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Regulation Of Cyclic-amp-dependent Protein Kinases During Skeletal Myogenesis

Ian Anthony Lorimer

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REGULATION OF cAMP-DEPENDENT PROTEIN KINASES DURING SKELETAL MYOGENESIS

by

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of the requirements for the degree of
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ABSTRACT

L6 myoblasts are a permanent rat skeletal myoblast cell line which differentiates in culture to form multinucleate myotubes. The regulation of the levels of cAMP-dependent protein kinases during myogenesis in L6 was studied, as these enzymes have been proposed to be involved in the control of this process. Measurement of the mRNA levels for the type I regulatory subunit (R_I) of cAMP-dependent protein kinase, and studies on the rates of transcription from the gene coding for this protein, showed that the increase in R_I which occurs during myogenesis is not regulated transcriptionally. Measurement of R_I half-lives showed that a decrease in the rate of R_I degradation during myogenesis probably causes its increase. Studies on the regulation of the catalytic subunit (C) of cAMP-dependent protein kinase showed that the increase in this subunit, which also occurs during myogenesis, is probably regulated at the level of transcription, and not by a change in its rate of degradation. On the basis of these results it is proposed that the increase in R_I during myogenesis is due to a decrease in its proteolysis caused by an increase in the amount of C available to bind with it. This type of mechanism may have relevance to the regulation of increases in other multisubunit complexes during differentiation.

The regulation of R_I was also studied in a spontaneously-transformed L6 cell line which is unable to differentiate. Measurement of R_I degradation rates in this cell line showed that R_I proteolysis is altered in the presence of certain types of cAMP analogues. As R_I from this cell line and normal L6 appeared to be structurally the same, it

is proposed that a change in some protease accounts for the altered R_I degradation. This change in proteolysis was not a common feature of myoblast transformation, as it was not observed in L6 myoblasts transformed by transfection of the Ha-ras oncogene.

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NOMENCLATURE

ATP	Adenosine 5'-triphosphate
8-Br-cAMP	8-Bromo-adenosine 3':5'-cyclic monophosphate
BSA	Bovine serum albumin
Bt_2cAMP	N^6, 2'-O-dibutyryladenosine 3':5'-cyclic monophosphate
C	catalytic subunit of cAMP-dependent protein kinase
cAMP	Adenosine 3':5'-cyclic monophosphate
CTP	Cytidine 5'-triphosphate
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminetetraacetic acid
GTP	Guanosine 5'-triphosphate
kDa	kilodalton
MES	2-(N-morpholino) ethanesulphonic acid
Mr	relative molecular mass
N^6-benzoyl cAMP	N^6-benzoyl adenosine 3':5'-cyclic monophosphate
PBS	Phosphate-buffered saline
R_I	Type I regulatory subunit of cAMP-dependent protein kinase
R_{II}	Type II regulatory subunit of cAMP-dependent protein kinase
R	Arginine
S	Serine
SDS	Sodium dodecyl sulphate
t1/2	Half-life
TBS	Tris-buffered saline (20 mM Tris-HCl, 146 mM NaCl, pH 7.4)
Tris	Tris (hydroxymethyl) aminomethane

CHAPTER 1

INTRODUCTION

1.1 MYOGENESIS

Most multicellular organisms arise from a single cell, the fertilized egg. During the course of development, this cell will go through a series of divisions to give rise to progenies which, although they are genetically identical, display different phenotypes. Eventually in an adult organism, cells will be specialized into distinct organs such as, for example, skin, muscle or brain. The regulation of this process, referred to as differentiation, remains one of the major unsol.ed problems of modern biology. A variety of approaches are currently being used to study this problem. These include studies of developmental mutants in lower eukaryotes such as Drosophila and Caenorhabditis, which are amenable to genetic manipulation. In higher organisms, where this kind of approach is often not possible, much research has focussed on the use of cultured cell lines to study differentiation.

One differentiation process which is readily studied using tissue culture techniques is skeletal myogenesis (reviewed by Sanwal, 1979; Pearson, 1981). Briefly, this process involves the fusion of myoblasts to form the multinucleate myotubes characteristic of mature muscle (figure 1.1). Accompanying this morphological change is a coordinated increase in the synthesis of muscle-specific proteins. This process has been studied either in primary cultures or in permanent cell lines. Primary cultures are typically isolated from the breast muscle of chick or quail. Myoblasts are separated from the bulk of contaminating

FIGURE 1.1

MORPHOLOGY OF L6 MYOBLASTS AND MYOTUBES

L6 rat skeletal myoblasts were grown for either two (upper photograph) or six days (lower photograph) in culture. Cells were then fixed and stained with Giemsa stain before being photographed. Typically greater than 95% of the myoblasts are fused into myotubes by day six.

fibroblasts by taking advantage of the stronger adherence of fibroblasts to tissue culture plates (Konigsberg, 1979). Advantages of primary cultures for the study of myogenesis are that these cells probably more closely mimic the myogenic process in the whole animal. In particular the maturation process, which involves muscle-specific protein isozyme switching after fusion, does not occur in permanent cell lines, but is evident in primary cultures. In addition primary cells are non-transformed, whereas permanent cell lines are generally considered to be at least partially transformed.

Disadvantages of working with primary cultures are the time required to isolate these for each experiment, the problem of reproducibility between different isolates and contamination with other cell types. The latter problem is particularly significant in studies on the influence of hormones on myogenic differentiation. All of these problems stem from the major drawback of primary cell culture, which is the inability to propagate these cells to high passage numbers. This also prevents the application of genetic analyses to myogenesis, which requires the expansion of single clones of myoblasts.

It is largely for this last reason that we have chosen to work with a permanent myogenic cell line. Several of these are currently available, including the mouse cell lines C2 (Yaffe and Saxel, 1977) and BC3H1 (probably a smooth muscle myogenic cell line; Schubert et al., 1974) and the rat cell lines L6 (Yaffe, 1968) and L8 (Yaffe and Saxel, 1977b). We have used the L6 cell line, which was originally isolated from a rat treated with the carcinogen methylcholanthrene.

At this point it is worthwhile to give a more detailed description

of myoblast differentiation as it occurs in L6, in order to provide a better perspective for interpreting the experiments outlined in this thesis. Aside from their myogenic potential, myoblasts are similar to fibroblasts. Some distinguishing features are their poorer adherence to plastic and a distinctive surface antigen of unknown function (Walsh et al., 1984). Upon plating myoblasts go through a proliferative phase, with a doubling time of 12-18 hours. Once they reach a high density on the surface of the tissue culture dish myoblasts withdraw from the cell cycle. This step appears to be a requirement for differentiation as myoblasts which are transformed with ras (Olson et al. 1987; this thesis) or src (Seth et al., 1983; Falcone et al., 1985) oncogenes and therefore do not withdraw from the cell cycle are unable to differentiate. A second line of evidence that cell cycle withdrawal is a necessary first step for differentiation comes from the non-fusing myoblast cell line BC3H1. This cell line will reversibly differentiate when stimulated to withdraw from the cell cycle (usually by lowering the concentration of serum in the media). Upon stimulation of the cells to reenter the cell cycle, the cells lose the differentiated phenotype (Olson and Spizz, 1986).

The next stages in L6 differentiation are fusion and biochemical differentiation. In vivo these two processes are tightly coupled temporally, so that it cannot be distinguished whether one precedes the other. This is also the case in L6. In several experimental systems these two processes have been uncoupled; mutants have been isolated in which fusion is uncoupled from the expression of some muscle-specific proteins (Rogers et al., 1978). Also calcium depletion and treatment

with cytochalasin B have been reported to block fusion without disrupting biochemical differentiation (Konieczny et al., 1982). The fusion process in myoblasts (reviewed by Wakelam et al., 1985) is unique for a mammalian cell type. Microscopically the cells are first observed to align in arrays before beginning to fuse. Myoblasts will only fuse with other myoblasts in culture; therefore there must be a specific cell-cell recognition system involved. The molecular basis of this is not known, although interactions between specific glycoproteins (Cates et al., 1984) or lectin-like molecules (Nowak et al., 1976) have been proposed. The fusion process has an absolute requirement for calcium (Konieczny et al., 1982), although it is not known what the precise reason for this requirement is. A wide variety of chemical compounds have been shown to block the fusion process (together with biochemical differentiation); a list of these is given in the review by Pearson (1981). Also hormones such as fibroblast growth factor (Gospodorowicz et al., 1976) and transforming growth factor (Massague et al., 1986) have been shown to regulate fusion and biochemical differentiation. These may be candidates for in vivo modulators of these processes.

The last event in L6 differentiation to be discussed is that of biochemical differentiation (as mentioned above, this does not imply that this follows the fusion process). Usually biochemical differentiation is used to refer to the increase in synthesis of the proteins characteristic of mature muscle. However it should be kept in mind that during myogenesis the synthesis of a set of proteins is down-regulated (Devlin and Emerson, 1978). It is also quite likely that

there are transient changes in the levels of some proteins around the time of onset of differentiation. These proteins might be those involved in signalling on and off states for the cell cycle and/or differentiation. Garrels (1979) has studied changes in protein synthesis during L6 differentiation by two-dimensional gel electrophoresis and shown that, as well as large increases and decreases in the levels of some proteins (>1000-fold), there were many small (on the order of two to three-fold) changes in the expression of others. Some specific proteins which are known to increase during differentiation are those of the contractile apparatus, including α-actin, myosin light and heavy chains, tropomyosin (Devlin and Emerson, 1978; Garrels, 1979) and desmin (Gard and Lazarides, 1980); the cytoplasmic enzyme creatine kinase (Turner et al., 1976), required to help meet the high energy requirements of mature muscle; and the acetylcholine receptor (Prives et al., 1976), a membrane protein required for signal transmission at the nerve-muscle synapse. Most evidence shows that the levels of these proteins are mainly regulated by changes in mRNA levels due to increases in rates of transcription (Medford et al., 1983; Jaynes et al., 1986). The process of myogenesis is therefore an interesting model for the study of mechanisms of coordinate gene expression. There is now good evidence that sequences upstream of these genes confer this inducibility during differentiation (Bouvagnet et al., 1987; Minty and Kedes, 1986). However there does not seem to be a common regulatory sequence shared by all these genes, so the mechanism of coordinate transcriptional regulation is not obvious at this point in time.

1.2 HORMONAL REGULATION OF MYOGENESIS

The major goal of our laboratory has been to identify the mechanisms by which early signals switch on (or off) the process of myogenesis. Broadly speaking, early signals which have been shown to regulate terminal differentiation processes are first, interactions with the extracellular matrix, and second, interactions with hormones. An example of the former is the stimulation of chondrogenesis by collagen matrices (Lash and Vasan, 1978). The focus of research described in this thesis is on the latter type of signal.

A variety of hormones have been shown or proposed to regulate myogenesis. A partial list includes prostaglandin E1 (Zalin, 1977), epinephrine and isoproterenol (Curtis and Zalin, 1981), insulin (Rutter et al., 1973), dexamethasone (Ball and Sanwal, 1980), transforming growth factor (Massague et al., 1986) and fibroblast growth factor (Gospodarowicz et al., 1976). However there have been several difficulties with these studies. First some were done with primary cultures where contaminating cell types could mediate the response of myogenic cells through their interaction with the exogenously added hormone. Second, different responses to hormones have been reported for different clones of permanent myogenic cell lines (Pearson, 1981). A third consideration is that all of these studies are done in media containing horse or fetal calf serum which contain various hormone species in varying and unknown amounts which could interact in an additive or antagonistic manner with the exogenously added hormone. Nevertheless these studies, if not definitive, have been useful in

suggesting a role for particular hormones in the control of myogenesis.

The mechanisms by which some of the hormones which have been shown to affect myogenesis act within the cell is not known and in others is poorly understood. Hormones where our understanding of their modes of action within the cell is at least partly understood include the steroids, which apparently interact directly with receptors in the nucleus (Yamamoto and Alberts, 1976), hormones using the phosphatidylinositol pathway (Nishizuka, 1984), and hormones which use cAMP as their "second messenger" within the cell. While all of these may be involved in varying degrees in the control of myogenesis, and may interact with each other to exert those controls, this thesis focusses primarily on aspects of cAMP-related events during myogenesis.

cAMP was originally investigated as a potential regulator of myogenesis by Wahrmann et al.(1973a, 1973b), Zalin and Montague (1974), and Epstein et al. (1975). These studies included two basic approaches: treatment of cells with cAMP analogues or hormones known to elevate cAMP levels; and measurement of cAMP levels in cells during differentiation. With the former approach, results were conflicting: Zalin and Montague (1974) reported a stimulation of fusion by cAMP elevating agents in primary chick myoblasts, whereas Wahrmann et al. (1973b) reported an inhibition of myogenesis using L6. Epstein et al. (1975) also reported an inhibition of fusion but considered it to be due to the growth inhibitory effects of cAMP analogues. Measurement of cAMP levels also gave conflicting results; a transient rise in cAMP levels was reported by some researchers (Zalin and Montague, 1974), whereas others reported a decrease in cAMP and adenyl cyclase activity

(Reporter, 1972) with fusion. A similar series of conflicting reports has been published concerning a role for cAMP in the regulation of the cell cycle (Pastan et al., 1975).

A role for cAMP in myogenesis was also suggested by studies which showed that cAMP was probably involved in the regulation of several other terminal differentiation events. Treatment with cAMP analogues (in combination with retinoic acid) has been shown to induce the differentiation of teratocarcinoma cells into parietal endoderm (Strickland et al., 1980). PC12 pheochromocytoma cells are induced to differentiate into neuron-like cells when treated with Bt_2cAMP (Schubert and Whitlock, 1977). Also somatic cell genetic techniques have been used to clearly show an involvement of cAMP and the cAMP-dependent protein kinases in the differentiation of Y1 adrenocarcinoma cells to form steroid-secreting cells (Rae et al., 1979).

1.3 cAMP AND THE cAMP-DEPENDENT PROTEIN KINASES

The idea that cAMP could function as a "second messenger" for certain hormones was first put forward by Sutherland (1971). Further research has supported this concept and also led to the identification of other second messengers; cGMP, Ca^{2+} and diacylglycerol. cAMP has now been shown to be involved in the control of a wide variety of biological processes. Some of the better understood processes which cAMP is known to regulate are glycogenolysis (Sutherland, 1971), lipolysis (Beebe et al, 1984) and gene regulation in prokaryotes (Adhya and Garges, 1982). There is also evidence that cAMP may be involved in the regulation of other processes ranging from the induction of meiosis

(Maller and Krebs, 1977; Matsumoto et al., 1983) to the control of short-term memory (Chen et al., 1986; Brunelli et al., 1976). It is not currently understood how cAMP can regulate so many different processes in so many different tissues, although it seems likely that this is due mainly to the presence of different substrates for the cAMP-dependent protein kinases in different tissues. cAMP levels within the cell are controlled by a complex regulatory system. In particular the enzyme adenylate cyclase, which catalyses the formation of cAMP from ATP, has been shown to be subject to a complex system of controls which tightly regulate its activity (Gilman, 1984). cAMP levels are also regulated by the cAMP phosphodiesterases which degrade cAMP to AMP. These enzymes are known to occur in several isoforms with distinct kinetic parameters and are also believed to be regulated by a feedback mechanism (Ball et al., 1980). A variety of techniques have been developed for the study of the role of cAMP in biological processes. In particular the use of cAMP analogues is widespread. These are usually cAMP molecules which are derivatized with hydrophobic substituents so that they can cross cell membranes (in some cases derivatization also makes them more resistant to hydrolysis by phosphodiesterases). A second approach has been the isolation of mutants which are defective in various enzymes involved in cAMP pathways. This approach has been particularly successful in S49 mouse lymphoma cells (Hochman et al., 1975) and in the yeast Saccharomyces cerevisiae (Matsumoto et al., 1983), but has also been employed in a number of other cell lines.

Probably all of the effects of cAMP within the cell are mediated by the cAMP-dependent protein kinases, enzymes first discovered in the

laboratory of Krebs (Walsh et al., 1968). The family of enzymes to which these belong, the protein kinases, is now known to include a large number of enzymes- it has been proposed that they may eventually number in the thousands (Hunter, 1987). Protein kinases are divided into two groups; those phosphorylating the serine and/or threonine residues of proteins and those phosphorylating tyrosine residues. The cAMP-dependent protein kinases fall into the former category. Using synthetic peptides, their substrate specificity has been investigated in more detail and it has been shown that they preferentially recognize the primary sequence R-R-X-S-X-in proteins, although higher order structures in substrates are also involved (Kemp et al., 1977).

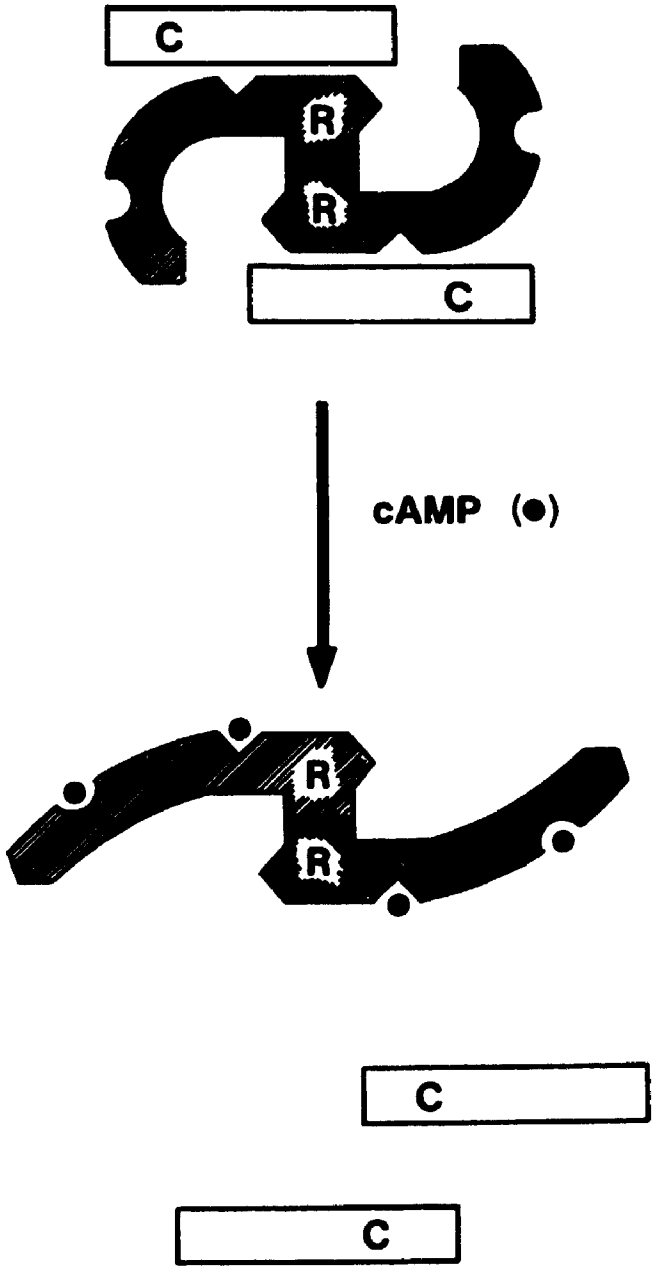
The cAMP-dependent protein kinases have a common subunit structure consisting of a dimer of regulatory subunits which binds two catalytic subunits (figure 1.2). Each regulatory subunit monomer has two distinguishable cAMP binding sites which bind cAMP in a cooperative manner (Rannels and Corbin, 1980; Ogreid and Doskeland, 1982). Binding of cAMP causes the release of catalytic subunit, which is then catalytically active. As the cAMP-dependent protein kinases were among the first protein kinases to be discovered and can be purified in relatively large amounts, they have been subjected to a great deal of analysis by chemical modification and kinetic techniques, which have been reviewed recently (Bramson et al., 1983). Currently their structure is also being investigated by site-directed mutagenesis and X-ray crystallography (Sowadski et al., 1985; Saraswat et al., 1986; Kuno et al., 1988).

Originally the cAMP-dependent protein kinases were thought to

FIGURE 1.2

SCHEMATIC REPRESENTATION OF cAMP-DEPENDENT PROTEIN KINASE ACTIVATION BY cAMP

The inactive form of cAMP-dependent protein kinase consists of a dimer of regulatory subunits which binds two catalytic subunits. Binding of cAMP to the regulatory subunits induces a conformational change which results in the dissociation of the catalytic subunits from the regulatory subunit dimer. The free catalytic subunits are then active in phosphorylating protein substrates. Adapted from Beebe and Corbin (1986).


C
R
R
C
cAMP (●)
R
R
C
C

exist in two different isozyme forms, denoted type I and type II according to their order of elution from ion exchange columns. Each of these contained an apparently identical catalytic subunit, but differed in the type of regulatory subunit. Molecular cloning has now refined our knowledge of different cAMP-dependent protein kinase isoforms. In mouse cells two genes for the catalytic subunit exist, both of which have been cloned and are designated C_α and C_β (Chrivia et al., 1988). The latter is expressed mainly in brain tissue. For the type II regulatory subunit there are also probably two genes coding for proteins designated $R_{II\alpha}$ and $R_{II\beta}$ (Jahnsen et al., 1986; Scott et al., 1987). Again $R_{II\beta}$ is expressed mainly in brain, but is also expressed in germ cells and in the ovary. There are also thought to be two genes for the type I regulatory subunit (Clegg et al., 1988). The mRNA for R_I has now been cloned from bovine (Lee et al., 1983), mouse (Uhler et al., 1986) and rat (Kuno et al., 1987) tissues and shows about 97 % homology at the amino acid level between these species. In addition to the different genes which give rise to different forms of cAMP-dependent protein kinases, post-translational modifications also give rise to heterogeneity. R_I, R_{II} and C subunits are all found in phosphorylated forms and the C subunit is also known to contain N-terminal myristate (Shoji et al., 1983). With the possible exception of phosphorylation of the type II regulatory subunit (an autophosphorylation), the reason for these post-translational modifications is not known.

Once the cAMP-dependent protein kinases are activated by cAMP, free catalytic subunit is capable of phosphorylating various cellular

proteins. It is generally thought that phosphorylation reactions mediate most, if not all, of the effects of cAMP in eukaryotic cells. However some researchers have proposed independent roles for the regulatory subunits - this will be discussed in more detail later. Covalent modification of proteins by reversible phosphorylation is now recognized as one of the major mechanisms for regulating protein activity in eukaryotic cells. In a sort of heirarchy of controls based on the rapidity of response, phosphorylation is considered to be intermediate between allosteric modifications (very fast) and protein synthesis or degradation (slow) (Krebs, 1986). It has been estimated that as many as one third of the proteins in a mammalian cell may be phosphorylated. As well many proteins are phosphorylated at more than one site, often by more than one protein kinase. This complexity has frustrated attempts to identify protein kinase substrates which are critical in the induction of a particular biological phenomenon. An additional difficulty is that often protein kinases exhibit less stringent substrate specificity in cell-free extracts than in vivo. Although a number of substrates have been identified for the cAMP-dependent protein kinases in vitro, it is not always known if theses serve as significant substrates in vivo. However, there are some notable exceptions. For example, the enzyme phosphorylase kinase (Pickett-Gies and Walsh, 1986) is known to be a substate in vitro and in vivo and its phosphorylation is clearly involved in the regulation of glycogen metabolism.

As well as alterations at the level of metabolism, cAMP has also been shown to regulate gene expression in eukaryotic cells. Two genes

from which transcription has been shown to be increased by cAMP are the phosphoenolpyruvate carboxykinase gene (Lamers et al., 1982) and the somatostatin gene (Montminy et al., 1986). Both of these genes have been shown to have upstream regions which confer this cAMP responsiveness (Wynshaw-Boris et al., 1984). Two basic mechanisms have been proposed for this effect on gene expression: in one, C subunit phosphorylates nuclear proteins, leading to changes in transcription; in the other, R subunit complexed with cAMP acts directly on regions of DNA to alter transcriptional rates, in a manner analogous to the lac repressor of E. coli. The latter mechanism received support from a report by Constantinou et al (1985) suggesting that R_{II} had DNA topoisomerase activity. However other laboratories have not succeeded in reproducing this work, and there is now considerable evidence for the first mechanism. An early piece of evidence was that protein induction was not seen in the kin^- mutants of S49 mouse lymphoma cells, which do not express detectable catalytic subunit (Steinberg and Coffino, 1979). More recently the protein inhibitor of cAMP-dependent protein kinase has been cloned and it has been demonstrated that its overexpression blocked cAMP effects on gene expression (Grove et al., 1987). Similar results have been reported by Buchler et al. (1988), who showed that the cAMP-dependent protein kinase inhibitor H8 and and a synthetic peptide corresponding to part of the Walsh protein kinase inhibitor both block changes in gene expression induced by cAMP. However these experiments may not be definitive, as it has been argued that phosphorylation of R_{II} by C might be required for R_{II} to regulate gene expression, so neither mechanism can be given unqualified support

at this point in time.

1.4 GOALS OF THIS STUDY

The long range goal of this research was to determine whether the cAMP-dependent protein kinases have a role in the control of myogenic differentiation, and, if so, to determine what that role is. Our initial approach to this problem was to determine the types of cAMP-dependent protein kinases present in L6 myoblasts and investigate any changes in their levels with differentiation. These studies showed that both type I and type II isozymes were present in undifferentiated and differentiated L6 and that there was a selective increase in the type I and/or free R_I subunit during differentiation (Rogers et al., 1985). This thesis descibes our investigation of the mechanism by which this increase occurs. Our results demonstrated that the increase in the levels of type I cAMP-dependent protein kinase during myogenesis is regulated by an apparently novel mechanism, which may have relevance to the regulation of other multisubunit complexes during myogenesis (Lorimer et al., 1987; Lorimer and Sanwal, 1988).

As well this thesis describes our research on the regulation of the cAMP-dependent protein kinases in a spontaneously-transformed L6 myoblast cell line (Lorimer and Sanwal, 1987) which is unable to differentiate. This work was also a continuation of previous studies in our laboratory which had initially shown that there were differences in the behaviour of the cAMP-dependent protein kinases in this cell line (Rogers, 1984). Here our long range goal was to determine the cause of this altered regulation and to establish whether or not the changes

were relevant to transformation and/or the loss of ability to differentiate.

CHAPTER 2

REGULATION OF R_I LEVELS DURING MYOGENESIS

2.1 INTRODUCTION

As one approach to determining what role cAMP-dependent protein kinases might play in the process of myogenesis, we have studied the regulation of the levels of these enzymes during this process. The work described in this chapter is a continuation of studies previously carried out in our laboratory which showed that there were changes in the amounts of the cAMP-dependent protein kinases during the differentiation of L6 myoblasts (Rogers et al., 1985). Assays for histone phosphorylating activity in the presence or absence of cAMP showed that there was about a three-fold increase in cAMP-dependent protein kinase activity during myogenesis. Both myoblasts and myotubes were found to contain type I and II cAMP-dependent protein kinases. As well, both contained a substantial amount of free R_I. These results were determined by separation of the isozymes by anion exchange chromatography and also by using a cAMP photoaffinity label to identify the different types of subunits. Using the same techniques it was shown that the increase in cAMP-dependent protein kinase activity was due to an increase in the type I isozyme. There was also apparently an increase in the amount of free R_I during myogenesis. No increase in R_{II} was detected.

It was these results that led us to focus on the type I isozyme, and particularly the type I regulatory subunit, in differentiating L6 myoblasts. In this chapter we descibe studies aimed at determining the

mechanism by which the increase in R_I levels occurs. At the end of chapter 3, the relevance of this increase to the process of myogenesis is discussed in the light of our findings on the regulation of both R_I and C.

Regulation of cAMP-dependent protein kinases has also been studied in several other differentiating cell lines. These include 3T3-L1 cells which differentiate into adipocytes in culture (Liu, 1982). It was shown that there is an increase in the type I isozyme during adipocyte differentiation, similar to the case in L6. However significant amounts of free R_I were not observed. A second cell type in which changes in the cAMP-dependent protein kinases with differentiation have been studied is Friend erythroleukemia cells (Schwarz and Rubin, 1983). In contrast to the situation in L6 and 3T3-L1 cells, the levels of type I isozyme were found to decrease with differentiation of Friend cells into erythroid-like cells. There was, however, an increase in the total amount of activity due to an increase in the type II isozyme.

Isozyme levels have also been studied during the differentiation of mouse spermatocytes (Conti et al., 1979). In this case, the levels of type I isozyme were found to decrease and the levels of type II isozyme to increase, as in Friend cells. A similar pattern of changes in isozyme profiles has also been reported to occur in differentiating rat Sertoli cells (Fakunding and Means, 1977).

2.2 MATERIALS AND METHODS

2.2.1 Materials

^{125}I (100 mCi/ml) was purchased from Amersham, Oakville, Ontario.

[α-^{32}P] dCTP (3000 Ci/mmol) and L-[^{35}S] methionine (>800 Ci/mmol) were purchased from New England Nuclear, Montreal. Affinity-purified goat anti-rabbit IgG antibody was from Cedar Lane laboratories, Hornby, Ontario. Forskolin was from Calbiochem-Behring, La Jolla, California. Bt_2cAMP, 8-Br-cAMP and 3-isobutyl-1-methylxanthine were from Sigma, St. Louis, Missouri. All other chemicals were from various commercial sources.

2.2.2 Cell Culture

A highly myogenic subclone of the rat myoblast cell line L6 (Rogers et al., 1985; Yaffe, 1968) was used. Cells were cultured in α-modified minimal essential media supplemented with 10% horse serum, 16 mM glucose and 50 µg/ml gentamycin unless stated otherwise. Cells were routinely plated at a density of 0.5-1.0 x 10^4 cells per cm^2. They showed greater than 95% fusion after 6 days in culture, as determined by the method of Morris and Cole (1972). Medium was changed every three days or at the time of addition of cAMP analogues and forskolin.

2.2.3 Production and Purification of Antibodies against R_I

R_I was purified from bovine or rat skeletal muscle by the method of Dills et al. (1975) using a cAMP-sepharose affinity column with an N^6etheno spacer synthesized also as described. Preparations from both tissues were homogeneous as judged by SDS-polyacrylamide gel electrophoresis, although occasionally small amounts of proteolytic products were observed. Antibodies were raised in rabbits and purified from whole serum using an R_I-sepharose column prepared by the method of Kapoor and Cho-chung (1983). Fractions containing the R_I were detected

by assaying for the ability to precipitate type I cAMP-dependent protein kinase activity. 10 µl (containing enough phosphotransferase activity to give about 30,000 cpm of labeled histone in a standard protein kinase assay) of type I cAMP-dependent protein kinase, partially purified by DEAE-cellulose chromatography, was added to 85 µl of 5 mM MES, pH 6.5, 9 mM NaCl, 15 mM β-mercaptoethanol and then incubated with 5 µl of each column fraction for 90 min on ice. S. aureus (25 µl of a 10% solution) was then added and pelleted after a 30 min incubation on ice. Activity remaining in 20 µl aliquots of the supernatant was then assayed by the method of Corbin and Reiman (1974).

2.2.4 Western Blotting

Samples for Western blotting were obtained by washing L6 monolayers once with cold PBS and then scraping them into 1% SDS heated to 90°C. After centrifugation for 20 min in a micro centrifuge, supernatant solutions were stored frozen at -20°C until use. Protein was quantitated using the Peterson modification (Peterson, 1977) of the Lowry assay (Lowry et al., 1951). 15 µg aliquots were run on 9% SDS-polyacrylamide gels as described by Laemmli (1970). Electrophoresed proteins were then electro-blotted onto nitrocellulose membranes for 75 min at 110 V using a Bio-Rad Trans-blot apparatus and stained with 0.0025% amido black in a solution of 50% methanol and 10% glacial acetic acid. The blots were then washed twice in H_2O for 10 min each and twice in TBS for 10 min each. Blots were blocked for 1 h at 37°C with 4% BSA in TBS and then incubated overnight at room temperature in the same buffer containing 1 µg/ml affinity-purified anti- R_I antibody. Blots were then washed four times with 0.01% Triton X-100 in TBS for 10

min each and incubated for 3 h in 4% BSA in TBS containing 0.1 μCi/ml of ^{125}I-labeled goat anti-rabbit IgG antibody. Blots were washed again as above, dried and autoradiographed at -70°C using Kodak XAR-5 film and Dupont intensifying screens.

2.2.5 Subcellular Fractionation

L6 cells grown for either two or six days in 150 mm tissue culture plates were fractionated as follows: The cells were washed successively with PBS, ice-cold 5mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), pH 7.4 containing 1 mM phenylmethylsulphonyl fluoride and 2 μg/ml leupeptin and then incubated on ice for 90 s in 1 ml/plate of the same buffer. Cells were then scraped off the plate and homogenized by passage through a 27 gauge 1/2" needle five times. Examination under a phase contrast microscope showed that this method of homogenization lysed essentially all of the cells but left the nuclei intact. NaCl was added to a final concentration of 150 mM and the homogenate was centrifuged at 800 x g for 10 min to sediment a crude nuclear pellet. The supernatant was again centrifuged at 40,000 x g for 30 min to pellet a crude membrane fraction. The nuclear and membrane fractions were resuspended in 0.5 ml of 1% SDS at 90°C and stored frozen. The supernatant from the second centrifugation (crude cytosolic fraction) was made 1% in SDS and boiled for 4 min before being frozen. Equivalent amounts from each fraction were then analyzed for R_I levels by Western blotting.

2.2.6 Isolation of Total and Polyadenylated RNA

Total RNA was isolated from L6 myoblasts by lysis in guanidine isothiocyanate and pelleting through a cesium chloride gradient as

described by Chirgwin et al. (1979). Polyadenylated RNA was isolated from total RNA using oligo-dT cellulose exactly as described by Maniatis et al. (1982).

2.2.7 Slot and Northern Blotting

RNA samples, quantitated by measurement of absorbance at 260 nm, were incubated for 15 min at 65°C in 6.15 M formaldehyde, 10 X SSC (1 X SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and spotted onto a Biodyne A nylon membrane (Pall Ultrafine Filtration Corporation, Glen Cove, New York) using a Schleicher and Schuell slot-blotter, according to the manufacturer's instructions. For Northern blotting, RNA samples were denatured with formaldehyde and electrophoresed in formaldehyde-containing 1.5% agarose gels as described by Maniatis et al. (1982). Electrophoresed RNA was then transferred to Biodyne A nylon membranes as described by Thomas (1982). This procedure was found to be much more sensitive (greater than ten-fold) than methods in which the RNA is denatured with glyoxal.

2.2.8 Hybridizations

Membranes were baked for 1 h at 80°C before use. The plasmid p62C12 (Lee et al., 1983) was digested with Pst I and the 770 kb cDNA fragment was purified by agarose gel electrophoresis and band interception with Schleicher and Schuell NA 45 anion exchange membrane. The fragment was ^{32}P-labeled to a specific activity of 0.8-2.0 x 10^9 cpm/µg using a "Prime Time" DNA labeling kit from International Biotechnologies, Inc., and purified from unincorporated label with a Schleicher and Schuell Elutip-d column or by chromatography on Sephadex G-50. (The latter technique was found to give better yields). RNA blots

were pre-hybridized at 42°C for 6 h in 50% formamide, 5 X SSC, 5 X Denhardt's solution (1 X Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 250 µg/ml herring sperm DNA, 0.1% SDS and then hybridized in the same buffer containing 10% dextran sulphate and 1 x 10^7 cpm/ml of labeled probe for 18 h at 42°C. Blots were washed two times with 2 X SSC containing 0.1% SDS at room temperature and then two times for 15 min each in 0.1 X SSC containing 0.1% SDS at 42°C before being dried and autoradiographed as above.

2.2.9 Determination of Total RNA and DNA

RNA and DNA were measured as described by Rowe et al. (1978). L6 monolayers growing in 100 mm plates were scraped into 3 ml of 1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 containing 65 µg/ml proteinase K, homogenized by passage five times through a 25 gauge needle and digested for 1 h at 45°C. Samples were extracted twice with an equal volume of 1:1 phenol/chloroform and twice with a half volume of chloroform alone before being precipitated with 0.1 M NaCl and 2.5 volumes of absolute ethanol at - 20°C. Precipitated nucleic acids were resuspended in 600 ul H_2O at a concentration of 170 µg/ml and RNA was separated from DNA by precipitation overnight at -5°C with two volumes of 3 M sodium acetate, pH 7.0, containing 5 mM EDTA. RNA in the pellet and DNA in the supernatant were quantitated by measurement of absorbance at 260 nm. The sodium acetate precipitation was quantitative over a range of RNA concentrations from 0-160 µg/ml at least. Agarose gel analysis showed that DNA was well separated from the lower molecular weight RNA. Cells from a separate plate of L6 were scraped into 1 ml of 1% SDS, 1 mM EDTA and 10 mM Tris-HCl, pH 7.5 at 90°C and

assayed for protein. A separate plate was also stained for determination of the extent of fusion.

2.2.10 ^{35}S-methionine labeling and immunoprecipitation

L6 cells growing in 35 mm tissue culture dishes were washed once with 0.15 M NaCl in 0.015 M sodium citrate and labeled for 6 h in methionine free media containing 10% horse serum, 292 µg/ml glutamine and 100 µCi/ml ^{35}S-methionine. Monolayers were then rinsed twice with 0.15 M NaCl in 0.015 M sodium citrate and chased with regular media containg 4 mM methionine. Samples were isolated at different intervals of chase by removing the media and scraping the monolayers into 0.5 ml 2 X RIPA buffer (1 X RIPA buffer is 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl and 10 mM sodium phosphate, pH 8.2). Samples were homogenized by passage five times through a 25 gauge needle and stored frozen at -20°C. 200 µl aliquots were thawed, diluted with an equal volume of H_2O and incubated on ice for 30 min with 25 µl of a solution of 10% fixed S. aureus cells. S. aureus cells were removed by centrifugation for 10 min in an Eppendorf microfuge and samples were incubated on ice for 90 min with 1 µg of affinity-purified anti R_I antibody. Samples were then reincubated with S. aureus cells for 30 min. S. aureus cells were then pelleted and washed three times with 1 X RIPA buffer. Samples were solubilized with 50 µl of Laemmli's buffer (Laemmli, 1970) and electrophoresed on 9% polyacrylamide gels. Gels were dried and autoradiographed as above. Scanning densitometry was performed using a Beckman DU-8 spectrophotometer with a gel scan module. Film responses were in the linear range when quantitation from autoradiograms was performed.

2.3 RESULTS

2.3.1 Preparation of Antibodies against R_I

In order to be able to quantitate R_I levels in crude L6 samples, we raised antibodies against purified bovine or rat R_I. Typical yields of R_I purified using the procedure of Dills et al. (1975) were 2 mg of pure R_I from about 2 kg of bovine muscle, or 0.8 mg of pure R_I from 1.4 kg of rat leg muscle. Antibodies were raised in rabbits and purified on either a bovine or rat R_I-sepharose column. An elution profile for the purification of anti-bovine R_I is shown in figure 2.1. Anti-rat R_I was found to give a stronger signal on Western blots, either because it had a higher affinity or recognized more antigenic determinants, and was therefore used exclusively with the Western blotting technique. Both antibody preparations precipitated R_I to the same extent in immunoprecipitations when used at the concentration described in Materials and Methods. Anti-bovine R_I was used mainly with this technique as it was obtained in higher yields. As well, both antibody preparations specifically precipitated a single band of Mr 47,000 on immunoprecipitations. However more than one band was labeled by the anti-rat R_I antibody in Western blots, as discussed below.

2.3.2 Increase in R_I during differentiation

Figure 2.2 shows a Western blot of total L6 protein probed with affinity-purified antibody raised against purified rat skeletal muscle R_I. A major band of 47 kDa and minor bands of 52 kDa, 41 kDa and 38 kDa are labeled. The 47 kDa band represents the R_I subunit, as it was shown earlier (Rogers et al., 1985) that a band of the same molecular weight

FIGURE 2.1

AFFINITY PURIFICATION OF ANTI-R_I ANTIBODY

Crude antisera (13 ml) from a rabbit immunized with bovine R_I was loaded onto a column of bovine R_I coupled to Sepharose 4B. After washing with 100 ml of TBS, bound antibody was eluted with 0.1 M glycine,, pH 2.8. Fractions (1 ml) were monitored for protein by measurement of absorbance at 280 nm. Fractions were also analysed for their ability to precipitate cAMP-dependent histone-phosphorylating activity. The amount of activity precipitated is indicated by the solid circles.

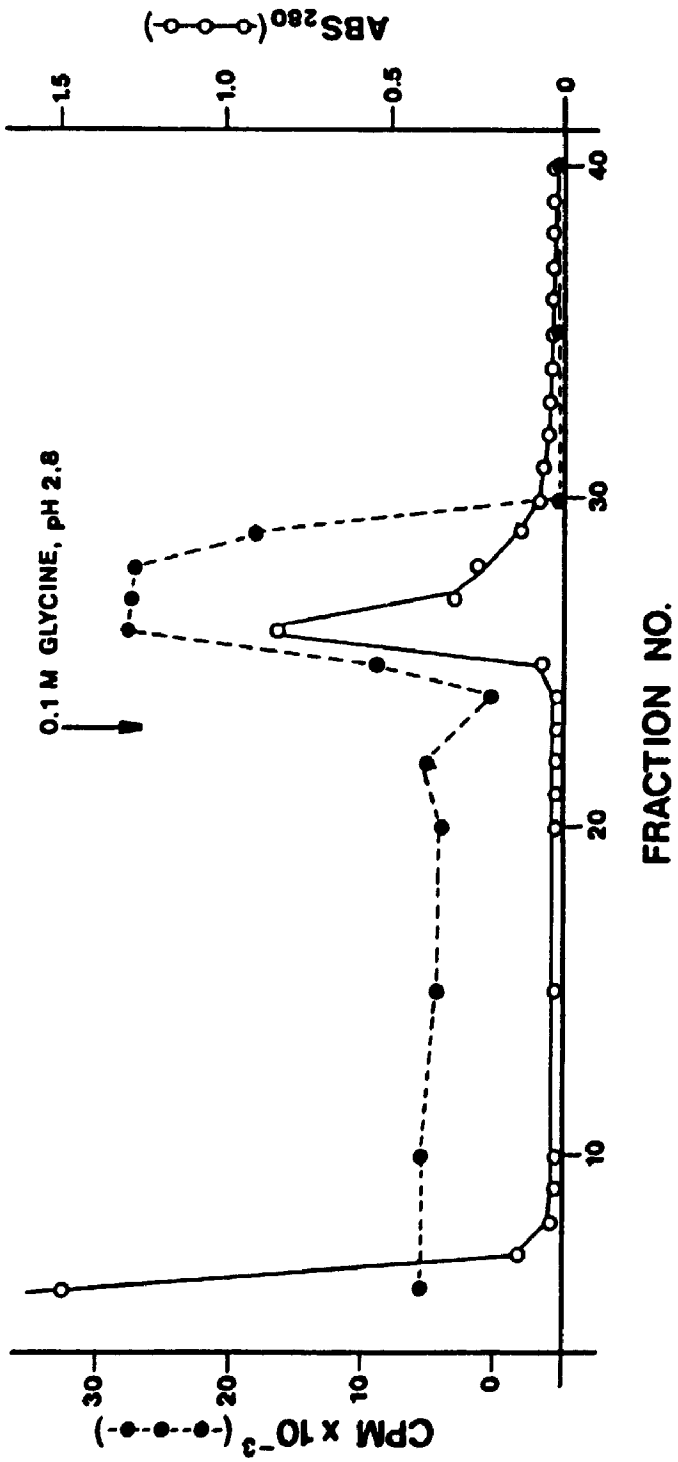
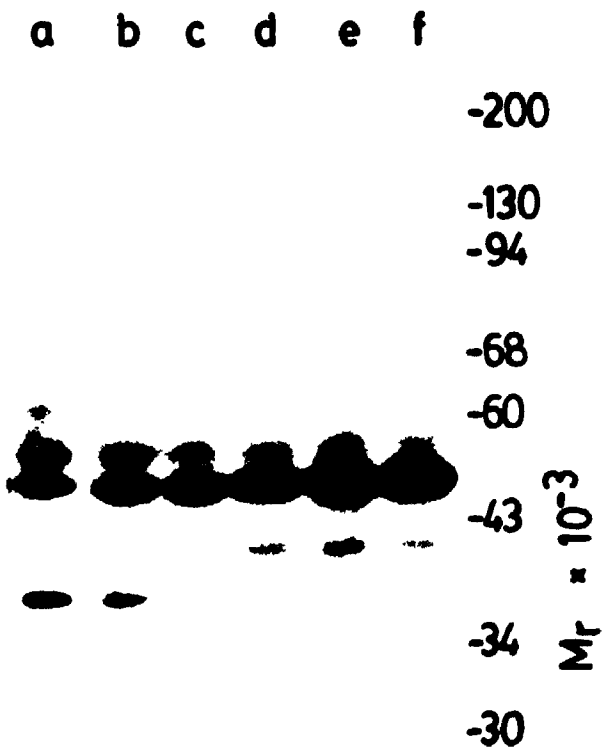

ABS280(-o-o-o-)
1.5
1.0
0.5
0
40
30
20
10
0.1 M GLYCINE, pH 2.8
CPM x 10-3 (-•--•--•-)
30
20
10
0
FRACTION NO.

FIGURE 2.2

WESTERN BLOT SHOWING CHANGES IN R_I LEVELS DURING L6 DIFFERENTIATION

Total L6 protein (15 µg) was isolated at different stages of differentiation, electrophoresed on SDS/polyacrylamide gels, transferred to nitrocellulose, and probed with anti-R_I antibody. Lanes a-f show samples from cells after 2-7 days in culture, respectively. The cells begin to fuse on day 4 and are typically greater than 95% fused on day 6.


a
b
c
d
e
f
-200
-130
-94
-68
-60
-43
-34
-30
Mr x 10-3

is labeled by the photoaffinity label, 8-azido cAMP. Scanning densitometry shows that the 47 kDa R_I band increases three-fold relative to total protein during L6 myoblast differentiation, in good agreement with results obtained earlier (Rogers et al., 1985). The 52 kDa band represents contaminating IgG heavy chains from serum used in the growth medium which reacts with the second antibody used in the Western blot procedure. This band was labeled when second antibody alone was used and it was found in later experiments that it could be removed by more extensive washing of the cells before lysis. The 41 kDa band most likely represents a proteolytic product of R_I as a band of the same molecular weight was labeled by cAMP photoaffinity label. We have not been able to establish the identity of the 38 kD band. Although several researchers have reported the existence of a proteolytic product of R_I of a similar size (Potter and Taylor, 1979; Beer et al., 1984), we were unable to test this possibility as this band does not appear in immunoprecipitates, preventing us from comparing the structure of this protein with R_I by procedures such as tryptic mapping. Analysis of several non-differentiating L6 lines (Seth et al., 1983; Cates et al., 1984), in which R_I levels do not change during growth, showed that the decrease in this band still occurred, and was therefore not coupled to the increase in R_I seen during differentiation.

2.3.3 Subcellular localization of R_I

There are several reports that cAMP-dependent protein kinase regulatory subunits can translocate to the nucleus upon activation by cAMP (Kapoor et al., 1984; Squinto et al., 1985) and it has been

proposed by several groups (Nagamine and Reich, 1985; Constantinou et al., 1985) that cAMP might regulate gene expression in this manner. To determine whether or not such a translocation of R_I took place during L6 differentiation, we fractionated L6 myoblasts and myotubes to determine where the increase in R_I was occurring within the cell. Accordingly, L6 cells were homogenized and separated into "nuclear", "membrane" and "cytosolic" fractions. Figure 2.3 shows the results of immunoblot analysis of these fractions in myoblasts and myotubes. Levels of R_I in the nuclear and membrane fractions were found to remain constant, with the increase in R_I only being evident in the cytosol. It therefore seems unlikely that R_I is translocated to the nucleus during myoblast differentiation.

2.3.4 Analysis of R_I mRNA levels during Myogenesis

A possible mechanism for the increase in R_I seen during myogenesis was that the increase was regulated transcriptionally. This seemed likely as the levels of several other proteins which increase during myogenesis had been shown to be regulated predominantly by this mechanism. An increase in transcription should result in an increase in the amount of the particular mRNA within the cell. Therefore we examined the levels of R_I mRNA during differentiation. For this purpose we used a cDNA clone of bovine testes R_I which was isolated by Lee et al. (1983). This clone contains the complete R_I coding sequence of bovine R_I. The R_I amino acid sequence was known to be well conserved between species (Steinberg, 1984; Shuntoh et al., 1988) so that this clone could be expected to hybridize with rat R_I mRNA at fairly high stringency. Figure 2.4 shows the autoradiogram of a slot blot in which

FIGURE 2.3

SUBCELLULAR LOCALIZATION OF R_I IN MYOBLASTS AND MYOTUBES

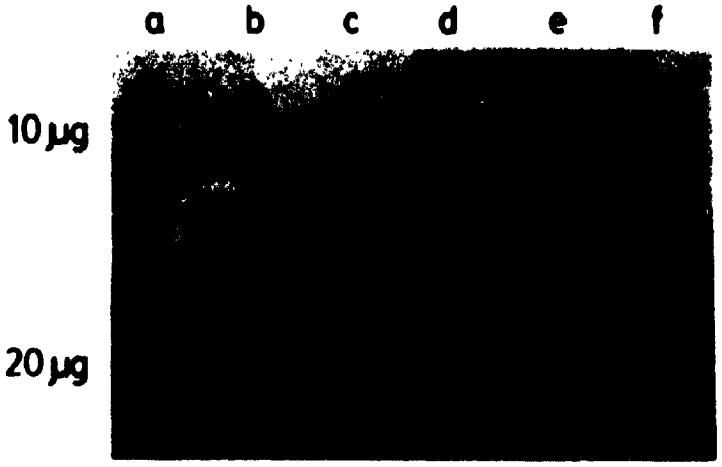
L6 myoblasts and myotubes were homogenized and fractionated into "nuclear", "membrane", and "cytosolic" fractions by differential centrifugation. Equivalent amounts of each sample were then analyzed for R_I levels by Western blotting. Lanes a-c show, respectively, the nuclear, membrane and cytosol fractions of L6 myoblasts. Lanes d-f show, respectively, the nuclear, membrane and cytosol fractions of L6 myotubes.


a b c d e f
-47 kDa

FIGURE 2.4

R_I mRNA LEVELS DURING L6 DIFFERENTIATION

10 or 20 µg of total L6 RNA were blotted onto a nylon membrane using a slot blot manifold and hybridized to a labeled R_I cDNA probe. Lanes a-f show R_I mRNA levels in L6 after 2-7 days in culture, respectively.


a
b
c
d
e
f
10 µg
20 µg

a 770 base pair fragment of the R_I cDNA clone has been hybridized to total L6 RNA samples from days 2-7 after plating, during which time more than 95% of the myoblasts fuse. Surprisingly, we could not detect any significant increase in the amount of R_I mRNA during myogenesis.

In order to correlate the slot-blot data (determined relative to total RNA) with immunoblot data (determined relative to total protein), we measured the levels of total protein, total RNA and total DNA during L6 differentiation. We found (figure 2.5) that RNA:DNA ratios did not change during differentiation. The same results were obtained when total RNA purified was correlated with numbers of nuclei in the sample. Nuclei were determined by staining one plate with Giemsa stain, counting nuclei under a microscope in a field of known size, averaging fields and calculating the total number of nuclei in the sample from this data. We did see an approximate doubling of protein:DNA ratios upon differentiation as reported by others (Sauro et al., 1985). Therefore if an increase in R_I mRNA were to account for the three-fold increase in R_I levels relative to total protein, we would expect to see an approximate six-fold increase in R_I mRNA relative to total RNA. As discussed above, this was not the case

Next we investigated the types of R_I mRNAs present in L6 cells by Northern blotting. Figure 2.6 shows a Northern blot in which total and polyadenylated RNA from L6 myoblasts were separated on an agarose gel, transferred to a nylon membrane and probed as described above for the slot-blot. The cDNA probe hybridized to three different mRNA species which appeared to be present in approximately equal amounts. The sizes of these mRNAs were estimated to be approximately 3.6, 3.2 and 1.7

FIGURE 2.5

LEVELS OF TOTAL RNA, DNA AND PROTEIN DURING L6 DIFFERENTIATION

Total RNA, DNA and protein were assayed at different stages of L6 differentiation. ●, DNA, O, total RNA, X, total protein. Points shown are the average of two separate determinations.

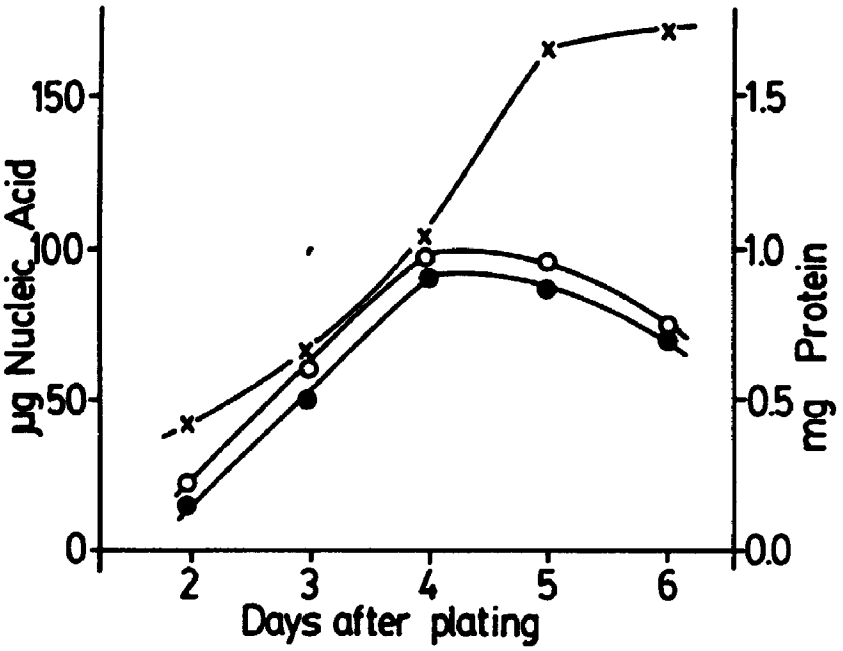
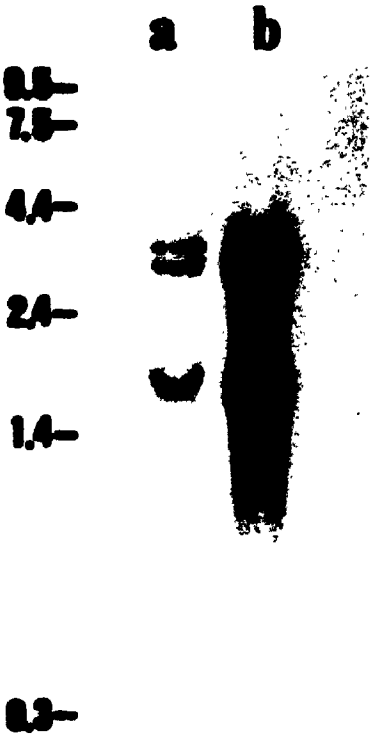

150
100
50
0
µg Nucleic Acid
1.5
1.0
0.5
0.0
mg Protein
2
3
4
5
6
Days after plating

FIGURE 2.6

NORTHERN BLOT OF TOTAL AND POLYADENYLATED RNA PROBED WITH R_I cDNA

L6 total RNA (lane a) or polyadenylated RNA (lane b) (20 μg per lane) was denatured with formaldehyde, electrophoresed on a 1.5% agarose gel, transferred to a nylon membrane, and hybridized to a labeled R_I cDNA probe. RNA size markers (shown in kilobases) were purchased from Bethesda Research Laboratories.


a
b
7.6
4.4
2.4
1.4
0.3

kilobases by comparing their migration to a set of standards of known size. All of these species were enriched for in the polyadenylated sample, showing that they were all processed to this extent. Since publication of this work, Oyen et al. (1987) have also described a similar pattern of bands in rat testes RNA probed with R_I cDNA, and also showed some changes in the relative intensities of the bands with development in this tissue. It seems unlikely that these different mRNA species are the product of different R_I genes. While two genes encoding similar R_I proteins have been identified in mouse (Clegg et al., 1988), the protein products of these genes migrate slightly differently on SDS/polyacrylamide gel electrophoresis and in L6 we have observed only one band for R_I labeled with polyclonal antibodies or the photoaffinity label 8-azido cAMP. This suggests that expression from only one R_I gene occurs in L6. (Most likely this is the $R_{I\alpha}$ gene, as expression from the $R_{I\beta}$ gene has only been observed in brain and testes (Clegg et al., 1988).) A more likely explanation for the presence of the different mRNA species is that multiple sites for polyadenylation are used during the transcription of the gene. Such a mechanism has been reported to give rise to multiple mRNA species with significant size differences (but coding for the same protein) in a number of cases, including mouse dihydrofolate reductase(Hook and Kellems, 1988) and alpha-amylase (Tosi et al., 1981), D. melanogaster actin (Bond and Davidson, 1986), bovine prolactin (Sasavage et al., 1982), chick ovomucoid (Gerlinger et al., 1982), and immunoglobulin heavy chain (Early et al., 1980). No known function for the use of different polyadenylation sites has been determined except in the case of immunoglobulin heavy chain genes,

where it has been observed that mRNAs for membrane and secreted forms of immunoglobulin M differ in their polyadenylation sites (Early et al., 1980). However instances where the use of different polyadenylation signals is developmentally regulated have been reported (Bond and Davidson, 1986). We therefore investigated whether or not there were any changes in the types or levels of particular R_I mRNA species during myogenesis. The results of this investigation are shown in the Northern blot depicted in figure 2.7. We did not detect any changes in either the types or levels of any of the three mRNA species during differentiation.

Finally we also investigated the actual rates of transcription from the R_I gene using the technique of run-off transcription. (These results are presented in detail in the following chapter on regulation of C.) We found that the rate of transcription from the R_I gene did not increase with differentiation, but rather decreased about four-fold. This decrease is probably due to the general decrease in rates of transcription between a growing population of myoblasts and quiescent myotubes.

Taken together the above results show that the increase in R_I levels was not regulated at the level of transcription as we first hypothesized. This led us to investigate possible post-transcriptional mechanisms by which the increase in R_I protein levels could be effected.

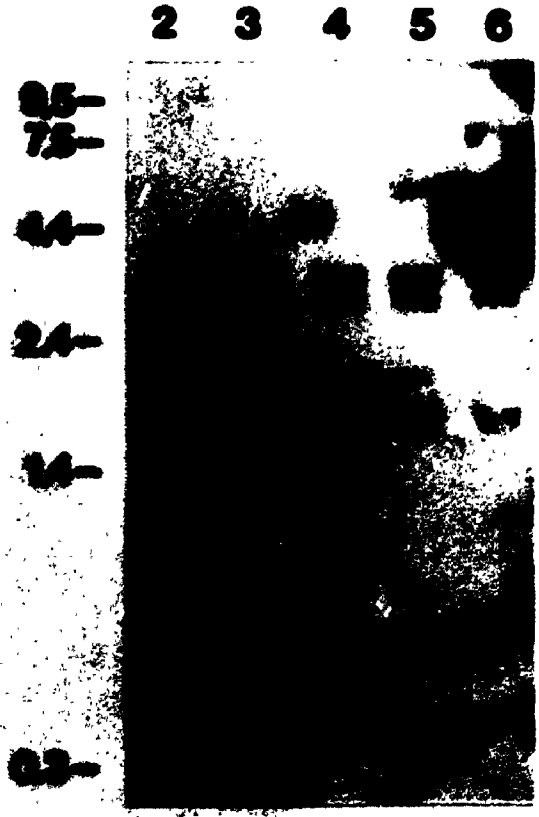
2.3.5 Measurement of the turnover rate of R_I

As one possibility for post-transcriptional control of R_I levels we compared the rates of R_I degradation in myoblasts and myotubes to

FIGURE 2.7

NORTHERN BLOT SHOWING R_I mRNA LEVELS IN TOTAL L6 RNA ISOLATED AT DIFFERENT STAGES OF DIFFERENTIATION

Total L6 RNA was isolated at different days after plating, electrophoresed and transferred to a nylon membrane. The membrane was then probed with labeled R_I cDNA.


2
3
4
5
6

see if the increase in R_I during differentiation could be caused by a change in its stability. L6 cells were labeled with ^{35}S-methionine and chased with media containing 4 mM unlabeled methionine for various periods of time. R_I was isolated by immunoprecipitation and polyacrylamide gel electrophoresis. The amount of radioactivity remaining in R_I was then quantitated by scanning densitometry of autoradiograms. A representative autoradiogram and plot of the corresponding scanning densitometry data are shown in figures 2.8 and 2.9. In both myoblast and myotubes R_I decayed exponentially with time and half-lives could therefore be calculated from the slopes of ln of radiolabeled R_I remaining versus time plots. The half-life of R_I was 3.2 h ± 0.1 h in myoblasts and 10 h ± 2 h in myotubes. (Slopes were calculated by linear regression; half-life values are the mean ± S.E. of three separate determinations.) A potential artefact in half-life determinations by the method used is that there may be reuse of labeled methionine during the chase if insufficiently high concentrations of unlabeled methionine are used in the chase, leading to an overestimation of the half-life value. This was not the case for myotubes as doubling the concentration of unlabeled methionine in the chase did not affect the half-life value determined (figure 2.10). These results show that R_I is degraded about three-times less rapidly in differentiated L6. This stabilization is specific as there is a slight increase in the rate of total protein degradation when L6 cells differentiate (Kaur, 1980). Further evidence for the specific nature of this stabilization came from studies of the degradation rates of C (described in the following chapter) which showed that C was degraded

FIGURE 2.8

IMMUNOPRECIPITATION OF R_I FROM ^{35}S-LABELED L6 MYOBLAST AND MYOTUBE EXTRACTS FOR HALF-LIFE DETERMINATIONS

Lanes a and b show immunoprecipitations of R_I from ^{35}S-labeled L6 extracts using preimmune serum (lane a) and immune serum (lane b). Other lanes show immunoprecipitations of R_I from myoblasts and myotubes after 1, 3, 5, 7 and 9 h of chase with media containing an excess of unlabeled methionine.

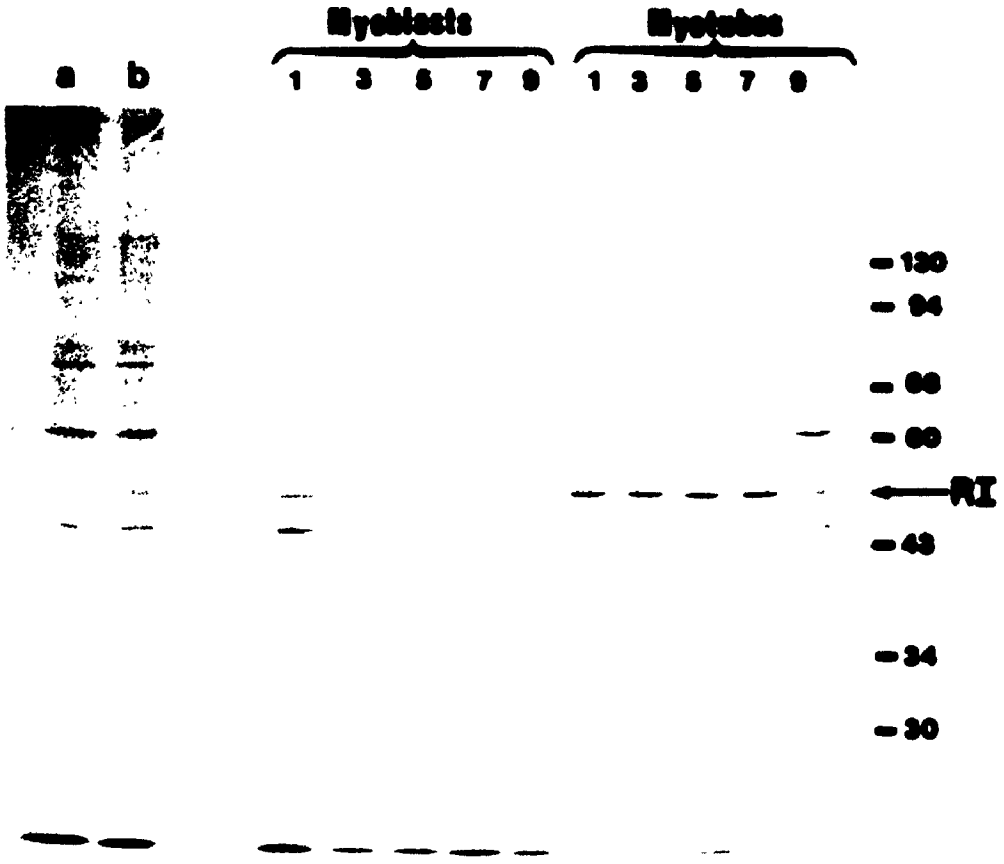

Myoblasts
Myotubes
a
b
1
3
5
7
9
1
3
5
7
9
120
94
68
60
RI
43
34
30

FIGURE 2.9

PLOT OF SCANNING DENSITOMETRY DATA FOR DETERMINATION OF R_I HALF-LIFE IN MYOBLASTS AND MYOTUBES

Autoradiograms of immunoprecipitated R_I were scanned using a Beckman DU-8 spectrophotometer with a gel scan module. Areas of the peaks were quantitated by weighing the excised peaks. Peak areas were then normalized to the peak area at 1 h of chase and the log values of these were plotted against time of chase. Slopes were calculated by linear regression. ●, myoblasts; ○, myotubes.

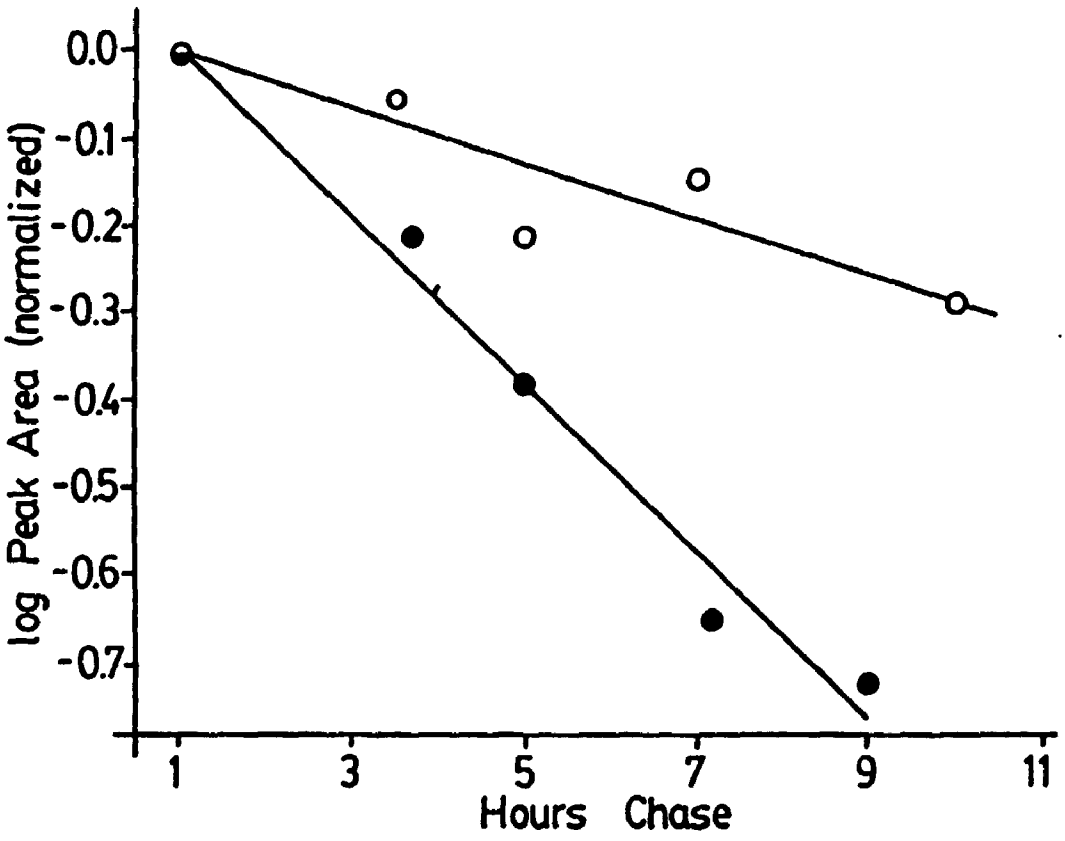
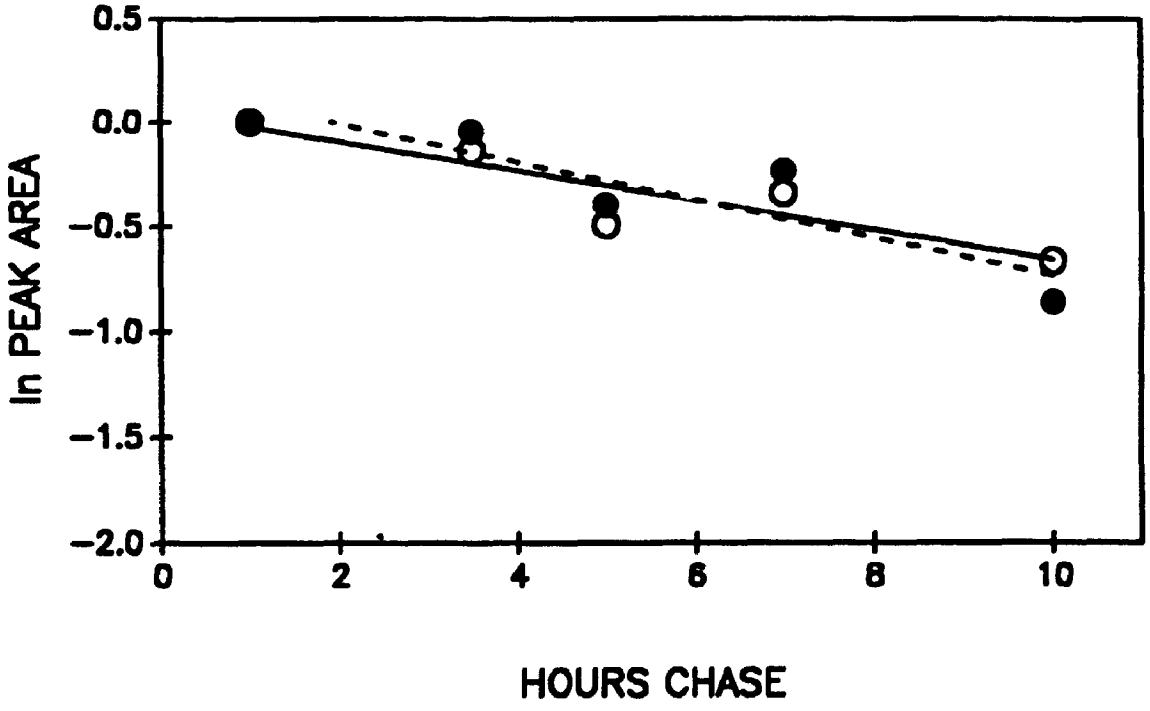

0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
log Peak Area (normalized)
1
3
5
7
9
11
Hours Chase

FIGURE 2.10

DETERMINATION OF R_I HALF-LIFE IN MYOTUBES USING DIFFERENT CHASE CONDITIONS

L6 myotubes were labeled with ^{35}S-methionine and then chased with media containing either 4 mM (O) or 8 mM (●) unlabeled methionine. Labeled R_I remaining was then immunoprecipitated and quantitated as described previously.


0.5
0.0
−0.5
−1.0
−1.5
−2.0
ln PEAK AREA
0
2
4
6
8
10
HOURS CHASE

more rapidly in myotubes, in contrast to the behaviour of R_I.

2.3.6 Effects of cAMP analogues on R_I levels and degradation rates

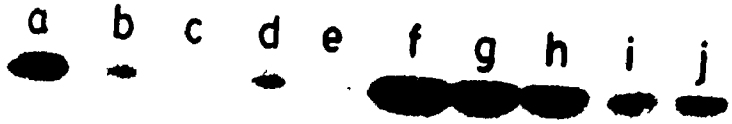
It has been reported that levels of regulatory subunit are decreased in an S49 mouse lymphoma mutant which lacks detectable C subunit (Steinberg and Agard, 1981). This was shown to be due to an increased rate of degradation of R_I. Increased degradation could also be induced when wild type S49 cells were treated with 8-bromo-cAMP. This report and also some of our own work on the regulation of R_I in a transformed cell line (discussed in chapter 4) suggested to us that changes in the association of R_I with C might mediate the change in R_I degradation rate seen during myogenesis.

Our first step towards investigating this possibility was to look at the effects of cAMP on the levels of R_I in myoblasts and myotubes. We used two different analogues, Bt_2cAMP and 8-bromo-cAMP, either separately or in combination, as Steinberg had reported opposing effects with these two analogues. As well we used forskolin, a compound which increases intracellular cAMP levels by stimulating adenylate cyclase (Seamon et al., 1981). Figure 2.11 shows an immunoblot of L6 samples, isolated after a 24 h treatment with these agents, which has been probed with anti-R_I antibody. All of the treatments caused a decrease in R_I levels compared to untreated controls in both myoblasts and myotubes. Some agents were more effective than others in this regard; in particular, forskolin or a combination of analogues were much more effective in myotubes than either Bt_2cAMP or 8-bromo-cAMP used singly. Both of the former treatments decreased R_I levels in myotubes below those seen in untreated myoblasts.

FIGURE 2.11

EFFECTS OF cAMP ANALOGUES AND FORSKOLIN ON R_I LEVELS IN MYOBLASTS AND MYOTUBES

L6 myoblasts and myotubes were treated for 24 h with cAMP analogues and forskolin. Equal amounts of protein (15 µg) were then analysed for R_I levels by Western blotting. Lanes a-e show myoblasts treated as follows: a, untreated; b, 0.25 mM Bt_2cAMP; c, 0.25 mM 8-Br-cAMP; d, 10 µM forskolin; e, 0.25 mM Bt_2cAMP plus 0.25 mM 8-Br-cAMP. Lanes f-j show the same treatments, respectively, for myotubes. Myotube cultures were obtained by growing L6 cells for 6 days. The cAMP phosphodiesterase inhibitor 1-isobutyl-3-methylxanthine was included in all treatments at a concentration of 0.1 mM.


a
b
c
d
e
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g
h
i
j

Since these results were consistent with the possibility that the change in R_I degradation rate during myogenesis was due to an alteration in its association with cAMP and/or C, we also studied the effects of cAMP on R_I half-lives in myoblasts and myotubes. Figure 2.12 shows the plots of R_I half-life data in myoblasts and myotubes treated with cAMP analogues or forskolin; the half-life data calculated from these plots are summarised in table 2.1. All of the treatments caused an increase in the rate of R_I degradation. Also there is a correlation between the different rates of degradation and the amount of R_I seen on the immunoblot (figure 2.11) descibed in the preceding paragraph. With respect to possible changes in the association of R_I with C during myogenesis, it is also significant that the difference between untreated and treated R_I half-lives is less in myoblasts than in myotubes.

2.4 DISCUSSION

Several mechanisms have been proposed by which increases in the levels of specific proteins are effected during myogenesis. The evidence now strongly favours the mechanism in which the changes in the levels of these proteins are controlled mainly at the level of transcription. Specific proteins for which this has been demonstrated are α-actin, myosin light chain, myosin heavy chain (Buckingham *et al.*, 1982; Medford *et al.*, 1983), desmin (Capetanaki *et al.*, 1984) and creatine kinase (Chamberlain *et al.*, 1985). However it appears that R_I is an exception; evidence from both the measurement of R_I mRNA levels and run-off transcription indicated that the increase in R_I is not

FIGURE 2.12

EFFECTS OF cAMP ANALOGUES AND FORSKOLIN ON R_I HALF-LIFE IN MYOBLASTS AND MYOTUBES

L6 myoblasts (upper plot) were treated for 18 h with 0.25 mM Bt_2cAMP and 0.1 mM 1-isobutyl-3-methylxanthine. These were also included in the labeling and chase media. O, untreated; ●, treated.

L6 myotubes (lower plot) were treated for 18 h as follows: O, untreated; ●, 0.25 mM Bt_2cAMP; ▲, 0.25 mM 8-Br-cAMP; △, 10 uM forskolin; □, 0.25 mM Bt_2cAMP plus 0.25 mM 8-Br-cAMP. 1-isobutyl-3-methylxanthine was also included in each treatment at a concentration of 0.1 mM. Analogues and forskolin were also included in the labeling and chase media.

Labeled R_I was immunoprecipitated and quantitated as described previously.

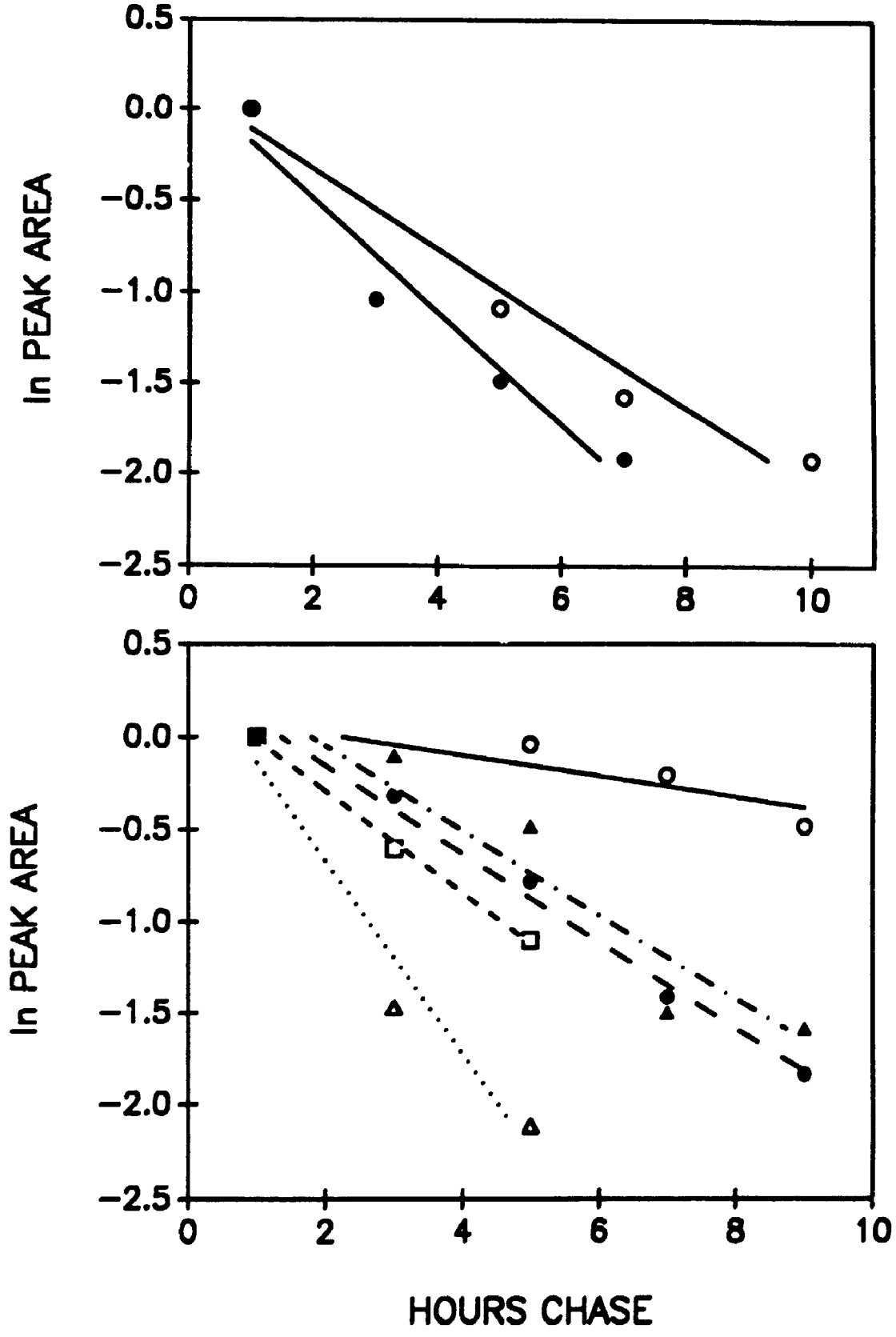

0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
ln PEAK AREA
0
2
4
6
8
10
0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
ln PEAK AREA
0
2
4
6
8
10
HOURS CHASE

TABLE 2.1

HALF-LIFE OF R_I IN MYOBLASTS AND MYOTUBES TREATED WITH cAMP ANALOGUES OR FORSKOLIN

	Half-life	
	Myoblasts	Myotubes
untreated	3.3 h	12 h
Bt_2cAMP	1.9 h	2.9 h
8-Br-cAMP	n. d.	3.0 h
Forskolin	n. d.	1.3 h
Bt_2cAMP +8-Br-cAMP	n. d.	2.5 h

Half-lives were calculated from the slopes of ln peak area versus hours chase plots using the formula

$$t1/2 = \ln 2 / \text{slope}$$

cAMP analogues were used at a concentration of 0.25 mM. Forskolin was used at a concentration of 10 µM. 1-isobutyl-3-methylxanthine was included in all treatments at a concentration of 0.1 mM. (n. d. - not determined).

regulated transcriptionally.

One mechanism by which protein levels can be regulated post-transcriptionally is by changes in degradation rates. An example of this is the protein p53, which is found in much higher levels in SV40-transformed cells than in non-transformed cells, apparently because of its association with the SV40 large tumour antigen which protects it from degradation (Deppert and Haug, 1986). We investigated the degradation rate of R_I in myoblasts and myotubes to see if changes in R_I degradation could lead to its increase during myogenesis. To accomplish this we used a pulse/chase procedure which is in widespread use for the study of protein turnover. In both myoblasts and myotubes we found that R_I was degraded exponentially and that highly reproducible half-life values could be calculated from the data. We consistently found that R_I was degraded approximately three times less rapidly in myotubes. This effect is contrary to the general trend seen during myogenesis; previous studies in our laboratory have shown that the rate of total protein degradation is increased slightly in myotubes. As well turnover of C was found to increase during differentiation (discussed in chapter 3). It therefore seems that a specific stabilization of R_I leads to the increase in its levels during myogenesis.

The next question we asked ourselves was how this specific stabilization of R_I might occur. Since it had been reported that cAMP and/or C could regulate the levels of R_I in S49 mouse lymphoma cells by changing its rate of degradation (Steinberg and Agard, 1981), we investigated whether this could also occur in L6 cells. We found that

in myoblasts and myotubes cAMP analogues or elevated levels of endogenous cAMP could decrease R_I levels by increasing R_I degradation. This was true for both of the cAMP analogues we tested, whereas in S49 cells it was only seen with 8-bromo-cAMP. Our results support the idea that the changes in R_I degradation during myogenesis are due to a change in its association with C. Also tney do not support the idea that a change in some protease causes the stabilization of R_I, as the results show that a protease is present in myotubes which can degrade R_I very rapidly, provided it is in the free state.

A change in the association of R_I with C could either be caused by a change in cAMP levels or a change in the amount of C present. Experiments are presented in the following chapter which, we believe, tend to support the second of these two possibilities.

CHAPTER 3

REGULATION OF C LEVELS DURING MYOGENESIS

3.1 INTRODUCTION

The purpose of the work described in this chapter is to compare the regulation of the catalytic subunit of the cAMP-dependent protein kinase with the regulation of R_I described in chapter 2. Both assays for cAMP-dependent protein kinase activity and analysis of isozyme levels by anion exchange chromatography had previously suggested that levels of C, as well as R_I, increased during myogenesis in L6 (Rogers et al., 1985). Studies of 3T3-L1 cells (Liu, 1982), Friend erythroleukemia cells (Schwartz and Rubin, 1983) and developing rat testes (Lee et al., 1976) also showed that an increase in C levels accompanies differentiation of these cell types or tissues, which suggests that an increase in C levels may be a common feature of many terminal differentiation processes.

As well as cDNA clones (Uhler et al., 1986a), genomic clones of C have also been isolated, so that some characteristics of the gene organization of this subunit has been delineated (Chrivia et al., 1988). There are two genes for C in mouse, which code for two distinct proteins, C_α and C_β (Showers and Maurer, 1986; Uhler et al., 1986b). These proteins are 91% similar in their deduced amino acid sequences. At least some of the organization of introns and exons is conserved between the two genes and both have been shown to have multiple start sites for the initiation of transcription (Chrivia et al., 1988). In this chapter some evidence is presented which suggests that expression

of the C_α gene may be developmentally regulated during myogenesis. As well, a model for the overall regulation of the type I cAMP-dependent protein kinase isozyme during myogenesis is proposed.

3.2 MATERIALS AND METHODS

3.2.1 Materials

[α-^{32}P]-UTP (3000 Ci/mmol) was purchased from Amersham Corp. DNase 1 was from Bethesda Research Laboratories. Proteinase K and RNase A were from Boehringer Mannheim. Rabbit anti-goat antibody was from Zymed Laboratories, Inc., and En^{3}Hance was from Du Pont-New England Nuclear. Other chemicals were as described in Chapter 2.

3.2.2 Measurement of C mRNA levels

Northern blotting was performed as described in Chapter 2. Hybridizations were also carried out as described previously except that a labeled 600 base pair Eco R1 fragment from the plasmid pMC1 (Uhler *et al.*, 1986a) was used. This sequence contains part of the C carboxyl terminal coding region and about 160 base pairs of 3' untranslated sequence.

3.2.3 Run-off transcription

(a) Purification of nuclei: Nuclei for run-off transcription were purified essentially as described by Medford *et al.* (1983). Cells growing on 150 mm tissue culture plates were washed twice with ice-cold PBS and then scraped into a total of 20 ml cold PBS. Four plates were used for preparations of myotube nuclei and ten were used for preparations of myoblast nuclei. Cells were pelleted by centrifugation at 1000 rpm for 5 min and then resuspended in 3 ml of lysis buffer

(25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 5 mM $MgCl_2$, 140 mM sucrose, 0.5% Triton X-100). After gentle vortexing, the cells were incubated 5 min on ice and then homogenized by passage 2 times through a 25 gauge needle. This preparation was then layered over 2 ml of 0.34 M sucrose, 0.5 mM $MgCl_2$ and centrifuged at 2000 rpm for 5 min at 4°C. The supernatant was then aspirated off and the pellet containing the nuclei was resuspended in 2 ml of lysis buffer. An aliquot of this, diluted appropriately, was then used to count nuclei with a hemocytometer. 1 X 10^7 nuclei were pelleted with a 20 s spin in a microcentrifuge. The pellet was then resuspended in 100 µl of freezing solution (50 mM Tris-HCl, pH 8.3, 40% glycerol (v/v), 5 mM $MgCl_2$, 0.1 mM EDTA), frozen in liquid N_2, and stored at -70°C until use.

(b) Run-off transcription reaction: Nuclei were pelleted by centrifuging for 10 s in a micro centrifuge and resuspended in freezing solution to a final volume of 100 µl. To this were added 50 µl of 4 X run-off transcription buffer (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl), 1 µl each of a 100 mM solution of ATP, GTP and CTP, 17 µl of H_2O and 30 µl (300 µCi) of ^{32}P -UTP. The reaction was allowed to proceed for 20 min at 30°C and was then terminated by the addition of DNase 1 to 50 µg/ml with a further 5 min incubation at 30°C. RNA was then purified by proteinase K digestion, phenol/$CHCl_3$ extraction and trichloroacetic acid precipitation exactly as descibed by Groudine *et al*. (1981). This lengthy procedure was necessary to reduce hybridization background so that long exposures could be used. Incorporation of ^{32}P was 3-5 fold greater for myoblast nuclei than for myotube nuclei, as observed by Medford *et al*. (1983). (Values for

yields of ^{32}P-RNA were 0.5 cpm per nucleus for myotubes and 2.4 cpm per nuclei for myoblasts.)

(c) Hybridization: The plasmids pUC13, pMC1 and p2RI (Uhler et al., 1986a), and pA1 (Cleveland et al., 1980) were purified using the alkaline lysis method of Maniatis et al. (1982) followed by banding on CsCl. pUC13 was a control plasmid for monitoring background hybridization, pMC1 was described at the beginning of this section , p2R1 is a plasmid containing carboxyl terminal coding cDNA for mouse R_I and pA1 is a plasmid which contains the entire coding sequence of chick β-actin. The plasmids were linearized by overnight digestion with a restriction enzyme. (Hind III was used for all plasmids except pA1 which was digested with Pst 1.) Plasmids were then repurified by extraction ith phenol/$CHCl_3$, extraction with $CHCl_3$ and precipitation with ethanol. After denaturation with NaOH, 6 µg per lane of each plasmid was spotted onto a Biodyne A nylon membrane using a Schleicher and Schuell slot blot apparatus according to the manufacturer's instructions.

Membranes were baked and prehybridized as described in Chapter 2. For the hybridization, the total reaction products from each run-off transcription reaction were denatured by heating at 100°C for 5 min and then hybridized for 48 h in a total of 5 ml hybridization buffer (containing 10% dextran sulphate; see chapter 2). Membranes were washed as described by Greenberg and Ziff (1984); after three 30 min washes in 2 X SSC at 65°C, they were incubated in 2 X SSC containing 10 µg/ml of RNase A for 30 min at 37°C and finally washed twice at 37°C with 2 X SSC containing 0.1% SDS. The membranes were then blotted dry and

autoradiographed under Saran wrap for 1-10 days at -70°C using DuPont intensifying screens.

3.2.4 Half-life Measurements

Labeling of L6 cells with ^{35}S-methionine and chase conditions with unlabeled methionine were as described in Chapter 2 except that the cells were labeled for only 4 h and longer periods of chase were used, as discussed in the results section of this chapter. Immunoprecipitations were also carried out as descibed in Chapter 2, but with the following changes: instead of anti-R_I antibody, extracts were incubated for 60 min with 1 µg of anti-C subunit antibody which had been raised in goat (a gift of M. Murtaugh); after this incubation, a second incubation with 5 µg of rabbit anti-goat antibody was carried out for 60 min on ice. The immunoprecipitation was then completed by incubation with S. aureus, pelleting and washing as described in Chapter 2. Samples were electrophoresed on 10% acrylamide gels. Gels were then treated with En^{3}Hance according to the manufacturer's instructions before being dried and autoradiographed. Films were scanned using an LKB densitometer.

3.2.5 Radioimmunoassay for cAMP

L6 cells growing in 100 mm tissue culture plates were washed once with ice-cold PBS and then scraped into 1 ml of cold 6% trichloroacetic acid. After incubation for 30 min on ice, insoluble material was pelleted by centrifugation for 15 min in an Eppendorf microcentrifuge. Pellets were stored frozen at -70°C until they were assayed for protein as described earlier. cAMP in the supernatant solutions (also stored at -70°C) was assayed using a cAMP radioimmunoassay kit from DuPont

according to the manufacturer's instructions.

3.3 RESULTS

3.3.1 Increase in C mRNA during differentiation

Figure 3.1 shows a Northern blot of total L6 RNA from days 2 to 7 after plating which has been probed with labeled C cDNA. Throughout differentiation a single band of approximately 2.6 kilobases is labeled. A band of this size has also been reported in both mouse (Uhler et al., 1986b) and bovine (Showers and Maurer, 1986) RNA and has been shown to code for the C_α isozyme. We did not detect a band in the 4.3-4.4 kilobase range which has been reported to be present in low levels in most mouse and bovine tissues (and more abundantly in the brain of these species) and which cross-hybridizes with the C_α probe under conditions of moderate stringency. This has been shown to code for a second catalytic subunit isoform, denoted C_β. Assuming that this pattern of different mRNA sizes holds in rat tissues, L6 myoblasts appear to contain only C_α. It is possible that C_β mRNA is present at levels below the sensitivity of the technique employed.

There is a marked increase in the amount of C mRNA with differentiation, which begins at day 4 and is maximal at day 6 in parallel with fusion. By scanning densitometry the increase was estimated to be about 4-fold (figure 3.1). Therefore it appears that the increase in kinase activity seen during myogenesis is regulated by this change in C mRNA.

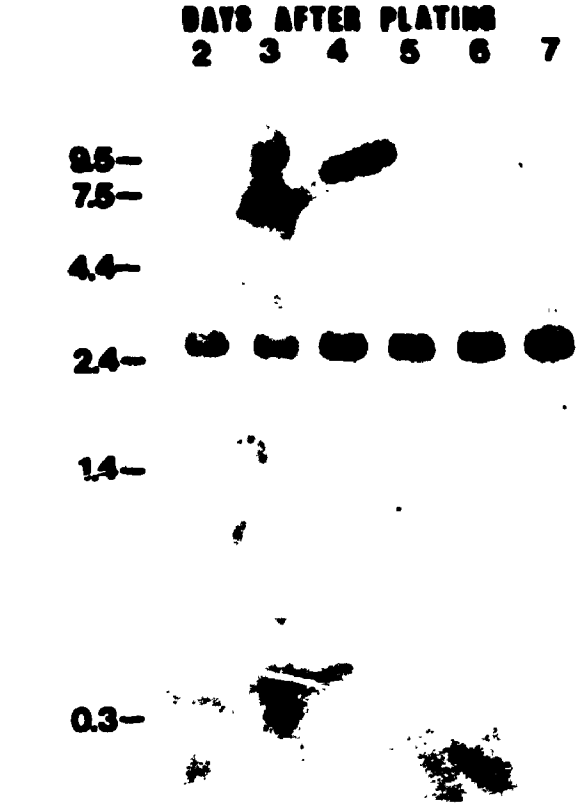
3.3.2 Transcription in Isolated Nuclei

There are several mechanisms by which increases in the levels of a

FIGURE 3.1

C MRNA LEVELS DURING L6 DIFFERENTIATION

Total RNA was isolated from L6 cells on days 2-7 after plating. RNA (20 µg per lane) was then denatured with formaldehyde, electrophoresed on a 1.5% agarose gel and transferred to a nylon membrane. The membrane was then probed with labeled C cDNA. The lower figure shows the quantitation of the increase in C mRNA by scanning densitometry. Values were normalized to the peak area for day 2 C mRNA.


DAYS AFTER PLATING
2 3 4 5 6 7
9.5
7.5
4.4
2.4
1.4
0.3

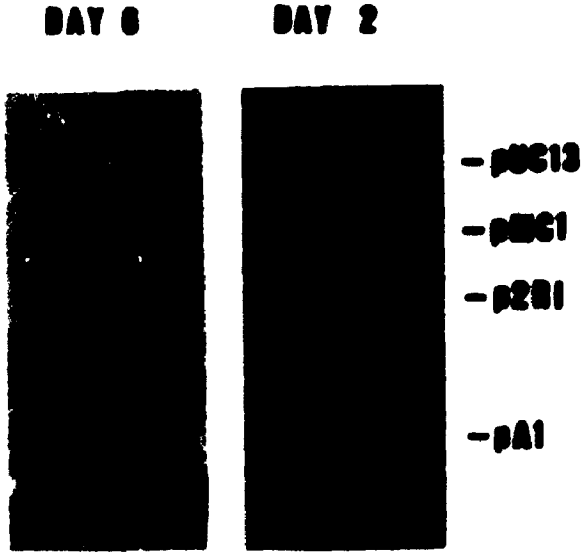

C mRNA LEVELS
0
1
2
3
4
5
1 2 3 4 5 6 7 8
DAYS IN CULTURE

particular mRNA can be effected. Most commonly this is due to a change in the rate of transciption. However, a decrease in the rate of degradation of a particular mRNA can also lead to an increase in its levels. To distinguish between these two possibilities we have attempted to measure the rates of transcription of C mRNA using the technique of run-off transcription (McKnight and Palmiter, 1979). In this procedure RNA transcripts which have been initiated before lysis of the cell are elongated, but no new initiation of transcription occurs. Therefore an estimate of the rate of transcription from a particular gene at the time of cell lysis can be determined. Figure 3.2 shows the results of one run-off transcription experiment in which labeled RNA transcribed from isolated day 2 and day 6 L6 nuclei has been hybridized to a nylon membrane on which plasmids containing cDNA corresponding to the mRNA for C, R_I and β-actin have been blotted. As mentioned in Materials and Methods, the total rate of RNA synthesis in myoblast nuclei was 3-5 fold greater than in myotubes, which is presumably a reflection of the comparative growth rates of the two cell types. Since we hybridized the total reaction products from each run-off transcription reaction, more ^{32}P-RNA was present in the day 2 hybridization; this accounts for the higher background on this membrane. Hybrization to a control plasmid, pUC13, was not seen in either the day 2 or day 6 hybridization. As a second control, we examined hybridization of labeled transcripts to the plasmid pA1 which contains a full length coding sequence for chick β-actin. For both day 2 and 6 nuclei a strong hybridization signal was obtained showing that labeled actin transcripts were synthesized in the run-off

FICURE 3.2

MEASUREMENT OF R_I AND C mRNA TRANSCRIPTION RATES IN MYOBLASTS AND MYOTUBES

Run-off transcription reactions were carried out using nuclei purified from day 2 myoblasts and day 6 myotubes. The labeled RNA products of the reactions were then purified and hybridized to blots of plasmids containing cDNA to C (pMC1), R_I (p2R1) and β-actin (pA1). pUC13 was also included as a control. The blots were then washed and autoradiographed for 10 days.


DAY 0
DAY 2
-pUC13
-pBC1
-p2B1
-pA1

transcription reaction. A decrease in the amount of transcripts with differentiation was observed. This is expected as levels of β-actin (i.e. non-muscle actin) mRNA have been shown to decrease during myogenesis (Buckingham et al., 1982). In the same experiment we also measured transcription levels for C mRNA (our main interest) and R_I mRNA (as a comparison with C). Unfortunately we were not able to detect a hybridization signal to the C cDNA-containing plasmid, in spite of the high concentration of high specific activity ^{32}P-UTP used in the run-off transcription reaction and the long film exposure used (10 days). This suggests that C mRNA is transcribed at a very low rate in both myoblasts and myotubes. We therefore could not determine whether the increase in C mRNA is regulated by an increase in transcription or by some other mechanism. We did observe a hybridization signal to the R_I cDNA-containing plasmid, suggesting that transcription from the R_I gene occurs at a faster rate than transcription from the C gene. The signal was about four-fold weaker in the day 6 hybridization. This is probably due to the overall decrease in the rate of transcription seen in myotube nuclei.

3.3.3 Immunoprecipitation and Measurement of C half-life

Although our results suggested that the increase in C activity during myogenesis was regulated by changes in C mRNA levels, we also chose to measure the half-life of C in myoblasts and myotubes as we had found that control at this level regulated R_I levels during myogenesis. For this we used an antibody which had been raised in goat against keyhole lympet hemocyanin-coupled bovine C by Dr. M. Murtaugh. Our own attempts to raise antibody in rabbits against C, either coupled to

keyhole lympet hemocyanin or oxidized with performic acid, were not successful. Figure 3.3 shows the results of an immunoprecipitation experiment done with the anti-C antibody. The antibody specifically immunoprecipitated a single band of M_r 40,000 from a ^{35}S-methionine-labeled L6 extract and could therefore be used in determinations of the rate of C degradation.

Our initial experiments, using chase periods of up to 9 h as with R_I, showed very little decrease in intensity of the labeled C band. This indicated that C was degraded very slowly in myoblasts, and we therefore chose to use much longer chase periods (up to 20 h) in further experiments. In these experiments it was found that C, like R_I, was degraded with exponential kinetics. Figure 3.4 shows a representative autoradiogram for the degradation of C in myoblasts and myotubes. A plot of the scanning densitometry data from one such experiment is shown in figure 3.6. The calculated half-life data, together with data on R_I half-lives for comparison purposes, is summarized in table 3.1. In myoblasts C was degraded with a half-life of 29 h ±5 h. This is almost a ten-fold slower rate than that determined for R_I in myoblasts. In myotubes C was degraded with a half-life of 14 h ±4 h, about twice as fast as in myoblasts. This is probably due to the overall increase in the rate of proteolysis with differentiation which has been observed previously in our laboratory (Kaur, 1980). This result, however is in contrast to the results obtained for R_I, which was found to be much more stable in myotubes. Interestingly, the rates of C and R_I are the same (within experimental uncertainty) in myotubes.

FIGURE 3.3

IMMUNOPRECIPITATION OF C FROM L6 CELLS

L6 cells were labeled with ^{35}S-methionine and C was immunoprecipitated from cell extracts. Lane a, pre-immune IgG; lane b, anti-C IgG.

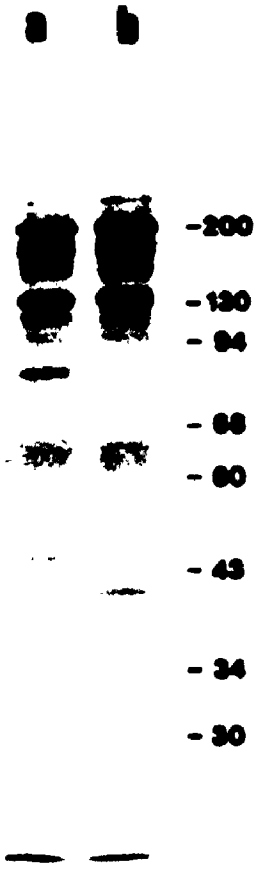

a
b
200
120
84
68
60
43
34
30

FIGURE 3.4

DETERMINATION OF C HALF-LIFE IN MYOBLASTS AND MYOTUBES

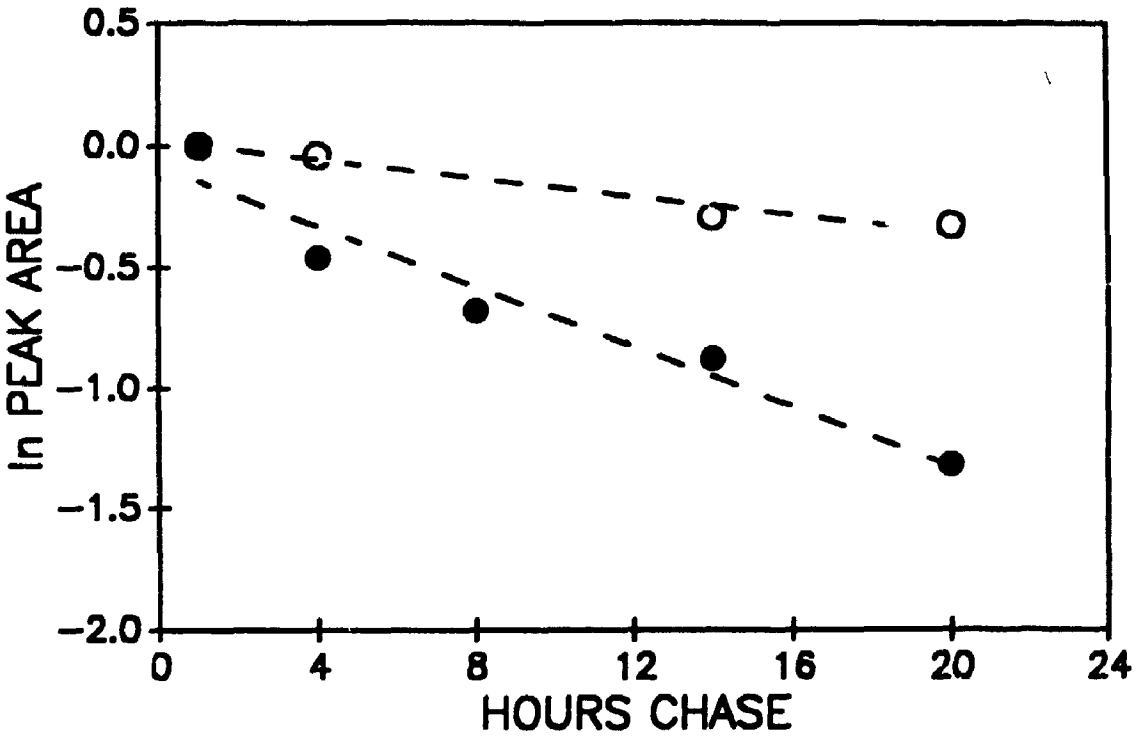
L6 myoblasts and myotubes were labeled with ^{35}S-methionine and then chased with media containing unlabeled methionine. Samples were isolated after 1, 4, 8, 14 and 20 h of chase. C was then immunoprecipitated and electrophoresed on 10% SDS/polyacrylamide gels.


Myoblasts
Myotubes
1 4 8 14 20 1 4 8 14 20
C

FIGURE 3.5

PLOT OF SCANNING DENSITOMETRY DATA FOR C HALF-LIFE DETERMINATION IN MYOBLASTS AND MYOTUBES

Labeled C remaining was quantitated by scanning densitometry of autoradiographs using an LKB densitometer. Peak areas were determined by weighing the excised peaks. Values were normalized to the peak area for 1 h of chase. The ln of these was then plotted against hours of chase, and slopes were determined by linear regression. O, myoblasts; ●, myotubes.


0.5
0.0
−0.5
−1.0
−1.5
−2.0
ln PEAK AREA
0
4
8
12
16
20
24
HOURS CHASE

To continue our comparison of C degradation with that of R_I, we also studied the effects of cAMP analogues on the degradation of C in myoblasts. The data shown in figures 3.7 and 3.8, and in table 3.1 are for myoblasts treated with a combination of Bt_2cAMP and 8-bromo-cAMP, together with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. As with R_I this treatment caused a marked increase in the rate of degradation of C; the half-life of C after this treatment was 9 h ±2 h, as compared to 29 h in untreated myoblasts. However degradation of free C is still much slower than degradation of free R_I. Interestingly the ^{35}S-labeled C bands were more intense after 1 h of chase in cAMP-treated myoblasts than in untreated cells chased for the same period. This suggests that there may be some increase in C synthesis to compensate for the increase in degradation, although further studies would be required to substantiate this. Several laboratories have previously reported studies on the regulation of C levels by cAMP. Alhanaty et al. (1981) have presented evidence for a proteolytic activity in brush border epithelial cells which was active against free C but not holoenzyme C. Hemmings (1986) has reported a decrease in C levels in porcine epithelial cells after long term treatment with hormones elevating intracellular cAMP, and proposed that the decrease was due to an increased proteolysis of C, although actual rates of proteolysis were not measured. Our results confirm this proposal, showing that cAMP can increase the rate of proteolysis of C in intact cells.

3.3.4 cAMP Levels during Myogenesis

One of our main reasons for studying C regulation during

FIGURE 3.6

DETERMINATION OF C HALF-LIFE IN MYOBLASTS TREATED WITH cAMP ANALOGUES

Myoblasts were treated for 6 h with 0.25 mM Bt_2cAMP, 0.25 mM 8-Br-cAMP and 0.1 mM 1-isobutyl-3-methylxanthine. Cells were then labeled and chased in media containing the same concentration of analogues. C was immunoprecipitated as described previously.

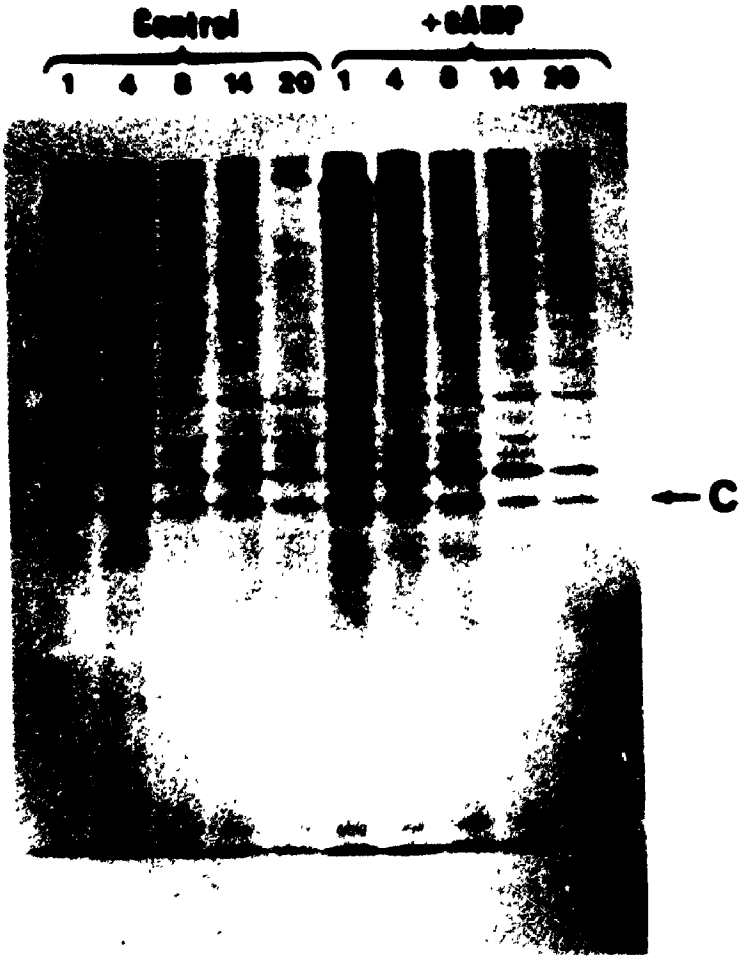

Control
+cAMP
1 4 8 14 20 1 4 8 14 20
C

FIGURE 3.7

PLOT OF SCANNING DENSITOMETRY DATA FOR THE DETERMINATION OF C HALF-LIFE IN MYOBLASTS TREATED WITH cAMP ANALOGUES

Labeled C was quantitated as described previously. O, untreated myoblasts; ●, myoblasts treated with 0.25 mM Bt_2cAMP, 0.25 mM 8-Br-cAMP and 0.1 mM 1-isobutyl-3-methylxanthine.

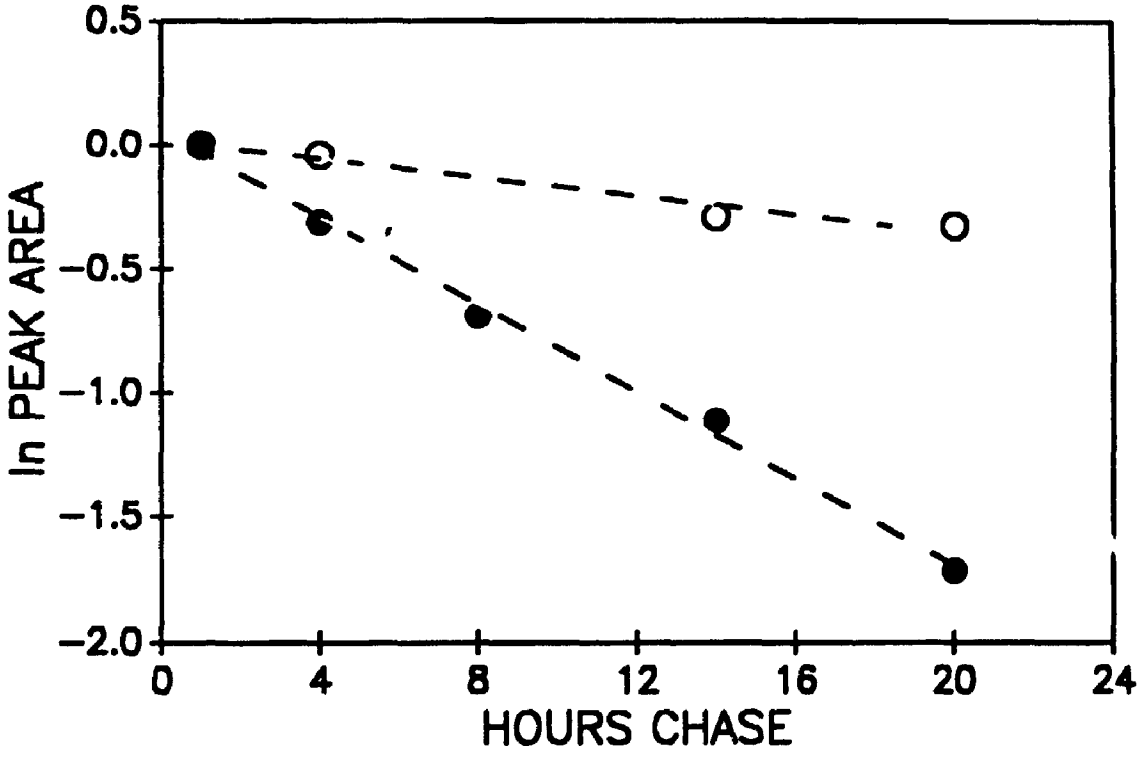

0.5
0.0
−0.5
−1.0
−1.5
−2.0
ln PEAK AREA
0
4
8
12
16
20
24
HOURS CHASE

TABLE 3.1

COMPARISON OF C AND R_I HALF-LIVES

	Half-life	
	C	R_I
Myoblasts	29 h ±5 h (4)	3.3 h ±0.1 h (3)
Myotubes	14 h ±4 h (3)	10 h ±2 h (3)
Myoblasts +cAMP	9 h ±2 h (3)	1.9 h ±0.3 h (3)

Half-lives were calculated from the slopes of ln peak area versus hours chase plots using the formula

$$t1/2 = \ln 2 / \text{slope}$$

Values shown are the mean ±standard deviation. Numbers in parentheses indicate the number of separate determinations. For the determination of C half-life in the presence of cAMP, myoblasts were treated with a combination of 0.25 mM Bt_2cAMP, 0.25 mM 8-Br-cAMP and 0.1 mM 1-isobutyl-3-methylxanthine. For the determination of R_I half-life in the presence of cAMP myoblasts were treated with 0.25 mM Bt_2cAMP and 0.1 mM 1-isobutyl-3-methylxanthine only. Differences discussed in the text were shown to be significant using t tests (at a level of 0.05).

myogenesis was that an increase in C levels was potentially a mechanism by which R_I could be stabilized during differentiation. A second possible mechanism by which R_I could be stabilized would be a decrease in cAMP levels during myogenesis. To determine whether such a decrease occurs, we measured cAMP levels during L6 differentiation using a sensitive radioimmunoassay procedure. The results are shown in figure 3.9. The levels of cAMP (expressed relative to total protein) do not follow a simple pattern; rather, there is an initial sharp decrease, probably density-dependent, followed by a transient increase around the time the cells reach confluence. After this the amount of cAMP decreases to almost undetectable levels. Therefore there is an overall drop in cAMP levels which lends support to the possibility that R_I levels (and the changes in R_I degradation) are due to changes in cAMP levels. However it should be borne in mind that these data are determined relative to total protein; it is difficult to determine an actual intracellular concentration and therefore it cannot be said whether these changes are significant in terms of activation of type I cAMP-dependent protein kinase. As well the conflicting reports from other laboratories on cAMP levels during myogenesis, discussed in Chapter 1, should be kept in mind.

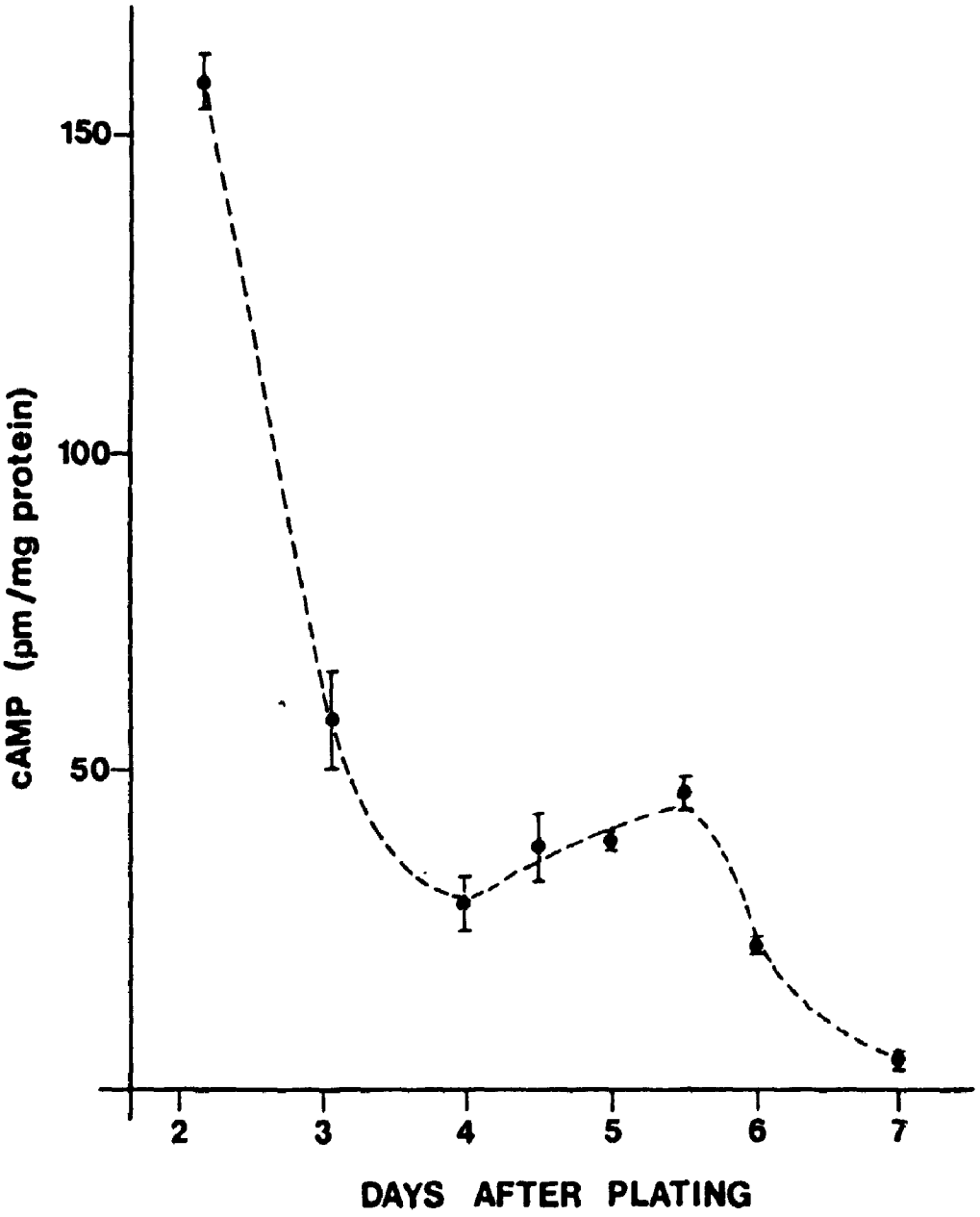
3.4 DISCUSSION

One significant point which arises from the work described in this chapter is the developmental regulation of C mRNA levels during myogenesis. Although we were not able to prove that this was due to a change in the rate of transcription from the C gene, this seems to be

FIGURE 3.8

cAMP LEVELS DURING L6 DIFFERENTIATION

cAMP was assayed at different stages of L6 differentiation by radioimmunoassay. Protein levels were determined by the method of Lowry *et al.* (1951). Fusion began on around day 5.


150
100
50
cAMP (pm/mg protein)
2
3
4
5
6
7
DAYS AFTER PLATING

the most likely explanation for the increase in C mRNA. Therefore it appears that C levels, unlike R_I levels, are regulated by the same mechanism as other proteins such as myosin heavy chain (Medford et al., 1983) and creatine kinase (Chamberlain et al., 1985) during myogenesis. We cannot at this point distinguish whether this increase is simply a result of differentiation or part of some signalling mechanism for the differentiation process. Concerning the former possibility, it may be that muscle requires more cAMP-dependent protein kinase for the hormonal control of glycogenolysis, an important source of energy for this tissue. Regulation of C mRNA levels during development has also been studied by Oyen et al. (1987), who showed that C mRNA levels increased in developing rat testes. As well, Uhler et al. (1986a) have measured the levels of C mRNA in a variety of mouse tissues and cell lines and shown that the mRNA is present in all of these at a fairly constant level of 10-25 molecules per cell.

With the data in this chapter on the regulation of C, and the data from the previous chapter on R_I regulation, it is possible to propose a model for the mechanism of regulation of the type I cAMP-dependent protein kinase during differentiation. The model we favour is depicted in figure 3.10. In this model the increase in R_I during myogenesis is due to a decrease in its rate of degradation, caused by an increase in its association with C. This increased association with C is due to an increase in the amount of C within the cell, so that more is available to complex with R_I. The evidence in support of this model is summarized as follows:

(i) During myogenesis there is an increase in C subunit mRNA, but no

FIGURE 3.9

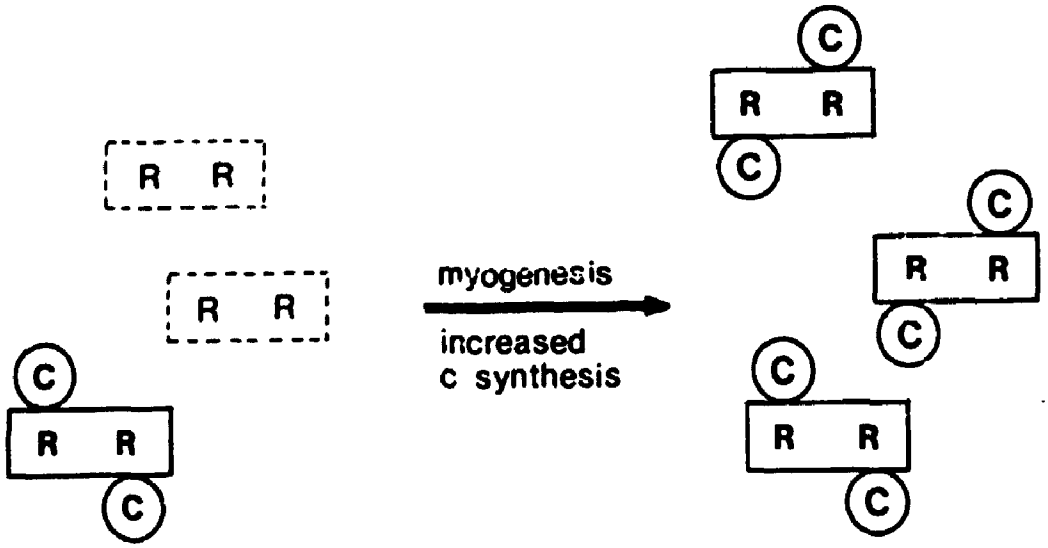
SCHEMATIC REPRESENTATION OF THE PROPOSED MECHANISM FOR THE REGULATION OF cAMP-DEPENDENT PROTEIN KINASE LEVELS DURING MYOGENESIS

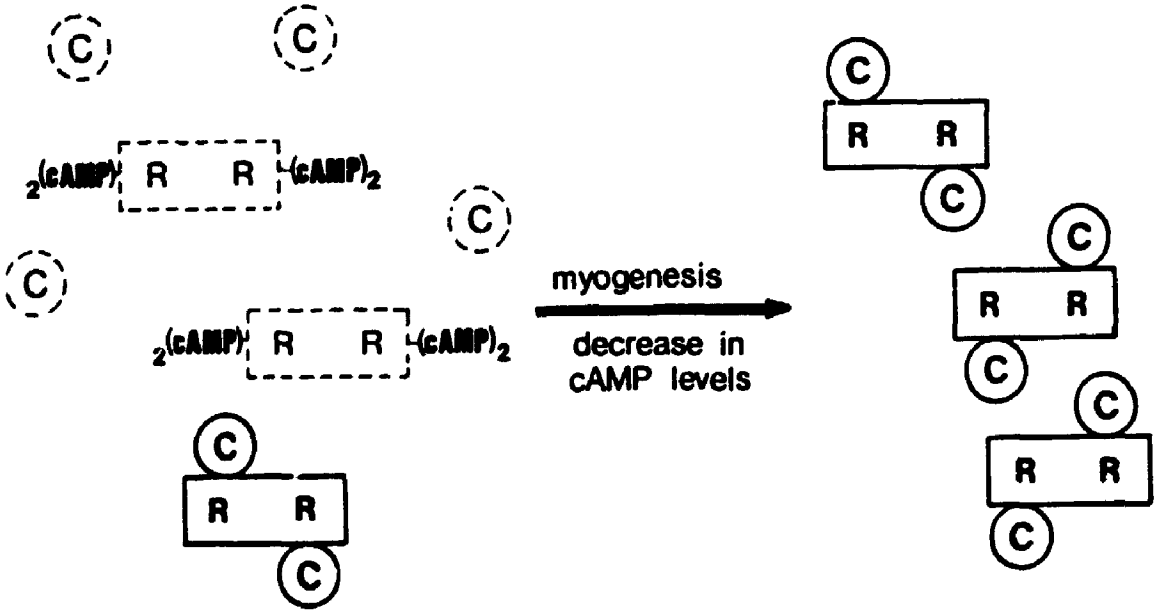
In myoblasts R_I is synthesized in much greater amounts than C. Excess R_I which does not bind with C is rapidly degraded. With differentiation, the synthesis of C increases. A greater amount of R_I is therefore able to bind with C, and as a consequence the overall rate of degradation of R_I decreases.

FIGURE 3.10

SCHEMATIC REPRESENTATION OF AN ALTERNATE POSSIBLE MECHANISM FOR THE REGULATION OF cAMP-DEPENDENT PROTEIN KINASE LEVELS DURING MYOGENESIS

In myoblasts R_I is dissociated from C by high levels of cAMP. Free R_I and C are rapidly degraded. With differentiation, R_I and C are able to associate again as cAMP levels decrease. R_I and C are therefore less susceptible to proteolysis and their levels increase. The mechanism of regulation of C was found to be inconsistent with this model.


R R
R R
C
R R
C
myogenesis
increased
c synthesis
C
R R
C
C
R R
C
C
R R
C


C
C
2(cAMP)
R R
(cAMP)2
C
C
2(cAMP)
R R
(cAMP)2
C
R R
C
myogenesis
decrease in
cAMP levels
C
R R
C
C
R R
C
C
R R
C

change in the levels of R_I mRNAs, indicating that C levels are probably regulated transcriptionally, but that R_I levels are regulated post-transcriptionally.

(ii) During myogenesis there is a marked stabilization of R_I, whereas the rate of C degradation increases, following the trend seen for total protein degradation.

(iii) The increase in R_I with myogenesis can be reversed by dissociating the type I holoenzyme with cAMP or cAMP analogues.

(iv) Dissociation of myotube type I holoenzyme with cAMP causes an increase in the rate of degradation of R_I, so that the half-life is slightly less than in myoblasts. Bt_2cAMP treatment of myoblasts causes a smaller relative change in the rate of R_I degradation than the same treatment does with myotubes.

During the course of this work a report published by Uhler and McKnight (1987) also suggested that this type of mechanism was involved in the control of type I holoenzyme levels. They studied the effects of overexpression of cloned C cDNA placed under control of the metallothionein promotor and transfected into 3T3 cells. It was shown that overexpression of C caused an increase in R_I without changing R_I mRNA levels. This would appear to be analogous to the events taking place during myogenesis, except that the increase in C expression was induced artificially rather than as part of the differentiation process.

One technique for studying the relative amounts of type I holoenzyme and free R_I is by separation of the two species using anion-exchange chromatography of cell-free extracts. This, in fact, was done

in our laboratory before the studies in this thesis were begun and it was reported that the ratio of free R_I to holoenzyme R_I did not change noticibly during myogenesis (Rogers et al., 1985). However there was a fair amount of variability in these results, suggesting that unknown factors during the cell lysis or subsequent handling of the extracts may have been causing artifactual changes in the association of R_I with C. The techniques used to obtain the results described in chapters 2 and 3 are generally less susceptible to artifacts, as they do not involve maintaining specific associations or activities after cell lysis. Potential artifacts in the measurement of the amount of association of R_I with C in tissue extracts have been discussed in detail by Corbin (1983).

A second possible mechanism for the regulation of type I cAMP-dependent protein kinase levels during myogenesis which we have considered is depicted in figure 3.10. In this model R_I stability (and consequently its levels) are also regulated by a change in its association with C, but in this case it is due to a decrease in cAMP levels during myogenesis, rather than an increase in the amount of C within the cell. It is important to distinguish whether this model, or the model described previously, is correct, as this model suggests a change in activity of the enzyme with myogenesis. This would have obvious implications as to a role for the type I cAMP-dependent protein kinase in myogenesis. However we feel that the first model, in which a change in C levels stabilizes R_I, is more likely for the following reasons:

(i) In the second model it would be expected that C mRNA levels would

not change during myogenesis. This was not the case, and the increase in C mRNA (4-fold relative to total DNA) is sufficient to explain the increase in R_I.

(ii) In the second model, free C would be present in myoblasts; it would be expected to be degraded at a rapid rate in myoblasts and a stabilization of it during myogenesis should cause its increase, as with R_I. Our results showed that C, in fact, is degraded very slowly in myoblasts, and that the rate of degradation increases in myotubes.

In spite of the above arguments we did observe significant changes in cAMP levels with myogenesis, so that this second model could be partly responsible for some of the change in R_I levels, if not the major factor.

Assuming that our first model is correct, we can propose a role for R_I in myogenesis; in myoblasts there is a rapidly-turning over pool of free R_I which, during myogenesis, combines with C which is being synthesized in increasing amounts. R_I thereby prevents any phosphorylating activity of C in the absence of a hormonal stimulus. By this mechanism R_I would always be in excess of C but, because of its rapid proteolysis in the free form, would not be in such a great excess that it would significantly effect activation by cAMP. This type of mechanism for maintaining R:C stoichiometry was originally suggested by Steinberg and Agard (1981) on the basis of their studies on an S49 mouse lymphoma mutant which did not express catalytic subunit, and also showed decreased levels of R_I. Our work supports this hypothesis and shows that this type of mechanism also functions during cellular differentiation. As well, our work suggests that this role is unique to

the R_I subunit in myoblasts as the levels of R_{II} do not change with differentiation (Rogers et al., 1985). This latter point is also supported by the findings of Uhler and McKnight (1987) who reported an increase in R_I but not R_{II} when C was artificially overexpressed in 3T3 fibroblasts.

CHAPTER 4

REGULATION OF R_I IN A SPONTANEOUSLY-TRANSFORMED L6 MYOBLAST CELL LINE

4.1 INTRODUCTION

Transformation of myoblasts has been shown to block the differentiation of these cells into myotubes. This has now been demonstrated using a number of viral oncogenes including mys, erb, fps and src (Falcone et al., 1985; Seth et al., 1983) and using the Ha-ras and N-ras oncogenes isolated from human tumours (Olson et al., 1987; this thesis). As well, spontaneously-transformed myoblasts have been isolated which are defective in differentiation (Kaufman and Parks, 1977; Seth et al., 1983). These cell lines are selected from a non-mutagenized parent population (hence the term spontaneous) either for their ability to grow in soft agar or by selecting for cells which overgrow fused myoblast cultures. It has been suggested that this spontaneous transformation is due to the expression of an endogenous C-type virus (Kaufman and Parks, 1977).

Previous work in our laboratory showed that there was an altered regulation of R_I in the spontaneously-transformed L6 myoblast cell lines JRU2 and JRU5 (Rogers, 1984). R_I in these cells appeared to be entirely in the free form and its levels increased upon treatment with Bt_2cAMP. In this chapter we show that this latter effect is due to a decreased proteolysis of R_I in the spontaneously-transformed cells (Lorimer and Sanwal, 1987). Experiments designed to determine the cause of this change in proteolysis are described. In addition, we have investigated R_I regulation in Ha-ras transformed L6 myoblasts in an

attempt to determine the relevance of changes in R_I regulation to the process of transformation.

4.2 MATERIALS AND METHODS

4.2.1 Materials

G418 was from Gibco Laboratories. Anti-ras antibody was from Triton Bioscience, CA. Ampholines were purchased from LKB. Thin layer chromatography plates were from Merck.

4.2.2 Cell Culture

The cell line JRU5 (Seth et al., 1983), a spontaneously transformed L6 cell line, was grown under the same conditions as L6 (see Chapter 2). Before use it was tested for the ability to grow in soft agar using the procedure of MacPherson (1973). After one week, JRU5 formed large colonies in soft agar, whereas L6 only divided once or twice on average in the same period of time. Friend erythroleukemia cells (clone 745a C110, originally from the Ontario Cancer Institute) were grown in Iscove's Modified Dulbecco's medium containing 10% fetal calf serum and 50 µg/ml gentamycin. Cells were subcultured every 2-3 days by adding them to fresh media at a concentration of 1×10^5 cells per ml. Large cultures of Friend cells were grown using spinner flasks.

4.2.3 Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (1975). Samples were prepared from 100 mm plates of L6 or JRU5 which had been labeled with ^{35}S-methionine as described in chapter 2. After removal of the labeling media, cells were scraped into 0.5 ml of boiling 1% SDS containing 1mM EDTA and 10 mM Tris-HCl,

pH 7.5. The extract was then centrifuged for 1 h at 100,000 X g and the supernatant was stored at -70°C until use. 50 µl of each extract was added to 200 µl of 2% Triton X-100, 2% sodium deoxycholate, 300 mM NaCl, 20 mM sodium phosphate, pH 8.2. To this were added 250 µl of H_2O and 16.5 µl of a solution of 10% BSA in PBS. The immunoprecipitation was then carried out as in chapter 2, using 2 µg of anti-bovine R_I per sample. The final washed pellet was solubilized with SDS and then treated with an excess of NP-40 (this removes bound SDS from the proteins) as described by Ames and Nikaido (1976). Samples were run in first dimension isoelectric focussing at 300 V for 18 h and for one more hour at 400 V using ampholytes with a pH range from 3.5-10. Gels were then removed from their tubes and either used immediately or frozen in SDS polyacrylamide gel sample buffer using a dry ice/ethanol bath and stored at -70°C until use. The tube gels were then loaded onto second dimension 9% SDS-polyacrylamide slab gels and electrophoresed. These gels were dried and autoradiographed for 6-7 days using DuPont intensifying screens.

4.2.4 Tryptic Mapping

Tryptic mapping was performed by a modification of the procedure of Zweig and Singer (1979). Instead of iodinating proteins in gel slices as described in this procedure, R_I was metabolically labeled with ^{35}S-methionine and isolated by immunoprecipitation and gel electrophoresis. The labeled R_I bands, detected by autoradiography of the dried gels, were excised and then digested with trypsin. To ensure a high level of incorporation of ^{35}S-methionine, confluent 10 cm plates of L6 and JRU5 were labeled for 16 h with 3 ml of labeling media

(chapter 2). The cells were then scraped into 0.5 ml of 2% Triton X-100, 2% sodium deoxycholate, 0.2% SDS, 300 mM NaCl, 20 mM sodium phosphate, pH 8.2 and the entire extract was then used in an immunoprecipitation as described in chapter 2. After trypsin digestion of the excised R_I bands, the extracted peptides were run in first dimension high voltage electrophoresis on cellulose thin layer chromatography plates using an acetic acid: formic acid: H_2O (15:5:80, pH 1.9) solvent. Second dimension thin layer chromatography was run using the solvent system 1-butanol: pyridine: acetic acid: H_2O (32.5:25:5:20) containing 7% PPO. Solvent was evaporated from the thin layer chromatography plates which were then autoradiographed as previously for 7 days.

4.2.5. In vitro Assay for ATP-dependent Protease Activity

Friend erythroleukemia cell extracts for protease assays were prepared according to Rieder et al. (1985), including the addition of leupeptin to the extract. ATP-dependent protease assays were carried out as described by Waxman et al. (1985). 100 µl of extract was added to 100 µl of 100 mM Tris-HCl, pH 8.0, 20 mM Mg^{2+}acetate, 1 mM dithiothrietol with or without 10 mM ATP and containing 1 µg of ^{125}I-BSA (100,000 cpm). After incubation for 1 h at 37°C, 25 µl of a 10% solution of BSA and 575 µl of 10% trichloroacetic acid were added. After a further incubation for 30 min on ice, protein was pelleted with a 15 min centrifugation in a micro centrifuge and 500 µl of the supernatant were counted in a gamma counter. Purified rat R_I and BSA were iodinated using the chloramine T method (Hunter and Greenwood, 1962). After iodination BSA was reduced by incubation with a 50-fold

molar excess of dithiothreitol for 4 h at 50°C under N_2 and then alkylated by treatment with a 100-fold molar excess of recrystallized iodoacetamide for 20 min at room temperature in the dark.

4.2.6 Transfection of L6 cells

L6 cells were transfected using the procedure of van der Eb (1973) except that a glycerol shock was used as described by Parker and Stark (1979). The plasmid used for transfection was pEJNEO (Bell et al., 1986), a gift of Dr. John Bell. This plasmid was constructed by ligation of the 6.6 kilobase Bam Hl fragment of pEJ (Tabin et al., 1982), which contains the human ras gene with an oncogenic gly→val point mutation at codon 21, into the Bam Hl site of pSV2NEO. 10 µg of pEJNEO and 30 µg of herring sperm DNA in 1 ml of transfection buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, 21 mM HEPES, pH 7.4) were sterilized by filtration and the DNA was then precipitated by the dropwise addition of 66.7 µl of 2M $CaCl_2$, followed by an 25 min incubation at room temperature. After removal of the media, the DNA precipitate was added to a 100 mm dish of low passage L6 cells which had been plated at 5 X 10^5 cells per plate the previous day. After incubation for 20 min at room temperature, 10 ml of media was added to the plate, which was then left for 4 h at 37°C. After this the media was removed, the dish was washed once with 10 ml of media, and the cells were treated with 15% glycerol in PBS for 3 min at 37°C. Cells were then quickly washed with media and left to recover in 10 ml of media at 37°C for 48 h. After this recovery period, the antibiotic G418 was added to a concentration of 800 µg/ml from a 100 mg/ml stock prepared in 100 mM HEPES, pH 7.4. Media and antibiotic were changed

after 4 days, and 6 days after the start of the G418 selection colonies were picked using cloning rings. These clones were expanded in the presence of 400 µg/ml of G418. Two clones chosen for further study, designated L6(ras1B) and L6(ras2A), were recloned from soft agar (both clones grew well in this medium). No colonies were found in a control transfection plate transfected with herring sperm carrier DNA alone. Several vials of each clone were stored frozen at -70°C, using a freezing solution of 60% α-modified minimal essential medium, 20% dimethyl sulphoxide and 20% fetal calf serum.

4.2.7. Southern Blotting

DNA was purified from L6, L6(ras1B) and L6(ras2A) as follows: Nuclei were purified by scraping the cells into media, pelleting them by centrifugation and washing them once with ice-cold PBS. Cells were then lysed by incubation for 1 min on ice in 1 ml of ice-cold 5 mM HEPES pH 7.4 and further homogenized by passing them through a 27 gauge 1/2" needle 5 times. NaCl was added to 150 mM and the nuclei were pelleted by centrifugation at 800 X g for 10 min. High molecular weight DNA was purified from the nuclear pellet exactly as described by Saris et al. (1986) except that the ether extractions were omitted. One 100 mm tissue culture plate was used for each separate preparation.

9 µg of DNA (assayed spectrophotometrically) was ethanol precipitated, resuspended in H_2O and digested for 12 h with 44 units of Bam H1. Samples were then extracted with phenol/chloroform, ethanol precipitated and electrophoresed on a 0.9% agarose gel. DNA was transferred to a nylon membrane as described by Maniatis et al. (1982) (without an acid cleavage step). The membrane was baked and

prehybridized as described in Chapter 2 for Northern blots. The blot was probed with ^{32}P-labeled pEJNEO, which had been repurified on a low-melting temperature agarose gel as described (Maniatis et al., 1982) and nick-translated to a specific activity of 1.8 X 10^8 using a nick translation kit from Bethesda Research Laboratories. Hybridization was carried out overnight at a concentration of 1 X 10^7 cpm/ml and the membrane was then washed and autoradiographed as described in Chapter 2 for Northern blots.

4.2.8 Western blotting

Western blots were done as described in Chapter 2 except that 50 µg of total cellular protein per lane was run on a 15% polyacrylamide gel and transferred to nitrocellulose. Anti-ras antibody was used at a concentration of 10 µg/ml and the second antibody used was goat anti-sheep IgG iodinated by the chloramine T method (Hunter and Greenwood, 1962). Because a high concentration of first antibody was required to obtain a positive signal, background labeling of the blots was high. A control blot, in which non-immune sheep IgG was used in place of the anti-ras antibody, was also done in order to distinguish specific from non-specific binding.

4.3 RESULTS

4.3.1 Regulation of R_I levels in JRU5

Previous work using photoaffinity labeling and cAMP binding assays of DE-52 column chromatography fractions had shown that there were several differences between the cAMP-dependent protein kinases of L6 and JRU5 (Rogers, 1984). First, JRU5 appeared to contain only free

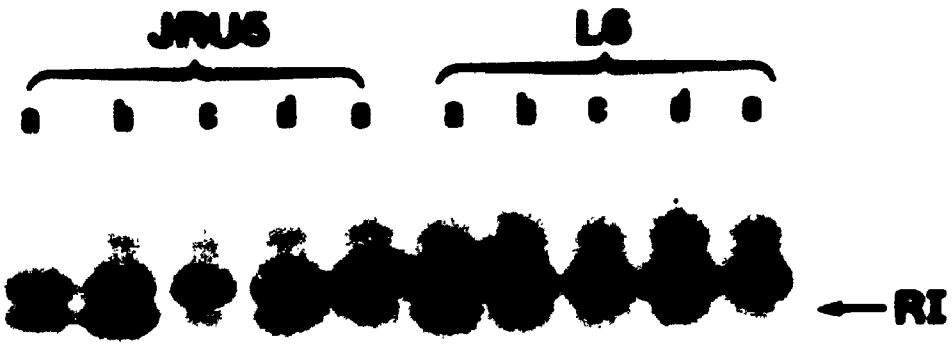
R_I, whereas L6 contained both free R_I and type I holoenzyme. Second, Bt_2cAMP treatment of JRU5 resulted in an increase in the cAMP binding activity of the free R_I peak. This increase was also seen in L6, but in this case was due to dissociation of the holoenzyme, which was clearly not the case in JRU5. Measurement of the cAMP binding affinities of L6 and JRU5 R_I showed that their affinities for cAMP were approximately the same, suggesting that the increase in binding activity in JRU5 was due to an increase in the amount of R_I. This result was confirmed by Western blotting (figure 4.1, lanes a and b). This showed that, while levels of R_I were the same in untreated L6 and JRU5, treatment of JRU5 with Bt_2cAMP caused a three-fold increase in R_I whereas in L6 the same treatment caused a decrease in the amount of R_I. We also examined the effects of other cAMP analogues and forskolin on R_I levels (figure 4.1, lanes c, d and e). As described in Chapter 2, treatments with Bt_2cAMP, 8-bromo-cAMP, a combination of these two analogues, and forskolin all decreased the levels of R_I in L6, although to varying degrees. In JRU5 the analogues varied markedly in their effects on R_I levels. While Bt_2cAMP increased R_I levels, 8-bromo-cAMP decreased R_I levels to almost undetectable levels, as in L6. Forskolin caused very little change in R_I levels and a combination of Bt_2cAMP and 8-bromo-cAMP caused only a small decrease in R_I levels (much less than in L6).

Preliminary experiments to determine the mechanism of the increase in R_I seen in Bt_2cAMP-treated JRU5 showed that the increase was not blocked by the transcription inhibitor actinomycin D. This result suggested some post-transcriptional mechanism for regulating the increase in R_I. We therefore chose to determine R_I half-lives in JRU5,

FIGURE 4.1

R_I LEVELS IN JRU5 AND L6 TREATED WITH DIFFERENT cAMP ANALOGUES AND FORSKOLIN

Day 2 JRU5 and L6 cells were treated for 40 h as follows: lanes a, untreated; lanes b, 0.25 mM Bt_2cAMP; lanes c, 0.25 mM 8-Br-cAMP; lanes d, 10 μM forskolin; lanes e, 0.25 mM Bt_2cAMP plus 0.25 mM 8-Br-cAMP. The phosphodiesterase inhibitor 1-isobutyl-3-methylxanthine was included in each treatment at a concentration of 0.1 mM. After treatment samples were isolated and Western blotted as described previously. The upper band seen on the blots is due to IgG heavy chains from the culture media which react with the second antibody used in the blotting procedure.


JPLUS
LS
RI

using the method described in chapter 2. Figure 4.2 shows plots of the scanning densitometry data from an experiment in which L6 and JRU5 were treated in parallel with Bt_2cAMP and the half-life of R_I was measured in each case. In untreated controls R_I was degraded at a slightly faster rate in JRU5 (t1/2 2.2 h) than in L6 (t1/2 3.2 h). Bt_2cAMP caused an increase in R_I degradation in L6, as discussed in chapter 2. However in JRU5 the same treatment caused about a three-fold stabilization of R_I (t1/2 6.4 h). Therefore changes in the rate of R_I degradation seemed to account for the changes seen in R_I levels in JRU5, as well as in L6.

We next examined the effects of different cAMP analogues and forskolin on the half-life of R_I in JRU5 to see if all of the alterations in R_I levels could be ascribed to changes in R_I half-lives. The data in figure 4.3 show that this was the case; changes in R_I degradation rates were different with different analogues and corresponded well with the changes in R_I protein levels observed (figure 4.1). 8-Bromo-cAMP greatly increased the rate of R_I degradation, whereas forskolin did not change the half-life significantly. Since it had been shown by Rannels and Corbin (1980) that cAMP derivatized at different positions recognized different cAMP binding sites on R_I, we asked whether another cAMP analogue modified at the same position as Bt_2cAMP could cause the same stabilization of R_I. (Bt_2cAMP is hydrolyzed to N^6-monobutyryl cAMP inside the cell (Kaukel and Hilz, 1972); this is the active form.) N^6-benzoyl-cAMP was also found to stabilize R_I and was somewhat more effective in this regard, perhaps because of its higher affinity for R_I (Beebe *et al.*, 1984).

FIGURE 4.2

R_I HALF-LIFE IN L6 AND JRU5 TREATED WITH Bt_2cAMP

L6 (panel A) and JRU5 (panel B) cells were untreated (O) or treated (●) with 0.25 mM Bt_2cAMP amd 0.1 mM 1-isobutyl-3-methylxanthine and then labeled and chased in media containing the same concentrations of analogues. Labeled R_I remaining was quantitated as described previously.

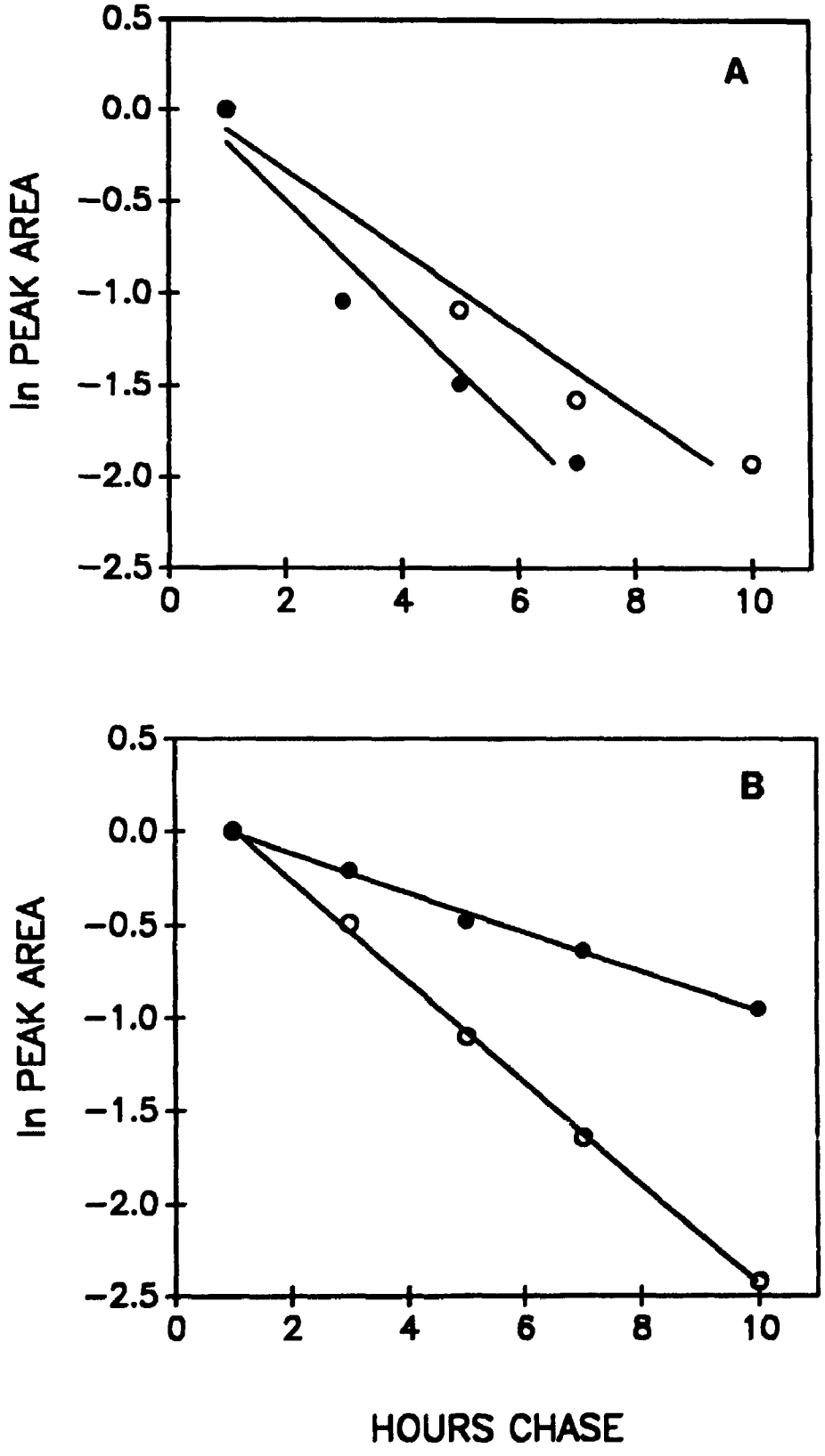
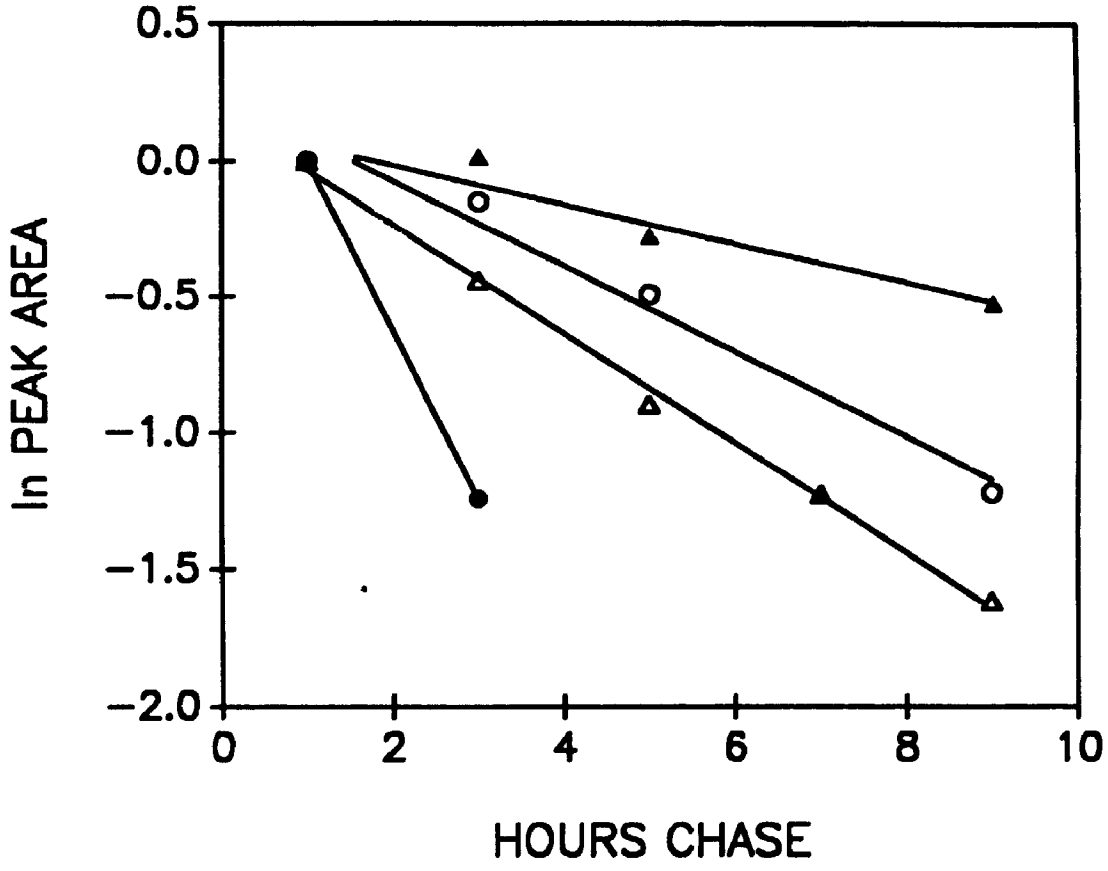

A
B
0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
0
2
4
6
8
10
ln PEAK AREA
HOURS CHASE

FIGURE 4.3

EFFECTS OF DIFFERENT cAMP ANALOGUES AND FORSKOLIN ON R_I HALF-LIFE IN JRU5

JRU5 cells were treated as follows: O, untreated; ▲, 0.25 mM N^6-benzoyl-cAMP; ●, 0.25 mM 8-Br-cAMP; Δ, 10 μM forskolin. 1-isobutyl-3-methylxanthine was also included in each treatment at a concentration of 0.1 mM.


0.5
0.0
−0.5
−1.0
−1.5
−2.0
ln PEAK AREA
0
2
4
6
8
10
HOURS CHASE

4.3.2 Comparison of R_I Structure in L6 and JRU5

The results described above demonstrate that treatment of JRU5 with a N^6-derivatized cAMP-analogue stabilized R_I, leading to an increase in its levels within the cell. Two possible explanations for this are either that JRU5 has some altered protease, or else that a modification of R_I structure has occurred in these cells. In this section experiments are described which were designed to test the latter possibility. As a general approach, we have compared R_I from L6 and JRU5 by two-dimensional gel electrophoresis and by tryptic mapping. Figure 4.4 shows the results of two-dimensional gel electrophoresis analysis of R_I from L6 and JRU5, either untreated or treated with Bt_2cAMP. The isoelectric points are the same in untreated L6 and JRU5. In each case there is a major spot and a minor more basic spot. These most likely represent the phospho and dephospho forms of R_I, respectively (Steinberg and Agard, 1981b). Both these spots are present in the Bt_2cAMP-treated samples as well, although the basic spots are much more prominent. The proportion of R_I in the dephospho form is greater in JRU5 than in L6. The conversion of phospho R_I to dephospho R_I with cAMP treatment has been reported previously by Steinberg and Agard (1981b).

Figure 4.5 shows tryptic maps comparing the structure of R_I from L6 and JRU5. In this technique, tryptic digests of a protein are separated on the basis of charge (at pH 1.9) in the first dimension, and then on the basis of hydrophobicity in the second dimension. With the procedure we followed, not all tryptic peptides will be detected, only those which contain methionine and are therefore labeled. Within

FIGURE 4.4

TWO-DIMENSIONAL GEL ELECTROPHORESIS OF R_I FROM L6 AND JRU5

Samples were isolated from L6 and JRU5 cells which were untreated or treated with 0.25 mM Bt_2cAMP and 0.1 mM 1-isobutyl-3-methylxanthine for 40 h. R_I was immunoprecipitated from the samples and the immunoprecipitates were then analysed by two-dimensional gel electrophoresis. Autoradiographs of the gels are shown with the first dimension isoelectric focussing oriented from right to left (basic side on the right) and the second dimension SDS/polyacrylamide gel electrophoresis oriented from top to bottom. A, untreated L6; B, treated L6; C, untreated JRU5; D, treated JRU5.

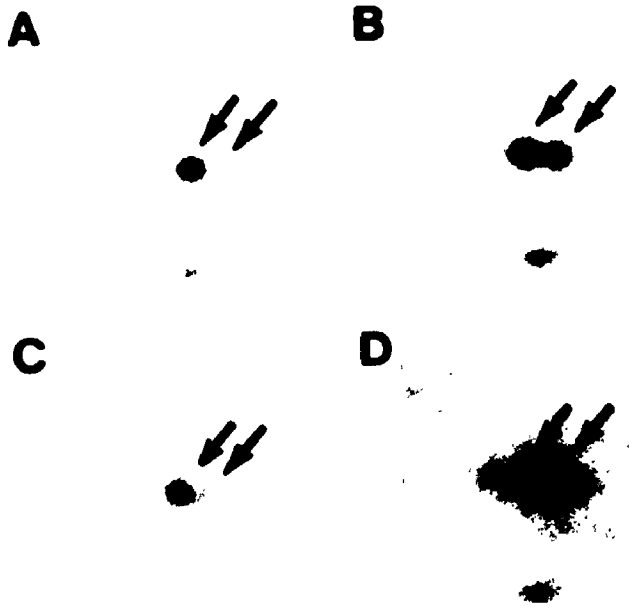
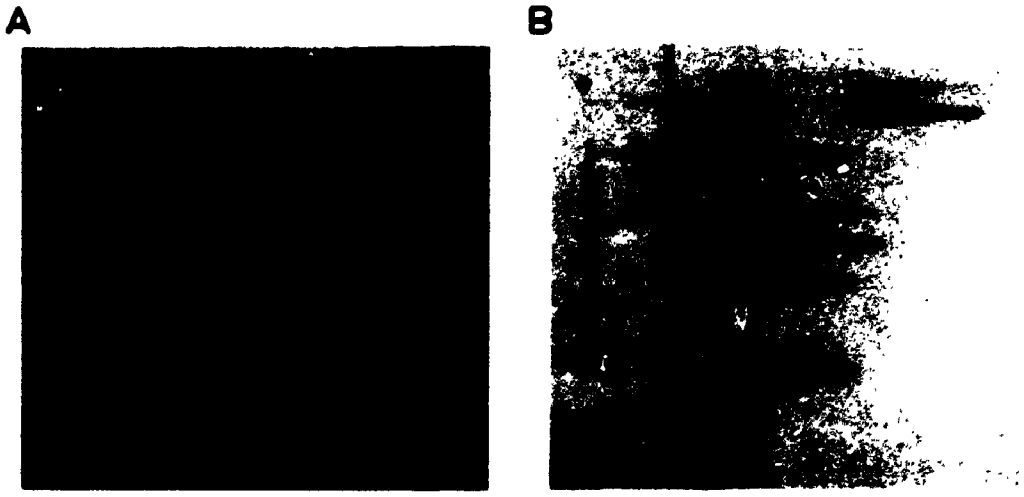

A
B
C
D

FIGURE 4.5

TRYPTIC MAPS OF R_I FROM L6 AND JRU5

Labeled R_I from L6 (A) and JRU5 (B) was isolated by immunoprecipitation and gel electrophoresis. Regions of the gels containing R_I were excised and digested with trypsin. The digests were then analysed by first dimension high voltage electrophoresis (run from left to right) and thin layer chromatography (run from top to bottom). Spots are smeared because of the large sample volume applied, which was necessary because of the weak signal obtained from the ^{35}S-methionine labeled peptides.


A
B

this limitation, the peptide maps of L6 and JRU5 R_I appear to be the same.

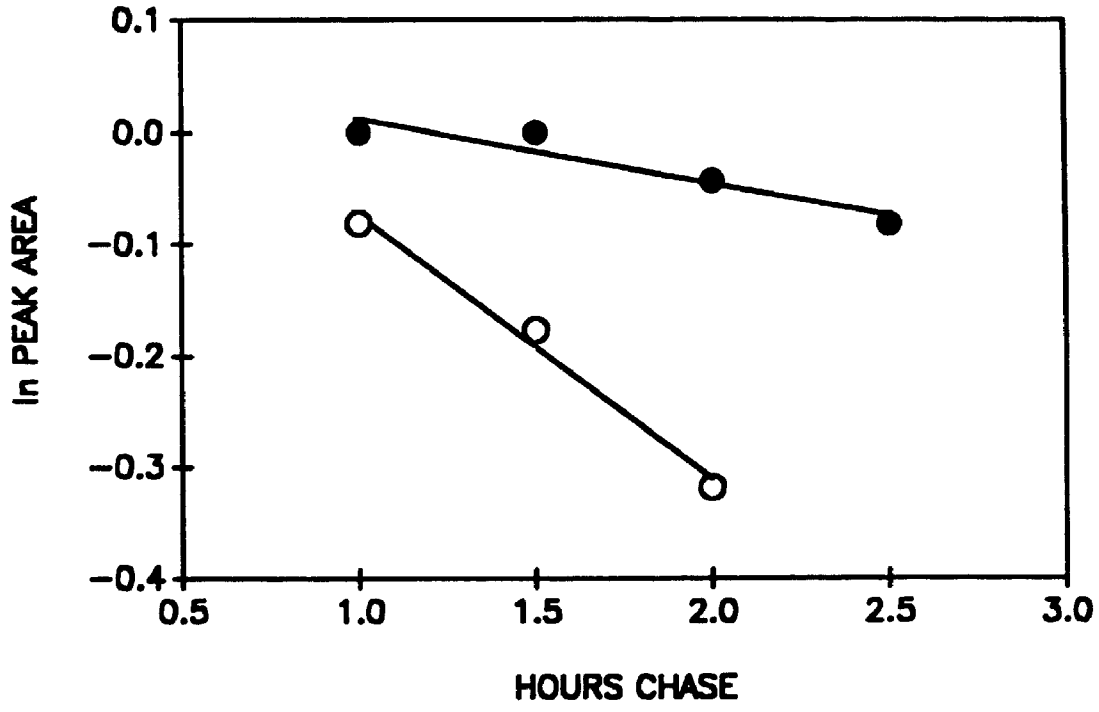
4.3.3 ATP-Dependent Proteolysis of R_I

A second possible explanation for the differences in proteolysis of R_I in JRU5 is that there is an alteration in some protease in this cell line. As an initial step, we have attempted to identify the type of protease involved in the degradation of R_I in the intact cell. We considered the possibility that an ATP-dependent protease was involved in the degradation of R_I, because this type of protease is thought to be involved in the breakdown of many cytosolic proteins in eukaryotic cells. Accordingly, we attempted to determine whether ATP was required for R_I proteolysis. This was done by using the pulse/chase procedure with ^{35}S-methionine described previously with the inclusion of inhibitors of ATP production in the chase. The inhibitors used were 2-deoxyglucose (an inhibitor of glycolysis) and dinitrophenol (an inhibitor of oxidative phosphorylation). Glucose-free medium was used in the chase so that the deoxyglucose would not be in competition with glucose in the medium. The concentrations of inhibitors used were those which were demonstrated by Gronostajski et al. (1984) to deplete intracellular ATP in fibroblasts by approximately 90% after 1 h. Since this treatment is obviously toxic to cells, only a short period of chase (up to 3 h) could be used. Therefore this technique is only useful for rapidly turning over proteins such as R_I, as changes in long-lived proteins would be too small to be detected using such a short period of chase. The results of one experiment are shown in figure 4.6. Using the shorter chase period we still determined a half-

FIGURE 4.6

EFFECT OF ATP DEPLETION ON R_I DEGRADATION

L6 cells were labeled with ^{35}S-methionine as described previously. Cells were then chased in the presence (●) or absence (O) of 0.5 mM dinitrophenol and 12.5 mM 2-deoxyglucose. Labeled R_I remaining at different periods of chase was then quantitated as described previously.


0.1
0.0
−0.1
−0.2
−0.3
−0.4
ln PEAK AREA
0.5
1.0
1.5
2.0
2.5
3.0
HOURS CHASE

life for R_I in agreement with our previous results (3.2 h). In two separate experiments it was found that the inclusion of ATP synthesis inhibitors in the chase inhibited R_I proteolysis approximately four-fold.

Since this result suggested the involvement of an ATP-dependent protease in R_I degradation (other possible explanations for the ATP dependence are considered in the discussion), we next investigated whether R_I could serve as a substrate for ATP-dependent proteases in vitro. We chose to use Friend erythroleukemia cells as a source of ATP-dependent proteolytic activity for several reasons: first, at the time this activity had only been observed in vitro in extracts of this cell type and reticulocytes, and the former was a more convenient source for us as we had previous experience in our laboratory with the culture of Friend cells. Also, we had found that in Friend cells R_I behaved in a similar fashion to R_I in L6 (i.e. Bt_2cAMP increased its rate of degradation; M. Mason, unpublished results). ATP-dependent proteolytic activity in Friend cell extracts was first reported by Rieder et al. (1985) and Waxman et al. (1985). As there was some disagreement between these reports concerning the subcellular distribution of this activity, we first ascertained whether the activity was present in the particulate or supernatant fraction of the cells. Using reduced, alkylated BSA as the substrate we found activity in both of these fractions (figure 4.7). We therefore chose to use a 15,600 X g supernatant, which contained all of the ATP-dependent protease activity, for further experiments. Figure 4.8 shows the results of an assay for the proteolysis of ^{125}I-labeled R_I using this fraction. In

FIGURE 4.7

SUBCELLULAR DISTRIBUTION OF ATP-DEPENDENT PROTEASE ACTIVITY IN FRIEND ERYTHROLEUKEMIA CELLS

Friend cells were lysed and centifuged at 15,600 X g for 15 min. Part of the supernatant was set aside and the rest was centrifuged at 100,000 X g for 1 h. The 15,600 X g and 100,000 X g supernatants were then assayed for their ability to hydrolyze reduced, alkylated BSA in the presence or absence of ATP.

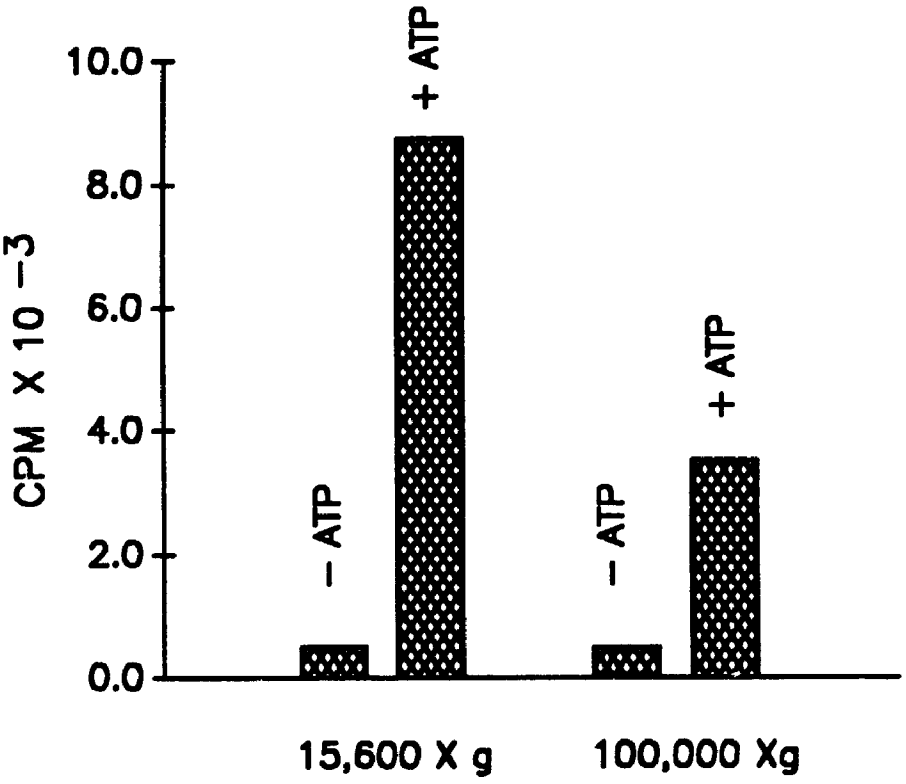
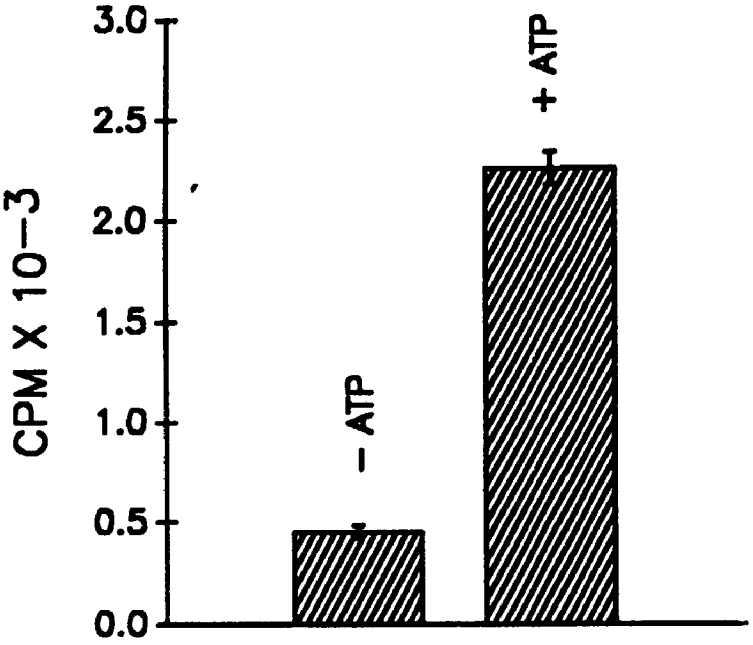

10.0
8.0
6.0
4.0
2.0
0.0
CPM X 10 −3
− ATP
+ ATP
− ATP
+ ATP
15,600 X g
100,000 Xg

FIGURE 4.8

ATP-DEPENDENT PROTEOLYSIS OF ^{125}I-LABELED R_I

Labeled R_I was incubated with 15,600 X g supernatant from Friend cells in the presence or absence of ATP. Release of TCA-soluble peptides was then assayed as described in the text.


CPM X 10−3
3.0
2.5
2.0
1.5
1.0
0.5
0.0
− ATP
+ ATP

the absence of ATP a very small amount of proteolysis of R_I to TCA soluble products was detected. Addition of ATP increased the amount of proteolysis 5-35 fold. This amount of activation was similar to that seen for reduced BSA although the overall amount of BSA hydrolyzed was greater, suggesting that it may be a better substrate for the ATP-dependent protease.

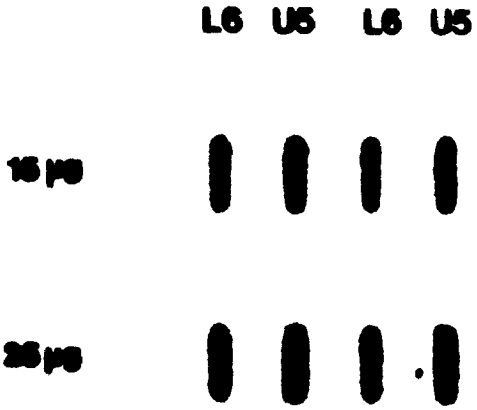
4.3.4 Regulation of R_I in ras-transformed L6

The transforming oncogene of the spontaneously-transformed cell line JRU5 is not known. If the changes in R_I proteolysis in JRU5 could be ascribed to the action of a specific oncogene, this would give us clues as to the events which caused the change in proteolysis and also give us a better idea of the relevance of these changes to the process of transformation. As one approach to determining what the transforming oncogene of JRU5 might be, we screened L6 and JRU5 RNA with various probes to see if any oncogenes or proto-oncogenes were expressed to a greater extent in the transformed line. By probing slot blots of total L6 and JRU5 RNA with different oncogene probes we found that there was a small but reproducible increase in the levels of Ha-ras mRNA in JRU5 (figure 4.9). This increase was estimated to be about 1.3-fold by scanning densitometry. Northern blotting showed that both L6 and JRU5 contained a single Ha-ras mRNA species with a size of approximately 1.3 kilobases (figure 4.10), which was more abundant in JRU5. We next examined the levels of ras protein in L6 and JRU5 by Western blotting using a commercially available antibody which had been raised against a synthetic peptide sequence common to all proteins of the ras family. Figure 4.11 shows that this antibody recognized a protein of Mr 23,000

FIGURE 4.9

LEVELS OF RAS mRNA IN L6 AND JRU5

Total L6 and JRU5 RNA was blotted onto a nylon membrane and probed with labeled fragments of Ha-ras DNA. The lower figure shows quantitation of the signals using scanning densitometry.


L6
U5
L6
U5

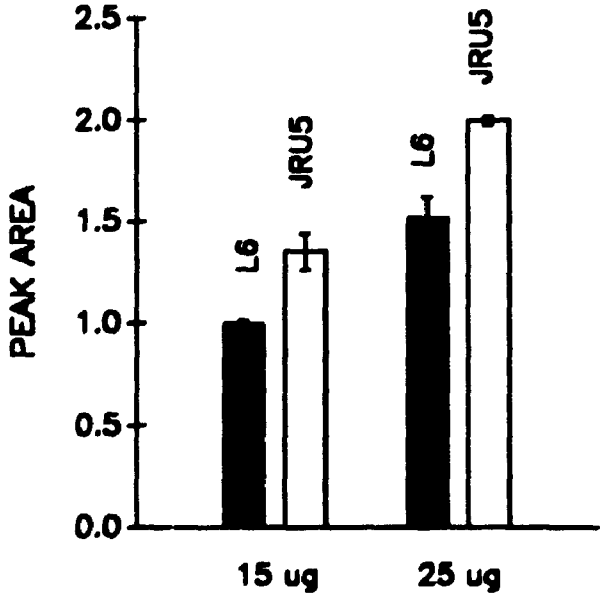

PEAK AREA
2.5
2.0
1.5
1.0
0.5
0.0
L6
JRU5
L6
JRU5
15 ug
25 ug

FIGURE 4.10

NORTHERN BLOT OF L6 AND JRU5 RNA PROBED WITH LABELED Ha-RAS DNA

Total L6 and JRU5 RNA (20 µg per lane) was denatured with glyoxal and electrophoresed as described by Maniatis et al. (1982). The RNA was then transferred to a nylon membrane and probed with labeled fragments of Ha-ras DNA. The migration of RNA size markers (Bethesda Research Laboratories) is shown on the left in kilobases.

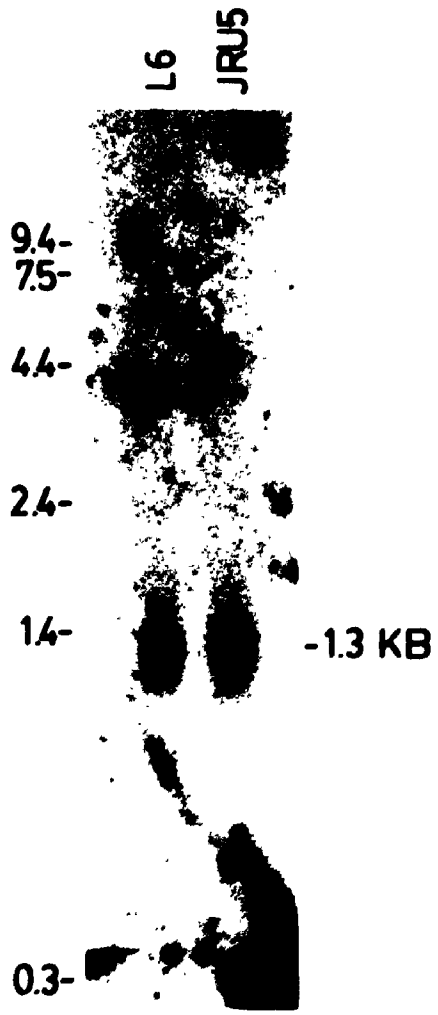
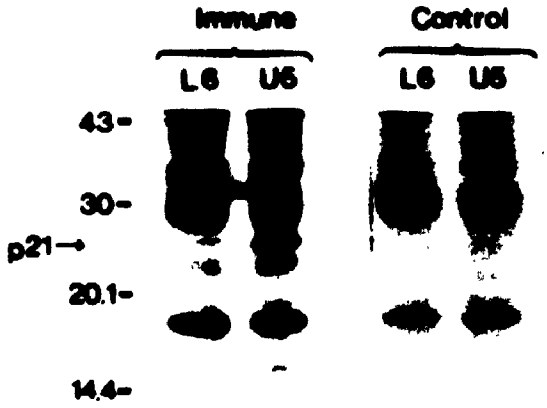

L6
JRU5
9.4-
7.5-
4.4-
2.4-
1.4-
-1.3 KB
0.3-

FIGURE 4.11

WESTERN BLOT SHOWING LEVELS OF RAS p21 IN L6 AND JRU5

Total L6 and JRU5 protein (50 µg per lane) was electrophoresed on a 15% polyacrylamide gel and transferred to nitrocellulose. The blot was then probed with anti-ras antibody (left) or non-immune IgG (right).


Immune
Control
L6
U5
L6
U5
43-
30-
p21→
20.1-
14.4-

in both L6 and JRU5, similar to the reported Mr of 21,000 for the product of the ras gene. This band was not labeled in a control blot done with non-immune sheep IgG. In JRU5, the levels of ras protein were about five-fold higher than in L6, as determined by scanning densitometry. We have not determined why the increase in ras protein levels is greater than the increase in mRNA levels. This could be due to a change in post-transcriptional regulation of Ha-ras protein, or the increased expression of another ras protein related to Ha-ras (N-ras or Ki-ras) which comigrates with Ha-ras.

Although not conclusive, these results suggested that one of the ras genes might be involved in the transformation of JRU5. We therefore chose to transform L6 cells by transfection with the Ha-ras oncogene to see if this would induce the same changes in R_I proteolysis we observed in JRU5. A standard transfection procedure was used in which a $Ca^{2+}PO_4$ precipitate of a plasmid is taken up and incorporated by cells. The plasmid used contained a dominant selectable marker (the G418 resistance gene) and the human gene encoding an oncogenic mutant of Ha-ras. The ras gene is under the control of its own promotor in this plasmid. The transfection yielded several colonies which, in addition to being resistant to G418, appeared to be transformed in that the cells were more rounded, appeared to grow faster, and grew past confluence. Two colonies, designated L6(ras1B) and L6(ras2A), were chosen for further study. These were both recloned from soft agar to ensure that they each represented a single clone. Figure 4.12 shows microphotographs of the two transformed L6 cell lines. Both transfectants are more refractile than the parent L6 cell line and show

FIGURE 4.12

MORPHOLOGY OF Ha-RAS TRANSFECTED L6 CELLS

Cells were grown on glass cover slips, fixed with formaldehyde and photographed using a phase contrast microscope. A, L6; B, L6(ras2A); C, L6(ras1B).

A

B

C

some membrane "ruffling" around their edges. Also, both transfectants were unable to fuse to form myotubes.

Figure 4.13 shows an autoradiograph of a Southern blot of L6, L6(ras1B) and L6(ras2A) samples. DNA was purified from each cell type, digested with Bam Hl, electrophoresed and then transferred to a nylon membrane. This was then probed with labeled pEJNEO. In the L6 lane, a single band of 4.0 kilobases was labeled which probably represents the endogenous Ha-ras gene, as a band of the same size has been identified as such in Bam Hl digests of mouse DNA (Dandekar et al., 1986). Both transfectants incorporated multiple copies of the plasmid, as shown by the large number of labeled bands in these lanes.

In order to demonstrate that the transfected Ha-ras gene was expressed, we performed Western blotting on total protein extracts of the transfectants (figure 4.14). In both transfectants the Mr 23,000 band was labeled more strongly than in L6, showing that ras was being expressed from the introduced human ras gene and that the protein product of this gene comigrated with the endogenous ras gene product. In L6(ras1B) the total expression of endogenous and oncogenic ras was four-fold higher than in L6, whereas in L6(ras2A) it was only about 1.4-fold higher.

Having characterized the transfectants, we next examined whether the alterations in R_I regulation seen in JRU5 were also evident in ras-transformed L6. Figure 4.15 shows a Western blot of L6, JRU5, L6(ras1B) and L6(ras2A) samples, either untreated or treated with Bt_2cAMP, which have been probed with anti-R_I antibody. In the control L6 and JRU5 samples, R_I behaved in the same way in response to Bt_2cAMP treatment as

FIGURE 4.13

SOUTHERN BLOT OF Ha-RAS TRANSFECTED L6 CELLS PROBED WITH LABELED pEJNEO

DNA from L6, L6(ras1B) and L6(ras2A) was digested with Bam Hl, electrophoresed on a 0.9% agarose gel and transferred to a nylon membrane. The membrane was then probed with labeled fragments of the plasmid pEJNEO. The migration of DNA molecular weight standards (Hind III digested phage DNA) is shown on the right.

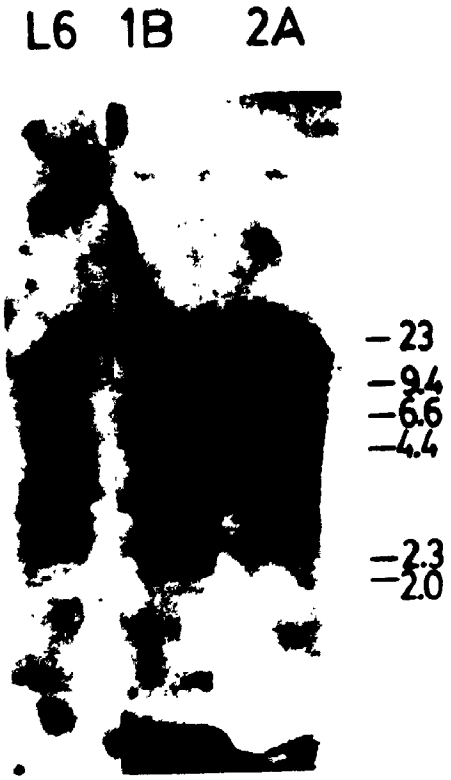

L6 1B 2A
23
9.4
6.6
4.4
2.3
2.0

FIGURE 4.14

LEVELS OF p21 IN RAS-TRANSFECTED L6 CELLS

Samples of L6, L6(ras1B) and L6(ras2A) total protein (50 µg per lane) were electrophoresed on a 15% acrylamide gel and transferred to nitrocellulose. The membranes were then probed with anti-ras antibody (left) or non-immune IgG (right). The migration of molecular weight standards (in kDa) is shown on the left.

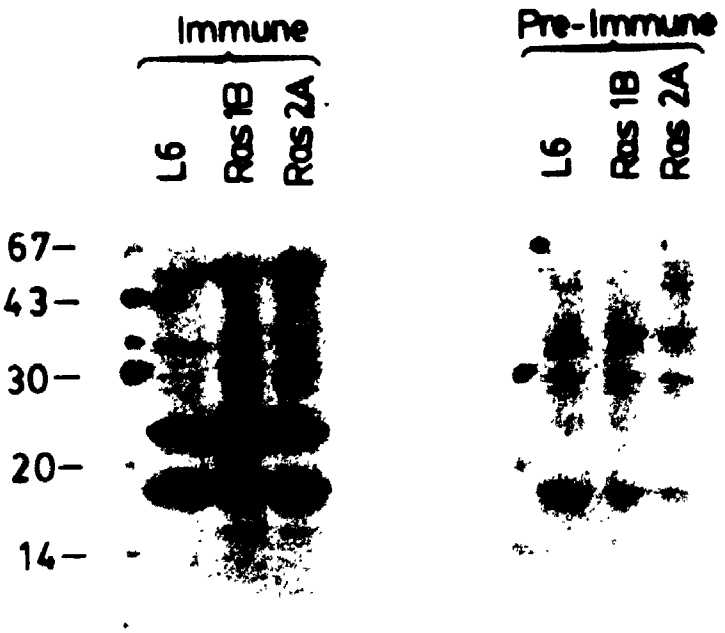
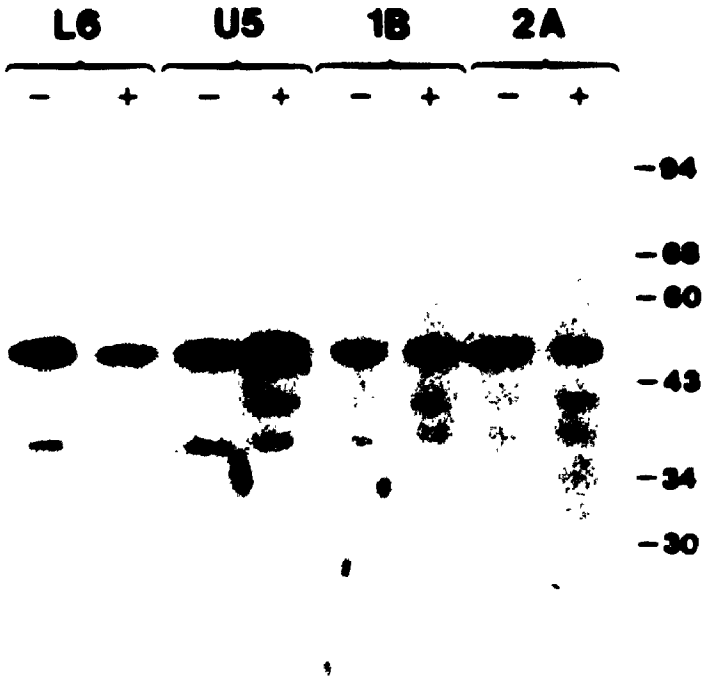

Immune
Pre-Immune
L6
Ras 1B
Ras 2A
L6
Ras 1B
Ras 2A
67–
43–
30–
20–
14–

FIGURE 4.15

R_I LEVELS IN Ha-RAS TRANSFECTED L6 CELLS TREATED WITH Bt_2cAMP

Cells were grown in the absence (-) or presence (+) of 0.25 mM Bt_2cAMP and 0.1 mM 1-isobutyl-3-methylxanthine for 40 h. Samples of total protein were then isolated and Western blotted as described, using antibody against R_I.


L6
U5
1B
2A
- + - + - + - +
94
68
60
43
34
30

described previously i.e. R_I levels decreased in L6, but increased in JRU5. The behaviour of R_I in the transfectants resembled neither of these. Levels of R_I were lower in untreated transfectant samples than in L6, and the levels were not affected at all by Bt_2cAMP treatment. Therefore transformation of L6 with the Ha-ras oncogene does not mimic the changes in R_I regulation seen in JRU5, but does alter R_I regulation in some other way.

4.4 DISCUSSION

In chapters 2 and 3 we showed that proteolysis was important in the regulation of R_I levels during L6 differentiation. The results in this chapter suggest that in the spontaneously-transformed L6 cell line JRU5, which does not differentiate, proteolysis of R_I is altered. Data from half-life studies and Western blotting showed that Bt_2cAMP increased R_I levels in JRU5 by decreasing its rate of proteolysis. This was opposite to the effect observed in L6. This behaviour of R_I in response to Bt_2cAMP has been described previously by Steinberg and Agard (1981) in S49 mouse lymphoma cells. Our results are novel in that we have observed differences in R_I proteolysis between the two closely related cell lines. This change in R_I behaviour led us to ask several questions: First, since different effects on R_I half-lives are seen with different analogues in JRU5, what are the effects of endogenous cAMP on R_I levels and half-lives in this cell line? Second, what is the specific biochemical change which causes the altered proteolysis of R_I? Third, what relevance, if any, does the change in R_I behaviour have to transformation?

With regard to the first of these questions, the observations of Rannels and Corbin (1980) on the binding specificity of different cAMP analogues is probably relevant. They have shown that each R_I monomer contains two cAMP binding sites, and that these binding sites show selectivity towards different classes of cAMP analogues. Site 1 was found to preferentially bind C^8-derivatized cAMP analogues, whereas site 2 preferentially bound N^6-derivatized analogues. When cAMP-dependent protein kinases are activated by endogenous cAMP (i.e. via activation of adenyl cyclase), both sites on R_I are probably occupied as binding of cAMP to the two sites has been shown to be cooperative under physiological conditions (Ogreid and Doskeland, 1982). Therefore the treatment in which a C^8-derivatized cAMP analogue and an N^6-derivatized analogue were used in combination, so that both sites of R_I would be occupied, probably most accurately reflects the effects of underivatized cAMP. In JRU5 this treatment caused only a slight decrease in R_I levels, whereas in L6 R_I levels were greatly reduced. Forskolin, which stimulates adenyl cyclase activity, also had very little effect on R_I levels or degradation rate, confirming that this was the effect of endogenous cAMP. It would appear that binding of cAMP to R_I in JRU5 does not cause an increase in its rate of proteolysis, in contrast to what is seen in L6. This might be explained by the fact that R_I does not appear to be associated with C at all in JRU5 (Rogers, 1984). However this cannot explain the pronounced stabilization of R_I when Bt_2cAMP is bound to it. The fact that a second N^6-derivatized cAMP analogue, N^6-benzoyl-cAMP, also stabilizes R_I in JRU5 suggests that occupation of site 2 alone induces a conformation of R_I which is

resistant to proteolysis.

We have investigated two hypotheses as to the biochemical changes which might cause the altered proteolysis of R_I in JRU5. First we investigated whether there were any alterations in R_I itself which could cause this. R_I could be altered in several ways, including the expression of a different isozyme, a mutation or a covalent or non-covalent modification. Although a second isozyme of R_I has been reported (Clegg et al., 1988), it seems unlikely that it is expressed in JRU5. The $R_{I\beta}$ isozyme is reported to migrate differently on one-dimensional SDS/PAGE, while R_I from L6 and JRU5 migrate identically. As well, the isozymes are reported to be about 80% similar in protein sequence so that differences in the isoelectric point and/or methionine-containing peptide maps of R_I from the two cell lines would be expected. Both of these were identical, as descibed in the results section of this chapter. It also seems unlikely that R_I is mutated in JRU5, partly because of the isoelectric focussing and tryptic mapping results, but also because the different proteolysis of R_I in response to different cAMP analogues has been suggested by a number of other studies. Bt_2cAMP has been reported to increase R_I levels in mouse neuroblastoma cells (Prashad, 1981), mouse S49 lymphoma cells (Steinberg and Agard, 1981) and also in primary rat oligodendrocytes (Beushausen et al., 1987). It is extremely unlikely that all of these cell types would have a mutated R_I. A more likely possibility is that R_I is somehow covalently or non-covalently modified in one of the cell lines. Although our results did not provide any evidence in support of this, neither of the techniques used allows us to exclude the

possibility that R_I is covalently-modified in a way that does not affect its net charge, or that R_I is modified non-covalently. The differences in the relative amounts of phosphorylated and non-phosphorylated R_I between Bt_2cAMP treated L6 and JRU5 probably do not give rise to the differences in proteolysis as Steinberg and Agard (1981) have shown that these two forms of R_I are degraded at similar rates in S49 cells.

As we did not find any evidence for an alteration in R_I, we began studies aimed at identifying proteases which might be involved in the degradation of R_I, following the hypothesis that a change in some protease caused the change in R_I behaviour in JRU5. Some previous results from our laboratory on cAMP phosphodiesterase had also suggested that there might be a change in intracellular proteolysis in JRU5 (Seth et al., 1983). Our findings suggest a proteolytic system with a high degree of specificity, such that it is able to distinguish between R_I with an analogue bound at site 1 or ar site 2.

At present, intracellular proteolysis is very poorly understood. This is especially true for the catabolism of cytosolic proteins, where no specific relationship between a cytosolic protein and a particular protease has as of yet been clearly established (Rechsteiner, 1987). However it has been hypothesized that ATP-dependent proteases may be responsible for the proteolysis of most cytosolic proteins (reviewed by Rechsteiner, 1987; Bond and Butler, 1987). These are high molecular weight, soluble enzymes which in some cases use ubiquitin to tag their substrates. Recently they have been reported to copurify with "prosomes", large complexes with a distinct morphology under the

electron microsope, and which are found in all mammalian cells investigated to date (Falkenburg et al., 1988; Arrigo et al., 1988). We have found two kinds of evidence which are consistent with the involvement of these proteases in the degradation of R_I; these are the ATP-dependence of R_I degradation in intact myoblasts and the ATP-dependent in vitro degradation of R_I by Friend cell extracts.

ATP-depletion using metabolic inhibitors has been used by many researchers to show ATP-dependence of general protein degradation (Gronostajski et al., 1985) and also the degradation of some specific proteins (Etlinger and Goldberg, 1977; Gronostajski et al., 1984). However, because of the large number of biochemical processes which use ATP, this type of experiment cannot be interpreted unambiguously. ATP depletion could inhibit the ATP-dependent proteases, but could also inhibit lysosomal proteases by not allowing cells to maintain the lower pH of the lysosomes. Also ATP depletion could inhibit ion transport, so that proteases such as the Ca^{2+}-dependent proteases would be inactivated. Another consideration is that ATP depletion is toxic to cells, so that proteolysis in this case is not being measured under normal physiological conditions.

There are also several problems in the interpretation of the results of in vitro degradation of R_I by Friend cell extracts. First, it is possible that this enzyme is active against any protein substrate when assayed under the conditions described in methods. Second, we did not find ATP-dependent protease activity in myoblast extracts, so that it is not clear how relevant this finding is to the degradation of R_I in myoblasts, as we were unable to detect ATP-dependent protease

activity in myoblast extracts in preliminary experiments. The absence of ATP-dependent protease activity in myoblasts may be due to the labile nature of these enzymes, which has been demonstrated in vitro.

In spite of the above reservations the simplest explanation for our observations is still that an ATP-dependent protease is involved in R_I degradation. Further work on the in vitro specificity of the Friend cell activity, using more highly-purified enzyme, would resolve some of these problems. Also other inhibitors could be used to further characterize the degradation of R_I in intact myoblasts. Much more knowledge on the structure and mechanism of action of ATP-dependent proteases would be required before we could address the question of whether there were some alterations in this enzyme in transformed cells such as JRU5. Recent success in the purification of ATP-dependent proteases (Hough et al., 1987) and in the production of antibodies against them (McGuire et al., 1988) should help increase the understanding of intracellular proteases greatly. Further comparative studies on R_I proteolysis in L6 and JRU5 could prove useful in studies of the specificity and regulation of enzymes involved in intracellular proteolysis.

The relevance of this change in R_I proteolysis to the process of transformation is not clear. As mentioned above, the effect in which Bt_2cAMP increases R_I levels has been observed in several other transformed cell lines (S49 lymphoma cells and neuroblastoma cells), but it has also been observed in non-transformed rat oligodendrocytes. Previous work also showed that R_I in src-transformed L6 behaved as in normal L6 (Rogers, 1984). Therefore the altered R_I behaviour is not

always coupled to transformation in myoblasts, and can occur in non-transformed cells. However the change in R_I proteolysis does seem to be coupled to spontaneous transformation of L6 myoblasts, as we had shown previously that it occurs in two independently isolated spontaneously-transformed L6 cell lines. This would suggest that the change in R_I proteolysis is due the specific action of some oncogene.

In order to clarify the relationship between transformation and the altered regulation of R_I, we attempted to identify oncogenes involved in the transformation of JRU5. The increased expression of p21, the ras gene protein product, which we observed in JRU5 suggested to us that one of the ras genes might contribute to the expression of the transformed phenotype in this cell line. It is generally believed that p21 proteins are activated by point mutations which alter their function rather than their expression. However, recent evidence suggests that the levels of expression of p21 may be more important than initially thought. Ricketts and Levinson (1988) have shown that overexpression of normal Ha-ras in rat-1 cells transforms these cells by the criteria of morphology and growth in soft agar. Levels of activated p21 also appear to be important in transformation. Paterson et al. (1987) have shown that a decrease of only about 50% in the expression of activated p21 in fibrosarcoma cells can cause these cells to revert to a non-transformed phenotype. Cohen and Levinson (1988) have demonstrated that, as well as the missense mutation at codon 12, the Ha-ras EJ oncogene has a point mutation in an intron which causes an ten-fold increase in its expression, and which contributes significantly to the transforming potential of this oncogene. It

therefore seems possible that the increased expression of p21 we see in JRU5 does contribute to the expression of the transformed phenotype. However, the possibility that the increased expression is only a secondary effect of the transformation of this cell line cannot be ruled out. In order to determine whether transformation by ras and altered proteolysis of R_I were linked, we transfected L6 cells with a plasmid containing the Ha-ras EJ oncogene. As described in the results section of this chapter, the transfected cells did not show the same changes in R_I behaviour which were observed in JRU5. Instead, expression of the Ha-ras EJ oncogene appeared to alter R_I regulation in some other unknown way, suggesting some indirect interaction between p21 and the cAMP-dependent protein kinases in the transformed myoblasts.

There are several possible reasons why the transfection did not cause the same changes in R_I behaviour seen in JRU5. Ha-ras may not be the transforming oncogene of JRU5. Another member of the ras family, or an unrelated oncogene could cause the changes in R_I behaviour. It is also possible is that the alterations in R_I behaviour are are not linked to transformation, but rather are due to some separate mutation or epigenetic event which has occurred in JRU5. However, we feel this last possibility is unlikely, for the reasons discussed in the previous paragraph.

CHAPTER 5

SUMMARY AND FUTURE PROSPECTS

The main goal of the studies described in this thesis was to determine the mechanism by which the levels of cAMP-dependent protein kinases are regulated in differentiating myoblasts and, as well, in spontaneously-transformed myoblasts which are unable to differentiate. These studies were undertaken with the view that an understanding of the regulation of these enzymes might provide clues as to their potential role in the regulation of myogenesis.

5.1 Regulation of cAMP-dependent protein kinase levels during myogenesis

The results of our investigation into the mechanism by which the increase in R_I levels occurs during myogenesis (Lorimer et al., 1987) showed that the increase was not regulated transcriptionally. Instead, the increase in R_I levels was apparently due to a specific decrease in the rate of degradation of this protein. Further studies showed that the change in the rate of degradation of R_I could be reversed by treatment with cAMP analogues, suggesting that the altered degradation rate was probably due to an increase in the association of R_I with C during myogenesis.

We considered two possible reasons for the postulated change in the association of R_I with C. One was that there was a decrease in cAMP levels during myogenesis which allowed R_I to bind C to a greater extent. The other was that there was an increase in the total amount of C during myogenesis, so that more would be available to bind to R_I. To distinguish between these two possibilities, we investigated the

mechanism of regulation of the levels of C during myogenesis (Lorimer and Sanwal, 1988). Our results showed that the increase in C was regulated by a different mechanism than the increase in R_I. The increase in C was apparently regulated transcriptionally, rather than by a decrease in its rate of degradation. As discussed in chapter 3, this evidence favours the second of the two explanations presented above; it appears that a transcriptionally-regulated increase in C causes the increase in R_I by binding to R_I and decreasing its rate of degradation.

This type of mechanism may have general significance for the coordinate regulation of multisubunit protein complexes during myogenesis or other differentiation processes. If this type of regulation is widespread, it could reduce the complexity of control mechanisms required to alter gene transcription during differentiation. Some other examples of this type of regulation during differentiation have been reported. It has been shown that the assembly of IgM during B cell differentiation is probably regulated by this type of mechanism (Dulis, 1982). Resting B cells synthesize only IgM heavy chains, which are rapidly degraded in the absence of light chain. Differentiation of these cells results in a transcriptionally-regulated increase in the synthesis of light chains which combine with the heavy chains, decreasing their rate of proteolysis, and thereby bringing about an overall increase in the amount of IgM complex. As well, the assembly of the membrane skeleton during avian and mouse erythropoiesis may be regulated similarly (Blikstad et al., 1983; Lazarides and Moon, 1984; Lehnert and Lodish, 1988). In this case α- and β-spectrin are

synthesized in excess of other membrane cytoskeletal components and stabilized by association with these. (However, this process differs from the regulation of cAMP-dependent protein kinase in that spectrin levels are probably also regulated at the level of transcription during erythropoiesis (Lehnert and Lodish, 1988).) There is also some evidence that assembly of the acetylcholine receptor may be regulated by a mechanism similar to this (Merlie and Lindstrom, 1983). These observations, and our own results, suggest that this type of regulation could be a common mechanism for effecting increases in the levels of many multisubunit complexes, both cytosolic and membrane-associated, during differentiation.

5.2 Future Prospects

As discussed above, it would be of interest to know how widespread the use of this type of regulatory mechanism is during differentiation. One question we have asked is whether cAMP-dependent protein kinase levels are regulated by this same mechanism during other terminal differentiation processes. To answer this question, we have examined the regulation of R_I in differentiating Friend erythroleukemia cells (M. Mason, I. Lorimer and B.D. Sanwal, unpublished results). These cells differentiate in culture to form cells resembling mature erythrocytes. It has been shown previously that the levels of type I cAMP-dependent protein kinase decrease during this differentiation process (Schwarz and Rubin, 1983). Our preliminary results show that the rate of degradation of R_I increases during differentiation of these cells. This suggests that a similar type of mechanism regulates R_I levels during Friend cell differentiation, except that in this case the

mechanism operates in reverse, decreasing the levels of R_I during terminal differentiation. Studies on the expression of R_I and C mRNA during Friend cell differentiation should be undertaken to confirm this possibility.

Another interesting area for future study would be to determine how this type of mechanism is involved in controlling the expression of different cAMP-dependent protein kinase isozyme types in different tissues. Our results, and those of Uhler and McKnight (1987), suggest that C preferentially associates with R_{II} in the cell, and that C begins to associate with R_I only when R_{II} is saturated. This is consistent with the observation that R_{II} has a higher affinity for C *in vitro* (Builder *et al.*, 1981). These findings suggest that the absence of type I isozyme in some tissues (such as bovine heart) may simply be due to a lower level of C expression. This possibility is supported by our finding that the levels of R_I mRNA are similar in bovine heart and muscle tissue, although type I holoenzyme is only found in bovine muscle.

Our results also emphasize the importance of intracellular proteolysis in the regulation of protein levels. It would be of interest to determine which protease(s) is involved in the degradation of R_I *in vivo*. Because the structure of R_I is fairly well understood and its half-life is readily measured in tissue culture cells, studies on R_I degradation may provide useful insights into the activity and specificity of intracellular proteases.

These studies on the mechanism of regulation of cAMP-dependent protein kinase levels have not revealed an obvious role for these

enzymes in the control of myogenesis. The increase in C mRNA, which apparently causes the change in holoenzyme levels, could be either a result of the differentiation process or part of a signalling mechanism involved in the control of differentiation. In order to determine definitively whether or not the cAMP-dependent protein kinases have a role in the control of myogenesis, genetic techniques would have to be used. We have previously attempted to isolate mutant myoblasts with defective cAMP-dependent protein kinases. However, these attempts were unsuccessful, probably because the cAMP treatment used to select for these mutants is not very toxic or growth-inhibitory to L6 myoblasts. An alternative approach would be to use molecular genetic techniques to make L6 cells with alterations in their protein kinases. This might be accomplished by using the antisense RNA technique (Weintraub et al., 1985) or by the transfection of dominant mutant genes coding for cAMP-dependent protein kinases. (Several of these have recently been isolated (Clegg et al., 1987; Levin et al., 1988).) We have made some preliminary attempts to inhibit R_I expression using antisense RNA, but further work is required before it can be determined if this approach will be successful or not.

5.3 Regulation of R_I in spontaneously-transformed myoblasts

Studies on the regulation of R_I in the spontaneously-transformed L6 cell line JRU5 showed that the proteolytic degradation of R_I was altered in this cell line (Lorimer and Sanwal, 1987). This was most evident in cells treated with N^6-derivatized cAMP analogues which are known to bind preferentially to the cAMP-binding site of R_I referred to as site 2 (Rannels and Corbin, 1980). Although other explanations are

possible, the simplest explanation for this seems to be that occupation of site 2 alone causes R_I to adopt a conformation which is resistant to proteolysis in JRU5, but not in L6. Additional evidence that R_I adopts different conformations when analogues are bound at different cAMP binding sites comes from the studies reported by Steinberg and Agard (1981b) which showed that the rates of dephoshorylation of R_I were different when cells were treated with different types of cAMP analogues. If this explanation is correct, it suggests that intracellular proteases are present in L6 and/or JRU5 which exhibit a high degree of specificity. The apparent loss of this proteolytic activity in JRU5 suggests that the activity of specific intracellular proteases can be modified in transformed myoblasts. Our finding that this altered proteolysis of R_I in JRU5 is not duplicated in Ha-ras transformed L6 cells indicates that the alteration is not a common property of all transformed myoblasts, but rather appears to be an effect of some specific mechanism of transformation.

5.4 Future Prospects

Further studies on proteolysis of R_I in L6 and JRU5 seem to be important for several reasons. As we have apparently identified a distinct difference in proteolysis of a particular physiological substrate in two closely related cell lines, further comparative studies in this system may be useful in the elucidation of general features of intracellular proteolysis. Also, further studies in this area might provide clues as to mechanisms by which cells become transformed, as it appears that the change in intracellular proteolysis is linked to spontaneous transformation of myoblasts. It is known that

some oncogenes can be activated by the disruption of controls which normally regulate their expression. To date, it has been shown that these changes in expression can occur by mutations which alter normal controls on transcription (Adams et al., 1985) or translation (Marth et al., 1988) of an oncogene. In theory, it is also possible that a change in some protease could change the normal regulation of the levels of an oncogene protein product, leading to cell transformation. This especially seems possible as several oncogene proteins (e.g. myc and fos) are known to have very short half-lives (Luscher and Eisenman, 1987; Curran et al., 1984).

There are several approaches which could be used to further characterize the apparent alterations in proteolysis in JRU5. The preliminary studies described in this thesis on the effects of ATP depletion, which suggested the involvement of an ATP-dependent protease, could be expanded upon. Other inhibitors could be used to further characterize R_I proteolysis in intact cells. As well, it is possible that R_I complexed with the one class of analogue could be degraded by a different pathway from that which degrades R_I complexed with the other class of analogue. Therefore, these inhibitor studies should be done in the presence of the different types of cAMP analogues as well. If there were differences in the proteolysis of R_I in the presence of the different types of analogues, this would provide clues as to the nature of the altered proteolysis of R_I in JRU5, which could be further investigated in vitro.

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