muscle-specific genes was induced in non-muscle cells by cell fusion experiments. Human amniocytes were shown to express muscle proteins such as MLC1, MLC2, and muscle creatine kinase (MCK) when fused with differentiated mouse muscle cells (Blau *et al.*, 1983). Later Wright (1984), also demonstrated the effect of cell fusion by inducing the expression of MLC1 and AChR

in Y1 mouse adrenal cells fused with differentiated chick skeletal myocytes. The question was better approached using the mesodermal stem cell line C_3H 10T1/2. These multi- potential cells could be induced to become myoblasts by a brief exposure to the demethylating agent 5-azacytidine (Taylor and Jones, 1979). Konieczny and Emerson (1984), proposed that one or a few closely linked loci were activated following hypomethylation by 5-azacytidine, and were responsible for establishing the myogenic lineage of 10T1/2 cells. This was supported by a later study. The 10T1/2 cells transfected with DNA from either 5-azacytidine-treated 10T1/2 cells or from myoblasts of a mouse myogenic cell line C2C12, expressed muscle markers. The conversion rate was 1 in 15,000, which was consistent with the transfection frequency of a single genetic determinant. This led to the conclusion that a single genetic locus was sufficient to convert 10T1/2 cells into determined myoblasts (Lassar *et al.*, 1986). The first myogenic regulatory gene was cloned by subtractive-hybridization (Davis *et al.*, 1987). Three cDNAs namely, myoA, myoD, and myoH were selected on the basis of a number of criteria. Only MyoD was able to induce multinucleate-synctia and expression of the MHC gene when transfected into proliferating 10T1/2 cells under the control of the Moloney sarcoma virus long terminal repeat(LTR) and, was named as the myogenic determination gene 1 (MyoD1). MyoD1 was also shown to induce myogenic colonies in mouse fibroblasts such as NIH 3T3, and Swiss 3T3, and in adipoblasts such as TA1, 3T3-L1 and 3T3-F442A. This suggested that MyoD1 gene encodes a master regulator for skeletal muscle differentiation. The 318 amino

acid long MyoD1 protein was shown to have a highly basic region (residue 102-134) which was just amino-terminal to a short segment (22 residues) that showed strong similarity to a domain of the *c-myc* protein in the mouse, chicken and humans.

Emerson and co-workers obtained evidence for another genetic locus *myd,* which was also able to function as a myogenic regulator. This led to the proposal that these genes (MyoD1 and *myd*) may function sequentially in a dependent myogenic regulatory pathway of cell lineage determination (Pinney *et al.*, 1988).

A third myogenic regulatory gene myogenin, involved in the stage between determination and terminal differentiation was identified using the rat myogenic cell line L6. Myogenin was shown to be transiently expressed at high levels in determined myoblasts prior to biochemical differentiation but following the signal to differentiate. The mouse homolog of the rat myogenin was identified from a non-fusing mouse muscle cell line BC_3H1 using the *myc* homology domain (Edmondson and Olson, 1989). Subsequently, MyoD1 related factors, *myf-5* (Braun *et al.*,1989) and MRF-4, also called herculin and *myf-6* (Rhodes and Konieczny, 1989; Braun *et al.*, 1990; Miner and Wold, 1990) were isolated independently. All these myogenic regulatory factors were expressed exclusively in skeletal muscle cells and were able to induce myogenesis when transfected into 10T1/2 cells.

The myogenic regulatory factors share $\approx$80% amino acid homology with each other within a segment of 70 amino acids comprising both the *myc* homology and the highly basic domain (Olson, 1990). A conserved motif within

amino acids 108-164 of the MyoD1 protein was found to be a helix-loop-helix(HLH) structure which plays a crucial role in DNA binding and dimerization (Murre *et al.*, 1989a). MyoD1 binds to its target as a heterodimer with two ubiquitously expressed immunoglobulin enhancer-binding proteins namely E12 and E47 (Murre *et al.*, 1989b). The HLH region appears to be essential for dimerization while the adjacent basic region seems to be required for DNA binding. These myogenic regulators might behave as transcriptional factors that activate muscle-specific gene expression during myogenesis (For a recent review see Weintraub *et al.*, 1991). HLH homo-and hetero-dimer binds to a consensus sequence referred to as E box (CANNTG) in the muscle-specific enhancers, first identified within the immunoglobulin gene enhancer (Church *et al.*, 1985). E box has been observed in most muscle-specific genes such as MCK, MLC 1/3, δ-subunit of the AChR, and human cardiac α-actin gene (Buskin and Hauschka, 1989; Lassar *et al.*, 1989; Brennan and Olson, 1990). Recently the TnI was also found to have the E box (Lin *et al.*, 1991).

A.3 ACTIN GENE EXPRESSION DURING MYOGENESIS

Actin is a major structural protein present in all eukaryotic and most of the prokaryotic cell types. Besides its role in generation of contractile force in muscle, actin plays an important role in non-muscle cells in processes such as cell shape determination, cell motility, and cytokinensis. Actin genes have been found to exist in extensive multigene families and were observed to vary in number between

different organisms. For example, the actin gene number varies from 4 in the nematode *Caenorhabdites elegans* to as many as 30 in man (Engel *et al.*, 1981). Even a simple organism like the slime mould *Dictyostelium discoideum* has as many as 17 such genes, which in some cases are developmentally regulated and in others (pseudogenes) are not expressed at all (McKeown *et al.*, 1981).

The actin genes in vertebrates code six major isoforms, usually grouped into two classes namely, muscle and non-muscle types. The isoforms are designated as α-, β-and γ- based on their mobilities on an isoelectric focusing gel. Cytoplasmic β-and γ-isoactins are the major isoforms in the non-muscle cells and are present in different proportions depending on the cell type and the state of differentiation (Tokunaga *et al.*, 1988). The muscle-specific actins are: α-skeletal, α-cardiac, and both γ-and α-smooth muscle actins. These different actin genes show extensive homology in their amino acid sequence and in their protein coding regions (Pearson *et al.*, 1981). Morphological differentiation of myoblasts to myotubes is accompanied by accumulation of the sarcomere-specific contractile protein isoforms (α-actin), with a coordinate down-regulation of the non-muscle actin isoforms (β- and γ- actins) (Devlin and Emerson, 1978).

A.3.1 α-actin gene regulation

α-skeletal actin is the major isoform of the skeletal muscle and α-cardiac actin is that of the adult heart. Cardiac and skeletal muscle α-actins diverge only by four neutral amino acid changes over 375 residues, nevertheless they have

distinct tissue specificities. The relative levels of both sarcomeric α-actin transcripts in cardiac and skeletal muscle depend on the species, the tissue, and the stage of development (Vandekerckhove *et al.*, 1986). Coexpression of the sarcomeric actin genes does not require proximity on the chromosome since cardiac and skeletal actins are encoded by distinct single-copy genes located on different chromosomes. In humans the skeletal actin gene is located on the long arm of the chromosome 1 and the cardiac actin gene is on the long arm of the chromosome 15 (Gunning *et al.*, 1984). A *cis* acting regulatory element referred to as the CArG box ($CC(AT)_6GG$) at -100 bp from the transcription start site was required for the tissue specific expression of the cardiac α-actin gene (Miwa *et al.*, 1987). Common regulatory factors were shown to bind the CArG boxes of the cardiac and the skeletal α-actin genes (Muscat *et al.*, 1988).

The tissue specificity of the human α-actin promoter was found to be mediated by simultaneous interaction of three different proteins with the DNA, namely, MyoD1, CArG-box binding factor and Sp1 (a GC box binding protein) (Sartorelli *et al.*, 1990). Recent studies on the avian α-actin promoter pinpointed the site of interaction of the myogenic regulators to an E box immediately adjacent to a previously identified CArG box (CArG3). However deletion and substitution experiments indicated that the E/CArG3 element by itself does not confer muscle-specific expression to a minimal promoter. Hence it was suggested that, direct and indirect pathways involving multiple *cis*-acting elements mediate the induction of α-

actin promoter by myogenin and MyoD1 (French et al.,1991). Though the molecular mechanism of α-actin activation has yet to be unravelled, it is accepted that the α-actin gene expression is controlled by *cis*-acting regulatory elements and the myogenic regulatory factors might be involved in transcriptional activation of the sarcomeric α-actin genes.

A.3.2 β-and γ-actin gene regulation

The non-muscle β-and γ-actins are highly homologous proteins, they differ only by 4 amino acids in the amino-terminal region of the total 375 residues (Vandekerckhove and Weber, 1978; Vandekerckhove *et al.*, 1981). The muscle-specific α-actins differ only at 24-25 amino acids from the non-muscle actins (Czonek *et al.*, 1983). The non-muscle actin genes are also located on different chromosomes namely, β-actin on chromosome 7 and γ-actin on chromosome 17 (Erba, 1988). Both β-and γ-actin genes were observed to be coexpressed in a wide range of tissues but the ratio of the levels of expression was found to be different for different tissues (Tokunaga *et al.*, 1988).

The mechanism by which the non-muscle actin genes are down-regulated during muscle differentiation has not been identified. The control might be either a transcriptional or a post-transcriptional event. In an attempt to distinguish between the transcriptional and post-transcriptional controls of the steady state levels of β-actin, Patterson *et al.* (1986), constructed a chimeric gene with the chicken β-actin gene promoter and the body of the human α-globin gene. Upon transfecting this

assembly into C2C12 myoblasts, α-globin mRNA was not down-regulated during differentiation. Instead it was constitutively expressed at all stages of myogenesis. It was therefore suggested that the steady-state level of β-actin gene was not controlled by the promoter element but probably by the sequence 3' to the gene promoter. Deletion analysis of the β-actin gene suggested that the domain responsible for the reduction in β-actin mRNA levels was a 40 bp sequence located just 5' to the polyadenylation signal of the β-actin gene. This 40 bp sequence was able to confer a β-actin pattern of expression to a cardiac α-actin gene when placed at the corresponding site adjacent to the polyadenylation signal. Nuclear run-on transcription assays had suggested that the observed decrease in the β-actin mRNA levels during myogenesis was due to a drop in the transcription rate of the β-actin gene (Deponti-Zilli *et al.*, 1988). Later observations confirmed that the down-regulation of β-actin gene during differentiation involved the sequences 3'to the β-actin gene promoter (Lahose and Arnold (1988). However, Lahose and Arnold did not observe a change in the transcription rate of the β-actin gene as monitored by nuclear run-on transcription assays. The half-life of the β-actin mRNA studied using the transcription inhibitor actinomycin D was reported to be 4 hours both in undifferentiated and differentiated state. Therefore it was suggested that post-transcriptional events such as, altered processing or transport of mRNA would probably be at the level at which β-actin gene expression was controlled in the rat C2 myogenic cell line (Lahose and Arnold, 1988). Sharp *et al.*, in 1989 studied the

regulation of the chicken β-actin gene by transfecting either, intact chicken β-actin or hybrids of the chicken α-and β-actin genes (α5'-β3' and β5'-α3') into BC_3H1 cells. Nuclear run-on experiments had showed no evidence for gene-specific changes in transcription rate of the transfected actin genes during differentiation. It was concluded that the regulatory function of the β-actin gene was located at the 3'half and the control of down-regulation during differentiation was not at the transcriptional level but probably at the post-transcriptional level of gene regulation. Recently the regulation of the endogenous β-actin gene in C2/7 cell line, a sub clone of the C2 cell line, was studied by nuclear run-on transcription assays. The results indicated an eight fold drop in the transcription rate of the β-actin gene during differentiation. Hence it was suggested that the β-actin gene was transcriptionally down-regulated (Cox *et al.*, 1990). Thus the present level understanding does not lead us to a consensus on the mechanism governing the down-regulation of the β-actin gene. No attempt has yet been made to understand the mechanism governing the repression of the γ-actin gene.

A.4 BC_3H1 CELL LINE

The BC_3H1 cell line was derived from a brain neoplasm induced by nitrosoethyl urea treatment in the C3H mouse (Schubert *et al.*,1974). BC_3H1 cells, despite their ectodermal origin behave like muscle cells in culture. This hypotetraploid cell line, when plated in a culture dish exhibits a flat morphology with

processes extending from the cell (typical fibroblastoid). Upon reaching confluency the cells detach slightly from the surface and become elongated and aligned beside each other, but they do not fuse to form myotubes. These elongated cells were observed to contract spontaneously, which is a characteristic of muscle cells in culture (Schubert *et al.*,1974).

It was originally thought that the BC_3H1 cell line was of smooth muscle type as it had several characteristics of smooth muscle cells such as the presence of myofilaments lacking Z bands, low thick filament to thin filament ratio, surface vesicles (Schubert *et al.*,1979) and its ability to synthesize vascular smooth muscle α-actin isoform in a developmentally regulated manner (Strauch and Rubenstein, 1984; Strauch *et al.*, 1986; Wang and Rubenstein, 1988). However, observations such as the expression of muscle creatine kinase (MCK), nicotinic acetylcholine receptor, skeletal muscle type voltage gated Ca^{2+} channel (Caffrey *et al.*, 1987), TnT, and sarcomeric muscle isoforms of MHC, MLC2, MLC3, α-TM and α-actin (Taubman *et al.*, 1989), suggest that cell line could be of skeletal muscle type. Taubman *et al.*, suggested that BC_3H1 cell line was a skeletal muscle cell line of ectodermal origin that is defective for commitment to terminal differentiation. More recently myogenin, a member of the MyoD family was found to be expressed in BC_3H1 cells (Edmondson and Olson, 1989). As MyoD family of regulators is specific to skeletal muscle type, myogenin expression strongly suggests the skeletal nature of this muscle cell line.

A.4.1 Actin expression in BC_3H1

The presence of actin isoforms at the protein level was reported by isoelectric focusing and tryptic peptide analysis of the NH_2 terminus (Strauch and Rubenstein, 1984). It was shown that the smooth muscle α-actin expression was induced within 72 hours following the treatment of a post-confluent cell monolayer with low serum medium. This was accompanied by a concomitant decrease in the level of expression of the non-muscle actins (β- and γ-). It was also reported that cell-cell contact was required for maximal induction of α-actin expression. Addition of serum back to the culture-medium of differentiated cells blocked the expression of α-actin gene, and was accompanied by an increase in the levels of the non-muscle actins (Strauch and Rubenstein, 1984). Dividing BC_3H1 myoblasts were shown to have high levels of a 2100 nucleotide actin mRNA which was identified as a mixture of similar size transcripts encoding both β-and γ-actins. Upon serum-withdrawal and cell-cell contact, the expression of both β-and γ-actin mRNA decreased by three fold and a concomitant six fold increase in the level of a smaller, 1500 nucleotide actin mRNA was observed. This smaller message was identified as the muscle-specific α-actin mRNA (Strauch *et al.*, 1986). As the expression of actin genes could be controlled simply by changing the culture conditions, it became apparent that the BC_3H1 cell line is an excellent model system to study the regulation of actin gene expression during differentiation.

The effect of cell-cell contact on expression of α-actin in BC_3H1 cells

has been recently analyzed (Strauch *et al.*, 1990). When a post-confluent monolayer of BC_3H1 cells was non-proteolytically dissociated using 0.1 % EDTA and re-plated in a low-serum medium, changes in cell shape involving conversion of bipolar highly refractive myocyte into tightly-adhered fibroblastoid cells were observed. This was accompanied by a partial inhibition in the α-actin gene expression. On the other hand, when the cells were dissociated with 0.25 % Trypsin and 0.1 % EDTA (proteolytically) and re-plated in a low-serum medium, they neither showed a fibroblastoid morphology nor a repression in α-actin expression. This suggested the involvement of trypsin-sensitive cell surface molecules in transmitting information about cell density for α-actin gene regulation.

The molecular basis of α-actin expression in BC_3H1 cells was investigated recently by Min *et al.*, (1990). Four 16 bp motifs containing *cis*-acting CArG regulatory elements were observed. A series of α-actin deletion constructs containing sequences upstream to the transcription start site were fused with human growth hormone reporter gene to study the role played by these regulatory elements. The results suggested that all four elements were required for tissue-specific expression, and that the core promoter between -372 and -143 was responsible for tissue restrictive expression. It was also speculated that a putative "cell density response element" might be located between base pairs -373 and -1074 (Min *et al.*, 1990).

No attempt has yet been made to study the regulatory mechanism

underlying the repression of the β-and γ-actin genes in BC_3H1 cells during differentiation.

A.5 EFFECT OF DIFFERENTIATION BLOCKING AGENTS ON ACTIN GENE EXPRESSION

Agents that disrupt the normal course of differentiation are useful tools to study the various steps involved in the differentiation process. The most commonly used agents are growth factors, oncogenes, and intracellular second messengers.

A.5.1. Growth factors

Acidic Fibroblast Growth Factor (FGF) was shown to block myogenic differentiation of BC_3H1 cells (Lathrop *et al.*, 1985). Addition of FGF to differentiated cells blocked the expression of α-actin but failed to induce the synthesis of β-and γ-actins. However, addition of serum resulted in a repression of α-actin synthesis and a maximal induction of β-and γ-actin expression. From these results it was concluded that repression of α-actin synthesis and induction of β-and γ-actin expression were under independent control (Wice *et al.*, 1987).

Epidermal growth factor (EGF) addition to differentiated BC_3H1 cells resulted in a repression of α-isoactin synthesis and in an induction in β-and γ-actin expression. In the cells treated with EGF, the β-and γ-actin proteins were selectively stabilised from degradation while the α-isoactin protein synthesis was reduced (Wang and Rubienstein, 1988). This observation again suggests an independent control for

both the genes.

A.5.2. Oncogenes

The products of proto-oncogenes play a central role in the regulation of cell proliferation and differentiation. They function at distinct steps in the intracellular growth factor cascades. For example, the product of the *ras* gene resembles the G protein that modulates adenylate cyclase, ion channels, and other membrane associated enzymes (Gilman, 1984). The product of the *c-myc* gene is a DNA binding protein that had been postulated to function as an intranuclear mediator of growth factor signals (Armelin *et al.*, 1984; Greenberg and Ziff, 1984). It is currently unknown whether one or more of these proto-oncogene products participate in the pathway for growth factor mediated regulation of myogenesis. It is known that the onset of myogenesis is preceded by a decline in the *c-myc* gene expression (Sejersen *et al.*, 1985). Transfection of the *c-myc* gene was shown to partially inhibit the myogenic differentiation of BC_3H1 cells (Schneider *et al.*, 1987). Varying effects have been observed in other muscle cell lines (Glaser and Wice, 1989). The activated Harvey-*ras* gene transfection completely blocked differentiation in BC_3H1 cells and other cell lines (Kelvin *et al.*, 1989a; Glaser and Wice, 1989). In a recent report, *ras* gene transfected C2 myogenic cells continued to express elevated levels of β-and γ-actin mRNA following withdrawal from the cell cycle (Olson and Capatanaki, 1989). E1A, the nuclear oncogene of the Adenovirus, was able to block myogenic differentiation of rat muscle cell lines C2 and L8, as monitored by repression of the

expression of muscle-specific genes such as skeletal and cardiac α-actin, MHC and creatine kinase (Webster *et al.*, 1988). Recently it was observed that the E1A gene blocked myogenic differentiation of 23A2 cells by repressing the expression of MyoD1 and Myogenin (Enkemann *et al.*, 1990). No attempt has yet been made to understand the mechanism of regulation of actin gene expression in cells transfected with oncogenes.

A.5.3. cAMP

Sutherland and colleagues in the 1950's discovered that cyclic adenosine 3'5'-monophosphate (cAMP) was synthesised in cells in response to extracellular signals such as hormones (Robinson *et al.*, 1971). The enzyme responsible for the formation of cAMP in the presence of ATP, Mg^{2+} and other cofactors was identified and named as adenylate cyclase (also called as adenyl cyclase or adenylyl cyclase) (Sutherland *et al.*, 1962; Schram and Selinge, 1984). Krebs and coworkers discovered that cAMP acts by activating a cAMP-dependent protein kinase (cAMP-dpk) (Walsh *et al.*, 1968).

The increase in the level of the intracellular cAMP leads to activation of cAMP-dpk, which catalyses the transfer of the γ-phosphoryl group of ATP to the hydroxyl group of a seryl or threonyl residue of a substrate protein (Krebs and Beavo, 1979; Nestle and Greengard, 1984). cAMP-dpk is multi-subunit enzyme, composed of two types of subunits, a regulatory subunit dimer and two catalytic unit monomers. The regulatory subunit inhibits the catalytic subunit and thus the $(R)_2C_2$ form of the

enzyme is inactive. cAMP activates the enzyme by binding to the subunit R, leading to disassociation of two active catalytic subunits (For a review Dudai, 1987). The catalytic subunit of cAMP-dpk has broad substrate specificity. The amino acid sequences around the phosphorylation site of several substrate proteins are of the type arg-arg/ser-x-ser/thr (Krebs and Beavo, 1979; Nestle and Greengard, 1984). The phosphorylation of substrate proteins by cAMP-dpk brings about or initiates, cellular response to the extracellular signal that has triggered the cAMP cascade.

The involvement of cAMP dependent PkA pathway in BC_3H1 differentiation was tested by Hu and Olson (1988). Undifferentiated cells were exposed to either activators of PkA such as permeable analogues of cAMP (dibutrylcAMP (dbcAMP) or 8-Bromo-cAMP (8-Br-cAMP)), or compounds that stimulate adenylate cyclase, eg., Forskolin, Prostaglandin E1 and cholera toxin. The elevation of intracellular cAMP levels was observed to reversibly inhibit the induction muscle-specific genes such as MCK and TnT. Serum mitogens such as, FGF and TGF-β were found to inhibit differentiation through mechanisms independent of cAMP. It was suggested that differentiation of BC_3H1 cells follows both cAMP-dependent and independent mechanisms (Hu and Olson, 1988). Cholera toxin also blocked proliferation and differentiation of BC_3H1 cells by raising intracellular levels of cAMP (Kelvin *et al.*, 1989b). The effect of cAMP on the expression of actin genes under cAMP treated conditions has not been demonstrated so far.

A.6 OBJECTIVES OF THE PRESENT STUDY

1. At present, it is not clear whether the down-regulation of β-and γ-actin genes during muscle differentiation is regulated at the transcriptional level or at a post-transcriptional level, such as mRNA stability. Hence, in this thesis, an attempt has been made to study the role of mRNA stability in the down-regulation process of β- and γ-actin genes. To investigate this, cAMP and E1A were used as tools.

2. It has been suggested that the α-actin expression is regulated at the transcriptional level during myogenic differentiation. Both cell-cell contact and serum starvation were found to be required for maximal induction of α-actin expression. But no information is available on the second messengers involved in these two activation processes. An attempt was made to investigate whether or not these two activation signals follow the cAMP-dependent signalling pathway.

3. The regulator of myogenesis, myogenin, must be subject to control during differentiation. The possible involvement of cAMP-dependent signal transduction pathway in myogenin gene expression was also investigated.

MATERIALS AND METHODS

B.1 CELL CULTURE

The myogenic cell line BC_3H1 was obtained from the American Tissue Type Culture Collections, Rockville, MD. The cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 20% fetal calf serum (FCS) (Gibco); 100 u Penicillin /ml and Streptomycin 100 μg/ml (Sigma) at 37°C in a 5% CO_2 enriched atmosphere.

The cells were plated at the rate of 100,000 cells/60mm or 280,000 cells/100mm dish (35 cells/mm^2) (Falcon), in serum-supplemented media, they became confluent by 4 days after plating. The signal to differentiate, by lowering the serum level in the media to 0.5% was given anytime between day 1 to day 4 as indicated in the results. Cells were rinsed twice with phosphate buffered saline (PBS)(137mM NaCl; 2.6mM KCl; and 8.3mM Na_2HPO_4 pH 7.2) and the low-serum media containing antibiotics was added. db-or 8-br-analogues (Boehringer Manheim) of cAMP was added to a final concentration of 1mM (100mM stock solutions were prepared in 1M HEPES and diluted to a 10mM stock with DMEM and sterilized by passage through a 0.2-micron filter (Millipore)) on days indicated in the results. In all cases the media was changed once in every three days.

Adenovirus 5 E1A transfected cell line BC_3E7 developed in our lab by Dr. R.W.H.Lee were grown in a media similar to that of BC_3H1 cells.

Pictures of cell morphology were taken using Leitz Diaplan scientific microscope.

B.2 RNA ISOLATION

RNA isolation was done following the procedure described by Chomczynski and Sacchi, (1987). Briefly: The cell monolayers were rinsed with PBS and dissolved in solution D (4M guanidinium thiocyanate; 25mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1M 2-mercaptoethanol) at the rate of 500 μl/60mm dish. To extract the RNA, 500 μl water-saturated Phenol (BRL), 100 μl chloroform:isoamyl mixture (49:1), and 50 μl of 2M sodium acetate at pH 4.0 was added. The samples were mixed well, incubated at 0°C for 15 min. and spun at maximum speed for 20 min. in a microcentrifuge at 4°C. The aqueous phase was transfered to a fresh 1.5 ml tube, and the RNA was precipitated by adding 500 μl of iso-propyl alcohol and incubating at -20°C for $\approx$1 hr. The samples were spun at maximum speed in a microcentrifuge for 15 min. at 4°C and the supernatant was discarded. The pellet was redissolved in 180 μl of sterile deionized water(dH_2O), and ethanol precipated as follows, 20 μl of 3M sodium acetate(pH 5.2) and 500 μl of 100% ethanol was added and the mixture was incubated at -20°C for 1 hour and the RNA was precipated by spinning at maximum speed in a microcentrifuge at 4°C for 15 minutes. The precipitate was rinsed with 75% ethanol, dried in Speedvac, dissolved in dH_2O and stored at -70°C. The concentration and purity of the RNA was determined by

measuring the OD_{260}/OD_{280} of an aliquot.

B.3 NORTHERN BLOTTING

B.3.1 Agarose gel electrophoresis

The RNA was electrophoresed as per Ausubel *et al.*, (1987): 5-10 μg of RNA was suspended in a premix such that the final concentration in the sample was, 1X MOPS; 6.5% Formaldehyde and 50% Formamide. The samples were denatured by incubating at 60°C for 15 minutes and 5μl of formaldehyde gel loading buffer (1mM EDTA, pH 8; 0.25% Bromophenol Blue; 0.25% Xylene Cyanol; 50% glycerol) was added. The samples were size-fractionated in an 1.2% agarose/formaldehyde gel. The gel was run with 1X MOPS running buffer (1X MOPS running buffer: 20 mM MOPS (pH 7.0); 0.8 mM sodium acetate; 1mM EDTA, pH 8.0) at a constant voltage of $\approx$ 5 V/cm. The amount of RNA loaded was verified by running a duplicate set and staining with 0.5 μg/ml of ethidium bromide(EtBr) in dH_2O. The ribosomal RNA bands were photographed under UV light.

B.3.2 Blotting, hybridization and washing

The gel was rinsed several times with dH_2O and soaked in 10X SSC (1X SSC: 0.15M sodium chloride; 0.15 M sodium citrate, pH 7.0) for 30 min. A piece of nylon membrane (DuPont) 5mm smaller than the gel was cut and presoaked in 10X SSC for 15-30 min. The blot was setup as follows: a wick was made from

Whatman 3mm chromatography paper, presoaked in 10 X SSC and disposed on to a glass plate such that the ends were in a plastic container having 10 X SSC, the gel laid upside down on the preset 3mm paper and laid over it a presoaked nylon membrane, 5 sheets of Whatman 3mm paper, precut to a size 5 mm smaller than the gel, and a stack of precut paper towels. A weight ($\approx$ 500 g) was then placed on a glass plate positioned on top of the transfer setup and the RNA was transfered for 15 to 18 hrs. After transfer the membrane was rinsed in 10 X SSC to remove agarose particles, and the RNA was fixed onto the nylon membrane by exposing to UV irradiation at 254 nm. The agarose gels were stained in dH_2O having 0.5μg/ml EtBr, for a minimum of 30 minutes and examined under UV light to verify complete transfer of the RNA.

The membranes were prehybridized for 5 to 6 hours at 60°C in hybridization buffer(1% sodium dodecyl sulphate (SDS); 1 M sodium chloride; 10% dextran sulphate; 100 μg/ml denatured salmon sperm DNA). The prehybrized membranes were then hybridized in the same buffer with 1 to 3 X 10^6 cpm of radiolabelled probe for 15 to 18 hours. The probes were labelled using random oligonucleotide primer extension kit (Pharmacia) with [^{32}P] dCTP as described by the manufacturer. The probe was purified by passing through a Sephadex G 50 (Pharmacia) column. The actin and tubulin cDNA's were generous gift of Dr.L. kedes, USC, and the myogenin cDNA was a kind gift of Dr.W. Wright, Univ. of Texas.

The membranes were washed to a final stringency of, 0.2 X SSC 0.1% SDS at 60°C and exposed to Kodak XAR film.

B.4 DOT BLOTTING

Five to 10 μg of RNA was suspended in TE (10 mM Tris HCl (pH 8.0); 1 mM EDTA (pH 8.0)), such that the final volume does not exceed 25 μl and 15 μl of 20 X SSC and 10 μl 37% formaldehyde were added. The samples were denatured by incubating at 60°C for 15 minutes. The nylon membrane was setup on the dot-blot manifold according to manufacturer's instructions (BioRad). After application of vaccum the samples were blotted onto the membrane, the wells were rinsed once with 6x SSC before and after blotting the RNA. The vaccum creates a cicular spot(dot) on the membrane in which the RNA is adsorbed. The apparatus was dismantled and the RNA was fixed to the membrane by exposing to UV light. The membrane was prehybridized, hybridized, washed and exposed to X-ray film as described in the section on Northern blotting.

After autoradiography, each dot was cut and counted in a scintillation counter for 10 minutes, using aqueous counting scintillant(ACS) (Amersham). For each probe two to three blank dots were counted seperately to provide the background readings.

B.5 PRIMER EXTENSION

B.5.1 Phosphate end labelling of oligonucleotide.

The synthetic 25 nucleotide primer, AB485 (a generous gift of P.E. Branton, McGill University), which hybridizes to the sequence from +56 to +81 relative to the E1A mRNA cap site was used.

The oligomer was diluted to a concentration of 1 OD/ml. The reaction mixture was prepared as follows: 1 μl of oligomer stock; 4 μl of ddH_2O; 35 μl of Kinase buffer 1 (20 mM Tris HCl, pH 9.5; 1mM spermidine (ICN); 0.1 mM EDTA, pH 8); 5 μl of Kinase buffer 2 (500 mM Tris HCl, pH 9.5; 100 mM $MgCl_2$; 50 mM DTT; 50% v/v glycerol); 5 μl of [γ^{32}P] ATP; 2 μl of T4 polynucleotide kinase (Pharmacia). The mixture was incubated at 37°C for 1 hour. The DNA was ethanol precipitated twice using 10μg of *E. Coli* tRNA (Boehringer) as a carrier. The pellet was washed with 70% and 90% EtOH, dried in Speedvac and resuspended in TE (pH 7.9).

B.5.2 Procedure

The RNA, isolated as described above, was further purified by another round of phenol-chloroform extraction. Primer extension reactions were done following the procedure described by Jones *et al.*, (1985), with minor modifications. Ten micrograms of RNA were ethanol precipitated, washed with 70% and 95% ethanol and then dried in a speedvac with no heat for 1 hour. The RNA pellet was resuspended in 8 μl of TE (pH 7.9) containing the radioactive oligonucleotide. To this 2 μl of TKE (10 mM Tris Hcl, pH 7.9; 1 mM EDTA, pH 8.0; 1.25 M KCl.)

was added and the RNA and DNA were allowed to hybridize at 60°C for 1 hour. The samples were allowed to reach room tempreature, and 25 μl of RT buffer (20mM Tris Hcl; 10mM $MgCl_2$; 5mM Dithiothreitol(DTT); 0.33 mM of each dNTP; 10 μg/ml of actinomycin D) containing 10 units of AMV reverse transcriptase (Pharmacia) was added and the reaction mixes were further incubated at 37°C for 1 hour. The nucleic acids were precipitated using 500 μl of 95% ethanol and sedimented by centrifugation. The pellet was washed with 70% and 95% ethanol and dried in a Speedvac. The extension products were resuspended in 3 μl of dH_2O and 9 μl of sequencing STOP buffer (USB)(95% formamide; 20mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol FF). The samples were denatured by boiling and 3 μl was loaded onto a 8% polyacrylamide urea gel as described by Ausubel *et al.*,(1987). The extended products were detected by autoradiography.

B.6 [^{3}H] URIDINE INCORPORATION ASSAY

The cells were plated at the rate of 35 cells/mm^2 in a multi-well dish and were grown for 3 days. On day three the cells were treated with various concentrations of actinomycin D (0.05 to 10 μg/ml) and incubated at 37°C for 30 minutes. The media was then replaced with one containing similar concentration of actinomycin D plus [^{3}H] uridine at a final concentration of 0.27 μM. The cells were incubated for one hour at 37°C. The wells in the multi-well dish were rinsed twice with PBS and the cells were lysed in 1% SDS; 10mM Tris-Cl(pH 7.5); 1mM EDTA,

and blotted onto glass fiber filter (Whatman GF/B). The filters were then washed thrice in an ice cold solution containing 10% TCA; 20mM sodium pyrophosphate. These filters were rinsed in 70% ethanol and air dried. The [^{3}H] uridine incorporation was estimated by scintillation counting.

RESULTS

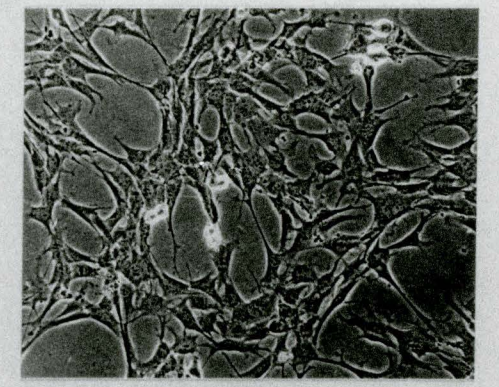
C.1 MORPHOLOGY

BC_3H1 cells were plated at 1x 10^5 cells/60 mm or 2.8 x 10^5 cells/100 mm plate (35 cells/mm^2) in DMEM supplemented with 20% fetal calf serum and antibiotics as described in Materials and Methods. The cells adhered to the bottom of the plate a few hours after plating and exhibited a typical fibroblast-like morphology. This morphology, seen in fig 1A, was maintained until they received a signal to differentiate. The differentiation signal was either cell-cell contact upon confluency or reduction in serum levels in the media from 20% to 0.5%. When the low-serum media was added to logarithmically growing cells, the cells advanced one or two more rounds of cell division before they withdrew from the cell cycle. After 3 to 4 days in low-serum media, the cells aligned to form streams of elongated, refractile cells (Fig. 1B), a characteristic morphology of muscle cells in culture. BC_3H1 cells when allowed to grow past confluency in high-serum media became elongated and aligned into streams of muscle like cells (Fig 1C).

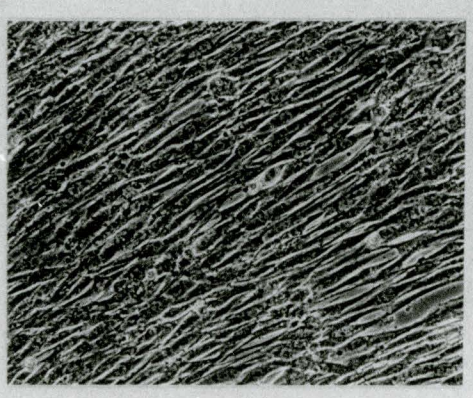
To study the effect of cAMP treatment on myogenic differentiation of BC_3H1 cells, logarithmically growing cells were treated with low-serum medium containing 1mM dbcAMP. The cAMP-treated cells did not become elongated and more refractile as the untreated BC_3H1 cells even after 6 days in culture, instead, they exhibited a less refractile, stellate morphology with long thin neurite-like processes

Figure 1. Morphology of undifferentiated and differentiated BC_3H1 cells. Panel A. Photomicrograph of dividing cells grown in 20% serum containing media for three days. Panel B. Morphology of cells that were allowed to grow in a media containing 20% serum for eight days. Panel C represents the morphology of the cells that were treated with low-serum media on the third day after plating.

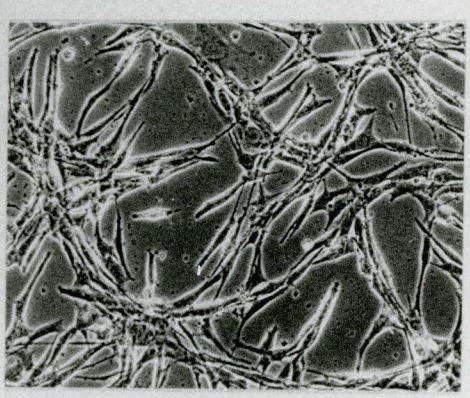
A



B



C



extending between cells (Fig 2A). Cells treated with 8-Br-cAMP (Fig. 2B), another analogue of cAMP, exhibited a morphology similar to that of dbcAMP treated cells.

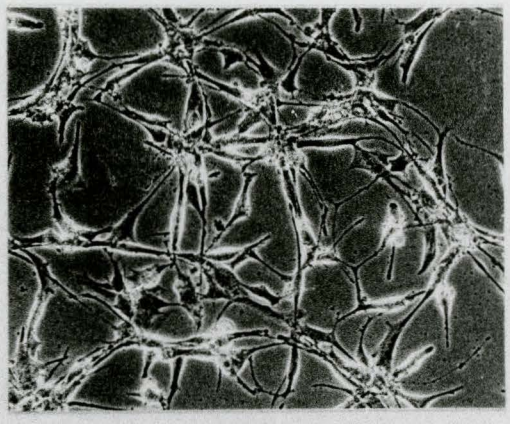
BC_3E7, an Adenovirus early region 1A-transfected cell line BC3E7, was developed in our lab by Dr R.W.H. Lee. This cell line was used to study the effect of the E1A gene on myogenic-differentiation of BC_3H1 cell line. The expression of E1A in BC_3E7 was demonstrated by primer extension analysis (Fig. 3). E1A was observed to be expressed at very low levels both in dividing and post-confluent BC_3E7 cells (lane 5&6), when compared to either 293 cells: an E1A transformed mouse kidney cell line (lane 2&3) or BC_3H1 cells infected with phenotypically *wt* Ad5dl309 virus (lane 4). No message was detected in uninfected BC_3H1 cells (lane 7).

Logarithmically growing BC_3E7 cells were flat and exhibited a fibroblast-like morphology (Fig 4A) similar to that of BC_3H1 cells. Unlike BC_3H1 cells, post-confluent BC_3E7 cells became round, flat, and contact inhibited (Fig 4B). When the dividing BC_3E7 cells were treated with low-serum medium, the cells exhibited a stellate-like morphology with long thin processes extending between them (Fig. 4C). We observed that serum-starvation of BC_3E7 cells resulted in extensive lifting of cells from the plate, so the experiments using BC_3E7 cells were conducted in high-serum medium.

C.2 ACTIN EXPRESSION IN BC_3H1

Figure 2. Effect of cAMP on the morphology of BC_3H1 cells. Three days after plating the cells were treated with low-serum media containing either db-cAMP(A) or 8-Br-cAMP(B) at a final concentration of 1mM and were maintained for an additional five days.

A



B

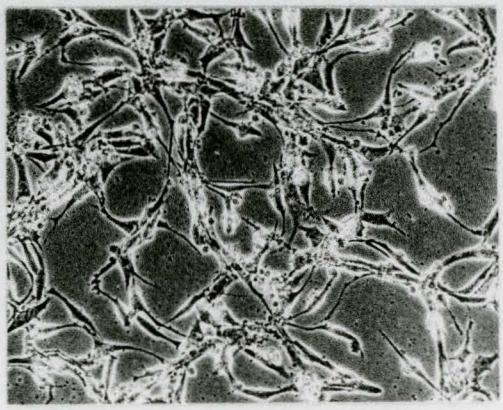


Figure 3. Primer extension analysis to detect the E1A message. Twenty micro grams of RNA (excepting lane 2) was annealed to a 25 nucleotide primer, complementary to the sequence from +56 to +81 relative to the E1A mRNA cap site. The extensions were carried out and the product was separated on a 8% polyacrylamide gel as described under Materials and Methods. Lane 1 shows the size markers, from top 90, 76, 71, 67, and 57 nucleotides. The arrow mark indicates the 81 bp long extended product. The bands around 60 bp in lanes 2 through 7 were contaminants from the primer. Both lanes 2 and 3 were RNA from 293 cells which served as a positive control, the only difference being, 10 μg was used for extension in lane 2. Lane 4 was RNA from BC_3H1 cells infected phenotypically *wt* adenovirus Adl309, the RNA was isolated 3 days after infection. Lane 5 and 6 were the RNA from dividing (day 4) and post-confluent (7 days after plating) BC_3E7 cells respectively. Lane 7 was RNA from uninfected BC_3H1 cells.

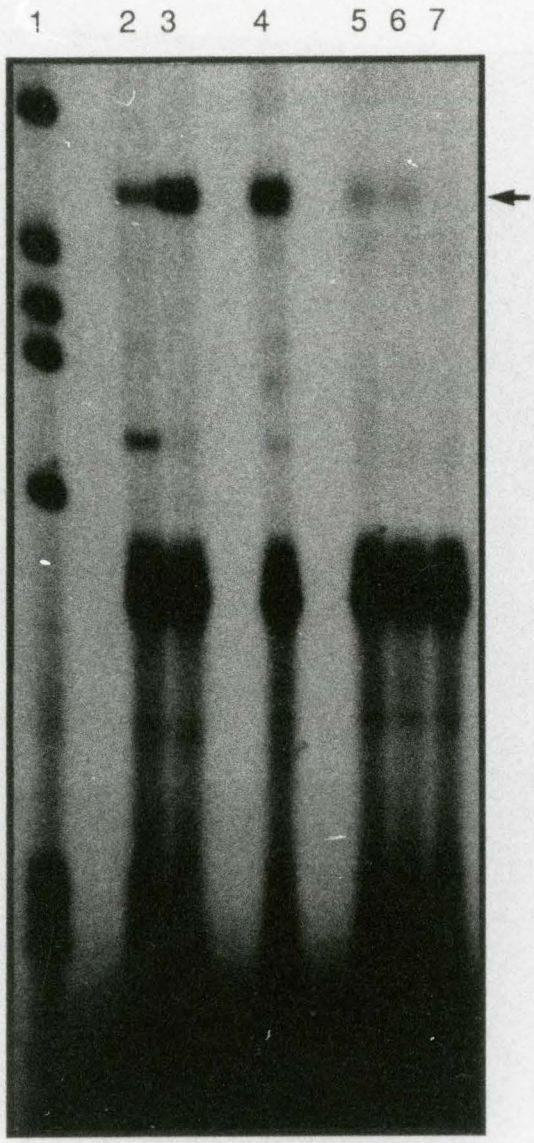
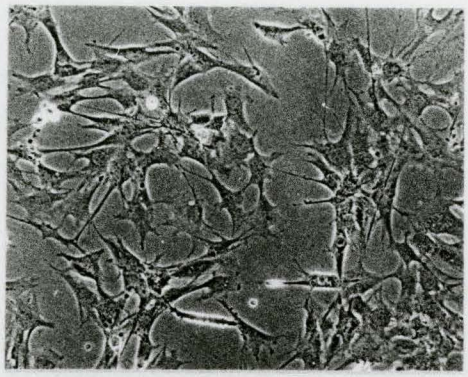
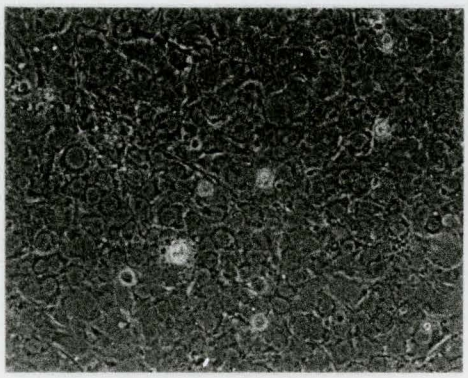

1
2 3
4
5 6 7

Figure 4. Morphology of BC_3E7 cells. Panel A is the morphology of dividing cells 4 days after plating. The cells in Panel B were grown in 20% media for nine days. Panel C represents the morphology of cells that were serum-starved on day four and maintained for an additional 5 days.

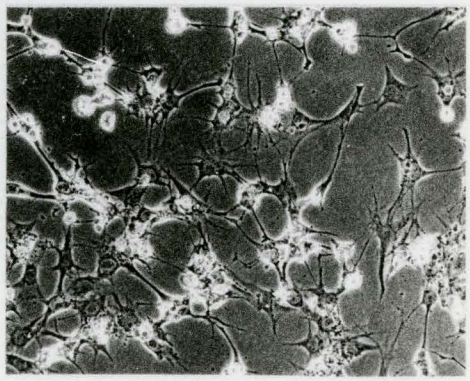
A



B



C



C.2.1 β-and γ-actin mRNA levels

During the normal course of differentiation of BC_3H1 cells β-and γ-actin mRNA levels had been reported to be down-regulated (Strauch and Rubenstein, 1984). This was confirmed in the present study. Logarithmically-growing cells were treated with low-serum medium 2 days after plating. RNA was isolated from the cells collected on different days after starvation and at various stages of differentiation. They were day 0 (the day of treatment: undifferentiated); day 1 (1 day after treatment: differentiating); and day 5 (5 days after treatment: differentiated). Northern analysis of the total RNA was done using a full-length actin cDNA probe (Gunning *et al.*, 1983) which cross-hybridizes to all three actin transcripts (α,β and γ). The results illustrated that the levels of both non-muscle actins (β-and γ-) were decreased from high levels on day 0 to very low levels on day 5, while the levels of muscle-specific α-actin increased as the cells differentiate (Fig. 5A). Both β-and γ-actin messages are the same size (2100 bp) and hence migrate to the same position in the gel. Unlike the coding region, the 3'untranslated(UT) regions of the actin genes do not have any homology, so β-and γ-actin messages could be studied individually using 3'UT region-specific probes (Ponte *et al.*, 1983). Northern analysis using 3'UT region-specific probes showed that the level both β-and γ-actin messages were reduced during differentiation (Fig 5B & 5C respectively).

To determine the effect of cAMP on the down-regulation of non-muscle actin genes, RNA was isolated from the cells maintained for five days in low-serum

Figure 5. Northern blot analysis for actin mRNA in BC3H1 cells: Effect of cAMP. Cells were treated with low-serum media in the presence or absence of 1mM dbcAMP on the second day after plating. The RNA was isolated from cells samples collected at the day of treatment (day 0), one day after treatment (day 1) and five days after treatment (day 5) indicated as 0, 1 and 5 above the Northern blots. Five micrograms of the RNA was blotted onto a Genescreen membrane and probed using a full-length actin cDNA probe which hybridizes to all three isoforms of actin (Panel A). The positions of α, β, and γ-actins are indicated. Panel B and Panel C are the autoradiograms of the Northern blots which were hybridized to the 3'UT region-specific probes of β-and γ-actins respectively. Panel D corresponds to the Ethidium bromide stained RNA used for Northern blotting in Panels A, B and C.

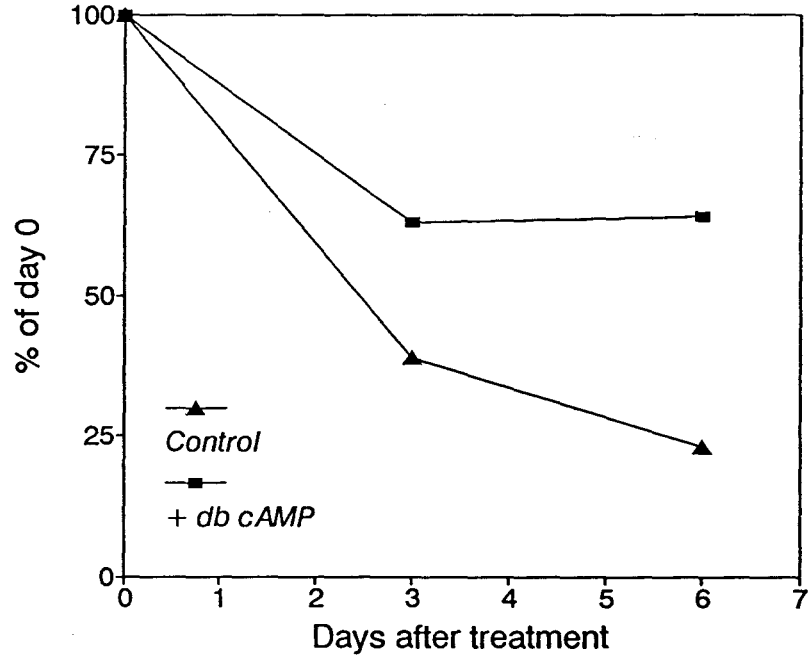
medium containing 1 mM db cAMP. Northern blot analysis using full-length actin probe showed that β-and γ-actins remained at higher level than the untreated cells (Fig. 5A). Analysis using gene-specific probes demonstrated that both the non-muscle genes were maintained at high levels (Fig. 5B and 5C respectively). Fig. 5D shows the Ethidium Bromide staining of the RNA used for all the Northern blots in Fig 5 indicating that equal amounts of RNA was loaded.

Quantitation of the β-and γ-actin message levels during differentiation was done by dot blot analysis using gene-specific probes as described under Materials and Methods. The mRNA levels were expressed as percentage of their initial levels on the day of treatment with low-serum medium (day 0). With serum-starvation alone, both β-and γ-actin levels declined to ≈25% of their initial levels, while with cAMP-treatment the message levels were maintained at no less than 70% of their initial levels even after six days of drug treatment (Fig. 6A and 6B). Treatment of cells with 8-Br-cAMP gave similar results (data not shown).

To confirm that cAMP did not block the down-regulation of all non-muscle genes during differentiation, the expression of β-tubulin was analysed. The β-tubulin gene was selected because, like β-and γ-actins the β-tubulin message has been shown to drop during myogenic differentiation (Gunning *et al.*, 1987). During differentiation of BC_3H1 cells, the level of β-tubulin mRNA was reduced to less than 25%, of its initial levels and the cAMP treatment did not have any significant effect on the β-tubulin mRNA levels (Fig. 7).

Figure 6. β-and γ-actin message levels in BC_3H1 cell line: Effect of cAMP. The cells were serum-starved on the second day after plating either in the presence or in the absence (control) of 1mM dbcAMP. The RNA was isolated from cell samples collected at, the day of treatment (day 0), one day after treatment (day 1) and six days after treatment (day 6). Five micrograms of RNA was blotted onto a Genescreen membrane and quantified by dot-blotting using actin gene-specific probes as described under Materials and Methods. The mRNA levels were expressed as percentage of initial levels on day 0. Panel A and Panel B corresponds to levels of β-and γ-actin mRNA, respectively.

A


100
75
50
25
0
% of day 0
Control
+ db cAMP
0 1 2 3 4 5 6 7
Days after treatment

B

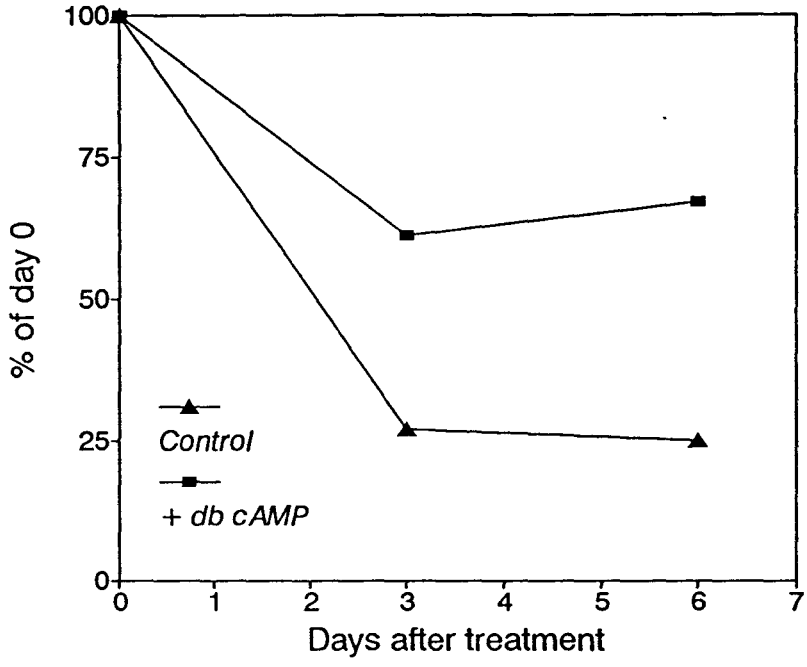
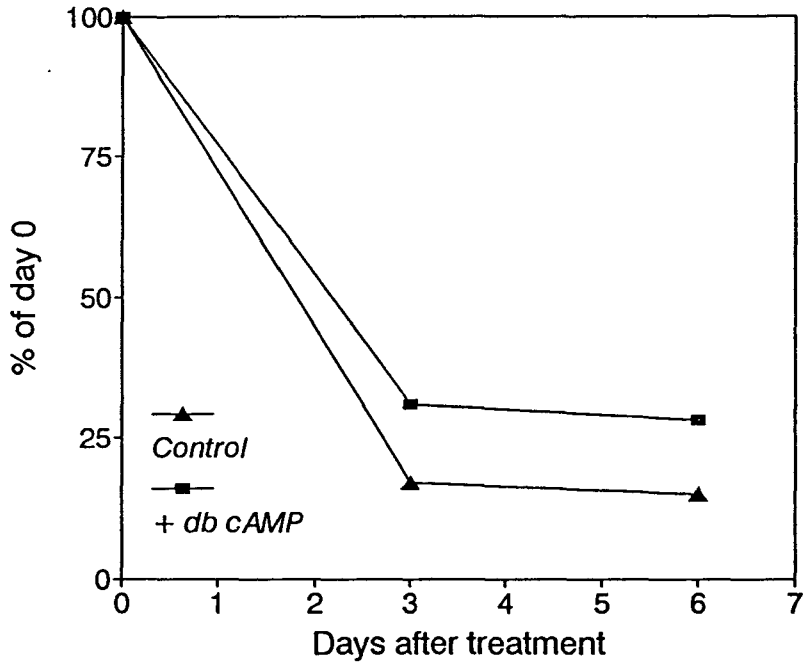

100
75
50
25
0
% of day 0
Control
+ db cAMP
0 1 2 3 4 5 6 7
Days after treatment

Figure 7. Effect of cAMP on the β-tubulin mRNA levels. The RNA for this experiment was obtained from the batch prepared for the experiment in Fig. 6. Five micrograms of RNA was blotted onto a Genescreen membrane and quantified by dot-blotting using chicken β-tubulin probe as described under Materials and Methods. The mRNA levels were expressed as percentage of initial levels on day 0.


100
75
50
25
0
% of day 0
Control
+ db cAMP
0
1
2
3
4
5
6
7
Days after treatment

The effect of E1A on actin gene expression was studied by Northern blot analysis of RNA from BC_3E7 cells grown in high-serum media. Higher levels of both β-and γ-actin mRNA was observed in BC3E7 cells even after 8 days in culture (Fig.8A), while a clear drop in the level of both messages was seen in BC_3H1 cells (Fig. 8B). Analysis of BC_3E7 RNA using gene specific probes for β-and γ-actin genes indicate that the levels of both messages remain high (Fig. 9A&B). Quantitation of the non-muscle actin message levels in BC_3E7 demonstrated that β-and γ-actin mRNA remained at $\approx$70-80% of their initial levels (Fig. 10).

To rule out the possiblity that E1A had a non-specific effect on all the non-muscle genes, expression of the house-keeping gene α-tubulin was analysed. β-tubulin was not used as a control because E1A was shown to activate its expression (Stein and Ziff, 1984). The α-tubulin gene expression had been used as a control for E1A transfections in earlier studies (Webester *et al.*, 1988). Fig 11 shows a Northern blot of RNA from BC_3H1 and BC_3E7 cells grown in serum-supplemented media. The result indicated no significant difference in the pattern of expression between the two cell lines.

C.2.2 Actin mRNA half-life determination

Earlier studies on BC_3H1 cells had suggested that β-actin may not be regulated at the transcriptional level (Sharp *et al.*, 1989). It is not known whether the regulation occurs at the level of mRNA stability. In the present study experiments were done to measure the changes in mRNA half-life during differentiation of BC_3H1

Figure 8. Effect of E1A on actin mRNA expression. The cells were maintained in 20% serum-containing media and the RNA was isolated from samples collected at day 4, 5, 6, 7, and 8 after plating. Northern blotting was performed using a full length actin probe which cross-hybridizes to all three actin isoforms. The positions of a, β-and γ-actins are indicated. Panel A, and Panel B correspond to RNA from BC_3E7 and BC_3H1 cells respectively.

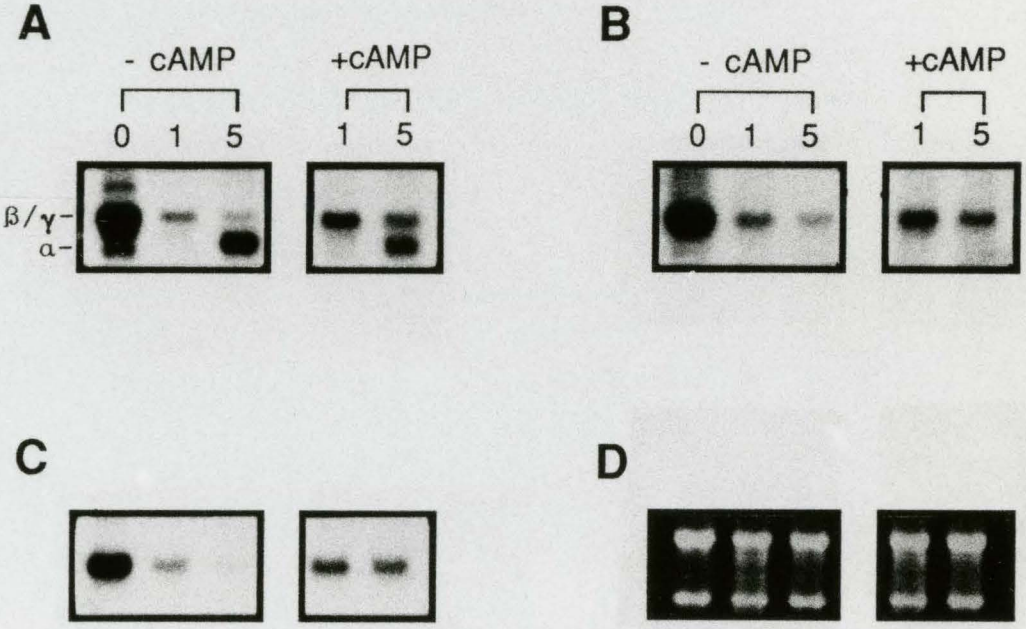

A
- cAMP
+cAMP
0 1 5
1 5
β/γ-
α-
B
- cAMP
+cAMP
0 1 5
1 5
C
D

Figure 9. Effect of E1A on the expression of β-and γ-actin mRNA. The BC_3E7 RNA for this experiment was from the same batch utilized for the experiment in Fig. 8. Panel A and Panel B are the Northern blots using 3'UT region probes of β-and γ-actin, respectively.

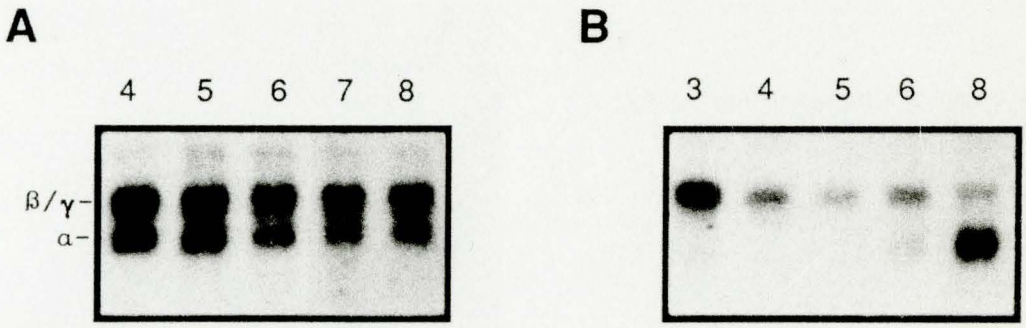

A
4 5 6 7 8
β/γ-
α-
B
3 4 5 6 8

Figure 10. Levels of β-and γ-actin mRNA in E1A transfected cell line. BC_3E7 cells were grown in 20% serum containing media and samples were collected at day 4, day 6, and day 8. RNA was isolated and the levels of β-(closed squares) and γ-(closed triangle) actin mRNA were quantified by dot-blotting as described in Materials and Methods.

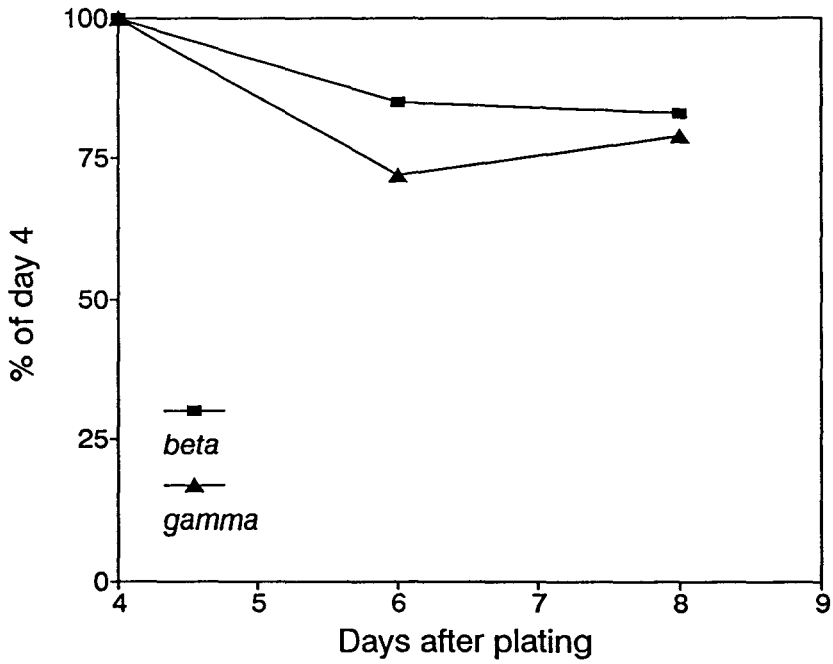
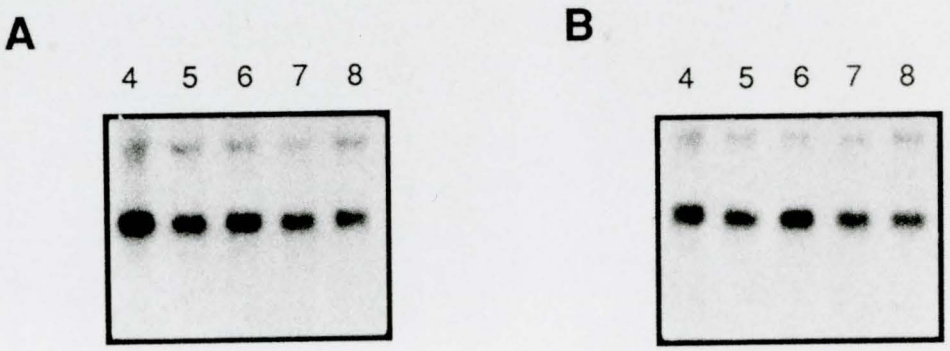

100
75
50
25
0
% of day 4
beta
gamma
4
5
6
7
8
9
Days after plating

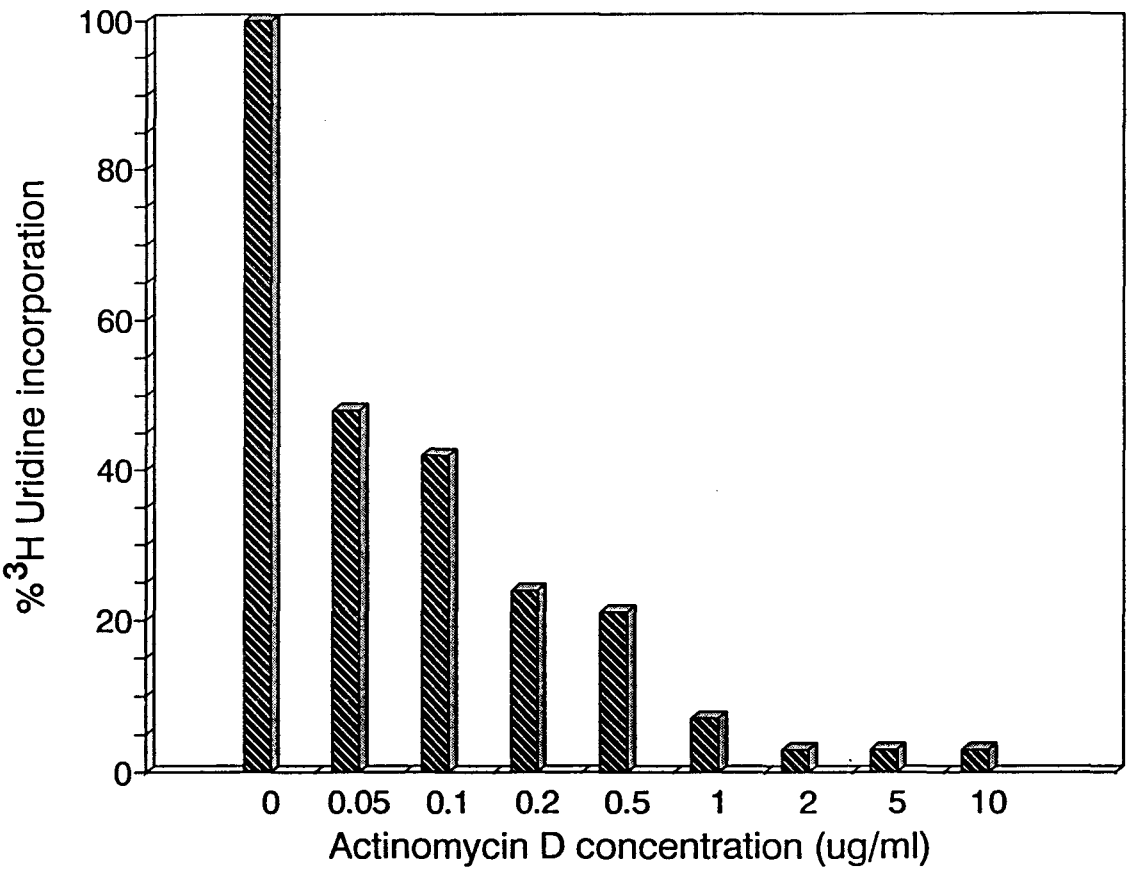
Figure 11. Effect of E1A on the expression of α-tubulin mRNA. BC_3H1 and BC_3E7 cell were grown in 20% serum containing media and the samples were collected at different days after plating. RNA was isolated and Northern blotting was carried out using the α-tubulin probe. Panel A and Panel B are the autoradiograms of Northern blots of RNA from BC_3H1 and BC_3E7 cells respectively.


A
4 5 6 7 8
B
4 5 6 7 8

cells. Pulse-chase experiments with [^{3}H] uridine were attempted first. The cells were pulse labelled with [^{3}H] uridine for 2 hrs and chased for 15 to 20 hours. Samples were collected at 1hr intervals and the radiolabelled RNA was isolated. Gene-specific probes for β-and γ-actins were denatured and fixed onto the nitrocellulose filter paper by dot-blotting. The amount of β-and γ-actin mRNA in each sample was determined by hybridizing the radioactive RNA to gene-specific probes. Non-specific binding was monitored by using denatured pBR322 on the nitrocellulose filter paper. After stringent washing, the amount of probe hybridized was determined by scintillation counting. However unfortunately, the background levels were found to be equal to or greater than 50 % of the actual signal. A different approach was thus attempted using Actinomycin D which blocks transcription of genes by binding to the GC residues in the DNA. An optimum working concentration of the drug was first determined by examining the effect of different ActinomycinD concentrations to block the incorporation of [^{3}H] Uridine in RNA, as described under Materials and Methods. The results indicated that at a concentration of 2μg/ml and above Actinomycin D repressed $\approx$97% of the[^{3}H] uridine incorporation (Fig. 12). All subsequent experiments were carried out at a concentration of 10μg/ml to ensure complete inhibition of transcription.

Cells were treated with Actinomycin D and RNA was isolated at different time intervals upto 12 hrs after addition. The amount of β-and γ-actin messages were quantified by dot-blot analysis using gene-specific probes, as outlined

Figure 12. Effect of various concentrations of Actinomycin D on [^{3}H] Uridine incorporation in BC_3H1 cells. BC_3H1 cells were grown in 20% serum containing media in a multi well dish. The cells were treated with varying concentrations (0.05 to 10.0 μg/ml) of Actinomycin D for 30 minutes. The medium in each well was replaced with one containing the same concentration of Actinomycin D plus 0.3 μM of [^{3}H] Uridine. The cells were incubated for an additional hour and the RNA was TCA precipitated and quantified as described under Materials and Methods. The incorporation rate is expressed as percentage of control(0 ug/ml).


100
80
60
40
20
0
% 3H Uridine incorporation
0
0.05
0.1
0.2
0.5
1
2
5
10
Actinomycin D concentration (ug/ml)

under Materials and Methods.

Figure 13 shows a representative autoradiograph of β-actin mRNA dot-blot at various time intervals under three different conditions such as, undifferentiated; differentiated; and cAMP treated. In logarithmically growing BC_3H1 cells the β-actin mRNA was present upto 12 hours after blocking transcription, probably due to long half-life (13A). In differentiated cells induced by serum-starvation for 6 days, the β-actin mRNA seemed to degrade more rapidly (13B). The β-actin mRNA of cells maintained in low-serum medium containing cAMP appears to be more stable than that of differentiated cells (13C). A similar pattern was observed for γ-actin under all three conditions (data not shown).

The amount of RNA was quantified as outlined under Materials and Methods and shown in Fig 14. The half-lives were mathematically calculated by linear regression analysis to ensure accuracy. The half-lives of β-actin mRNA were, 19 hrs in dividing cells (open squares), and 6 hours in differentiated cells (closed triangles). In cells treated with cAMP β-actin mRNA was observed to have a higher half-life 11 hours, (closed squares), as compared to the differentiated cells (Fig 13B). The half-lives of γ-actin were found to be 38, 7, and 12 hours for undifferentiated, differentiated, and cAMP treated cells respectively (Fig 15).

Stability measurements of β-and γ-actin mRNA in BC_3E7 cells are shown in fig.16 and fig. 17 respectively. In dividing cells β-and γ-actin mRNA had a half-life of 12 and 10 hours respectively (open squares). The half-lives of β-and γ-

Figure 13. Stability of β-actin mRNA in BC_3H1 cells. Transcription of RNA in BC_3H1 cells was blocked using Actinomycin D at a concentration of 10 ug/ml. The samples were collected from 0 to 12 hours after addition of Actinomycin D. The RNA was dot-blotted onto a genescreen membrane and probed using β-actin 3'UT region. The resulting autoradiograms are shown. Panel A represents RNA from

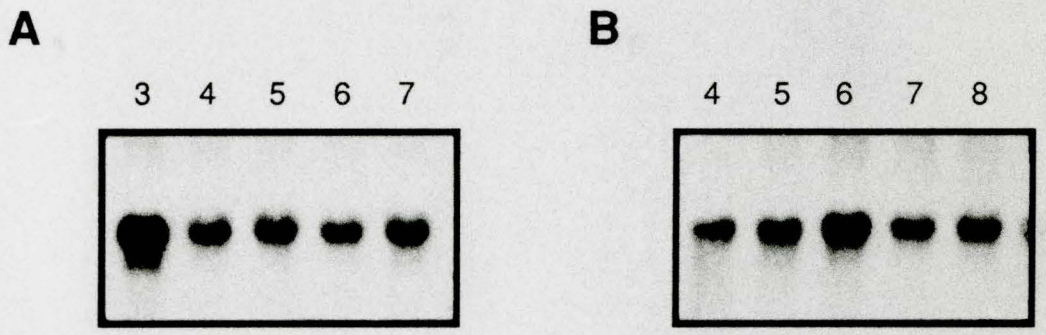
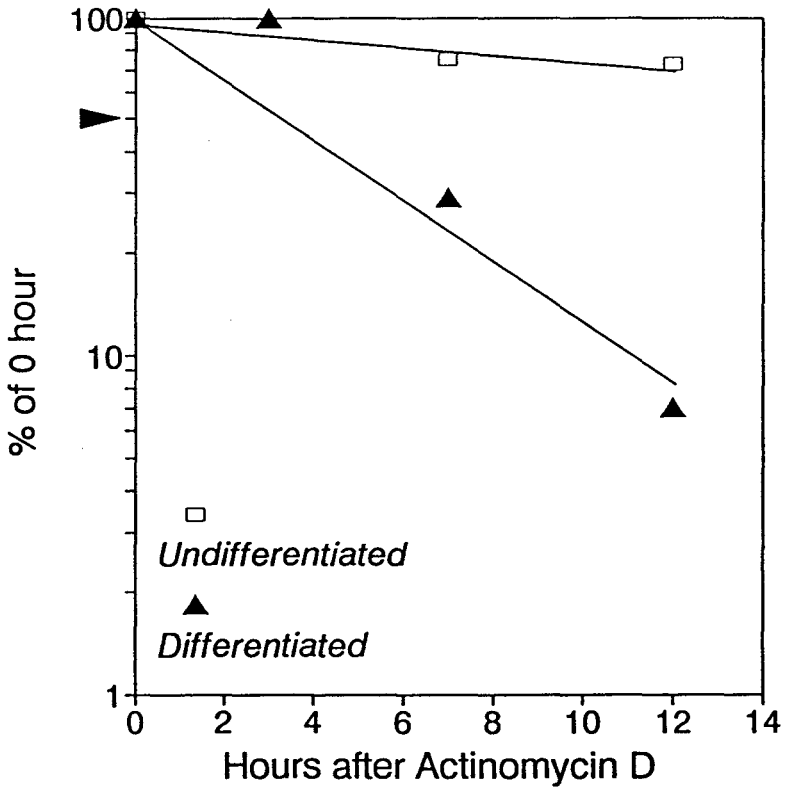

A
3 4 5 6 7
B
4 5 6 7 8

Figure 14. Half-life of β-actin mRNA in BC_3H1 cells: Effect of cAMP treatment. The signal obtained by dot-blotting (Fig. 13) was quantified by scintillation counting as described in the text. The graphs were drawn as percentage of initial levels at 0 hours. The arrow head indicates the 50% level on the logarithmic scale. Panel A shows the effect of differentiation on the half-life of β-actin mRNA. Panel B shows the effect of cAMP treatment on the half-life of β-actin message.

A


100
10
1
% of 0 hour
0
2
4
6
8
10
12
14
Hours after Actinomycin D
Undifferentiated
Differentiated

B

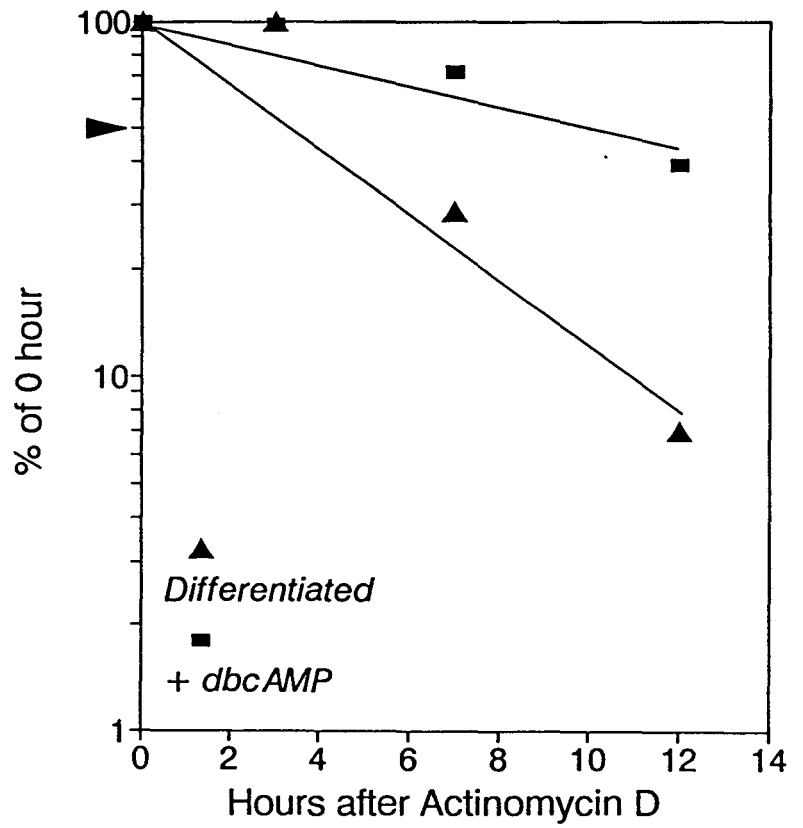
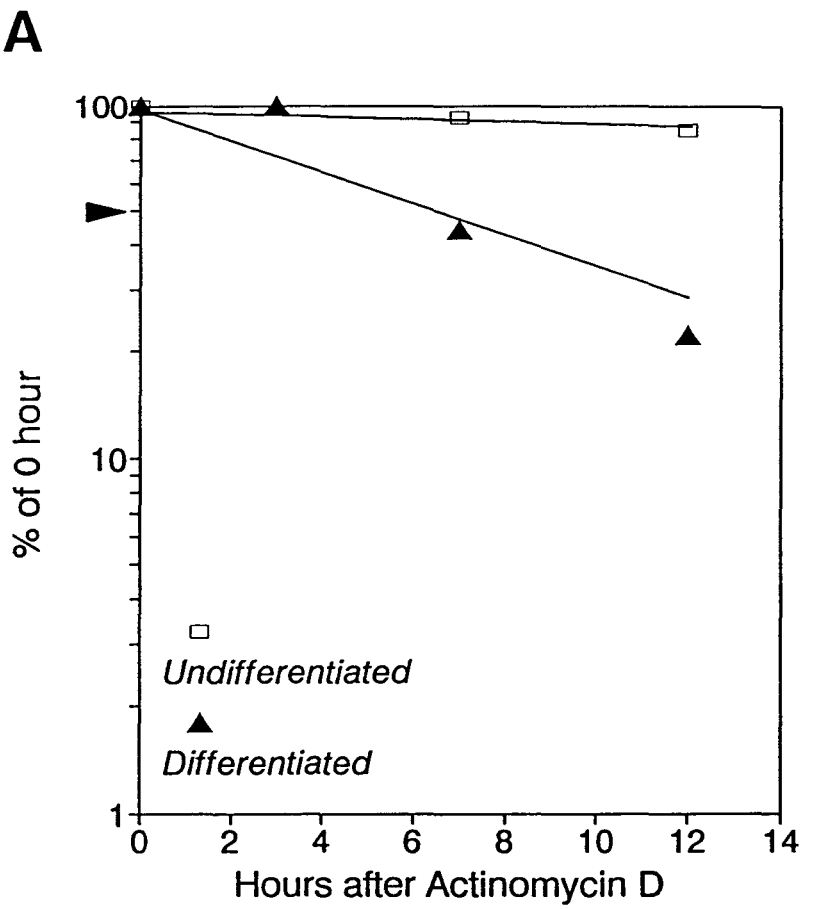

100
10
1
% of 0 hour
0
2
4
6
8
10
12
14
Hours after Actinomycin D
Differentiated
+ dbcAMP

Figure 15. Half-life of γ-actin mRNA in BC_3H1 cells: Effect of cAMP treatment. The RNA for this experiment was obtained from the same batch used for Fig. 13. The RNA wasblotted onto a gene screen membrane and hybridized to 3'UT region-specific γ-actin probe and the signal was quantified as described in the text. Panel A and Panel B shows the effect of differentiation and the effect of cAMP treatment on the half-life of γ-actin mRNA, respectively. The arrow head corresponds to the 50% level on the log scale.


A
100
10
1
% of 0 hour
Undifferentiated
Differentiated
0
2
4
6
8
10
12
14
Hours after Actinomycin D

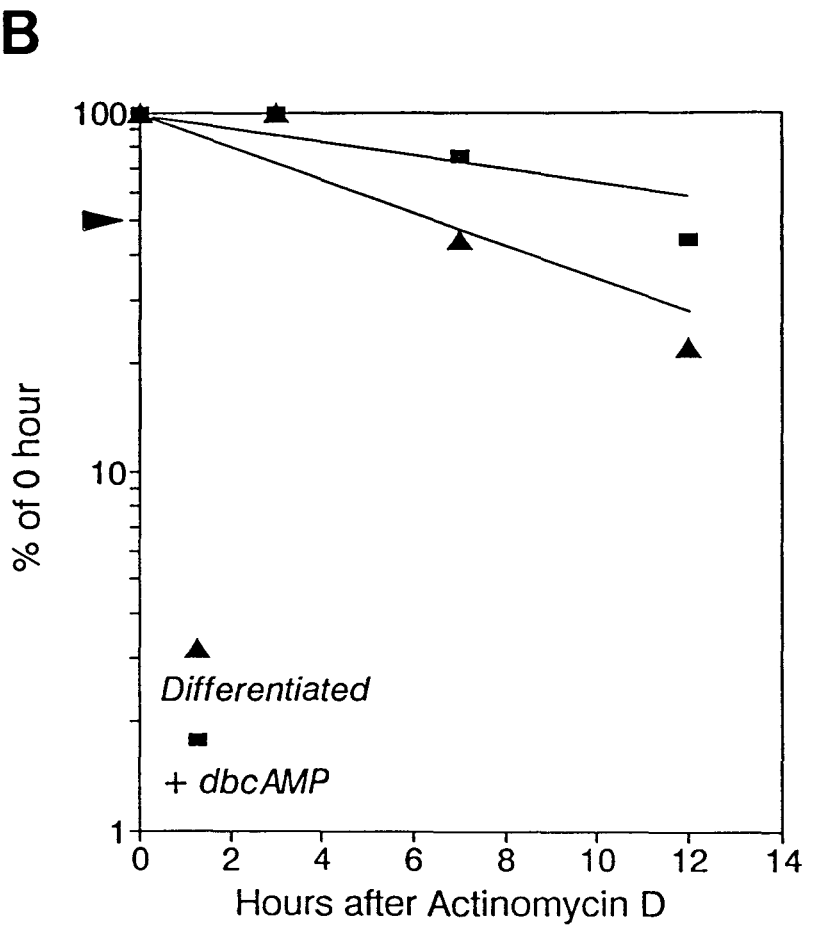

B
100
10
1
% of 0 hour
Differentiated
+ dbcAMP
0
2
4
6
8
10
12
14
Hours after Actinomycin D

Figure 16. Half-life of β-actin mRNA in BC_3E7 cells. The cells were grown in 20% serum-containing media. Transcription was blocked using Actinomycin D at a concentration of 10 μg/ml, in dividing (4 days after plating) and post-confluent (6 days after plating) cells and samples were collected at various time points between 0 to 12 hours. The RNA was isolated, and analyzed as described in Fig. 14. The arrow head indicates the 50% mark on the logarithmic scale.

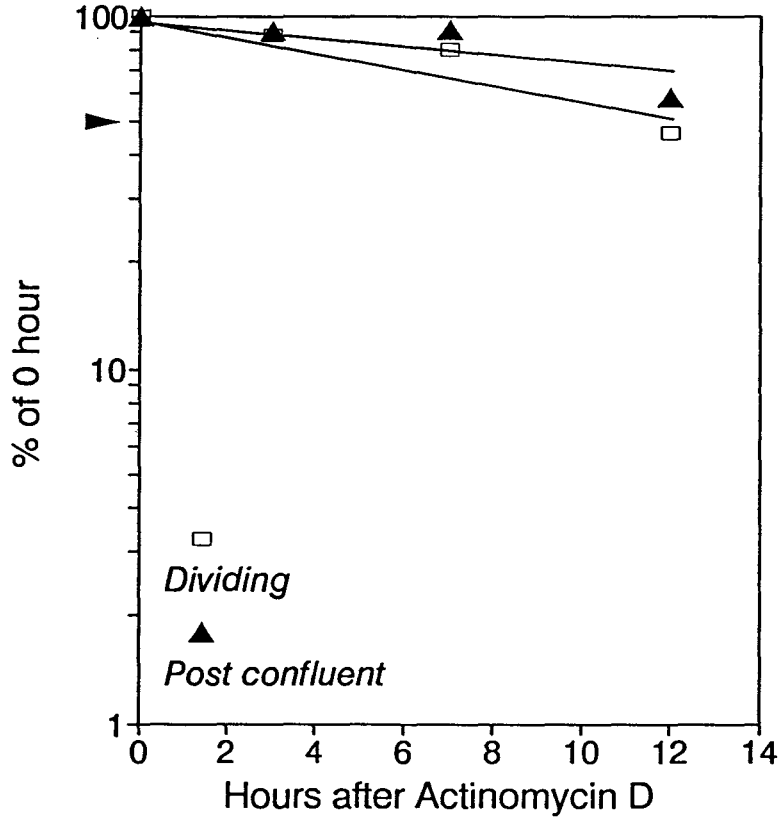
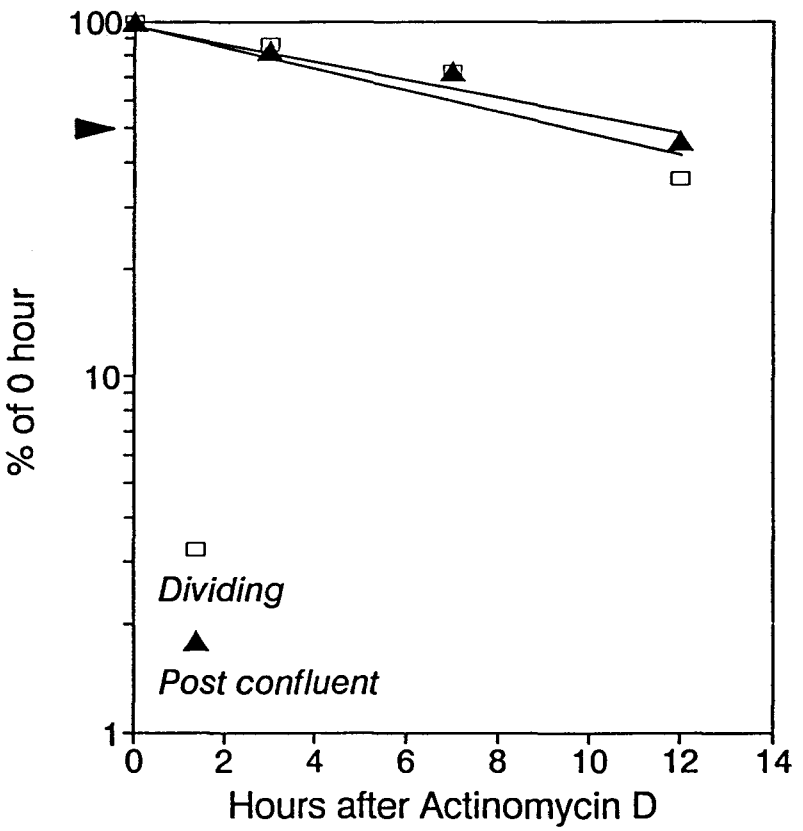

100
10
1
% of 0 hour
Dividing
Post confluent
0
2
4
6
8
10
12
14
Hours after Actinomycin D

Figure 17. Half-life of γ-actin mRNA in BC_3E7 cell line. The RNA for this experiment was from the same batch used for experiment in Fig. 16. The RNA was analyzed for the levels of γ-actin mRNA by dot-blotting as described in Fig. 15. The 50% level in the graph is indicated by an arrow head.

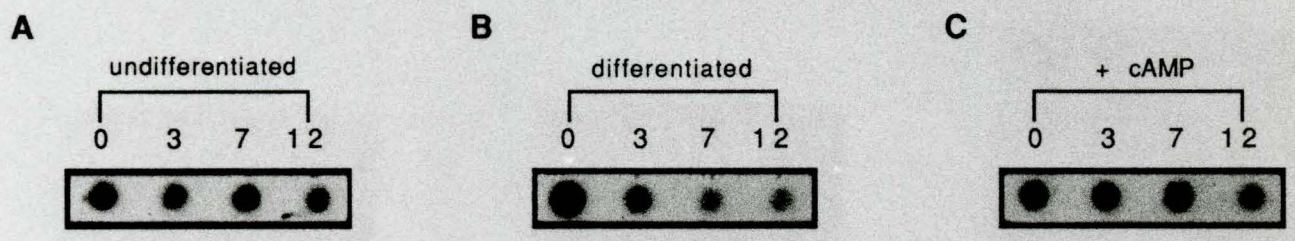

100
10
1
% of 0 hour
Dividing
Post confluent
0
2
4
6
8
10
12
14
Hours after Actinomycin D

actin mRNA in confluent cells were essentially similar and were calculated to be 16 and 11 hours for β-and γ-actins respectively (closed triangles).

C.2.3 . α-ACTIN EXPRESSION IN BC_3H1

When BC_3H1 cells were given the signal to differentiate on day 3 by serum-starvation the α-actin expression was observed to be activated within 48 hrs. Fig. 18A, shows a Northern blot of RNA collected from cells at different days after treatment with low serum medium. When the cells were treated with low-serum medium in the presence of cAMP, the expression of muscle-specific isoform of actin was not found to be repressed but instead, only a delay in the onset of expression was observed (Fig. 18B). During normal differentiation maximal induction of α-actin expression in BC_3H1 was suggested to involve two signals namely, cell-cell contact and serum-starvation (Strauch and Rubenstein, 1984). It is possible that cAMP is not able to inhibit both the signals thereby distinguishing them. A series of experiments were done in which the cells were induced to differentiate by serum-starvation at different days after plating in the presence or absence of cAMP analogues. By day 1 (early log) cells did not yet make cell-cell contact. By day 3(log) had estabilished partial cell contact, whereas by day 4(confluent) the cells had extensive cell-cell contact. Northern analysis of the RNA showed that, the expression of α-actin was completely inhibited in cells treated with cAMP on day 1 (Fig. 18A), whereas the α-actin expression was delayed but still expressed in cells treated on day 3 or 4 (Fig.18

Figure 18. Effect of cell density and cAMP on the α-actin mRNA expression in BC_3H1 cell line. The cells were treated with low-serum containing media in the presence or absence of 1 mM dbcAMP. The cell samples were collected at various days after treatment as indicated in Panel A. RNA was isolated from the samples and was subjected to Northern blotting analysis using a full-length actin cDNA probe. The positions of α-, β-and γ-actin mRNA are indicated. Panels A, B and C correspond to the autoradiograms of Northern blots performed using the RNA isolated from the cells which were treated on day 1, day 3 or day 4 after plating, respectively.


A
undifferentiated
0 3 7 12
B
differentiated
0 3 7 12
C
+ cAMP
0 3 7 12

B&C respectively).

The effect E1A on α-actin expression was also interesting. A Northern blot analysis of the RNA from BC_3E7 cells showed that, α-actin mRNA was expressed during all stages of growth ie., dividing and post-confluent cells (Fig. 8A). Under similar conditions the BC_3H1 cells showed a clear developmental pattern of α-actin gene expression (Fig.8B)

C.3 MYOGENIN mRNA EXPRESSION IN BC_3H1

The expression of the myogenic regulatory gene was studied in serum-starved BC_3H1 cells. Myogenin mRNA expresson peaked by 2 days after serum-starvation (Fig. 19), in agreement with previously published results (Brunetti and Goldfine, 1990). The data presented in this thesis and others findings in the lab have shown that cAMP blocks the expression of muscle-specific genes.

To investigate if cAMP affects the expression of the master regulator (myogenin) which is expressed prior to muscle-specific genes, RNA from cAMP treated cells was probed for myogenin mRNA by a northern blot analysis. The expression of the myogenin mRNA was however activated following the signal to differentiate both in the presence and absence of cAMP, suggesting that cAMP had no effect on myogenin transcription.

The RNA from BC_3E7 cells through different days in culture was analysed by Northern blotting. The results (Fig. 20A) show that E1A inhibits the

Figure 19. Effect of cAMP treatment on myogenin mRNA expression. The BC_3H1 cells were plated at concentration of 1 x 10^5 cells /100 mm^2 plate. They were treated with low-serum medium three days after plating either in the presence or in the absence of 1 mM dbcAMP. The cell samples were collected at various days after plating as indicated. The RNA was isolated and the myogenin mRNA was detected by Northern blotting analysis.

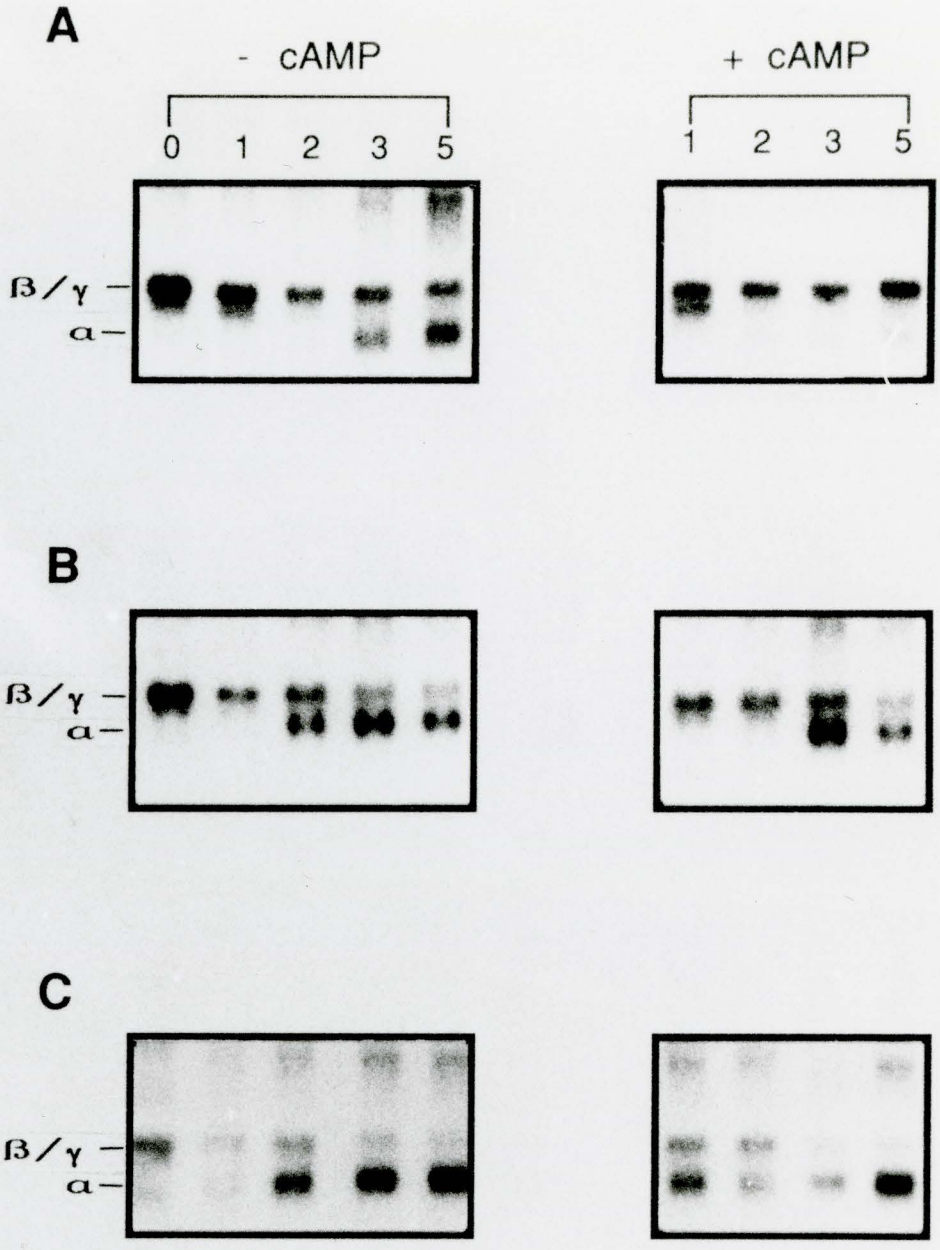
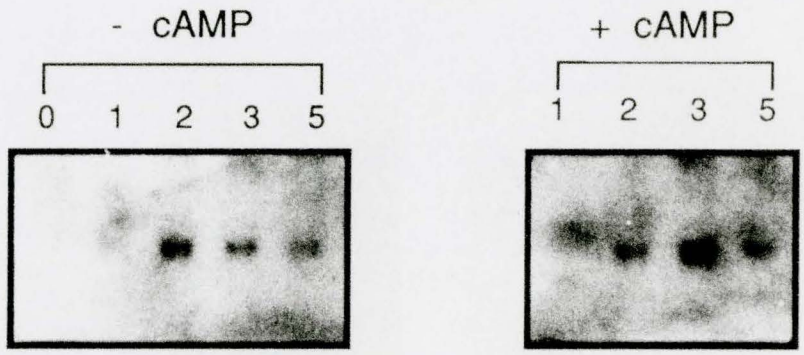

A
- cAMP
+ cAMP
0 1 2 3 5
1 2 3 5
ß/γ
a
B
ß/γ
a
C
ß/γ
a

Figure 20. Effect of E1A on myogenin mRNA expression. BC_3E7 and BC_3H1 cells were plated in 20% serum containing media, and the cell samples were collected on day 3, 5 and 9 after plating. RNA was isolated and subjected to Northern blotting using a myogenin cDNA as the probe. Panel A and Panel B corresponds to the autoradiograms of the Northern blots using the BC_3E7 and BC_3H1 RNA respectively.


- cAMP
0 1 2 3 5
+ cAMP
1 2 3 5

expression of the myogenin gene, while BC_3H1 cells exhibited the expected pattern of myogenin regulation (Fig. 20B).

DISCUSSION

Differentiation of muscle cells from mononucleated myoblasts to multinucleated myotubes is accompanied by changes in cell morphology and gene expression. This includes coordinate activation of muscle-specific genes and the coordinate down-regulation of non-muscle genes. Studies so far suggest that activation of muscle-specific genes such as MCK, troponin, MHC, and α-isoform of actin are mediated at the level of transcription, whereas no consensus has been reached on the mechanism of down-regulation of non-muscle genes such as β-and γ-isoforms of actin. The present study was conducted in an attempt to understand the mechanism underlying the actin gene expression, with emphasis on the regulation of β-and γ-actins.

D.1 DIFFERENTIATION BLOCKING AGENTS AFFECT CELL MORPHOLOGY

Treatment of BC_3H1 cells with cAMP induces characteristic morphological changes, in agreement with an earlier report on L6 cells (Wahrmann *et al.*, 1973). Hu and Olson(1988) also showed that cAMP treatment of dividing BC_3H1 cells promotes a less-refractile and stellate phenotype. cAMP is known to be potential inducer of neuronal differentiation. The BC_3H1 cells treated with cAMP had morphological characters similar to those of neuronal cells in culture such as neurite-like processes with structures resembling growth cones.

cAMP has been shown to induce morphological transformation of human medulloblastoma clonal line TE671 to neuronal cells characterized, by neurite outgrowth and expression of a human neuronal nicotinic receptor (Seigel and Lukas, 1988). The cAMP analogues were shown to promote the survival of the neonatal rat sympathetic neurons and the effect was similar to that of nerve growth factor (NGF) (Rydel and Greene, 1988). Recent observations have indicated that cAMP could induce pluripotent embryonic carcinoma cells to neuron-like cells as monitored by development of neuronal morphology and expression of neurofilament proteins (Sharma *et al.*, 1989). BC_3H1 cells were originally derived from a brain tumour (Schubert *et al.*, 1974) and were suggested to be a skeletal muscle cell line of ectodermal origin that were defective for terminal differentiation (Taubman *et al.*, 1989). Whether cAMP could induce these ectodermal cells towards a neuronal pathway of differentiation, is an interesting question to be confirmed experimentally.

The E1A oncogene-transfected BC_3H1 cells maintained a round morphology upon confluency and did not differentiate. In the earlier E1A transfection studies carried out on L8, C2 and 23A2 myogenic cell lines (Webster *et al.*, 1988; Enkemann *et al.*, 1990) the E1A transcripts could be detected by a conventional Northern blot, while in BC_3E7 cells E1A mRNA were detected only by using a more sensitive primer extension method. Low levels of E1A were thus able to cause a morphological change in BC_3H1. Whether expression of different levels of E1A in BC_3H1 cells would have varying effects on morphology and differentiation needs to

be determined experimentally.

D.2 REGULATION OF ACTIN GENE EXPRESSION

During myogenic differentiation of BC_3H1 cells induced by treatment with low-serum medium, the β-and γ-actin mRNA levels were observed to be down-regulated to very low levels by 5 days after treatment. This was accompanied by the up-regulation of the muscle-specific α-isoform. Quantification of the myoblast-specific (β-and γ-) actin mRNA using gene-specific probes showed that after 6 days of treatment the levels dropped to 25% of the initial levels in myoblasts which was in agreement with an earlier observation (Strauch *et al.*, 1984).

D.2.1 β-and γ-actin gene regulation involves message stability

The effect of differentiation-inhibiting agents such as cAMP and E1A on the actin gene expression was investigated in the present study. Studies in our laboratory and by others (Hu and Olson, 1988; Kelvin *et al.* ,1989b) showed that cAMP blocks the expression of a variety of muscle-specific genes such as MCK and AChR in BC_3H1 cells. E1A was also observed to block myogenesis of C2, L8 (Webster *et al.*, 1988), 23A2 cells (Enkeman *et al.*, 1990) and BC_3H1 (Mymryk *et al.*, submitted). Careful observations on the behaviour of actin genes were not made in the above studies.

Treatment of BC_3H1 cells with cAMP was observed to inhibit the decrease in the levels of β-and γ-actins. Studies in the past have shown that cAMP

could activate expression of a variety of genes such as phosphoenol pyruvate carboxykinase (Hod and Hanson, 1988), lactate dehydrogenase (Jungman *et al.*, 1983), somatostatin, parathyroid hormone, enkephalin and *c-fos* (Montminy *et al.*, 1986). In all the above mentioned genes cAMP was shown to affect their expression both at transcriptional and at post-transcriptional (message stability) levels.

The regulatory mechanism controlling β-and γ-actin transcript levels in BC_3H1 is the next obvious question. Previous studies by Sharp *et al.*, (1989) on the regulation of chicken β-actin gene expression in the BC_3H1 cells suggested that, changes in transcription rate do not account for the drop in the level of the transfected chicken β-actin mRNA during differentiation. Hence, in the present study, the role of mRNA stability in the regulation of non-muscle actin genes was investigated. During normal differentiation, the half-life of β-actin decreases significantly ($\approx$70%) which was similar to the drop in the actual mRNA levels (75%). The half-life of β-actin mRNA in differentiated cells observed in this study was in agreement with a previously reported value $\approx$8 hrs (Lahose and Arnold, 1988). The half-life of γ-actin was also observed to decrease during differentiation ($\approx$80%) which was similar to the drop in its mRNA levels $\approx$75%. Our results on γ-actin mRNA suggest the presence of a highly stable message with a half-life of $\approx$ 38 hrs. Such long half-lives are not unexpected, as β-globin mRNA has a half-life greater than 24 hrs in erythroid cells (for a recent review see Atwater *et al.*, 1990). However, it may be possible that Actinomycin D selectively stabilises γ-actin in dividing cells as it does for transferrin

receptor mRNA in the presence of iron (Mullner and Kuhn, 1988). This may not be true because the half-life of γ-actin mRNA in differentiated cells was significantly lower and comparable with that of β-actin mRNA. These results could be confirmed by repeating the stability measurements either by using different transcription blocking agents such as 5,6-Dichloro-1-ribofuranosyl- benzimidazole (DRB). DRB has recently been claimed to be more suitable for assaying the decay of long-lived mRNA, due to its low cytotoxity and side effects (Helms and Rottman, 1990), or by employing a different technique for half-life determination such as the pulse-chase labelling. The results presented here suggest that, message stability plays an important role in the down-regulation of both β-and γ-actin gene expression during normal differentiation of BC_3H1 cells. This is the first report where γ-actin gene expression during myogenesis has been clearly documented.

The next step was to assess the role of mRNA stability in the cAMP regulated non-muscle actin gene expression. Cells maintained for six days in low-serum medium containing cAMP, had 2.3 fold more β-actin mRNA and three fold higher levels for γ-actin than the cells maintained in low-serum medium alone. The stability of the message increased by approximately two fold for both β-and γ-actin transcripts. This observation is contrary to a recent finding by Ohara *et al.*(1991), in primary smooth muscle cells, where they observed that, treatment with forskolin selectively destabilised β-actin. The above mentioned study was done using the transcription blocking agent DRB. It is not clear why such a difference in gene

regulation exits for a particular gene in different cell lines, however, it could be due to the difference in the transcription inhibitor used, because the experiments carried out using Actinomycin D in the same primary smooth muscle cells under similar conditions did not yield a comparable result (Ohara *et al.*, 1991). According to the results presented here, the elevation of β-and γ-actin mRNA levels by cAMP cannot be completely attributed to the increase in message stability, as the actin mRNA levels were 2.5-three fold higher in cAMP treated cells while the message stability increased only by 1.8 fold. Hence it might be possible that the promoter activity of β-and γ-actin genes are positively regulated to some extent by cAMP. This explanation is plausible but circumstantial at present and a conclusive explanation will not be possible until the transcription rates of non-muscle actin genes are measured by nuclear run-on assays.

Adenovirus E1A transfection of BC_3H1 cells was also observed to maintain higher levels of β-and γ-actin mRNA up to 8 days in culture. The half-life of β-and γ-actin message was also high up to 8 days after plating. Webster *et al.*, observed a normal down-regulation of β-and γ-actin mRNA levels in E1A transfected C2 cell line (Webester *et al.*, 1988). This discrepancy could be explained by the fact that BC_3E7 cells express low levels of E1A than the transfected C2 cell line (Mymryk *et al.*, submitted).

The observations in the present study, on the role of message stability in the β-and γ-actin regulation agree with the observations of Lahose and Arnold,

(1988) and Sharp *et al.* (1989). The present conclusion is however contrary to the reports of Depont-Zilli *et al.* (1988), and Cox *et al.* (1990), where a transcriptional control was suggested. Whether, changes in transcription rates are also involved or not remains to be determined experimentally by nuclear run-on transcription assays, nevertheless the ability of the differentiation blocking agents to maintain higher levels of β-and γ-actin mRNA by altering the message half-life strengthens the present conclusion that changes in mRNA stability plays an important role in the non-muscle actin regulation.

Sequences in the non-coding regions of a mRNA are likely to play a role in determining the stability of that mRNA (for a review see Brawerman, 1987). Gay *et al.*, (1987) have reported that the sequence responsible for altered stability of β-tubulin resides within the 106 bases at the 5'region of the mRNA. Other studies have suggested that secondary structures near or at the 3'terminus may influence the susceptibility of eukaryotic mRNA to attack by nucleases (Brawerman, 1987). For example, the stability of histone mRNA during the cell cycle can be attributed to short, conserved stem loop structures at the extreme 3'terminus (Atwater *et al.*, 1990)

The 3'untranslated region at the β-and γ-actin mRNA contains conserved AU-rich sequences(Tokunaga *et al.*, 1988), which appears to be the most striking feature common to several short-lived RNAs (Atwater *et al.*, 1990). Whether the sequences in the 3'UT region are specifically involved in determining the stability of the non-muscle actin messages still remains to be established.

Evidence suggests that polyadenylation to the 3'end of newly synthesised RNA might be involved in regulating several processes: nuclear processing and transport, modulation of mRNA translation efficiency, and mRNA turnover (Atwater *et al.*, 1990). Although most of the evidence is correlative, some data suggests that poly(A) protects mRNA from rapid degradation. The half-lives of globin mRNAs with poly(A) tracts of varying sizes have been measured in Xenopus oocytes (Nudel *et al.*, 1976), and mRNA with fewer than 30 A residues were 10 fold less stable than molecules with 32 or more A residues. Conversely histone mRNA which is normally not polyadenylated and has a very short half-life, was 10 times more stable in oocytes when artificially polyadenylated (Peltz *et al.*, 1989). Human growth hormone and vasopressin mRNAs exhibit elongation of their poly(A) tracts in response to glucocorticoids and osmotic stress respectively (Atwater *et al.*, 1990). Hence poly(A) elongation correlates with stabilisation of these mRNAs, suggesting that mechanisms exist to regulate poly(A) removal, addition and consequently the rate of mRNA turnover. Cytoplasmic mRNA exists as a ribonucleoprotein complex. Among the proteins bound to mRNA are the poly(A) binding proteins (PABP) (Blobel, 1973). Experiments done *in vitro* support the hypothesis that poly(A) in conjunction with PABP protects mRNA from rapid nucleolytic attack (Bernstein *et al.*, 1989). Now, an interesting question is whether β-and γ-actin regulation during differentiation involves changes in poly(A) length, and if cAMP treatment mediates changes in poly(A) tail or PABP binding leading to the observed changes in message

stability. Further experimental evidence is necessary to confirm these hypotheses.

D.2.2 α-actin activation involves two different inductive signals

Differentiation of BC_3H1 cells is accompanied by the up-regulation of the muscle-specific α-actin gene expression (Strauch and Rubenstein, 1984). They also reported that maximal-induction of α-actin expression requires both the inductive signals namely, cell-cell contact and serum-starvation. In the present study, the effect of cAMP levels on induction of α-actin expression was analysed by treating the cells under different densities with cAMP, in an attempt to differentiate the two possible signals, i.e., serum-starvation in the absence of cell-cell contact, and serum starvation in the presence of cell-cell contact. Surprisingly, cAMP was able to repress α-actin activation in cells which were prevented from making cell-cell contact, while there was only a delay in the onset of α-actin expression in cells observing cell-cell contact. This suggests that, of the two inductive signals, only serum-starvation was sensitive to intracellular cAMP levels while the cell-cell contact signal was not sensitive to increase in cAMP levels. The recent report that a trypsin sensitive cell surface molecule was needed for transmitting information about cell density for the muscle-specific α-actin expression in BC_3H1 cells (Strauch *et al.*, 1990) and the finding that a putative "cell density responsive element" might be located between -373 and -1074 in the 5' flanking region of vascular α-actin gene (Min *et al.*, 1990) suggest that cell density could serve as an independent signal.

The E1A transfected BC_3H1 cells were observed to express muscle

specific α-actin both in dividing and in contact-inhibited quiescent cells, while the expression of some muscle-specific genes such as MCK and AChR were repressed whereas that of few other genes such as MLC and tropomyosin are not affected. Earlier observations have shown that E1A could repress the expression of muscle-specific gene α-actin in myogenic cell lines L8, C2, and 23A2 (Webster *et al.*, 1988; Enkemann *et al.*, 1990). Though this discrepancy could be attributed to the difference in cell line, a more plausible explanation would be the difference in the amount of E1A expressed, since it was demonstrated by Mymryk *et al.*, (submitted) that expression of elevated level of E1A in BC_3H1 would repress the expression of muscle-specific α-actin genes. This observation is consistent with the earlier finding that varying concentration of E1A could have different effects on transcriptional trans-activation (Brunet and Berk, 1988; Adami and Babiss, 1990).

D.3 MYOGENIN GENE EXPRESSION IS NOT BLOCKED BY cAMP

An important issue to be explored is the pathway through which cAMP and E1A control the actin and mRNA levels in BC_3H1 cells. It is, at present, not clear whether the increase in stability of β-and γ-actin mRNA and the inhibition of α-actin induction are primary and direct consequences of the elevation of the intracellular cAMP concentration, or an indirect effect mediated through another gene product.

One possible direct target for cAMP and E1A action could be

myogenin, a regulatory factor thought to be involved in controlling the expression of many of the muscle-specific genes in BC_3H1 cells (Edmondson and Olson, 1989). Surprisingly, myogenin expression was found to be inducible upon serum-starvation in both cAMP-treated and -untreated cells. The myogenin protein was observed to have consensus phosphorylation sequences for cAMP dependent protein kinase around amino acid Ser 77, 79, 107 and Thr 108, 108 so it is likely that the myogenin gene product might be negatively-regulated through cAMP dependent protein phosphorylation (Muthuswamy *et al.*, manuscript in preparation). Whether, myogenin phosphorylation actually plays a role in cAMP action needs to be confirmed experimentally. These observations suggests that cAMP might be acting downstream of myogenin transcription in blocking myogenic-differentiation of BC_3H1 cells (Muthuswamy *et al.*, manuscript under preparation)

E1A transfection was observed to block the expression of the myogenin gene under the described conditions, in agreement with the findings reported earlier (Enkemann *et al.*, 1990). Whether or not myogenin has an exclusive role in controlling differentiation in BC_3H1 cells could be questioned, because in the absence of myogenin expression in BC_3E7 cells, synthesis of muscle-specific α-actin was still activated. While in the cAMP-treated cells, where myogenin was activated upon serum-starvation, muscle-specific genes such as MCK, AChR, MLC and tropomyosin were repressed. This might mean that some muscle-specific genes that carry *cis*-regulatory sequences can be activated independently of the presence of myogenin.

Recently French *et al.*, reported that chicken α-cardiac gene activation could follow myogenin dependent and independent activation pathway (French *et al.*, 1991). This observation also suggests the involvement of more than one pathway of muscle-specific gene activation.

The results presented in this thesis suggest that the mechanism by which the non-muscle actin genes were down-regulated during differentiation of BC3H1 cells involve changes in mRNA stability. A raise in intracellular cAMP levels and transfection with the E1A oncogene were observed to elevate the levels of non-muscle actin mRNA by increasing the mRNA half-life. Though cAMP was observed to block myogenic differentiation of BC_3H1 cells, it did not affect the transcription of the myogenic-regulatory gene, myogenin, suggesting that cAMP action is independent of myogenin expression. cAMP was also able to differentiate between cell-cell contact and serum-starvation, the signals which activate the expression of α-actin. This suggest that the signals inducing α-actin transcription might be following different pathways. The results support the notion that muscle-specific gene activation could be mediated by more than one activation pathway which might be functioning in concert with one another. Whether or not the down-regulation of non-muscle genes during differentiation is also regulated by one of these pathways is still an unanswered question.

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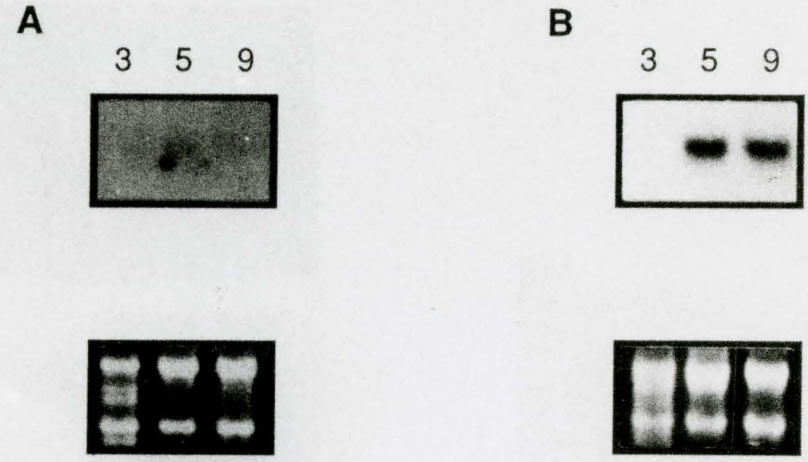
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