REGULATION OF RIBOSOME BIOGENESIS DURING MYOGENESIS OF RAT MYOBLASTS

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REGULATION OF RIBOSOME BIOGENESIS DURING MYOGENESIS OF RAT MYOBLASTS

by

© Frederik Andre Jacobs, B.Sc., M.Sc.

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ABSTRACT

The ribosome is an organelle which translates the genetic information provided by the nucleus into proteins. Because of its central role in gene expression, the production of the numerous ribosomal components must be coordinated for the efficient assembly of the ribosome so that it may perform effectively. The rat-derived muscle cell line L6-5 provides an excellent system in which to study ribosome biogenesis since, after terminal differentiation, ribosome accumulation rates are much reduced.

The synthesis rates of rRNA and ribosomal proteins (r-proteins) were investigated in proliferating L6-5 myoblasts and terminally differentiated myotubes. The rRNA and r-proteins were coordinately produced in myoblasts, with little detectable turnover of the individual components. In myotubes, however, the rate of rRNA synthesis was reduced by approximately 75% while the r-proteins continued to be synthesized at levels near those observed for myoblasts. The production of rRNA and r-proteins, which was coordinate in myoblasts, became uncoupled in myotubes.

Various possible explanations of the uncoupling of rRNA and r-protein production in myotubes were explored. It was determined that the levels of the r-protein mRNA's (rp-mRNA's) were similar in myoblasts and myotubes, suggesting that their translational efficiencies were unaltered by terminal differentiation. It therefore

appeared that the rate of r-protein synthesis was determined by cellular rp-mRNA levels.

To ascertain whether altered rp-mRNA metabolism accounted for the uncoupling of r-protein and rRNA synthesis in myotubes, the half lives of the rp-mRNA's were measured. The results reveal that the stability of rp-mRNA's were similar in myoblasts and myotubes, with half lives of approximately 11 hours. The data indicate that the rate of r-protein synthesis was regulated at the level of r-protein gene transcription, which suggests that rRNA and r-protein production was uncoupled at the transcriptional level in myotubes.

The uncoupling of rRNA and r-protein gene transcription in myotubes was demonstrated by measuring their transcription rates in vitro. It was determined that rRNA genes in myotubes were transcribed at approximately 30% of the rate found in myoblasts when compared to rp-mRNA genes. These results directly demonstrated the differential expression of these two gene families in L6-5 cells. The implications of these findings with respect to the regulation of ribosome biogenesis and myogenesis are discussed.

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CHAPTER ONE

Introduction

A) Preamble

The fate of an organism is often determined by how successfully it is able to exploit its environment. Production of a particular gene product or a change in morphology may lead to survival or a more efficient use of the organism's resources. The response may be a homeostatic one, which optimizes the organism's growth, or may be a differentiation one, which alters the organism's phenotype. The former response is one primarily of modulating the expression of a relevant set of genes and is generally reversible. The latter response is normally irreversible (or is part of cycle) and usually requires the induction or repression of larger families of genes.

In higher eukaryotes, homeostatic regulatory mechanisms remain important for survival. The development of differentiated tissues, however, allows for more efficient and specialized functions and has led to the high degree of success of complex multicellular organisms. The differentiation and development of specialized cell types poses complex gene regulatory problems. Not only do specific gene families have to be induced or repressed, but these events must also be controlled in a temporal manner. The development of a zygote into a fully mature individual

is a graphic demonstration of the overall effects of gene regulation in development.

The regulation of gene expression during development goes beyond the control of phenotypic differentiation. Many constitutive or 'housekeeping' genes are also modulated. Prime examples of such genes are those specifying the components of the ribosome. The cell produces this organelle, which plays a central role in gene expression, at varying rates, depending upon its state of differentiation as well as its metabolic condition and rate of growth. The complexity of the ribosome, its mode of biogenesis and its importance to the cell imply a sophisticated regulatory system for its turnover. We have chosen to study the regulation of ribosome biogenesis in one such differentiating system, the rat myoblast.

B) Regulation of Gene Expression

DNA appears to be the irreducible inheritable genetic material and it is at this level that the mechanism of gene regulation is most frequently investigated. The regulatory mechanisms for gene expression, however, extend beyond the control of gene transcription. They extend through to the levels of translation and degradation of the gene products. The various steps in gene expression can be segregated as follows:

1) pre-transcription; where the decision is made as to

which genes are expressed.

2) transcription; where the RNA polymerases initiate and transcribe RNA copies of specific genes.

3) processing; where the RNA transcripts are cleaved, spliced and modified to meet the requirements for their eventual use.

4) transport; where the RNA products are shipped to the appropriate sub-cellular locations.

5) translation; where mRNA's are translated into proteins.

6) post-translation; where the protein products are modified and transported to their destinations.

7) degradation; where both the RNA and protein products are disposed of when their usefulness has ended.

i) Pre-Transcription

The regulation of gene transcription is at the chromosomal level, where the complex of DNA and associated proteins allows for the activation of specific genes and the repression of others. This usually takes the form of positive control of genes which are normally in a 'switched off' or attenuated mode (Derman, Krauter, Walling, Weinberger, Ray & Darnell, 1981). Up to a million fold change in transcription rates has been noted in some systems as a result of gene induction (Mather, & Perry, 1981).

a) Primary Structure of Genes

The primary structure of DNA consists of a sequence of 4 nucleotides (de Robertis & de Robertis, 1980). In addition to specifying gene products, the sequence of these nucleotides determines the mechanisms by which gene expression is regulated. Since the advent of rapid DNA cloning and sequencing techniques, the acquisition of the precise primary DNA structure of specific genes has become almost trivial (see for example van Beveren, Galleshaw, Jonas, Berns, Doolittle, Donogue & Verma, 1981) However, DNA sequencing studies alone have not clearly indicated how specific genes are selected for expression.

Outside the actual gene's coding sequence are DNA sequences which are critical for the specific initiation of the gene's transcription. For example, the primary structure of the chick alpha-2(I) collagen gene allows several mutually exclusive hairpin loops to form in the 5' flank of the gene, some of which include the putative promoter sequences (Vogeli, Ohkubo, Sobel, Yamada, Pastan, & de Crommbrugghe, 1981). The various conformations in which the promoter finds itself may affect the transcriptional state of the collagen gene during embryogenesis (de Crommbrugghe & Pastan, 1982).

b) DNA Methylation

Much interest has been generated over the finding of methylated DNA and the role it may play in gene regulation (Manes & Menzel, 1981). The conservation of methylation sites upon DNA replication, which are mostly at the 5' position of cytosine in 'CG' pairs, (Wigler, Levy & Perucho, 1981) and the non-random, tissue specific distribution of the methylation sites (Razin & Riggs, 1980 and Doerfler, 1983) point to a potential regulatory role in gene transcription. Other sites of methylation include m^7G and m^6A (Adams, McKay, Craig & Burden, 1979) but appear to be functionally insignificant (Doerfler, 1983).

The extent of 'CG' methylation of specific genes, which can vary from 35 to 70% in vertebrates, has been inversely correlated with the degree of expression of those genes (Naveh-Many & Cedar, 1981). For example, ovalbumin, conalbumin and ovomucoid genes in egg laying hens are only hypo-methylated in the tissue (the oviduct) in which they are expressed (Mandel & Chambon, 1979). The temporal relationship between the methylation of alpha-globin genes in the chick erythrocyte and its developmentally controlled de-induction during embryogenesis also suggests that gene methylation has an important role in gene regulation (Weintraub, Larsen & Groudline, 1981, Doerfler, Kruczek, Eick, Vardimon & Kron, 1982, and Weatherall & Clegg, 1982 and Busslinger, Hurst & Flavell, 1983). However, the

picture is not entirely clear since examples have been described where gene induction is not temporally related to gene methylation, such as in the case of the induction of delta-crystallin genes during chick embryogenesis (Grainger, Hazard-Leonards, Samaha, Hougan, Lesk & Thomsen, 1983).

Gene methylation may act as a gene switch via a methylated DNA binding protein (Huang, Wang, Gamma-Sosa, Shenoy & Ehrlich, 1984). However, this mechanism appears insufficient since, for example, the conalbumin gene which is hypo-methylated in the oviducts of egg laying hens, still requires hormone for expression (Mandel & Chambon, 1979). These potential gene switches, in any case, appear only to function for genes transcribed by RNA polymerase II, since the transcription of rRNA genes is unaffected by its state of methylation (Macleod & Bird, 1983 and Pennock & Reeder, 1984).

c) Chromatin Structure

The organization of chromatin structure is thought to play a role in gene expression. Although chromatin is responsible for packaging DNA into a micron-sized nucleus, the wide range of morphology of chromatin observed suggests a more active role in gene regulation (Igo-Kemenes, Horz & Zachau, 1982). The first level of DNA organization is the chromatosome, which embodies the nucleosome core of pairs of histones H2a, H2b, H3 and H4, histone H1 and approximately

200 base pairs (bp) of DNA. The histones in the nucleosome may be modified in a variety of ways (Isenberg, 1979) but only histone methylation appears to be positively correlated with gene transcription (Nelson, Perry & Chalkley, 1979).

The phasing (or sequence specific localization) of nucleosomes has been postulated as a potential gene regulatory mechanism (Igo-Kemmenes et al., 1982). However, the finding of phased nucleosomes on Simian Virus 40 (SV40), for example, appears to result from a very specific 'nucleosome gap' near the origin of replication (Wasylyk & Chambon, 1980). The significance of nucleosome phasing in nuclear chromatin is unclear and has been claimed by some authors to be an artifactual observation (Fittler & Zachau, 1979 and McGhee & Felsenfeld, 1983).

Digestion of chromatin with DNase I has been used as a tool to probe the relationship between chromatosome structure and function. Three general classes of DNase I sensitivities have been described for chromatin, each differing by approximately an order of magnitude (McGinnis, Shermoen, Heemskerk & Beckendorf, 1983). The bulk of the chromatin DNA, which is not being transcribed, has been described as being insensitive to DNase I, probably because of its condensed nature.

DNase I sensitive material appears to be actively transcribed or 'open' DNA. Digestion of these structures often yields uniformly sized DNA fragments of multiples of

150 to 200 bp, corresponding to the amount of DNA associated with one chromatosome. The actual distance between nucleosomes can vary by up to 80 bp in different tissues (Finch & Klug, 1976), although no correlation with transcription levels has been recorded (Humphries, Young & Carroll, 1979).

DNase I hyper-sensitive chromatin regions have been associated with the 5' flanking sequences of transcribing genes, and contain the putative transcription initiation, promoter and enhancer sequences. Furthermore, these DNase I hyper-sensitive regions appear to be almost devoid of nucleosomes (a nucleosome gap) and perhaps therefore are most exposed (McGinnis et al., 1983). How these regions lose their chromatosome structures may be related to the generation of histone H2a and H2b derivatives with ubiquitin which turn over very quickly (Goldstein, Scheid, Hammerling, Boyse, Schlessinger & Niall, 1975 and West & Bonner, 1980).

The presence of non-histone proteins such as the High Mobility Group (HMG) proteins HMG-14 and HMG-17 bound to chromatin can alter the chromatin's DNase I sensitivity (Weisbrod, Groudine & Weintraub, 1980). These proteins have been shown to preferentially bind single stranded DNA, inferring an open chromatin structure and an easy access for other regulatory proteins, RNA polymerases, and DNase I. The description of HMG-14 and HMG-17 proteins, however, does not begin to catalogue the DNA binding proteins found in the

nucleus (Reeck, Isackson & Teller, 1982).

d) Gene Amplification

Full production of new gene products such as globin in reticulocytes or ovalbumin in chick oviducts, may take hours or days to achieve because of the time required to build up sufficient stores of translatable mRNA's (Chrisholm, 1982). However, when large amounts of RNA transcripts are required quickly, the cell may resort to gene amplification by generating multiple copies of the required gene. Gene amplification such as described for the chorion genes in Drosophila (Spradling & Mahowald, 1981 and Osheim & Miller, 1983) or the rRNA genes in Xenopus (Rochaix, Bird & Bakken, 1974) during oogenesis occurs by a variety of mechanisms.

ii) Transcription

The transcription of all nuclear genes appears to be closely linked with the nuclear matrix. In tissues which transcribe unique genes, such as ovalbumin in the chick oviduct or beta-globin in reticulocytes, the DNA being transcribed is found preferentially associated with the nuclear matrix (Hentzen, Rho & Bekhor, 1984). The association is reversible, since ovalbumin genes in the chick oviduct are associated with the nuclear matrix only when being induced by hormone (Ciejek, Tsai & O'Malley, 1983).

Three RNA polymerases, each generating different classes of RNA, have been identified in the nucleus. RNA polymerase I (or A) transcribes nucleolar genes, chiefly those for 18s, 5.8s and 28s ribosomal RNA's. RNA polymerase II (or B) transcribes DNA which yield mostly mRNA's, while RNA polymerase III (or C) transcribes the smallest genes, including those for tRNA's and 5s rRNA (Chambon, 1975).

a) RNA Polymerase I

The transcriptional activity of RNA polymerase I has been localized in the fibrillar centers of the nucleolus by immunocytochemical techniques (Scheer & Rose, 1984). The initiation requirements of RNA polymerase I transcription are species specific and are encoded in the 5' flanking region of the gene (Mishima, Financsek, Kominami & Muramatsu, 1982 and Rothblum, Reddy & Cassidy, 1982). Two initiation factors for RNA polymerase I have been recently described which are required for the species specific initiation of rRNA transcription in mouse L cells and HeLa cells (Miesfeld & Arnheim, 1984).

The promoter and initiator sequences of RNA polymerase I transcribed genes such as those for mouse rRNA genes are contained in the region 22 bp up-stream to 2 bp up-stream of the transcription start site. The 5' flank of mammalian rRNA genes also contain at least 5 additional

conserved sequences which may further regulate their transcription rate (Miller & Sollner-Webb, 1981 and Yammamoto, Takakusa, Mishima, Komihami & Muramatsu, 1984).

No consensus sequences have yet been described for transcription termination with RNA polymerase I. Instead, studies with cells treated with proflavin, a chemical which interferes with RNA secondary structure, indicate that the transcripts' secondary structures dictate the termination sites (Nielson, Carin & Westergaard, 1984). Premature termination, a common prokaryotic regulatory mechanism, or varied rates of elongation do not appear to be significant factors in modulating transcription in eukaryotes (Darnell, 1982).

b) RNA Polymerase II

Messenger RNA and some of the small RNA's (such as the U series of RNA's and small cytoplasmic RNA's (scRNA's)) are transcribed by RNA polymerase II (or B). Most of the final products are capped on their 5' ends (Banerjee, 1980) and usually contain a 3' poly-adenylated tail (Corden, Wasylyk, Buchwald, Sassone-Cori, Kedinger & Chambon, 1980). Reports indicate that initiation is controlled by a 'TATAA' consensus sequence approximately 20 to 35 bp up-stream of the actual transcription start site (Nevins, 1983). Initiation is precise and the first nucleotide transcribed is immediately capped (Contreras & Fiers, 1981).

The requirement for the 'TATAA' (or Goldberg-Hogness) box is not absolute since the deletion of this sequence from the Herpes Simplex virus (HSV) thymidine kinase gene, for example, still allows transcription. The 5' initiation site, however, is no longer precise and transcription proceeds at a reduced rate (McKnight & Kingsbury, 1982). Although the 'TATAA' sequence is believed to play a crucial role in precisely locating the initiation site (Nevins, 1983), certain Adenovirus (Baker & Ziff, 1981), SV40 (Fiers & Contreras, 1978) and Polyoma virus (Soeda, Arrand, Smolar, Walsh & Griffin, 1980) genes which are efficiently transcribed do not contain this sequence.

Further up-stream of the initiation site exist other elements which promote transcription, such as the 'CCAAT' box, found approximately 80 bp up-stream (Benoist, O'Hare, Breathnach & Chambon, 1980 and Efstradiadis, Lamedico, Rosenthal, Kolodnar, Tizard, Perler, Villakomaroff, Naber, Click & Broome, 1980). Although the CCAAT box promotes the transcription of some genes *in vivo*, its deletion from the *beta*-globin or conalbumin genes has little effect on their transcription *in vitro* (Wasylyk, Kedinger, Corden, Brison & Chambon, 1980 and Grosveld, Shewmaker, Jat & Flavell, 1981).

Regions 200 to 300 bp up-stream of the initiation site of the gene may contain tissue-specific control sequences which promote initiation (Derman *et al.*, 1981, Groudine, Peretz & Weintraub, 1981 and Nevins, 1983). For example,

the alpha-amylase gene is transcribed in both the salivary glands and the pancreas in mouse (Hagenbuchle, Bovey & Young, 1980). Although the final protein product is the same in both tissues, the primary transcripts and the resulting mRNA's are different because they are transcribed from tissue specific start sites from the same gene (Young, Hagenbuchle & Schibler, 1981). Similar tissue specificity is also seen in the transcription of the insulin and chymotrypsinogen genes of the rat (Walker, Edlund, Boulet & Rutter, 1983). These and other 'enhancer' type sequences, which are correlated with the DNase I hyper-sensitive regions discussed earlier, may provide an entry point for RNA polymerase II (Khoury & Gruss, 1983). However, the transcription enhancement sequences can also appear in the internal sequences of the expressed gene such as in the human IgM genes (Mills, Fisher, Kuroda, Ford & Gould, 1983).

The actual initiation of transcription requires several initiation factors in addition to the RNA polymerase II enzyme complex (Matsui, Segall, Weil & Roeder, 1981). These factors appear mostly to suppress random initiation by the polymerase.

In contrast to the specificity of initiation of transcription, most authors suggest that termination is an approximate event, yielding primary transcripts with heterogeneous 3' ends (Hofer, Hofer-Warbinek & Darnell, 1982 and Nevins, 1983). Most RNA polymerase II transcription units

contain an 'ATAAA' sequence approximately 10 to 30 bp before the transcript terminus, the deletion of which can yield unstable run-on transcripts (Fitzgerald & Shenk, 1981). Deletions around (but not including) the 'ATAAA' sequence yield stable transcripts terminating 11 to 19 bases beyond this sequence.

Histone mRNA's are an example of eukaryotic mRNA's which do not contain the 'ATAAA' or a 3' poly(A) sequence. The transcription of their genes yield functional albeit short-lived mRNA's (Hentschel & Birnstiel, 1981). Histones, however, prove to be a special case since their genes contain highly conserved sequences at the 3' termini which have been shown to be required for proper termination and processing (Birchmeister, Grosschedl & Birnstiel, 1982).

The transcription of host cell genes can be inhibited by some viruses. For example, the polio virus specifically inhibits one of the initiation factors required for nuclear gene transcription but permits transcription of its own genes (Crawford, Fire, Samuels, Sharp & Baltimore, 1981). Similar transcription initiation control is seen in the SV40 T antigen (Khoury & May, 1977) and the E4 gene of Adenovirus (Nevins & Winkler, 1980).

c) RNA Polymerase III

RNA polymerase III (or C) transcribes the smallest genes found in eukaryotes, including those for the tRNA's

and the 5s rRNA (Bogenhagen, Sakonju & Brown, 1980). In the case of the 5s rRNA gene, initiation is controlled by a promoter in the center of the transcribed sequence (Sakonju, Bogenhagen & Brown, 1980 and Bogenhagen et al., 1980). In Xenopus oocytes, the 40 bp internal promoter is recognized by a pair of 40k dalton proteins (Sokonju, Brown, Engelke, Ng, Shastry & Roeder, 1981) (42k dalton in somatic cells (Pelham, Wormington & Brown, 1981)) which have been determined to be one of the transcription factors required by RNA polymerase III (Ng, Parker & Roeder, 1979 and Honda & Roeder, 1980). The control region and the transcription factor direct transcription to start approximately 50 bp upstream of the promoter site. Since the 40k dalton protein also tightly binds the 5s rRNA, it forms a feed-back mechanism which regulates the transcription of the 5s rRNA gene. The 5s rRNA:protein complex is very stable and is dissociated only when the 5s rRNA is incorporated into a maturing ribosome, leading to the degradation of the 40k dalton protein (Garret & Noller, 1982). The concentration of 5s rRNA not incorporated into the ribosome therefore regulates the transcription of the 5s rRNA gene. This type of regulation is reminiscent of the type found in abundance in the regulation of r-proteins during prokaryotic ribosome production (Nomura, Dean & Yates, 1982).

While the intra-genic control region defines the general initiation site for RNA polymerase III genes, the 5'

flanking region is still required for precise initiation. Deletions in the 5' flank of the Xenopus 5s rRNA gene results in altered initiation sites clustered around the natural one (Sakonju et al., 1980).

Termination of RNA polymerase III transcription is thought to be controlled by a cluster of thymidines at the 3' terminus (Brown & Gurdon, 1977) since deletions in the 3' flank up to but not including the thymidine cluster did not affect termination (Bogenhagen et al., 1980). Accurate termination of RNA polymerase III genes can be efficiently and precisely executed by the purified polymerase alone (Cozzarelli, Gerrard, Schlessinger, Brown & Bogenhagen, 1983).

iii) RNA Processing

Primary transcripts are usually not co-linear with the final RNA products and must therefore undergo a considerable amount of splicing and cutting, requiring complex processing systems (Breathnach & Chambon, 1981). A variety of mechanisms exist to accomplish these tasks, including specific RNases and small nuclear RNA's (snRNA's), postulated to bring the splice sites together (Nevins, 1983). Nuclear proteins are implicated in the maturation process of the RNA; indeed the RNA would be degraded if they were not present to form a heteronuclear ribonucleo-protein (hnRNP) complex (Busch, Reddy, Rothblum & Choi, 1981).

Despite the enormous amount of processing which must occur, the rate limiting step in forming most mature RNA's is its transcription (Sobel, Yamamoto, de Crombrugghe & Pastan, 1981).

a) Processing of RNA Polymerase I Products

The processing of pre-rRNA transcripts, which can be 45s or larger, to yield 28s, 18s and 5.8s products proceeds though a number of steps (Perry, 1976). Unlike RNA polymerase II products (discussed below), there do not appear to be consensus sequences specifying cleavage sites (Crouch, 1984). The retained sequences of the rRNA termini are highly conserved between species as disparate as yeast and HeLa cells but are different for each cleavage site (Khan & Maden, 1977 and Veldman, Klootwijk, de Jong, Leer & Planta, 1980). Little conservation is seen in the intervening sequences, including the cleaved nucleotide. It therefore appears that the secondary structure of the rRNA plays an important role in precisely locating the cleavage sites. The kinetics of the pre-rRNA cleavage reactions are remarkably quick, with half lives of approximately 2 seconds (Cech, Zaug, Grabowski & Brehm, 1982).

Quaternary structures of rRNA's with r-proteins are critical for the proper cleavage of the rRNA's (Craig & Perry, 1970). In addition, it has been observed that the small nuclear RNA 'U3' is hydrogen bonded to the 32s rRNA.

intermediate and may be responsible for the accurate processing of the 3' end of the 5.8s rRNA (Zieve & Penman, 1976 and Crouch, 1984).

The methylation of the preserved rRNA sequences is also required for the proper processing of pre-rRNA (Perry, 1976). The absence of such methylation, as observed during heat shock of CHO cells, for example, leads the pre-rRNA to wastage pathways (Bouche, Raymal, Amalric & Zalta, 1981). The processing of rRNA in terms of ribosome biogenesis is further discussed in Section 'C-ii'.

b) Processing of RNA Polymerase II Products

The first post-transcriptional event to occur to RNA polymerase II transcipts is the capping of the 5' end of the hnRNA. No nuclear RNA polymerase II transcripts have been described without the 5'm^7G cap (Salditt-Georgieff, Harpold, Chen-Kiang & Darnell, 1980 and Nevins, 1983), even those which have been prematurely terminated (Babich, Nevins & Darnell, 1980). The role of the 5' cap may be two fold: (1) to protect the transcript from exonucleolytic degradation during processing (Nevins, 1983), and (2) to enhance the initiation of translation (Banerjee, 1980).

In addition to the m^7G cap, the RNA polymerase II transcripts are often methylated at other sites, usually on the N^6 position of the penultimate 5' nucleotide (Shatkin, 1976). The appearance of m^6A residues in other parts of the

hnRNA appear to dictate the preserved sequences and the splicing kinetics of processing (Stoltzfus & Dane, 1982, Nevins, 1983 and Camper, Albers, Coward & Rottman, 1984). Other methylation sites include the 2'-O-ribose position in the 5' penultimate residues of RNA polymerase II transcripts (Shatkin, 1976).

The addition of adenylate residues (the poly(A) tract) to the the 3' termini of RNA polymerase II transcripts also occurs early during hnRNA processing (often within 120 seconds of transcription (Salditt-Georgieff, Harpold, Sawicki, Nevins & Darnell, 1980)), perhaps with the aid of the U4 snRNP complex (Berget, 1984). The presence of cordycepin, which inhibits polyadenylation, still allows proper processing of the hnRNA, although the final products usually do not appear in significant amounts in the cytosol (Darnell, Philipson, Wall & Adesnik, 1971 and Zeevi, Nevins & Darnell, 1981).

The precise role of the 'AUAAA' consensus sequence found near the 3' termini of most RNA polymerase II products has been correlated with a polyadenylation site within 20 bases of the 3' end of the transcript. It may also specify the terminal cleavage site (Nevins, 1983). Alternative polyadenylation sites are available to some transcripts such as the immunoglobin mu heavy chain mRNA's, where distinct mRNA species are generated (Early, Rogers, Davis, Calame, Bond, Wall & Hood, 1980).

Nuclear transcribed RNA polymerase II products often contain introns and must be spliced to produce a translatable mRNA product. The exon/intron locations have consensus sequences about the two sites which are to be spliced together:

5'exon-$\frac{A}{C}$AG/$\underline{GU}\frac{A}{G}$AGT · intron · $(py_6)X\underline{AG}/G\frac{G}{U}$-exon3'

(Breathnach, Benoist, O'Hare, Gannon & Chambon, 1978 and Breathnach & Chambon, 1981). The concensus sequence indicated shows 4 highly conserved bases (underlined) which mark the termini of the intron (slashed). This so called 'GU-AG splice rule' has been found to hold for most eukaryotic mRNA's (Mount, 1982). The sequences of the introns are generally not important to the fidelity of the splice since large deletions made in the introns of rabbit beta-globin hnRNA's not affecting the consensus sequence still allowed accurate splicing (Hamer & Leder, 1979).

It has been recognised, however, that these splice site consensus sequences occur more often than the actual splice sites, inferring the presence of other controlling factors. Different conformations of the myosin light chain primary RNA transcript in chick, for example, allow different splice sites to be brought together, producing two distinct mRNA's (LC_1 and LC_3) from one gene in the same tissue (Nabeshima, Fuji-Kuriyama, Muramatsu & Ogata, 1984). Similarly, the calcitonin gene yields different mRNA's from the same gene by different processing pathways in the rat hypothalamus and

thyroid glands (Amara, Jonas, Rosenfeld, Ong & Evans, 1982).

Processing of RNA is not the exclusive domain of the nucleus. For example, maternally supplied mRNA's in the Xenopus oocyte are translationally blocked (Wells, Shoman, Klein & Raff, 1981). Upon fertilization of the egg, the internal repetitive sequences of the mRNA's are spliced out, producing messages which can then be translated (Anderson, Richter, Chamberlin, Price, Britten, Smith & Davidson, 1982 and Colman, 1983).

Small nuclear ribonucleo-protein particles (snRNP's) have been implicated in the processing of hnRNP's (Busch et al., 1982). Not only do anti-snRNP antibodies inhibit the splicing of transcripts (Yang, Lerner, Steitz & Flint, 1981), but U1 snRNP has been shown to bind to the 5' splice site (in reference to the intron) of beta-globin hnRNP's (Mount, Pettersson, Hinterberger, Karmas & Steitz, 1983). The lack of good in vitro hnRNP processing systems has impeded the understanding of this type of splicing, although details are slowly being elucidated (Hardy, Grabowski, Padgett & Sharp, 1984).

c) Processing of RNA Polymerase III Products

The third general type of RNA splicing is exemplified by nuclear tRNA's. These RNA's display no consensus sequences either at the splice junction or within the intron (Johnson, Ogden, Johnson, Abelson, Dembeck & Itakura, 1980).

In all tRNA's studied which contained introns, the site of the intron is at the same position in the anticodon loop. This suggests that the tertiary structure of the remaining part of the pre-tRNA assumes the conformation of the mature tRNA, allowing the splices of different pre-tRNA's to be made with one endonuclease (Peebles, Gegenhei & Abelson, 1983).

iv) Transport

Once the RNA transcript is processed, it is exported from the nucleus via an energy and cyclic-AMP dependent process (Moffet & Webb, 1981) requiring specific transport proteins (Moffett & Webb, 1983). The nuclear complement of the proteins associated with the mRNA is partially unique since some of these proteins are exchanged for cytoplasmic proteins when the mRNA's are exported (Schweiger & Kostka, 1980). Ribosomal RNA as ribosomal subunits are similarly exported through the nuclear pores (Wunderlich, 1981). Immature transcripts or fragments of RNA generated from hnRNA processing are not exported, suggesting that the transport process is quite specific (Nevins & Darnell, 1978 and Piper, 1979).

The export of tRNA's from the nucleus also requires a specific mediator. Not only does the mediator exhibit saturatable kinetics, but its specificity is very high: a single base change made in the highly conserved loop IV of

Xenopus Met-tRNA$_i$ results in a 20 fold reduction in the export of the tRNA (Zasloff, 1983).

Negative regulation of nuclear transport may occur since late Adenovirus mRNP's inhibit the export of nuclear mRNP's. It is possible, however, that this may be due to a saturation of the transport system by Adenovirus mRNP's (Beltz & Flint, 1979).

v) Translation

a) Messenger Ribonucleoproteins

Before a discussion about translation and its regulation can be engaged, it is necessary to characterize the mRNA as it exists in the cytosol. Messenger RNA in eukaryotic cells exist as mRNA:protein complexes (mRNP's), of which approximately 25% is RNA. Despite the large protein complement, these EDTA stable complexes are sensitive to RNase digestion and are usually able to hybridize with oligo(dT)-cellulose (Preobrazhensky & Spirin, 1978).

The mRNP's purified from polysomes contain 6 to 9 major proteins varying from 15k to over 100k daltons. One protein (70k to 78k daltons) has been identified as a poly(A) binding protein (Favre, Morel & Scherrer, 1975). Also, tissue specificity of some of the mRNP proteins has been demonstrated (Darnbrough & Ford, 1981). Recent work has demonstrated that specific subsets of mRNP proteins are bound to specific mRNA's (Ruzdijic, Bag & Sells, 1984).

The mRNP's purified from the free or non-polysomal fraction (previously known as 'informosomes') differ considerably in both stability and specific protein content from the polysomal mRNP's (Olsnes, 1971). The role of the free mRNP's appears to be both a 'stepping stone' for the mRNA's from the nucleus to the polysomal fraction (de Robertis, 1983) and a temporary holding stage for non-translating mRNA's (Darnbrough & Ford, 1981). The free mRNP's are thought to be untranslatable in vivo, although some of the translation results in vitro are equivocal (Villringer, Kuhn, Schneider, Andus, Michels, Northemann, Krupp, Walckhof, Gross, Klump, Kroll, Branlant & Heinrich, 1983). However, protein-free mRNA's obtained from free mRNP's are indistinguishable from those purified from polysomal mRNP's by all testable criteria (Jenkins, Kaumeyer, Young & Raff, 1978 and Infante & Heilmann, 1981).

b) Initiation and Regulation of Translation

Translation of eukaryotic mRNA's can be divided into three phases: initiation, elongation and termination. The initiation step is the rate limiting portion of translation (Austin & Clemens, 1980). Formation of the initiation complex requires hydrolysis of ATP and GTP and may therefore be an excellent site for translational regulation (Trachsel, Erni, Schreier & Staehelin, 1977 and Ochoa, 1983). Two basic types of regulation can occur at the translational

level: control of the overall efficiency of initiation and the discrimination of specific mRNA's chosen for translation (Maitra, Stringer & Chauduri, 1982).

Initiation of translation begins with the formation of the ternary complex (eIF-2:GTP:Met-tRNA$_i^{Met}$) and its subsequent binding to the 40s ribosomal subunit (the quaternary complex) (Weissbach & Ochoa, 1976). Phosphorylation of the alpha subunit of eIF-2 inhibits the quaternary complex formation (de Haro & Ochoa, 1978). The recycling of eIF-2 and thus regulation of the rate of formation of the quaternary complex is performed by the eIF-2 stimulatory factor (Siekierka, Mauser & Ochoa, 1982). The coordinate production of alpha- and beta-globin mRNA's with haemin in rabbit reticulocytes is regulated in this fashion via a haemin control repressor (Austin & Clemens, 1980).

At least 7 other defined factors are involved in the initiation of translation, although most only enhance the rate of initiation (Maitre et al., 1982). Some of these factors (such as eIF-4A and eIF4B) play a discriminatory role, allowing one mRNA to initiate more efficiently than another (Darnell, 1982). Additional unkown factors are required, however, to allow the initiation complex to recycle properly.

The efficiency of initiation is also affected by components of the mRNA itself, such as the 5' cap (Shatkin,

1976) and the 3' poly(A) tail (Brawerman, 1976). The precise cap structure ($m^7G^{5'}ppp^{5'}XYZ$), where the penultimate bases X, Y and Z may be variously methylated, influences the efficiency of initiation. Such discrimination is seen in the initiation rates between the two globin mRNA's in the rabbit reticulocyte and is used to coordinate their translation (Lockard, 1978 and Hunt, 1980)

The poly(A) tract found on the 3' end of most mRNA's may enhance the stability of the initiation complex, either directly or through a poly(A) binding protein (Setyono & Greenberg 1981). It was found that the initiation of polyadenylated mRNA's was specifically and competitively inhibited by oligo(A) but not by other homopolymers in vitro and that this inhibition did not occur when the mRNA was added as its native mRNA:protein complex (Spieth & Whitely, 1981 and Jacobson & Favreau, 1983).

The observation of unique protein complements in polysomal and free mRNP's has led many authors to suggest that these proteins regulate the translatability of a variety of mRNA's (Olsnes, 1971, Bag & Sells, 1979a, Bag & Sells, 1979b, Darnbrough & Ford, 1981 and Vincent, Akhayat, Goldenberg & Scherrer, 1983). In general, it has been found that polysomal mRNP's are translatable and free mRNP's are not (Vincent, Goldenberg, Standart, Civelli, Imaizumi-Scherrer, Maudrell & Scherrer, 1981). Movement between these two mRNP pools has been reported in such instances as

heat shocked CHO cells, where the polysomal mRNA's were actively sequestered in the free mRNP pool (Enger, Campbell & Hanfers, 1975) and during Drosophila embryogenesis, where the Tl mRNA was actively redistributed during development (Fruscoloni, Al-Atia & Jacobs-Loren, 1983). Also, the free mRNP form of the myosin heavy chain mRNA in fetal calf myoblasts was redistributed to the polysomal pool and translated after myogenesis (Buckingham, Cohen & Gros, 1976).

Recently, translation control RNA's (tcRNA's) have been described which inhibit the translation of specific mRNA's in vivo (Bag, Hubley & Sells, 1980, Bag, Maundrell & Sells, 1982 and Villringer et al., 1983). It is not clear whether these tcRNA's inhibit translation of the mRNA by direct hybridization (Heywood & Kennedy, 1976 and Bag et al., 1982) or by association via a protein complex (Kennedy, Siegel & Heywood, 1978). More recently, a 19s RNP has been described in duck erythrocytes which specifically associates with non-translating mRNP's (Schmid, Akhayat, Martins, de Sa, Puvion, Koehler & Scherrer, 1984). The particle, coined a 'prosome', appears to contain a previously identified 7s tcRNA and has been observed in various tissues of other species (Schmid, Koehler & Setyona, 1983). Other factors may be involved with the conversion of free mRNP's into a translatable form such as phosphorylation (Preobrazhensky & Spirin, 1978) or ADP-ribosylation (Elkaim, Thomassin,

Niedergang, Egly, Kempf & Mandel, 1983) of the mRNP proteins.

c) Elongation and Termination of Translation

The elongation and termination steps do not appear to be major sites of translational control. Examples exist, however, where the apparent rate of elongation is stimulated by hormones in such tissues as the placenta and liver (Ilan, Pierce, Hochberg, Folman & Gyves, 1984).

Termination of protein synthesis is specific, effected by the termination codons on the mRNA. Proteins which are prematurely terminated (as a result of a translation error) are quickly degraded (Wharton & Hipkiss, 1984). No evidence of regulation at this point has been reported (Moldave, David, Hutchison, Laidlaw & Fisher, 1982) even though a specific 'release factor' with GTPase activity is required for termination (Goldstein, Beaudet & Caskey, 1970).

vi) Post-translational Processing

As with the newly transcribed RNA, proteins are frequently processed before the final mature product is functional. Over 100 different protein modifications have been catalogued (Wold, 1981). The deamination of asparagine residues in alpha- and beta-crystallin proteins, for example, serve to greatly increase the life time of these eye lens proteins (van Kleef, de Jong & Hoenders, 1975). A discussion of the various protein modifications and their

functions and regulation is beyond the scope of this introduction.

vii) Degradation

a) Protein

The terminal phase in the scheme of gene regulation is the degradation of the final products. Two major routes appear to be available to the cell by which proteins and other cellular products are broken down. The first is by lysosomal autophagy, where cellular components are incorporated into lysosomes and enzymatically digested (Hershko & Ciechanover, 1982). The second is by a variety of specific cytoplasmic degradation systems, the best characterized of which is the conjugated ubiquitin pathway (Hershko & Ciechanover, 1982). Despite the fact that the actual degradation of cellular products is an enthalpic process, both degradation routes are ATP dependent. This infers that these systems are under regulatory control.

The non-selective degradation of protein in rat liver has long been known to occur upon the depletion of exogenous levels of insulin and amino acids (Mortimore & Mondon, 1970). In addition, glucagon, an antagonist of insulin, accelerates this degradation, probably by lowering intracellular amino acid levels (Schworer & Mortimore, 1979). This response is a general metabolic one, where the protein pool is mobilized for energy production under

starvation conditions.

The regulated degradation of specific proteins can also occur. The levels of glutamine in cultured hepatoma cells determine the rate of degradation of glutamine synthetase, for example (Freikopfcassel & Kulka, 1981). The selectivity of the degradation systems is developmentally regulated during erythropoiesis in rabbits, where most of the developing erythrocytes' cellular structures and proteins are specifically degraded, leaving the hemoglobin complexes and associated enzymes intact (Rapoport, Rosenthal, Schewe, Schultze & Muller, 1974 and Chalevekis, Clegg & Weatherall, 1975). How the specific concerted degradation in this or other systems is achieved is still unclear (Hershko, Ciechanover, 1982).

b) RNA

Several features of the mRNA have been implicated in affecting the rate of degradation of mRNP's. The presence of both the 5'm^7G cap and the 3' poly(A) tract appear to inhibit degradation by exonucleases (Darnell, 1982 and Nevins, 1983). Early work with histone and globin mRNA's suggest that the poly(A) tract increases the stability of the mRNA in the cytoplasm (Huez, Marbaix, Burny, Herbert, Leclerq, Cleuter & Chantrenne, 1977 and Huez, Marbaix, Gallwitz, Weinberg, Devos, Herbert & Cleuter, 1978), although these results are not consistent with the finding of others (Deshpande, Chatterjee & Roy, 1979 and Palatnik,

Storti, Capone & Jacobson, 1980).

Specific factors may affect the stability of selected mRNA's. The presence of the hormone estrogen, for example, dramatically increases the half life of vitellogenin mRNA by over 30 fold in Xenopus liver (Brock & Shapiro, 1983). In contrast, the histone mRNA's in HeLa and mouse myeloma cells appear to be actively degraded upon the cessation of DNA synthesis (Borun, Gabrielli, Ajiro, Zweidler & Baglioni, 1975 and Sittman, Graves & Marzluff, 1983).

C) Ribosomes

Ribosomes are pivotal organelles involved in gene expression since these cellular machines translate the genetic information into their protein products. They are also a rate limiting step in gene expression since the cytoplasmic mRNP pool which they translate is in excess (Walden & Thach, 1982). Among its many requirements, the ribosome must be able to recognize a translatable mRNP, find the initiator codon of the message, and match amino acids to the corresponding codons with high fidelity as the message is being read (Haselkorn & Rothman-Denes, 1973). The ribosome achieves these feats with the help of a multitude of initiation and elongation factors as well as energy in the form of GTP and ATP (Maitra et al., 1982). Because the ribosome is responsible for this most important step in gene expression, it has been extensively studied for several

decades in both prokaryotes and eukaryotes (see for example the reviews by Chambliss, Craven, Davies, Kahan & Nomura, 1980 and Warner, Tushinski & Weijksnora, 1980).

i) General Characteristics

Ribosomes occur ubiquitously in the cytoplasm of all cells. They are either 'free floating' or attached to membranes such as the endoplasmic reticulum via specific attachment proteins call ribophorins (Kreibich, Ulrich & Sabatini, 1978 and Kreibich, Freienstein, Pereyra, Ulrich & Sabatini, 1978). The location of ribosomes plays a role in directing the eventual destination of the protein being translated. For example, secreted proteins are often synthesized on membrane-bound ribosomes, with the elongating peptide being injected through the membrane (Blobel & Sabatini, 1971). Cytoplasmic and nuclear proteins are synthesized mostly on free ribosomes.

The mammalian ribosome contains almost 80 r-proteins and 4 RNA species (Cox, 1977 and Wool, 1979). Each protein, with few exceptions, and each RNA species is uniquely and stoichiometrically represented (Wool, 1979). The ribosome, which normally exists as an 80s particle, is composed of 2 subunits (Blobel & Sabatini, 1971). The larger 60s subunit consists of approximately 50 proteins and 3 rRNA's (28s, 5.8s and 5s), while the 40s subunit contains the remaining 30 proteins and the 18s rRNA (Wool, 1979).

ii) Ribosomal Proteins

The majority of r-proteins are basic and range in molecular weight from 8k to over 55k (Terao & Ogata, 1975). A few acidic r-proteins have been described (Sanchez-Madrid, Reyes, Conde & Ballesta, 1979). Some of the r-proteins have been sequenced in the case of the rat (Wittman-Liebold, Geissler, Lin & Wool, 1979, Lin, McNally & Wool, 1983 and Torczynski, Bollon & Fuke, 1983). The r-proteins are numbered according to their relative mobility on a standardized gel system and are prefixed with 'L' (large) or 'S' (small), depending upon which subunit they populate (McConkey, Bielkas, Gordon, Lastick, Lin, Ogata, Reboud, Traugh, Traut, Warner, Welfle & Wool, 1979). The distribution of the r-proteins between the subunits varies depending upon the system and the investigator, making a standardized nomenclature difficult. An exact inventory of r-proteins is not possible with current methods since some of the proteins dissociate to some extent from the ribosome (Hardy, 1975) or become oxidized (Welfle, Goerl & Bielka, 1978) upon purification.

Most of the r-proteins are essentially unmodified except, for example, the small subunit protein S6 and some of the acidic r-proteins, which exist in several phosphorylated forms (Gressner & Wool, 1974, Anderson, Gundholm & Sells, 1975 and Sanchez-Madrid et al, 1979).

Other basic r-proteins may also be phosphorylated (Wool, 1979), acetylated (Liew & Gornall, 1973) or methylated (Scolnik & Eliceiri, 1979). The modification of r-proteins appears to occur on mature ribosomes and may modify its activity. The degree of S6 phosphorylation, for example, tends to reflect the metabolic state of the cell (Thomas, Siegmann, Kubler, Gordon & de Asua, 1980).

The genes of some r-proteins number up to 20 copies in the rat and are found scattered over the entire genome (Monk, Meyuhas & Perry, 1981 and Faliks & Meyuhas, 1982). Despite the multiplicity of r-protein genes, only one gene appears to be transcribed while the rest are silent (pseudogenes) (Dudov & Perry, 1984). The primary hnRNA transcripts for r-proteins are large and undergo several processing steps in the nucleus (Meyuhas & Perry, 1980). The final mRNA's generally are 50% larger than the length of the coding sequence.

The r-protein mRNA's are monocistronic (Nabeshima, Imai & Ogata, 1979) and are translated predominantly on 'free' cytoplasmic polysomes (Nabeshima, Tsurugi & Ogata, 1975). The newly translated r-protein product is transported to the nucleolus where it is incorporated into the maturing ribosome (Warner, 1974 and Maden & Salim, 1974). Although un-incorporated r-proteins are concentrated in the nucleolus, virtually no cytoplasmic pool of r-proteins can be detected (Wool & Stoffler, 1976). No specific mechanism

for the transport of r-proteins has been demonstrated (Warner et al., 1980). In studying the assembly of ribosomes, it has been observed that only newly synthesized r-proteins are incorporated into the ribosome in vivo and are generally not recycled (Tsurugi, Morita & Ogata, 1974).

iii) Ribosomal RNA

Three of the rRNA's (28s, 18s and 5.8s) are transcribed as a single 45s rRNA precursor in the nucleolus by RNA polymerase I. In the rat, the rRNA genes number approximately 150 tandem repeats (Brimacombe & Kirby, 1968) but can number as high as 20,000 in some amphibia (Sinclair & Brown, 1971). Portions of the rat liver rRNA genes have been sequenced (Rothblum et al., 1982 and Subrahmanyam, Cassidy, Busch & Rothblum, 1982). The 45s pre-rRNA, even as it is being transcribed, is specifically methylated in the sequences destined to become mature rRNA's (Maden & Salim, 1974 and Liau & Hurlbert, 1975). Indeed, if methylation does not occur, the 45s precursor is degraded (Vaughan, Soeiro, Warner & Darnell, 1967).

The 5s rRNA is transcribed by RNA polymerase III. The 5s rRNA genes are located outside the nucleolus (McReynolds & Penman, 1974) and are arranged in tandem repeats of approximately 830 copies in the rat (Quincey & Wilson, 1969). Significant processing of the 5s rRNA does not appear to occur (Perry, 1976) and the rRNA is quickly

incorporated into the 90s pre-ribosomal particle (Winicov, 1975).

The 45s or larger pre-rRNA is processed in the nucleolus (Dabev, Dudov, Hadjiolov & Stoykova, 1978). Four major cleavage events occur, as diagrammed in Figure 1-1 (Perry, 1976, Hadjiolov & Nikolaev, 1976 and Hadjiolov, 1977). The cleavages are generally sequential events starting from the 5' end and, with subsequent trimming, eventually eliminate approximately 50% of the primary rRNA transcript during the maturation process (Perry, 1976). In most eukaryotic systems, no splicing of the pre-rRNA's occurs.

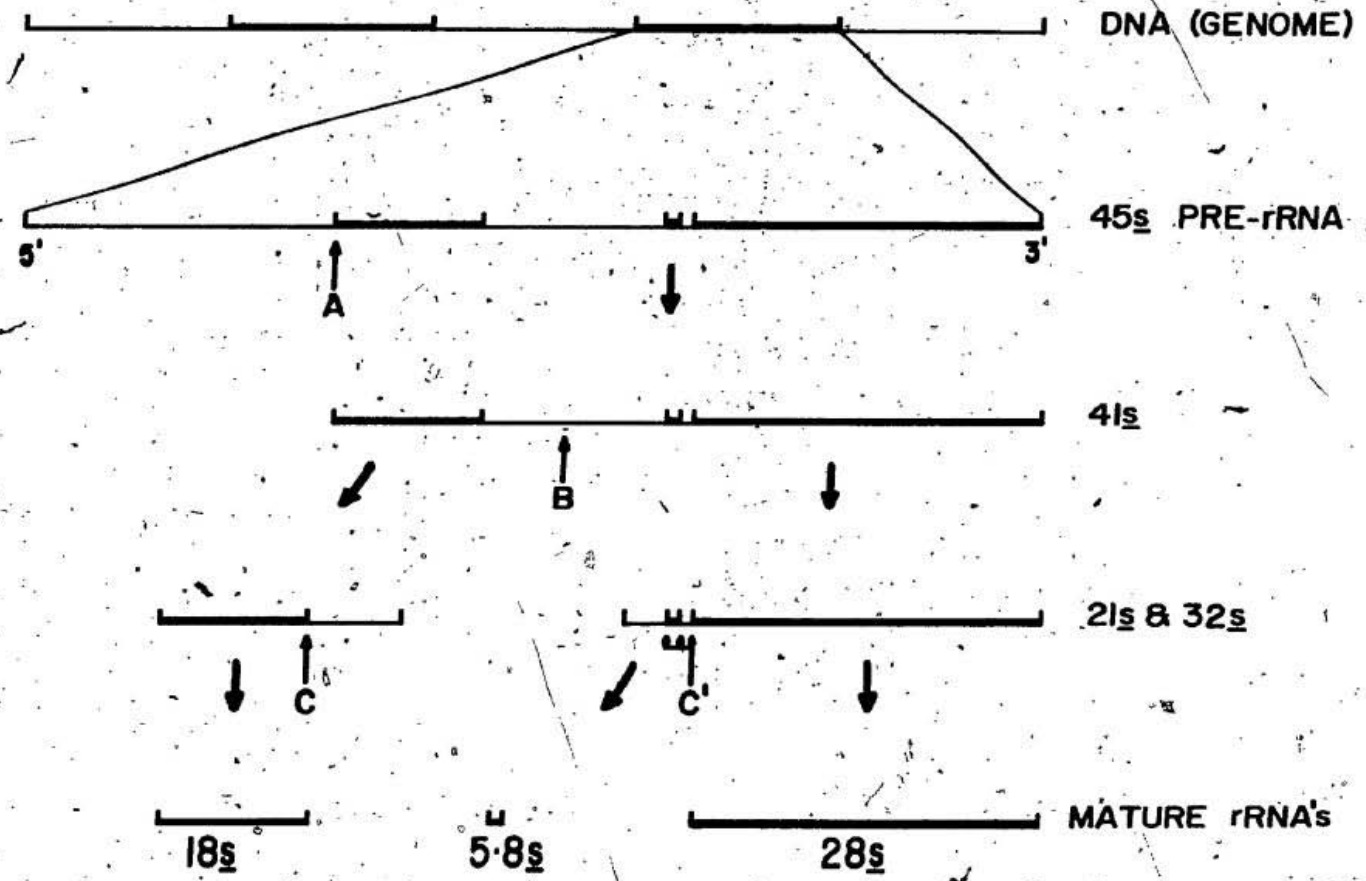
The processing of the 45s pre-rRNA's has several potential control points. During ribosome maturation in rat liver, significant pools of partially processed rRNA can accumulate in the nucleolus (Rizzo & Webb, 1972). In a cell free pre-rRNA processing system, nuclei, which supported in vivo like processing and transport, maintain the 32s and 28s rRNA precursor pools until the 45s rRNA precursor pool is depleted (Schumm, Niemann, Payaloort & Webb, 1979).

The processing of the 45s pre-rRNA to the final 28s, 18s and 5.8s rRNA's requires the presence of the r-proteins and other nuclear specific proteins (Craig & Perry, 1970). Indeed, a large number of the r-proteins and nuclear specific proteins decorate the pre-rRNA transcript as it is being transcribed (Wool, 1979 and McKnight, Hipskind &

Figure 1-1.

Scheme for Ribosomal RNA Processing.

The common cleavage sites of mammalian pre-rRNA are indicated by arrows lettered in the most common order of cleavage. The top line represents tandem repeats of the rRNA gene, with the heavy lines representing the transcribed portion. The subsequent lines indicate the various stages of processing of the RNA transcript most commonly used in the rat liver, with the heavy lines indicating the final rRNA products. Lines are not to scale. Modified from Hadjiolov & Nikolaev (1976) and Hadjiolov (1977).


DNA (GENOME)
45s PRE-rRNA
5'
3'
A
41s
B
21s & 32s
C
C'
MATURE rRNA's
18s
5·8s
28s

Reeder, 1980) and appear to be added in a specific order (Todorov, Noll & Hadjiolov, 1983).

iv) Coordinate Regulation of Ribosomal Proteins and Ribosomal RNA

The regulation of ribosome biogenesis requires the orchestration of the synthesis, processing and transport of its components. Synthesis occurs in three areas of the cell: the r-proteins in the cytoplasm, the 5.8s, 18s and 28s rRNA's in the nucleolus and the 5s rRNA in the nucleoplasm. Not only are these areas physically distinct, but a permeability controlled nuclear membrane interposes the system. The requirements for the regulation of the ribosomal components are therefore diverse and must be coordinate for efficient cell growth and maintenance (Warner et al., 1980).

a) The Regenerating Rat liver

The rat liver has been the system of choice for many workers who study ribosome production for several reasons. It is a large and easy tissue to manipulate, it responds quickly to major assaults such as poisons, starvation and surgery and it has a high and modulated potential for protein synthesis (Bucher & Malt, 1971). Furthermore, many of the results obtained from the study of the regulation of ribosome production in the rat liver appear to be applicable to most other mammalian systems.

The mechanisms of coordinate regulation of a system can be elucidated by perturbing the system in some manner and then observing how the system compensates for the perturbation. Most studies with the coordinate regulation of ribosome production employ partially hepatectomized rats. After approximately 70% of the liver is removed from the animal, the remaining liver responds with massive hypertrophy for the first 6 to 12 hours. DNA synthesis occurs by 24 hours followed by mitosis and cytokinesis by 30 hours (Bucher & Malt, 1971). The number and the rate of appearance of ribosomes increases during the first 12 hours after partial hepatectomy (Tsurugi, Morita & Ogata, 1972). Tsurugi et al (1972) also recorded that the net rate of synthesis of the RNA and protein moieties of the ribosome are coordinately elevated during the early phases of liver regeneration.

Further analysis of the r-proteins reveal that they are produced in stoichiometric amounts, both in vivo and when total mRNA from regenerating rat livers is translated in vitro under saturating conditions (Nabeshima et al., 1979). The increased specific translation of r-proteins results from a 2 to 3 fold increase in the concentration of r-protein specific mRNA's (rp-mRNA's), rather than from a higher efficiency of their translation (Nabeshima & Ogata, 1980). More recently, Faliks & Meyuhas (1982) have shown with specific rp-mRNA cDNA probes that cellular levels of 6

rp-mRNA's (S16, L7, L13, L18, L30 and L32) coordinately increase by approximately 2 fold by 12 hours following partial hepatectomy, before falling to control values by 48 hours..

The increased rate of appearance of ribosomes in the regenerating rat liver is also influenced by post-transcriptional events. In a detailed study of ribosomal precursors, it was found that the relative amounts of the various ribosomal precursors in the nucleolus differ between resting and regenerating rat liver cells (Dudov & Dabeva, 1983). As noted earlier, maturation of pre-rRNA can undergo a variety of intermediate processing steps (Dabeva et al., 1978). In the regenerating rat liver, many of the higher molecular weight rRNA precursors do not accumulate significantly (Dudov & Dabeva, 1983). Instead, the 45s rRNA precursor is quickly processed to the 32s rRNA before the final 28s rRNA product, while the 18s rRNA appears directly from the 45s rRNA precursor (see Figure 1-1). In addition, the rate of ribosome processing increases by approximately 100% and the transport of mature ribosomal subunits from the nucleus is accelerated. Generally, the increased rate of appearance of ribosomes in the rat liver during regeneration results from enhanced coordinate transcription, translation and processing of its various components. Thus, the increased demand for new ribosomes in the regenerating rat liver is met with little wastage and tight coordination.

b) Other Rat Liver Studies

The regulation of rRNA and r-protein synthesis in the rat liver has also been investigated with the aid of transcription and translation inhibitors. These studies were designed to dissect the coordinate regulation of pre-rRNA and r-protein synthesis. Low levels of Actinomycin D specifically inhibit the transcription of rRNA while permitting mRNA and protein synthesis to proceed (Wilson & Hoagland, 1967). Treatment of regenerating rat liver with low levels of Actinomycin D *in vivo* inhibits pre-rRNA transcription but not r-protein translation (Tsurugi & Ogata, 1979). Instead, the r-proteins are synthesized at rates similar to controls, imported into the nucleolus and quickly degraded by a thiol protease (Tsurugi, Oyanagi & Ogat, 1983). The drug Actinomycin D therefore uncouples the normally coordinate synthesis of rRNA and r-proteins, resulting in the unscheduled degradation of the latter.

After administration of low levels of Actinomycin D to the rat liver, r-protein synthesis continues unabated for up to 12 hours (Tsurugi *et al.*, 1983). If the half-lives of the rp-mRNA's are not unusually long (and the kinetics of r-protein transcription suggest that they are not (Toku, Nabeshima & Ogata, 1983)), it may then be assumed that the transcription rates of the rp-mRNA's are not significantly altered by the inhibition of

rRNA transcription. Also significant is the observation that most of the r-proteins are still synthesized stoichiometrically under these conditions (Toku et al., 1983). Actinomycin D therefore appears to uncouple the coordinate production of rRNA and r-protein at the transcriptional level.

The uncoupling of the r-protein translation with rRNA transcription can also be achieved with the protein synthesis inhibitor, cycloheximide (Muramatsu, Shimada & Higashinakagawa, 1970). The 45s pre-rRNA transcription rate remains unaltered when the synthesis of r-proteins is inhibited by the drug (Stoyanova & Hadjiolov, 1979). However, little of the 45s rRNA yield functional ribosomal subunits since it is degraded for lack of r-proteins and other nuclear proteins required for its proper maturation (Craig & Perry, 1970). It therefore appears that transcription of rRNA is not regulated by high turn-over proteins which would also be absent under these conditions (Stoyanova & Dabeva, 1980).

The coordinate expression of rRNA and r-protein synthesis normally observed in the rat liver can be uncoupled under certain circumstances. Since the inhibition of the synthesis of one component does not significantly alter the synthesis of the other, their regulation occurs independently. However, since their regulation is coordinate under normal circumstances, an unidentified

common regulatory component or system must be involved which is subverted by the drugs Actinomycin D and cycloheximide. The identification of this component is one of the key questions being pursued in the study of ribosome production (Warner et al., 1980).

c) Other Mammalian Studies

The regulation of ribosome biogenesis has been investigated in many other mammalian systems. Specific inhibition of pre-rRNA synthesis with low levels of Actinomycin D or protein synthesis with cycloheximide in these mammalian systems generally yield similar results to those observed in the rat liver. For example, HeLa cells (Warner, 1977), Ehrlich ascites cells (Iapalucci-Espinoza & Franze-Fernandez, 1979) and mouse 3T3 cells (Tushinski & Warner, 1982) treated with low levels of Actinomycin D have severely repressed rRNA precursor transcription rates but nearly normal r-protein translation rates. In all cases, the r-proteins are quickly degraded.

Similarly, inhibition of r-protein translation with cycloheximide does not severely reduce rRNA precursor transcription in HeLa cells (Willems, Penman & Penman, 1969), mouse L cells (Craig & Perry, 1970), cultured lymphocytes (Cooper & Gibson, 1971) or Ehrlich ascites cells (Iapalucci-Espinoza & Franze-Fernandez, 1979). Although the pre-rRNA is degraded in these cases, the pathways have not

been studied in detail (Dudov & Dabeva, 1983).

The coordinate regulation of ribosome production during the conversion of mouse 3T3 and 3T6 cells from a resting to a growing state (nutritional shift-up) provides a different set of conditions for the investigation of ribosome biogenesis. Nutritional shift-up causes a 2 to 3 fold increase in the cellular content of ribosomes, as seen by new rRNA synthesis (Johnson, Abelson, Green & Penman, 1974). In addition, the ribosomes (measured as rRNA) are more stable in growing cells than in resting cells (Abelson, Johnson, Penman & Green, 1974).

The r-proteins in these two fibroblast lines are also synthesized in increased amounts upon nutritional shift-up (Tushinski & Warner, 1982). Analysis of the rp-mRNA metabolism during the nutritional shift-up of 3T6 cells, however, suggests that the total amount of rp-mRNA does not significantly increase (Geyer, Meyuhas, Perry, & Johnson, 1982). In both metabolic states, the amount of general mRNA's associated with the polysomes accounts for 70% of the total mRNA's found in the cell. However, over 85% of the total cellular rp-mRNA's are associated with polysomes in growth stimulated cells while only about 50% of the total cellular rp-mRNA's are associated with translating polysomes in resting cells. Therefore, the increased rate of r-protein synthesis in 3T6 after nutritional shift-up is achieved by altering the size of the translatable rp-mRNA

pool and not by the increase in the total amount of the rp-mRNA. Since increased ribosome production upon growth stimulation occurs with little wastage of either rRNA or r-proteins, coordinated regulation of these components is achieved by a different mechanism than in the regenerating rat liver (Tushinski & Warner, 1982).

Another in vivo example of growth stimulation is provided by contralateral nephrectomy in the mouse. After surgery, the remaining kidney undergoes hypertrophy and an increase in the ribosome pool size to compensate for the loss (Malt & Lemaitré, 1968). However, the increased ribosome pool size is accomplished not by an increased rate of ribosome accumulation as in the regenerating rat liver, but by an increased half-life of the ribosome particle (Melvin, Kumar & Malt, 1980).

Nutritional shift-up or shift-down experiments with transformed cells (such as virally transformed CHO cells or anchorage independent HeLa cells) show that these cells respond to growth stimulation differently than normal cells. Transformed cells produce ribosomes at 'growing cell' levels regardless of their nutritional state, and can die under starvation conditions by an over-production of ribosomes (Stanners, Adams, Harkins & Pollard, 1979). Although these cells have lost the ability to control the rate of production of ribosomes under these conditions, the rates of

pre-rRNA and r-proteins synthesis remain coordinate (Warner, 1977).

From these and other studies, it appears that the coordinate regulation of pre-rRNA and r-protein synthesis can be uncoupled in higher eukaryotes. The mechanism of coordinate regulation must be different from the one found in yeast, for example, which exhibits a tight coordinate regulation of pre-rRNA and r-protein synthesis even when one component is specifically inhibited (Warner et al, 1980). Yeast cells, being single cell eukaryotes, must be able to respond quickly to changes in the environment in order to survive. A directly coupled coordinate regulatory system for the production of ribosomes provides a rapid response to changing nutritional conditions. Multicellular systems, on the other hand, are less sensitive to environmental changes and do not require tight, direct regulation. As seen with the examples discussed above, the indirect coordinate regulation of pre-rRNA and r-protein synthesis is adequate under normal circumstances.

D) Differentiation and Gene Expression

Differentiating cells in culture provide well controlled systems for the study of gene regulation (Kreider & Schmoyer, 1975, McBurney, 1976, Rheinwald & Beckett, 1980 and Tarella, Ferrero, Gallo, Pagliardi & Ruscetti, 1982). However, most studies investigate the regulation of genes

specific to the phenotype of the system and not of the genes involved with the process of differentiation itself. Of equal importance to the induction of specific phenotypic genes is the regulation of such house-keeping genes as those for the ribosomal components.

The requirement for protein synthesis, which dictates the size of the ribosome population in the cell, changes considerably in various differentiating cell types (Warner et al, 1980). In muscle cells, the rate of protein synthesis is high in both the proliferating myoblast and the differentiated myotube forms. Mammalian myotubes are not increasing in size and their ribosomes turn over very slowly (Singer & Kessler-Icekson, 1978), thus their accumulation rates must be modulated upon differentiation of the myoblast (Krauter, Soeiro & Nadal-Ginard, 1979). The myoblast therefore provides an opportunity to study the coordinate regulation of ribosome biogenesis in a differentiating cell system which requires a high protein synthesis capacity in its differentiated state.

The L6 myoblast cell line was chosen as the differentiation model in which to study ribosome metabolism in this thesis for the following reasons:

1) It has been in continuous cell culture since it was cloned (Yaffe, 1968) and expresses a stable phenotype and genotype (Richler & Yaffe, 1970).

2) The L6 myoblasts can be induced to differentiate in a concerted manner without resorting to starvation conditions or exposure to harsh chemicals (Richler & Yaffe, 1970).

3) The L6 myotubes exhibit morphological features of myotubes found *in vivo* (Yaffe, 1968) as well as expressing muscle related products such as myosin heavy chain (Benoff & Nadal-Ginard, 1979 and Medford, Nguyen & Nadal-Ginard, 1983), myosin light chain, muscle (*alpha*-) actin (Shani, Zevin-Sonkin, Saxel, Carmon, Katcoff, Nudel & Yaffe, 1981), creatine phosphokinase, myokinase (Schubert, Tarikas, Humphry, Heinemann & Patrick, 1977), glycogen phosphorylase (Shainberg, Yagil & Yaffe, 1971) and a variety of sarcoplasmic reticulum proteins (Zubrzycka-Gaarn, Campbell, MacLennan & Jorgensen, 1983). (This list is by no means exhaustive since cross-hybridization studies between L6 myoblast and myotube cDNA's revealed thousands of unidentified unique sequences in both myoblasts and myotubes (Merlie, Buckingham & Whalen, 1977, Leibovitch, Leibovitch, Harel & Kruh, 1979 and Zevin-Sonkin & Yaffe, 1980.) In addition, the L6 myotubes exhibit similarities to rat muscle cells *in vivo*, including the formation of functional synaptic junctions with rat neural components (Kidokoro & Heinemann, 1974).

The differentiation of L6 cells proceeds through several steps before the final myotube phenotype appears

(Richler & Yaffe, 1970). It has long been understood that muscle differentiation involves the concurrent cessation of DNA synthesis, the induction of muscle specific genes and cell fusion (Yaffe, 1971). Recent work with L6 and a temperature sensitive mutant of L6 has suggested that differentiation follows at least two consecutive genetic programs. Each time an L6 cell enters the G1 phase of the cell cycle, it either continues with the cell cycle or enters the first differentiation program, the 'commitment' phase (Nadal-Ginard, 1978). During the commitment phase, the cell remains in G1 but begins the differentiation program. The irreversible decision to continue the differentiation program, which is affected by the availability of growth factors in the medium (Pinset & Whalen, 1984), is not taken immediately. The exact point at which the commitment to differentiation appears to be irreversible is after the induction of muscle-specific genes but before cell fusion. This was clearly seen in a temperature sensitive differentiation mutant of L6, where a transient induction of muscle-specific genes without irreversible differentiation was observed (Nguyen, Medford & Nadal-Ginard, 1983). The second genetic program is followed after the commitment to differentiation becomes irreversible and leads to the full expression of the differentiated phenotype.

The metabolic states of the rapidly proliferating myoblasts and the non-dividing myotubes are quite different since the former is pre-occupied with growth while the latter must express the differentiated phenotype. The study of the regulation of a component common to both states, such as ribosome biogenesis, under these circumstances may lead to new insights into the differentiation process. The L6 myoblast provides such a system, in which the differentiation characteristics are well defined but its ribosome metabolism is poorly understood.

E) Objectives

The purpose of this study is to determine the modes of coordinate regulation of the components of the ribosome during ribosome biogenesis in the L6-5 myoblast and how the coordinate regulation is affected by myogenesis.

The main objectives of the thesis are as follows:

1) measurement of the rate of r-protein synthesis and turnover, both en masse and individually in myoblasts and myotubes. These data will be compared with the rate of rRNA synthesis and turnover (objective 2).

2) measurement of the rate of rRNA synthesis and turnover in myoblasts and myotubes. These results with those from objective 1 will indicate the degree of overall and coordinate regulation of ribosome biogenesis and how it is affected by the differentiation of the L6 myoblast.

3) measurement of the relative levels of translating rp-mRNA's in myoblasts and myotubes. These measurements will indicate if the translation of r-proteins is regulated in any unusual fashion when comparing rRNA and r-protein turnover before and after differentiation of the L6 myoblast.

4) measurement of rp-mRNA half lives in myoblasts and myotubes. These measurements will determine if the stability of the rp-mRNA's are affected by the differentiation of the L6 myoblast.

5) measurement of the relative rates of rp-mRNA and rRNA synthesis in myoblasts and myotubes. These measurements will determine if the expression of rRNA and r-proteins are coordinated at the level of transcription and if the coordinate regulation is affected by the differentiation of the L6 myoblast.

6) an ancillary project was undertaken to investigate the properties of a poly(A) containing histone H4 mRNA sub-species, which like the rp-mRNA's, codes for a small basic protein which is imported into the nucleus. The description of this poly(A)-containing mRNA species is unique and is further discussed in Appendix A.

CHAPTER TWO

Materials and Methods

A) Growth and Maintenance of L6-5 Cultures

The L6 myoblast used in these studies was originally obtained from Dr. C.P. Stanners of McGill University, Montreal. The cell line was sub-cloned and a single cell derived clone, L6-5, was chosen for its fast growth and rapid differentiation properties. The cells were grown on plastic tissue culture dishes in Growth Medium (alpha-MEM (alpha-Modified Essential Medium) containing fetal bovine serum (10%, v/v), 5k international units per ml of penicillin, 5 ug per ml of streptomycin and $NaHCO_3$ (0.2%, w/v)). The Growth Medium was sterilized by ultra-filtration through a sterile 0.22 micron filter (Millistack, Millipore). A controlled environment of 37°C, CO_2 (6%, v/v in air) and 100% humidity was provided for the cells.

Replating of the proliferating myoblasts was performed in a sterile laminar flow hood. The tissue culture dish containing the cells was treated with a minimum amount of a sterile solution containing Hank's balanced salts, trypsin (0.25%, w/v), 10 mM EDTA and 10 mM Hepes (pH 7.2) at 37°C for 3 minutes. The cells released were diluted with Growth Medium to approximately 2×10^5 cells per ml and plated onto new tissue culture dishes (25 ml of cell suspension per 15 cm diameter tissue culture dish). The cells attached to

the dish within 30 minutes and proceeded to grow with little detectable lag. The mean generation time of the L6-5 myoblast was approximately 16 hours. The cells were fed fresh Growth Medium every second day. At confluency, a 15 cm diameter tissue culture dish contained approximately $2x10^7$ cells.

Differentiation of L6-5 myoblasts was initiated when the myoblasts were 70% confluent by changing the Growth Medium to Differentiation Medium (alpha-MEM containing donor horse serum (2.5%, v/v), 5 international units per ml of penicillin, 5 ug per ml of streptomycin and $NaHCO_3$ (0.2%, w/v)). The Differentiation Medium was changed every second day. Fully differentiated myotubes were obtained within 4 days. At this time, more than 90% of the nuclei were syncytial.

Stocks of L6-5 cells were maintained in liquid nitrogen or in a -70°C freezer. These stocks were prepared by centrifugation (1kxg for 3 minutes) of freshly trypsinized myoblasts and resuspending the pelleted cells in Growth Medium containing dimethyl sulphoxide (10%, v/v) at a concentration of $5x10^6$ cells per ml. One ml aliquots of the cell suspension were placed in 2 ml cryo-vials and immersed in ice for 30 minutes. The vials were then wrapped in several layers of paper towel and placed at -70°C overnight before being removed to a liquid nitrogen freezer.

To revive a frozen stock of L6-5 myoblasts, a cryo-vial containing the cells was quickly thawed and centrifuged (1kxg for 3 minutes). The pelleted cells were resuspended in Growth Medium and plated onto a plastic tissue culture dish. Each 1 ml stock was used to seed a single 15 cm diameter tissue culture dish. Survival of the cells upon recovery was normally 60 to 90%.

All tissue culture materials were obtained from Flow Laboratories.

B) Determination of DNA, RNA and Protein Accumulation Rates

The rates of DNA, RNA and protein accumulation in L6-5 cells were determined by measuring the incorporation of the appropriate radioactively labelled precursor into TCA insoluble material. L6-5 cells at various stages of differentiation were labelled for 2 hours with 2 uCi per ml of [^{3}H]thymidine (greater than 10 Ci per mmol), 5 uCi per ml of [^{3}H]uridine (greater than 35 Ci per mmol) or 20 uCi per ml of [^{3}H]lysine (greater than 80 Ci per mmol) (all isotopes obtained from New England Nuclear). The cells were lysed with a solution containing Nonidet P-40 (0.5%, v/v), 10 mM Tris HCl (pH 7.6), 100 mM NaCl and 5 mM Mg acetate and the concentration of the nuclei in the lysate was determined with a hemocytometer. SDS from a stock solution (20%, w/v) was added to the lysate to a final concentration of 1% (w/v)

before the lysate was homogenized by several passages through a tuberculin syringe. Aliquots of the homogenate were spotted onto filter paper discs (2.4 cm diameter, #540, Whatman) and allowed to dry. The filters were washed twice in 100 ml of a solution containing TCA (20%; w/v), thymidine, uridine and lysine (0.1% each, w/v) over a period of 15 minutes. The filters containing samples labelled with [^{3}H] lysine were given an intermediate wash with TCA (20%, w/v) at 95°C for 5 minutes. The filters were then briefly rinsed with ethanol, washed with ether for 15 minutes and rinsed again in ethanol. After the filters had been dried, they were placed with 10 ml of a solution containing Omnifluor (20%, w/v) dissolved in toluene and the radioactivity was determined by liquid scintillation counting.

C) Preparation of Subcellular Fractions of L6-5

Differential centrifugation was used to obtain nuclear, polysomal and post-polysomal fractions from L6-5 lysates. A 15 cm diameter tissue culture dish of L6-5 cells was rinsed with 3 ml of an ice-cold solution of Hepes Buffered Saline (HBS) containing 10 mM Hepes (pH 7.2) and 150 mM NaCl and lysed with 1.5 ml of Lysis Buffer containing 250 mM NaCl, 10 mM Tris HCl (pH 7.6), 5 mM Mg acetate, Nonidet P-40 (0.5%, v/v), 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM aurintricarboxylic acid, Emetine hydrochloride (0.02%, w/v),

10 mM beta-mercaptoethanol and 5 units per ml Human Placental RNase Inhibitor (Amersham). The lysate was homogenized with a tight fitting glass Dounce homogenizer until the nuclei were judged to be substantially free of attached cytoplasmic debris by microscopic examination. The homogenate was layered over 0.5 volumes of a solution containing glycerol and Lysis Buffer (0.25:0.75) and centrifuged (2kxg for 15 minutes, Busch & Daskal, 1980). The nuclear pellet thus obtained was resuspended in 0.05 volumes of the original homogenate volume of Lysis Buffer. Polyvinylsulphate (PVS) (0.01 volumes of a stock solution (1%, w/v)) and DNase I (0.002 volumes of a 100,000 units per ml stock solution) were added to the suspension. The nuclear suspension was incubated at 37°C for 1 hour before the addition of SDS (0.05 volumes of a stock solution (20%, w/v)). The digest was stored at -70°C.

To prepare the polysomal and post-polysomal fractions, the post-nuclear supernatant was further centrifuged (10kxg for 20 minutes) to obtain a post-mitochondrial supernatant. After the addition of PVS (0.01 volumes of a stock solution (1%, w/v)), the post-mitochondrial supernatant was layered over 0.25 volumes of a solution containing sucrose (30%, w/v) and 0.1 mg per ml of PVS dissolved in Lysis Buffer and centrifuged (176kxg for 70 minutes) to obtain the polysomal pellet (Ogata & Terao, 1979). Two ml of a solution containing 10 mM Tris HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA

and SDS (1%, w/v) were added to the polysomal pellet before the pellet was stored at -70°C. PVS (0.01 volumes of a stock solution (1%, w/v)) was added to the supernatant of the polysomal pellet before it was centrifuged (176kxg for 18 hours) to obtain the post-polysomal pellet (Bag & Sells, 1979a). The pellet was also stored in 2 ml of a solution containing 10 mM Tris HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA and SDS (1%, w/v) at -70°C.

D) Isolation of Ribosomal Subunits from L6-5 Cells

Ribosomes were obtained for analysis from 70% confluent myoblasts and 4 day myotubes which had been incubated for 2 hours in medium containing 2 uCi per ml of [^{3}H]lysine (greater than 80 Ci per mmol, New England Nuclear). Polysomal pellets were prepared from L6-5 cells as described in Section 'C'. However, since the polysomes were later to be dissociated, Emetine hydrochloride was omitted from the Lysis Buffer. The polysomal pellet obtained from one 15 cm diameter tissue culture dish of L6-5 cells was resuspended in 500 ul of a solution containing 10 mM Tris HCl (pH 7.6), 5 mM $MgCl_2$, 0.2 mM Puromycin and Triton X-100 (0.5%, v/v) and incubated at 37°C for 15 minutes. After the addition and dissolution of 30 mg of KCl, the sample was centrifuged (10kxg for 5 minutes) and the supernatant was layered on an iso-kinetic sucrose gradient.

Iso-kinetic gradients of 15 to 30% (w/v) sucrose were prepared with an exponential gradient maker (Model XPO77, Hoefer Scientific Instruments) (Noll, 1967). The reservoir chamber of the gradient maker contained 35 ml of a solution containing sucrose (32.5%, w/w), 10 mM Tris HCl (pH 7.6), 5 mM $MgCl_2$ and Triton X-100 (0.5%, v/v). The mixing chamber contained 39.6 ml of a solution containing sucrose (15%, w/w) dissolved in the same buffer. A 35 ml 'SW27' polyallomer centrifuge tube (Beckman Instruments) was filled from the bottom with the sucrose gradient and cooled to 2°C before the sample was layered on top.

After centrifugation in a Beckman 'SW27' rotor (27krpm (132kxg) at 2°C for 12 hours), the gradient was sampled from the top through a flow-cell in a spectrophotometer, monitoring the absorbance at 260 nm. Fractions of 400 ul were collected and the radioactive content of a 5 ul sample was determined by liquid scintillation counting.

E) Preparation of Ribosomal Proteins

i) Ribosomal Proteins from L6-5 Cells

Ribosomal proteins were extracted from whole lysates of L6-5 cells for analysis by 2 dimensional gel electrophoresis (Krauter, Soeiro & Nadal-Ginard, 1980). A 15 cm diameter tissue culture dish of L6-5 cells which had been incubated for 54 hours in medium containing 5 uCi per ml of [^{14}C] lysine (greater than 0.3 Ci per mmol) and for 30

minutes in medium containing 200 uCi per ml of [^{3}H]lysine (greater than 80 Ci per mmol, both radioisotopes from New England Nuclear) was briefly rinsed with 3 ml of ice-cold HBS and lysed with 1.5 ml of Lysis Buffer (Section 'C'). After the lysate was homogenized with a Dounce homogenizer, DNase I (0.001 volumes of a 100,000 units per ml stock solution) was added and the homogenate was incubated at 37^{o}C for 1 hour. The homogenate was then slowly acidified by the addition of HCl (0.1 volumes of a 2.5 M stock solution). The acidified suspension was placed on ice for 1 hour and then centrifuged (10kxg for 20 minutes). To the supernatant, 5 volumes of acetone were added and the solution was stored at -20^{o}C overnight. After centrifugation (10kxg for 20 minutes), the pellet was washed twice with 95% ethanol and dried on ice under vacuum. The pellet was immediately resuspended in the Sample Buffer used for the first dimension of the 2 dimensional gel electrophoresis system for r-proteins (Section 'F-i') at a concentration of approximately 1x10^{8} cpm per ml and stored at -70^{o}C.

ii) Ribosomal Proteins from Rat Liver

Rat liver r-proteins were used as markers for the localization of the labelled L6-5 r-proteins in the 2 dimensional gel electrophoresis system for r-proteins (Section 'F'). Ribosomal proteins from rat liver were

prepared from rat liver polysomes (Ogata & Terao, 1979). Livers, obtained from 250 g male Sprague Dawley rats which had been fasted for 18 hours, were immediately placed in Lysis Buffer (Section C) (5 ml per gm wet tissue weight. After the liver was minced with a pair of scissors, it was homogenized with a Potter Elvehjem homogenizer in a motor driven Teflon pestle at 200 rpm for 2 minutes. The homogenate was centrifuged (10kxg for 10 minutes) and the supernatant carefully decanted and stored on ice. The pellet was resuspended in the original volume of Lysis Buffer and was homogenized and centrifuged as before. The supernatant was decanted and combined with the previous supernatant stored on ice. To the combined supernatants, KCl (0.3 volumes of a 2.5 M stock solution) and deoxycholate (0.15 volumes of a freshly prepared stock solution (10%, w/v)) were added. The mixture was then layered over 0.25 volumes of a solution containing sucrose (30%, w/v) dissolved in Lysis Buffer. After centrifugation (176kxg for 70 minutes), the pellet was resuspended in the original tissue weight equivalence of 1 volume of Lysis Buffer.

To calculate the yield of polysomes obtained, the following formula was applied to account for the ferritin contamination in rat liver (Wilson & Hoagland, 1965):

$$OD_{260} - (1.59) \times (OD_{320}) = OD_{260}\text{net}$$

One OD_{260}net unit (1 cm path length) of polysomes was equivalent to 80 ug of

polysomes per ml. Yields of polysomes were approximately 5 mg per gm of original rat liver.

Ribosomal proteins were prepared from rat liver polysomes by a procedure modified from Welfle et al., (1978). Polysome suspensions of approximately 5 mg per ml in Lysis Buffer were slowly acidified with the addition of HCl (0.1 volumes of a 2.5 M stock solution) and further purified and prepared as described above for L6-5 r-proteins (Section 'E-i'). Rat liver r-proteins were dissolved in Sample Buffer for the first dimension of the 2 dimensional gel electrophoresis system for r-proteins (Section 'F-i') at a concentration of approximately 20 mg per ml and stored at -70°C.

F) Two Dimensional Gel Electrophoresis of Ribosomal Proteins

Ribosomal proteins were separated on a 2 dimensional polyacrylamide gel electrophoresis system (Kaltschmidt & Wittmann, 1969, Lastick & McConkey, 1976 and Ogata & Terao, 1979).

i) First Dimension

Electrophoresis of r-proteins in the first dimension was carried out in a tube gel apparatus (Model GT-3, Hoefer Scientific Instruments). The first dimension Running Gel contained 6 M urea, 0.5 M boric acid, 0.4 M Tris base, 20 mM EDTA, acrylamide (8%, w/v), N,N'-methylene-bis-acrylamide

(0.2%, w/v) and TEMED (0.3%, v/v) and was adjusted to pH 8.6. The Stacking Gel contained 6 M urea, 0.1 M boric acid, 2 mM EDTA, acrylamide (3%, w/v), N,N'-methylene-bis-acrylamide (0.2%, w/v) and TEMED (0.4%, v/v) and was adjusted to pH 6.7. The Reservoir Buffer contained 0.15 M boric acid, 0.12 M Tris base and 3 mM EDTA and was adjusted to pH 8.2. The Sample Buffer contained Stacking Gel buffer, sucrose (25%, w/v) and beta-mercaptoethanol (1%, v/v).

The first dimension gel was cast in a siliconized glass cylinder (0.3 cm id x 12 cm), which contained by height 90% Running Gel, 5% Stacking Gel and 5% sample space. After degassing 10 ml of Running Gel under vacuum for 5 minutes, ammonium persulphate (10 ul of a freshly prepared stock solution (10%, w/v)) was added. The Running Gel was quickly pipetted into a glass cylinder with care to avoid introduction of air bubbles. The Running Gel was overlayed with 50 ul of butanol and allowed to polymerize overnight. The Stacking Gel (1 ml) was similarly degassed before the addition of flavin mononucleotide (10 ul of a stock solution (1%, w/v)). After removal of the butanol overlaying the polymerized Running Gel, the Stacking Gel was pipetted into the glass tube and polymerized by exposure to intense ultraviolet light for 1 hour.

After assembly of the tube gel apparatus with the Reservoir Buffer in place, samples of 400 ug rat liver carrier r-proteins and more than 1×10^6 cpm of labelled L6-5

r-proteins in a total volume of 20 to 50 ul of Sample Buffer were layered on top of the Stacking Gel. In a separate gel, 10 ul of Sample Buffer containing 40 ug of horse cytochrome C (TCA free grade, Sigma Chemicals) were loaded to serve as a visual marker protein (Leader, 1980). Electrophoresis proceeded towards the cathode at 0.5 mA per gel for 2 hours to stack the gel and then at 2 mA per gel until the cytochrome C marker was within 1 cm of the bottom of the gel. After lubricating the inside wall of the glass cylinder containing the gel with a solution of Triton X-100 (1%; v/v), the gel was extruded from the glass cylinder into 25 ml of a solution containing acetone (70%, v/v) and placed at -20°C overnight. The gel was then placed into 25 ml of a solution of acetone containing 100 mM beta-mercaptoethanol and allowed to completely dehydrate at -20°C for at least 12 hours.

ii) Second Dimension

Electrophoresis in the second dimension was carried out in a slab gel apparatus (Model SE 600, Hoefer Scientific Instruments). The second dimension Running Gel contained 6 M urea, 48 mM KOH, 0.9 M acetic acid, acrylamide (15%, w/v), N,N'-methylene-bis-acrylamide (0.5%, w/v) and TEMED (0.3%, v/v) and was adjusted to pH 4.6. The Stacking Gel contained 3 M urea, 24 mM KOH, 26 mM acetic acid, 10 mM beta-mercaptoethanol and agarose (1%, w/v) and was adjusted

to pH 5.2. The Reservoir Buffer contained 0.19 M glycine and was adjusted to pH 5.2 with acetic acid.

Running Gel (100 ml) was degassed for 5 minutes before the addition of ammonium persulphate (1 ml of a freshly prepared stock solution (30%, w/v)). The Running Gel was then pipetted into the slab gel form. While the Running Gel polymerized, the dehydrated first dimension gel was rehydrated in a solution containing Stacking Gel (lacking agarose) and 100 mM beta-mercaptoethanol for 30 minutes. The first dimension gel was then cemented onto the top of the second dimension slab gel with the Stacking Gel containing molten agarose. This formed a stacking gel of several mm once the agarose had solidified. Saturated $FeCl_3$ in glycerol (50%, v/v, 10 ul) was layered over the stacking gel to serve as a visual tracking dye for the electrophoresis of the second dimension (Bernabeu, Martinez, Vazquez & Ballesta, 1979). Electrophoresis was performed towards the cathode at 6 V per cm until the tracking dye reached the bottom of the gel. The slab gel was then removed and prepared for transfer to a nitrocellulose membrane (Western Blot).

iii) Western Blot

To analyze the r-proteins separated on the 2 dimensional gel system for r-proteins, the r-proteins were electrophoretically transferred (electro-blotted) onto a

nitrocellulose membrane (Type HA, Millipore) (Towbin, Staehelin & Gordon, 1979). Upon completion of electrophoresis of the second dimension, the slab gel was soaked in Transfer Buffer containing ethanol (20%, v/v) and acetic acid (1%, v/v) for 30 minutes. The gel was then assembled in a sandwich in the following order: 1 Scotch-Brite pad (3M), 2 layers of chromatography paper (3MM, Whatman), 1 nitrocellulose sheet (Type HA, Millipore), slab gel, 2 layers of chromatography paper and 1 Scotch-Brite pad, all cut to the same size and all pre-soaked for 15 minutes in Transfer Buffer. The sandwich was then placed in an Electroblot Chamber (E-C Apparatus). Transfer occurred at a potential of 10 V per cm towards the cathode at room temperature for 1 hour with constant recirculation of the Transfer Buffer.

The nitrocellulose membrane was then stained with a filtered solution containing amido black (0.1%, w/v), ethanol (40%, v/v) and acetic acid (10%, v/v) for 5 minutes and destained in 3 changes with a solution containing ethanol (90%, v/v) and acetic acid (1%, v/v) over 1 hour. The individual stained r-proteins were identified by comparison with a standard map of rat liver r-proteins (McConkey et al., 1979). The individual r-protein spots were removed with a paper punch to scintillation vials. The membranes were allowed to dissolve overnight in 10 ml of

Beckman HP Fluor before being counted in a Beckman 'LS9000' liquid scintillation counter using a 'Dual Label Data Reduction Package'. The results obtained were dpm values for [^{3}H] lysine and [^{14}C] lysine, corrected both for quench and channel spillover.

G) Purification of RNA from L6-5 Cells

The procedures used for RNA purification and analysis were modified from Maniatis, Fritsch & Sambrook (1982) unless otherwise noted.

i). RNA from Subcellular Fractions

RNA was purified from the nuclear, polysomal and post-polysomal fractions obtained from L6-5 lysates (Section 'C'). Each fraction, which contained 2 ml of a solution containing 10 mM Tris HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA and SDS (1%, w/v), was digested with Proteinase K (500 ug per ml) at 37^{o}C until the pellets were dissolved. The digests were then extracted with phenol and chloroform in 15 ml screw-cap pyrex tubes at room temperature (Palmiter, 1974). Phenol (1 volume, Ultrapur, Bethesda Research Laboratories), which was first equilibrated with Tris base (0.1 volume of a 1 M stock solution), was added to the digest and the mixture was gently agitated for 5 minutes. Chloroform (1 original volume) was then added and, after brief mixing, the phases were separated by centrifugation (2kxg for 5 minutes). The organic phase was

carefully removed and discarded, leaving the interface material behind. The aqueous phase was extracted 2 more times with chloroform (1 volume). After the final chloroform extraction, the aqueous phase was removed, again leaving the interface material behind and the RNA in the aqueous phase was precipitated by the addition of ammonium acetate (0.5 volumes of a 7.5 M stock solution) and ethanol (2 volumes) and stored at -20°C covernight (Maxam & Gilbert, 1980).

RNA content and purity were assessed spectrophotometrically by measuring absorbances of the RNA samples dissolved in a solution containing 10 mM Tris HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl and SDS (1%, w/v) at 260, 280 and 320 nm. The amount of RNA was calculated using the following formula:

$$(OD_{260}-OD_{320})x40 = \text{ug per ml.}$$

An $OD_{260}:OD_{280}$ ratio of 2 was considered to indicate pure RNA.

ii) RNA from Whole Cells

RNA was prepared from whole cells by a total denaturation procedure (Glisin, Crkvenjakiv, & Byus, 1974 and Ullrich, Shine, Chargwin, Pictet, Tischer, Rutter & Goodman, 1977). A 15 cm diameter tissue culture dish of cells was rinsed with 3 ml of ice-cold HBS and lysed in 2 ml of a solution containing 6 M guanidinium isothiocyanate

(Fluka Chemicals), Sarkosyl (1%, w/v), 50 mM Tris HCl (pH 7.6), 0.1 M beta-mercaptoethanol and $CsCl_2$ (40%, w/v). The lysate was gently homogenized in a tight fitting Dounce homogenizer to shear the DNA before being layered over a 2 ml cushion containing $CsCl_2$ (97%, w/v or r.i.=1.71) and 10 mM Tris HCl (pH 7.5). After centrifugation (147kxg for 20 hours at 20°C), the RNA pellet was dissolved in 300 ul of a solution containing 10 mM Tris HCl (pH 7.6), 1 mM EDTA and SDS (1%, w/v) and precipitated with ammonium acetate (150 ul of a 7.5 M stock solution) and ethanol (1 ml) and stored at -20°C overnight.

iii) Oligo(dT)-Cellulose Chromatography of RNA

Poly(A) enriched RNA was prepared from purified RNA samples by oligo(dT)-cellulose chromatography (Bantle, Maxwell & Hahn, 1976). Oligo(dT)-cellulose (approximately 200 mg, Type III, Collaborative Research) was first decanted of its fines in a solution containing 10 mM Tris HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA and SDS (1%, w/v) before being pipetted into a 0.7 cm id x 10 cm column. The column was washed with 1 column volume of a solution containing 0.2 M NaOH, followed by 10 column volumes of Binding Buffer containing 10 mM Tris HCl (pH 7.6), 500 mM NaCl, 10 mM EDTA and SDS (1%, w/v).

The RNA sample to be chromatographed was dissolved in a solution containing 10 mM Tris HCl (pH 7.6), 1 mM EDTA and

SDS (1%, w/v) at a concentration of no greater than 1 mg per ml. The sample (up to 2 mg) was heated to 65°C for 1 minute before the addition of NaCl (0.1 volume of a 5 M stock solution). The sample was quickly cooled to room temperature in an ice-water bath and then passed through the oligo(dT)-cellulose column 3 times. The column was washed with 10 bed volumes of Binding Buffer. The sample volume plus the first 2 ml of Binding Buffer wash was kept as the poly(A) poor fraction. The fraction bound to the oligo(dT)-cellulose was eluted with 4 bed volumes of Elution Buffer containing 1 mM EDTA (pH 7.6) and SDS (0.1%, w/v). The column was re-equilibrated with 5 bed volumes of Binding Buffer before the next sample was applied.

The eluted poly(A) enriched RNA fraction was bound to and eluted from the oligo(dT)-cellulose column two more times, although the unbound fractions from these steps were usually discarded. The final bound fraction was eluted and the RNA precipitated with 2 volumes of ethanol and stored at -20°C overnight.

H) Electrophoresis of RNA

RNA samples of up to 25 ug were subjected to electrophoresis in an agarose (1.2%, w/v) gel. The gel buffer contained 25 mM Mops (pH 7.4), 5 mM Na acetate and 2 mM EDTA. Samples of RNA were precipitated in 0.5 ml Eppendorf tubes with ammonium acetate (0.5 volumes of a

7.5 M stock solution) and ethanol (2 volumes) and stored at -20°C overnight. The pellets were rinsed once with 70% ethanol and dissolved in 20 ul of Sample Buffer containing freshly deionized formamide (50%, v/v, Section 'K-iii'), gel buffer (25%, v/v) and formalin (25%, v/v) and heated at 60°C for 15 minutes. Tracking dye (5 ul) containing glycerol (50%, v/v), bromophenol blue (0.1%, w/v) and xylene cyanol (0.1%, w/v) was added to the samples before they were loaded onto the gel. Electrophoresis proceeded towards the anode at a potential of 5 V per cm until the bromophenol blue dye traversed 80% of the gel. The agarose gel was then placed in 5 volumes of an aqueous solution containing 2 ug per ml of ethidium bromide to stain the RNA. The gel was subsequently photographed with an MP-4 Polaroid Land camera (Polaroid Corp.) fitted with a Wratten #42 filter using ultraviolet transillumination.

I) Plasmid DNA's

Plasmid cDNA and genomic clones obtained from a variety of sources were used for hybridization with specific RNA species. The plasmids and some of their relevant properties are listed in Table 2-1. Methods used for the preparation of DNA plasmids were similar to those outlined by Maniatis et al. (1982) unless otherwise indicated.

Table 2-1. Recombinant Plasmid DNA's.

Recombinant Plasmid	Designation	Source	Probe for	Type	Vehicle	Drug Marker	Restriction Enzyme
pS16[a]	p9	Mouse	rp-S16	cDNA	pMB9	Tet	Bam Hl
pL10[a]	p31	Mouse	rp-L10	cDNA	pMB9	Tet	Bam Hl
pL18[a]	p60	Mouse	rp-L18	cDNA	pMB9	Tet	Bam Hl
pL30[a]	p50	Mouse	rp-L30	cDNA	pMB9	Tet	Bam Hl
pL32[a]	p24	Mouse	rp-L32	cDNA	pMB9	Tet	Bam Hl
pgL32[b]	p3AH3.4	Mouse	rp-L32	genomic	pBR322	Amp	Bam Hl
pMHC[c]	p24	Chick	MHC	cDNA	pBR322	Amp	Eco Rl
pH4[d]	pF0108A	Human	histone H4	Genomic	pBR322	Amp	Eco Rl
p-pre18s[e]	p2.0	Rat	pre-18s rRNA	Genomic	pBR322	Amp	Eco Rl
p-pre28s[e]	p59	Rat	pre-28s rRNA	Genomic	pBR322	Amp	Eco Rl

a) Meyuhas & Perry (1980).

b) Dudov & Perry (1984).

c) Freyer & Robbins (1983).

d) Plumb, Stein & Stein (1982).

e) Subrahmanyam, Cassidy, Busch & Rothblum (1982).

Drug markers: Tet Tetracyclin, 15 ug per ml.
Amp Ampicillin, 50 ug per ml.

i) Growth and Maintenance of Bacterial Plasmid Stocks

A hybrid strain of Escherichia coli, HB101, was used as a host for the production of plasmids (Boyer & Rouland-Dussoix, 1969). The bacteria were grown at 37°C with vigorous agitation in autoclaved Luria-Bertani (LB) medium containing 125 mM NaCl, yeast extract (0.5%, w/v) and tryptone (1%, w/v) which was adjusted to pH 7.2.

The growth vessels were screw capped erlenmeyer flasks with a test-tube side arm which allowed the growth rate of the bacteria to be directly monitored with a Klett meter (Klett Manufacturing Corp.) fitted with a #52 green filter.

ii) Transformation of HB101 Bacteria with Plasmids

HB101 bacteria were transformed with recombinant DNA plasmids to generate large amounts of plasmid (Mandel & Higa, 1970). To render the bacteria competent for transformation, an 1 ml sample of an overnight (stationary) culture of HB101 bacteria was used to inoculate 100 ml of LB medium. The bacteria were grown to a density of 50 Klett units (approximately 5×10^7 bacteria per ml) before the culture was chilled on ice for 10 minutes. After centrifugation (5kxg for 10 minutes), the pellet was resuspended in 0.5 of the original culture volume of an ice-cold, sterile solution containing 50 mM $CaCl_2$ and 10 mM Tris HCl (pH 8.0) and placed on ice for 15 minutes. After

centrifugation (5kxg for 5 minutes), the bacteria were resuspended in 1/15 of the original culture volume of the same ice-cold sterile solution and dispensed in 0.2 ml aliquots into sterile 1.5 ml Eppendorf tubes. The aliquots were stored at 4°C overnight.

The recombinant DNA plasmid to be used for transformation was dissolved at a concentration of 0.4 ug per ml in a sterile solution containing 10 mM Tris HCl (pH 7.6) and 1 mM EDTA. A 100 ul aliquot of the DNA solution was mixed with 1 aliquot of competent HB101 bacteria and stored on ice for 30 minutes. The mixture was then heated to 42°C for 2 minutes before 1 ml of LB medium at 37°C was added. After incubation at 37°C for 30 minutes, the bacterial culture was plated as a lawn on an LB plate (LB medium containing agar (1.5%, w/v) and a suitable antibiotic selection marker (see Table 2-1) in a 10 cm diameter petri dish) and incubated at 37°C overnight.

Antibiotic resistant colonies were tested for the presence of the desired recombinant plasmid (Barnes, 1977). Individual colonies were picked and used to inoculate a 5 ml solution containing LB medium and the appropriate antibiotic and the bacteria were allowed to grow at 37°C overnight. A 1.5 ml aliquot of the culture was put into an 1.5 ml Eppendorf tube and centrifuged (10kxg for 1 minute). The remaining culture was stored at 4°C.

The pelleted bacteria were resuspended in 100 ul of a

solution containing 10 mM Tris HCl (pH 8.0) and 10 mM EDTA at room temperature for 5 minutes. The bacteria were lysed by the addition of 100 ul of a solution containing 0.1 M NaOH and SDS (1%, w/v) before being heated at 68°C for 1 hour. The lysate was cooled and centrifuged (10kxg for 5 minutes). To the supernatant, 100 ul of a solution containing glycerol (50%, v/v) and bromocresol green (0.1%, w/v) was added. A 10 ul aliquot of the mixture was then subjected to electrophoresis on a DNA agarose gel (Section 'J').

The remaining overnight cultures of successful transformants were used for the inoculation of preparative scale cultures and for the production of long term storage stocks. Long term storage of plasmid bearing HB101 bacteria was possible after mixing a stationary culture of the transformed bacteria with 1 volume of sterile glycerol and storing 1 ml aliquots of the mixture at -20°C or at -196°C.

iii) Large Scale Purification of Plasmid DNA

The purification of plasmid DNA from transformed HB101 bacteria was performed using a high salt and alkali lysis procedure (Birnboim & Doly 1979). A 5 ml aliquot of an over night culture of HB101 bacteria bearing the desired plasmid was used to inoculate a 500 ml solution of LB medium containing the appropriate antibiotic. The bacterial culture was incubated at 37°C with vigorous agitation until

it reached a density of 100 Klett units (approximately $1x10^8$ bacteria per ml). Plasmid production was then amplified by the addition of chloramphenicol (500 ul of a 170 mg per ml stock solution in ethanol) and incubated at 37°C overnight with constant agitation.

The bacteria were recovered by centrifugation (5kxg for 5 minutes), washed with 25 ml of a solution containing 10 mM Tris HCl (pH 7.6) and 10 mM EDTA and resuspended in 4 ml of a solution containing 25 mM Tris HCl (pH 7.6), 10 mM EDTA and 2 mg per ml of freshly prepared lysozyme (Boehringer Mannheim). After incubating the suspension on ice for 30 minutes, the bacteria were lysed by the addition of 8 ml of a solution containing 0.2 M NaOH and SDS (1%, w/v). The lysate was placed on ice for 5 minutes before 6 ml of a solution containing 3 M sodium acetate (made to pH 4.8 by the addition of acetic acid) was added and the mixture was gently inverted several times and stored for 1 hour on ice. After centrifugation (16kxg for 30 minutes at room temperature), the plasmid in the supernatant was precipitated with ethanol (2 volumes) and stored at 4°C for 1 hour. The precipitate was recovered by centrifugation (10kxg for 20 minutes) and resuspended in 10 ml of a solution containing 10 mM Tris HCl (pH 7.6) and 100 mM NaCl before the plasmid was precipitated again by the addition of 20 ml of ethanol and stored at 4°C for 1 hour. The pellet was recovered by centrifugation (10kxg for 20 minutes),

dissolved in 4 ml of a solution containing 10 mM Tris HCl (pH 7.6), 10 mM EDTA and 100 units per ml of RNAase A (pre-boiled for 5 minutes, Boehringer Mannheim) and incubated at 37°C for 1 hour. SDS (0.5 volumes of a stock solution, 20%, w/v) was added to the incubation mixture which was then extracted with phenol and chloroform (Section 'G-i'). After removal of the aqueous phase, it was made up to a final volume of 11.5 ml with water. Sodium acetate (0.375 ml of a 4 M stock solution, pH 6.3) and ethanol (16 ml) were then added and the DNA was allowed to precipitate at room temperature for 1 hour before being recovered by centrifugation (10kxg for 20 minutes).

To further purify the plasmid DNA, it was chromatographed on a Biogel A 15-m (Bio-Rad) column. A 0.7 cm id x 10 cm column filled with Biogel A 15-m was washed with 10 column volumes of a solution containing 0.3 M ammonium acetate, 10 mM Tris HCl (pH 7.6) and 1 mM EDTA. The plasmid DNA, dissolved in 400 ul of a solution containing 10 mM Tris HCl (pH 7.6) and 1 mM EDTA, was centrifuged (10kxg for 5 minutes) and the supernatant was applied to the Biogel column. The plasmid was eluted with the Biogel column buffer and was collected in 500 ul fractions. Each fraction was assayed for plasmid DNA by running a 5 ul aliquot on a DNA agarose gel (Section 'J'). Fractions containing purified plasmid DNA were pooled and the plasmid was precipitated with 2 volumes of ethanol and

stored at -20°C overnight. The yield of DNA was determined spectrophotometrically by measuring absorbances at 260 nm of the plasmid samples, dissolved in a solution containing 10 mM Tris HCl (pH 7.6) and 1 mM EDTA, using the formula:

$$(OD_{260}) \times 50 = \text{ug DNA per ml.}$$

Plasmid DNA was stored in a solution containing 10 mM Tris HCl (pH 7.6) and 1 mM EDTA at a concentration of approximately 1 mg per ml at 4°C.

iv) Nick-Translation

DNA plasmids were labelled to high specific activity by (^{32}P) nick-translation (Rigby, Dieckmann, Rhodes & Berg, 1977). Reaction mixtures contained 0.25 ug of the desired plasmid, 2.5 units of DNase I, 50 pg of DNA polymerase I, 25 uM each of dATP, dGTP and dTTP, 50 mM Tris HCl (pH 7.8), 5 mM $MgCl_2$, 10 mM beta-mercaptoethanol and 50 uCi [alpha-^{32}P]dCTP in a total volume of 25 ul. The reaction was allowed to proceed for 1 hour at 15°C before it was halted by the addition of 1 volume of chromatography buffer (below). Commercial nick-translation kits and [alpha-^{32}P]dCTP (greater than 3,000 Ci per mmol) obtained from New England Nuclear or Amersham were used.

The nick-translated DNA was purified from unincorporated radioactive material by 'spin-column' chromatography. Sephadex G-50 (medium, Pharmacia) was suspended in a solution containing 10 mM Tris HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl and SDS (1%, w/v) and was packed by centrifugation (4kxg for 5 minutes) into an 1 ml plastic syringe fitted with a siliconized glass wool plug. The nick-translation reaction mixture, diluted with 1 volume of the chromatography buffer, was applied to the column and washed through with an additional 200 ul of buffer by centrifugation (4kxg for 5 minutes). The eluate (approximately 300 ul) contained more than 90% of the nick-translated material, leaving the unincorporated radioactive material in the column. The resulting specific activity of the labelled DNA plasmids were between 2 and $5x10^8$ cpm per ug.

J) Electrophoresis of DNA

Plasmid DNA samples were analyzed by electrophoresis in an agarose (0.8%, w/v) gel. The gel buffer contained 40 mM Tris acetate (pH 7.8), 2 mM EDTA and 0.2 ug per ml of ethidium bromide. DNA samples (approximately 0.5 ug dissolved in 10 ul of gel buffer) were mixed with 0.1 volume of a solution containing glycerol (25%, v/v), bromophenol blue (0.1%, w/v) and xylene cyanol (0.1%, w/v). The samples were loaded onto the gel and electrophoresis proceeded towards the anode at a potential of 5 V per cm until the bromophenol blue dye traversed 80% of the gel. Since the gel contained ethidium bromide, it was directly photographed with an MP-4 Polaroid Land camera fitted with a Wratten #42 filter using ultraviolet transillumination.

K) Northern Blots

To qualitatively and quantitatively assay specific RNA species in a diverse RNA population, the RNA was resolved by agarose gel electrophoresis and immobilized by electro-blotting the RNA onto a solid support. The RNA was then available for hybridization with specific nick-translated

DNA plasmids, generating a 'Northern Blot'. Two different support media were used to bind RNA electro-blotted from agarose gels.

i) APT-Paper

The first method of immobilizing RNA onto a solid support employed aminophenylthioether modified paper (APT-paper) which was freshly diazotized (DPT-paper) (Seed, 1982). APT-paper was prepared by reacting 5 sheets of 20 cm^2 filter paper (Type 589WH, Schleicher & Schuell) with 100 ml of a solution containing 350 mM NaOH and butanediol diglycidylether (30%, v/v, Aldrich Chemicals, also called bisoxirane) in a Super Sealobag (Philips Electronics) with constant agitation at room temperature overnight. After discarding the reaction mixture, a solution containing 11.5 ml of aminophenylthioether and 40 ml of acetone was sealed in the bag with the filter paper and agitated at room temperature for 24 hours. The APT-paper was then washed twice sequentially with ethanol and 0.1 M HCl for 15 minutes each. After a final rinse with ethanol, the APT-paper was air dried and stored in a Super Sealobag in the dark.

Just before use, the APT-paper was diazotized in 100 ml of a solution containing 1.2 M HCl and 25 mg $NaNO_2$ at 0°C for 15 minutes. After brief rinses first in ice-cold water and then in Transfer Buffer containing 25 mM KH_2PO_4 (pH 5.0), the DPT-paper was ready for use. Once the paper

was activated, it had to be used immediately since the reactive diazo groups have short half-lives.

After the RNA gel to be electro-blotted had been stained and photographed, it was washed with 2 changes in 200 ml of Transfer Buffer for 20 minutes. A piece of freshly activated DPT-paper and 2 pieces of chromatography paper (3MM, Whatman) were cut to the size of the gel to be transferred and were soaked in Transfer Buffer for 10 minutes. The blot was assembled in much the same manner as described for the Western blot for r-proteins (Section 'F-i'), except that the DPT-paper was on the anode side of the gel. Transfer of the RNA was carried out at a potential of 4 V per cm at 4°C overnight with recirculation of the Transfer Buffer. After transfer, the gel was immersed in 200 ml of an aqueous solution containing 2 ug per ml of ethidium bromide for 30 minutes and checked by ultraviolet transillumination for the efficiency of transfer. The DPT-paper was vigorously washed with Transfer Buffer and rubbed with a gloved hand to remove any residual agarose. The blot was then ready for pre-hybridization.

ii) Gene Screen^{tm}

The second Northern blot support used was a Gene Screen membrane (New England Nuclear). After soaking the membrane in Transfer Buffer containing 25 mM NaH_2PO_4 (pH 6.5) for 15 minutes, the blot was ready to be assembled and electro-

blotted in the manner described for DPT-paper. After transfer, the membrane was vigorously washed with Transfer Buffer and baked for 2 hours at 85°C in vacuum. The Gene Screen^{tm} blot was then ready for pre-hybridization.

III) Pre-Hybridization

After the RNA had been transferred to a solid support, the blot was pre-hybridized before being hybridized with specific labelled DNA plasmids (Thomas, 1980). The blots were pre-hybridized in a solution containing formamide (50%, v/v, freshly deionized), 5x Modified Denhardt's solution, 5x SSC, 100 ug per ml of poly(U), 100 ug per ml of yeast tRNA, SDS (0.2%, w/v) and glycine (1%, w/v; glycine was used with DPT-paper only). The formamide (Ultrapur, BRL) was deionized by constant agitation with 0.2 volumes of dual ion exchange resin (RG501-X8, Bio-Rad) at 42°C for 1 hour. Modified Denhardt's solution (1x) contained Ficoll (0.02%, w/v), poylvinylpyrrolidone (0.02%, w/v), 5 mM Mops (pH 7.0) and 1 mM EDTA and was kept as a 50x stock. SSC (1x) contained 0.15 M NaCl and 0.3 M tri-sodium citrate (pH 7.0) and was kept as a 20x stock. Poly(U) and yeast tRNA were denatured by adding aliquots of a 10 mg per ml stock solution directly to the deionized formamide before the other reagents were added. The pre-hybridization solution was sealed in a Super Sealobag with the Northern blot (10 ml of pre-hybridization solution per 100 cm^2 blot) and placed

at 42°C overnight on a rotator.

iv) Hybridization

Hybridization was carried out in the same solution used for pre-hybridization except that the (^{32}P)-labelled nick-translated plasmid was included. In the case of DPT-paper, glycine was omitted from the hybridization buffer. Sufficient nick-translated plasmid to yield a final concentration of 1 to 5 ng per ml of hybridization solution was denatured by incubation in a minimum volume of 0.2 M NaOH for 15 minutes before the addition of the hybridization solution. The pre-hybridization solution in a Super Sealobag was then replaced with an equal volume of the hybridization solution and the bag was re-sealed. Hybridization was carried out at 42°C for at least 72 hours on a rotator.

After hybridization, the blot was washed with 3 changes of 500 ml of a solution containing 2x SSC and SDS (0.1%, w/v) at room temperature for 60 minutes, followed by at least 3 changes of 500 ml of a solution containing 0.2x SSC and SDS (0.1%, w/v) at 55°C for 60 minutes or until the washes contained less than 50 cpms of Cerenkov radiation per 10 ml of wash solution.

v) Autoradiography of Radioactive Blots

After the Northern blot had been washed and dried, it was wrapped in plastic wrap and exposed to Kodak X-Omat

XAR-5 film with a Cronex Lightning-Plus screen (Dupont) to enhance the radioactive signal in a light-tight film holder at -70°C for a suitable length of time. The film was developed and fixed with Kodak GBX developer and fixer, following the manufacturer's instructions.

In some cases, the RNA electro-blotted onto the support membrane had been labelled in vivo with [^{3}H]uridine. To visualize these RNA samples, the support membrane was treated with Spray En^{3}Hance (New England Nuclear) before autoradiography was performed.

To estimate the amount of radioactive material which had hybridized to the blots, autoradiographs were quantitated by scanning densitometry (Model GS 30Q, Hoeffer Scientific Instruments). Care was taken to ensure that the signals on the autoradiographs were linear with the radioactivity on the blot.

L) Quantitation of Specific RNA from L6-5 Cells

The relative amounts of rp-mRNA's in subcellular fractions of L6-5 cells were determined by hybrid-selection (Lichter, Sierra, Clark, Wells, Stein & Stein, 1982). L6-5 cells (70% confluent myoblasts and 4 day myotubes) were incubated for 54 hours in medium containing 100 uCi per ml of [^{3}H]uridine (greater than 30 Ci per mmol, New England Nuclear). The cells were lysed and subcellular fractions prepared (Section 'C-i'). RNA samples purified from the

nuclear, polysomal and post-polysomal fractions of the lysate (Section 'G-i') were chromatographed on oligo(dT)-cellulose (Section 'G-iii'). The RNA in the poly(A) enriched fractions was precipitated with 2 volumes of ethanol at -20°C overnight.

Plasmids to be used for the hybrid-selection of specific RNA transcripts were first linearized at a concentration of 1 ug per ul of a solution containing 10 mM Tris HCl (pH 7.6), 50 mM NaCl, 10 mM Mg acetate, 1 mM dithiothreitol, 50 ug per ml of bovine serum albumin and 1 unit per ul of the appropriate restriction enzyme (Table 2-1) at 37°C overnight. The incubation mixtures were made to 1% with SDS from a stock solution (20%, w/v) and extracted with phenol and chloroform (Section 'G-i'). The plasmids were precipitated with ammonium acetate (0.5 volumes of a 7.5 M stock solution) and ethanol (2 volumes) and stored at -20°C overnight.

Equal amounts of 5 different linearized rp-cDNA plasmids (1.5 ug each per assay) were dissolved in water and combined (a total of 7.5 ug of plasmid DNA per 25 ul of water). In a separate vial, 7.5 ug of linearized pMB9 per assay was dissolved in 25 ul of water. NaOH (0.02 volumes of a 10M stock solution) was added to the samples and allowed to incubate at room temperature for 30 minutes. While the plasmids were incubating, APT-paper filters (2 cm diameter) were activated and mounted in an ice-cold Swinnex

filter unit (Millipore). After the addition of Hepes (1/ volume of a 2 M stock solution) to the plasmid solution, it was passed through the freshly activated DPT-paper filter (7.5 ug of total plasmid per filter) several times before the filter was washed with 5 ml of a solution containing 2x SSC and glycine (10%, w/v).

The filters were pre-hybridized as described in Section 'K-iii', except that the 2x SSC was substituted for 5x SSC and the pre-hybridization was performed at 37°C. The labelled poly(A) enriched RNA from each subcellular fraction was dissolved in 500 ul of hybridization buffer (Section 'K-iii'), except that the 2x SSC was substituted for 5x SSC. The pre-hybridization solution was replaced with the hybridization solution containing the labelled RNA, with each individual hybridization containing a filter with the 5 combined rp-mRNA cDNA plasmids and a filter containing the plasmid pMB9 as a control. Hybridization of the filters proceeded at 37°C for 72 hours.

Upon completion of the hybridization, the filters were washed 3 times with 100 ml of a solution containing 2x SSC and SDS (0.1%, w/v) at room temperature for 1 hour followed by 3 washes with 100 ml of a solution containing 0.1x SSC and SDS (0.1%, w/v) at room temperature for 1 hour. The filters were incubated with 1 ml of a solution containing 2x SSC and 10 ug per ml of RNase A for 1 hour at room temperature and then washed 3 times with 100 ml of a solution containing

2x SSC and SDS (0.1%, w/v) at room temperature for 1 hour. Following the final wash, the RNA was eluted from the filters by incubating the filters with 1 ml of water at 95°C for 5 minutes. The water was transferred to a liquid scintillation vial and the radioactive content determined in the presence of Aquasol (New England Nuclear).

M) Nuclear Transcription

Nuclear transcription assays were performed with nuclei prepared from 70% confluent myoblasts and 4 day myotubes (Alterman, Ganguly, Schulze, Marzluff, Schildkraut & Skoultchi, 1984 and Marzluff & Huang, 1984). L6-5 cells were rinsed with 3 ml of ice-cold HBS before being lysed with 2 ml of a solution containing Nonidet P-40 (0.3%, v/v), 10 mM Tris HCl (pH 7.6), 150 mM NaCl, 3 mM Mg acetate, 3 mM $CaCl_2$, 0.1 mM EDTA and 1 mM dithiothreitol. After the lysate was homogenized with 10 quick strokes of a tight fitting Dounce homogenizer, it was layered over 1 volume of a solution containing sucrose (20%, w/v), 10 mM Tris HCl (pH 7.9), 5 mM Mg acetate, 0.1 mM EDTA and 1 mM dithiothreitol and centrifuged (2kxg for 10 minutes) in a swinging bucket rotor. The nuclear pellet from 2 plastic tissue culture dishes (15 cm diameter) of 70% confluent myoblasts or 1 dish of 4 day myotubes was resuspended in 100 ul of a solution containing 50 mM Tris HCl (pH 8.0), 5 mM Mg acetate, 0.1 mM EDTA, 5 mM dithiothreitol and 25%

glycerol (v/v) and was either used immediately or stored at -196°C.

The nuclear transcription assay was performed in a siliconized 5 dram vial containing 200 uCi of [alpha-^{32}P]UTP (410 uCi per mmol, lyophylized, Amersham), 50 ul each of a solution containing 4 mM ATP, 2 mM each of GTP and CTP, 0.2 mM UTP and 1 mM dithiothreitol and of a solution containing 500 mM KCl, 10 mM Mg acetate and 1 mM dithiothreitol and 100 ul of nuclei suspension. To determine the time course of the assay, a series of reaction mixtures were incubated at 25°C for various periods of time. The reactions were terminated by the addition of 1 volume of a solution containing 10 mM Tris HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl and SDS (1%, w/v) and homogenized by several quick passages through a tuberculin syringe. Aliquots (5 ul) of the lysates were spotted onto filter paper discs (2.4 cm diameter, #540, Whatman) and allowed to dry. The filters were then washed and their radioactive content determined by liquid scintillation counting (Section 'B').

Subsequent reactions used for the hybrid-selection procedure were incubated at 25°C for 60 minutes after which time the reactions were terminated by the addition of 200 ul of a solution containing 10 mM Tris HCl (pH 7.6), 1 mM EDTA and SDS (1%, w/v). Each mixture was homogenized by several quick passages through a tuberculin syringe. After the addition of 50 ug of yeast tRNA from a 10 mg per ml stock

solution and Na acetate (0.1 volumes of a 3 M stock solution, pH 4.8), phenol (1 volume) was added and the mixture heated to 55°C for 5 minutes. Chloroform (1 original volume) was then added and the mixture was cooled to room temperature and centrifuged (10kxg for 5 minutes). The material in the aqueous phase was precipitated by the addition of ethanol (2 volumes) and stored at -20°C overnight.

The material recovered by centrifugation (10kxg for 5 minutes) was resuspended in 400 ul of a solution containing 10 mM Tris HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl and SDS (1%, w/v). The solution was subjected to gel filtration in a 0.7 cm id x 30 cm column containing Sephadex G-50 (Medium, Pharmacia Fine Chemicals) in the same buffer. Fractions of 500 ul were collected and the radioactively labelled material in the void volume of the column was pooled, precipitated with ethanol (2 volumes) at -20°C overnight and centrifuged (10kxg for 5 minutes).

Plasmids to be used for hybrid-selecting specific RNA transcripts were first linearized and purified as described in Section 'L'. After extraction with phenol and chloroform, the aqueous phase was retained and NaOH (0.02 volumes of a 10 M stock solution) was added. The plasmids were allowed to denature in the NaOH solution for 20 minutes before being centrifuged (10kxg for 5 minutes). The supernatant was diluted with 5 to 10 volumes of a solution

containing 10x SSC and immediately applied to a Gene Screen^{tm} membrane.

The membrane was prepared by soaking the membrane in a solution containing 10x SSC for 20 minutes before it was mounted in a 'Hybridot' template (Bethesda Research Laboratories) with a filter paper underlay (Type 589WH, Schleicher & Schuell). The denatured plasmids were applied to the template, loading no more than 10 ug of plasmid per well. The plasmids were filtered through the membrane under vacuum and the wells were washed through with .2 ml of a solution containing 10xSSC. The membrane was then dried and baked at 85^{o}C under vacuum for 3 hours.

The membrane was divided into portions required for the individual hybridizations and pre-hybridized in a solution containing formamide (50%, v/v, freshly deionized), 2x Modified Denhardt's solution, 5x SSC, 100 ug per ml of HB10I bacterial DNA (sheared by sonication and denatured by incubation with 0.2 M NaOH for 15 minutes), 10 ug per ml of poly(U) and SDS (0.2%, w/v) at 52^{o}C on a rotator overnight. To make the hybridization solution, the (^{32}P)-labelled RNA was dissolved in pre-hybridization buffer at a concentration of 10^{6} cpm per ml. Dot blots with plasmids containing sequences complementary to rRNA were incubated with hybridization solution containing 10^{4} cpm of (^{32}P)-labelled RNA per ml. The pre-hybridization solution sealed with the dot blots was replaced with the hybridization solution

(100 ul of hybridization solution per cm^2 of membrane) and the bag was resealed. Hybridization proceeded at 52°C on a rotator for 72 hours.

After hybridization, the blots were washed 3 times with 100 ml of a solution containing 2x SSC 1 mM EDTA and SDS (0.2%, w/v) at 52°C over a period of 1 hour and 3 times with 100 ml of a solution containing 0.2x SSC and SDS (0.1%, w/v) at 52°C over a period of 1 hour. The radioactivity hybridized to individual plasmid dots was quantitated by liquid scintillation counting.

N. Miscellaneous

Glassware which was used for RNA and DNA work was baked at 190°C overnight before use to eliminate possible traces of RNase. All disposable glassware was siliconized by treatment with a solution containing dimethyldichlorosilane (2%, w/v) in trichloroethane (BDH) before baking. All plastic-ware used was disposable and was considered to be RNAase free. All solutions were passed through a 0.45 micron filter and those which could be autoclaved were. Water was glass distilled from demineralized water and was autoclaved before use. Other utensils such as rubber policemen and polycarbonate ultracentrifuge tubes were treated with a solution containing 1 M NaOH for 5 minutes and rinsed with copious amounts of autoclaved water before use. All radioactive counting was performed to a standard

deviation of less than 1% of the total counts.

O) Enzymes

The following enzymes were used in these experiments:

DNase 1, EC 3.1.21.1 (Flow Worthington)

Lysozyme, EC 3.2.1.17 (Boehringer Mannheim)

Proteinase K, EC 3.4.21.14 (Sigma Chemicals)

RNase A, EC 3.4.21.4 (Boehringer Mannheim)

RNase T1, EC 3.1.4.8 (Boehringer Mannheim)

Restriction endonucleases:

Bam H1, EC 3.1.23.6 (Bethesda Research Laboratories)

Eco R1, EC 3.1.23.13 (Bethesda Research Laboratories)

Trypsin, EC 3.4.21.4 (Flow Laboratories)

P) Abbreviations

Units used in text are Standard International (SI) units.

alpha-MEM: Minimum Essential Medium, Eagles (alpha modification)

APT: o-Aminophenylthioether

CHO: Chinese Hamster Ovary cells

cpm: radioactive counts per minute

DNA: Deoxyribonucleic acid

dpm: radioactive disintegrations per minute

DPT: Diazophenylthioether

EDTA: Ethylenediaminetetraacetic acid

HBS: Hepes Buffered Saline (Section 'C')

Hepes: N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid

id: inside diameter

krpm: revolutions per minute times one thousand

kxg: indicates centrifugal force in units of gravity times one thousand

LB: Luria-Bertani medium (Section 'I-i')

Mops: Morpholinopropanesulfonic acid

mRNA: Messenger RNA

OD_{nnn}: optical density at 'nnn' nanometers wave length

oligo(dT): oligodeoxythymidylic acid (approximately 15 units long)

poly(A): Polyadenylic acid (greater than 200 units long)

poly(U): Polyuridylic acid (greater than 200 units long)

PVS: Polyvinylsulfate

r.i.: Refractive Index

RNA: Ribonucleic acid

SDS: Sodium dodecyl sulphate

SSC: Saline Sodium Citrate Solution (Section 'K-iii')

TCA: Trichloroacetic acid

TEMED: N,N,N',N'-Tetramethylethylene diamine

Tris or Tris base: Tris(hydroxymethyl)aminoethane

tRNA: Transfer RNA

v/v, w/v: Indicates volume by volume or weight by volume

CHAPTER THREE

Results

The L6-5 myoblast is an *in vitro* model of myogenesis which can be employed to study gene regulation during differentiation. When the cells differentiate into myotubes, they stop proliferating and alter their metabolic state, which provides for an opportunity to investigate the regulation of a set of housekeeping genes coding for ribosomes. The findings of others, which revealed that the rate of ribosome accumulation decreased once the myoblasts differentiated, have not indicated the nature of the regulatory mechanisms involved (Krauter *et al.*, 1979 and Krauter *et al.*, 1980). An analysis of the metabolism of the components of the ribosomes in terms of their rates of synthesis and decay and sub-cellular distribution, should provide a greater understanding of how ribosome biogenesis is regulated in this system.

The study of ribosome biogenesis in differentiating myoblasts begins with a general characterization of the cells. Information on the morphological and physiological changes which occur during myogenesis will provide the context in which ribosome production will be examined.

A) Physiology of L6-5 Differentiation

i) Morphology

Since the L6 cell line tends to drift and become polymorphic (Richler & Yaffe, 1970), a clone of L6 was isolated and its growth characteristics defined. A sub-clone L6-5 was chosen for study because of its short generation time and its ability to differentiate rapidly. Microscopic inspection of the myoblasts revealed them to be mono-nucleated, bi-polar spindle shaped cells with visible tonofilaments (Figure 3-1, MB). After the cells had been exposed to Differentiation Medium for 2 days, the cells appeared elongated and had aligned themselves with each other (Figure 3-1, MI). Between 2 and 3 days in Differentiation Medium, the cells fused to form syncytial myotubes, with large numbers of nuclei clustered together (Figure 3-1, MT). The myotubes formed the final phenotype of the morphological transformation seen in vitro as a result of the terminal differentiation process. The myotubes could be maintained for 5 or 6 days before they began to detach from the plastic tissue culture dish.

A small number of cells in the myotube cultures, however, did not fuse (Yaffe, 1971). These cells proliferated at a very low rate and, since the tissue culture dish was confluent with myotubes, did not readily attach to the dish. As a result, they represented an insignificant proportion of the cell mass in

Figure 3-1.

Differentiation of L6-5 Myoblasts.

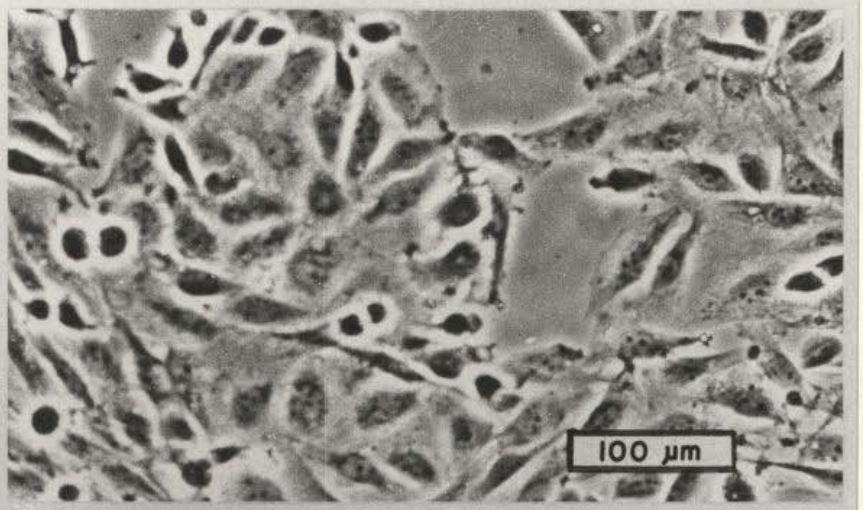
L6-5 cells were grown and induced to differentiate on Lab Tech tissue culture slides (Flow Laboratories). At various stages of differentiation, slides were washed with HBS and fixed with a solution containing glutaraldehyde (1%, v/v) in HBS for 5 minutes. After 1 wash in HBS, the slides were wet-mounted in a solution containing glycerol (50%, v/v) and photographed using a phase contrast microscope.

MB: myoblasts

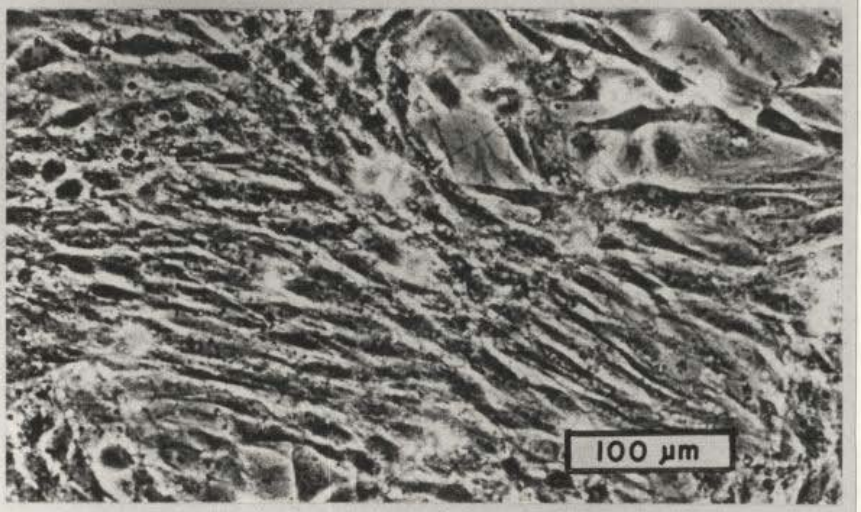
MI: committed myoblasts

MT: myotubes

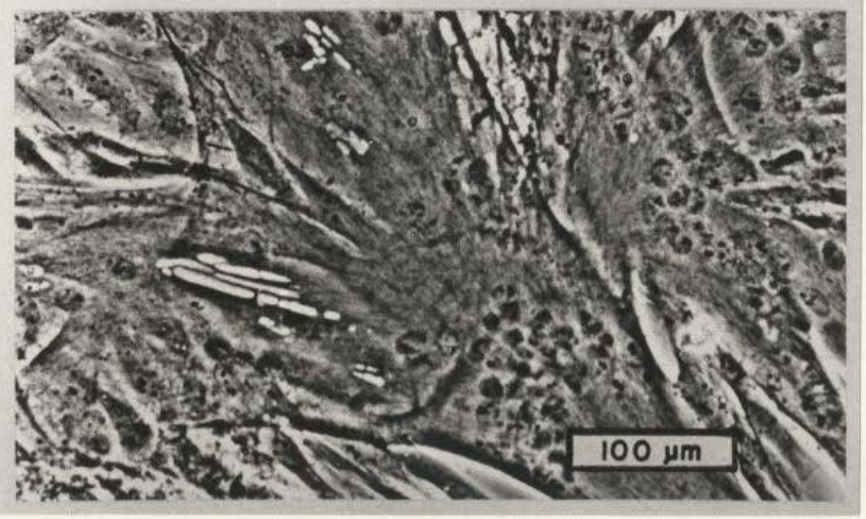
MB



MI



MT



mature myotube preparations.

ii) DNA, RNA and Protein Accumulation Rates

After induction of differentiation, the L6-5 myoblast redirects its priorities from proliferation to differentiation. The differentiating cell no longer divides and the processes involved with cell growth are turned off (Yaffe, 1974). To characterize the metabolic shifts caused by the genetic differentiation program, the rates of DNA, RNA and protein accumulation were monitored on sequential days of differentiation. L6-5 cells (70% confluent myoblasts and 1 through 4 day myotubes) were incubated for 2 hours in medium containing radioactively labelled precursors for DNA, RNA or protein ([^{3}H]thymidine, [^{3}H]uridine or [^{3}H]lysine respectively). After determining the number of nuclei per sample, the TCA insoluble radioactivity per 10^6 nuclei was determined and the value plotted against days of differentiation (Figure 3-2).

The rates of accumulation of DNA, RNA and protein changed as the L6-5 cells differentiated. In the case of DNA, the rate of incorporation of [^{3}H]thymidine into TCA insoluble material increased 1 day after differentiation was induced before it decreased to less than 15% of the rate found in myoblasts. The much lower rate of [^{3}H]thymidine incorporation into TCA insoluble material in maturing myotubes coincided with the fusion of these cells

Figure 3-2.

Rates of DNA, RNA and Protein Accumulation in Differentiating L6-5 Cells.

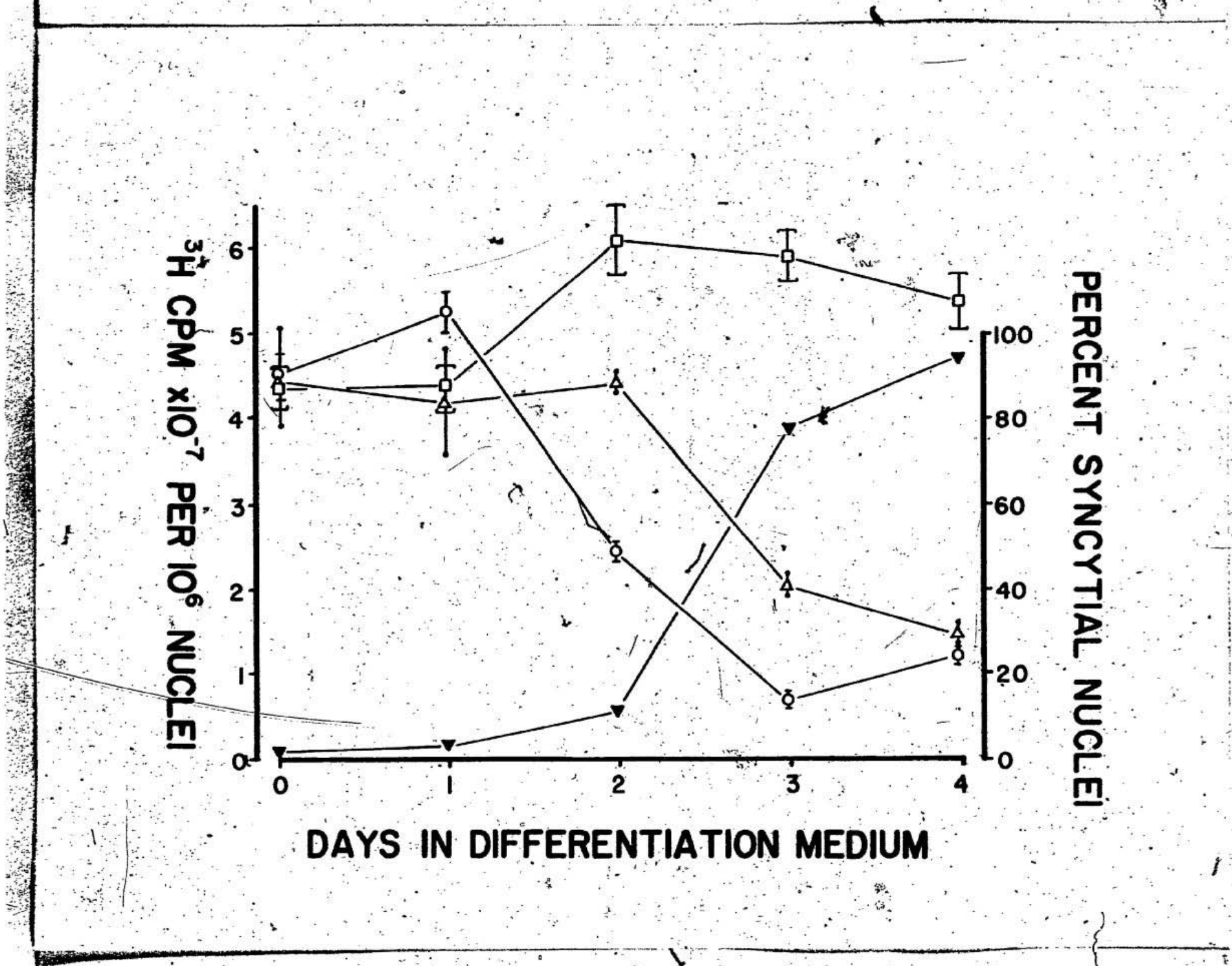
L6-5 cells (70% confluent myoblasts and 1 through 4 day myotubes) were incubated for 2 hours in medium containing 2 uCi per ml of [^{3}H]thymidine, 5 uCi per ml of [^{3}H]uridine or 20 uCi per ml of[^{3}H] lysine before being lysed in Lysis Buffer. After the number of nuclei were determined, TCA insoluble counts were determined per 10^6 nuclei. The values obtained for [^{3}H]uridine and [^{3}H] lysine were normalized to the values obtained for [^{3}H]thymidine by factors of 1.25 and 2.5 respectively. The error bars represent the standard deviations of 4 replicates. The number of syncytial nuclei were determined by counting nuclei in 4 microscope fields of L6-5 cells for a total of approximately 500 nuclei and expressed as a percent of total nuclei.

○ cpm x10^{-7} [^{3}H]thymidine

△ cpm x10^{-7} [^{3}H]uridine

□ cpm x10^{-7} [^{3}H]lysine

▼ Percent Syncytial Nuclei


^{3}H CPM x10^{-7} PER 10^6 NUCLEI
PERCENT SYNCYTIAL NUCLEI
DAYS IN DIFFERENTIATION MEDIUM
0
1
2
3
4
0
1
2
3
4
5
6
0
20
40
60
80
100

(Figure 3-1). Nuclei in myotubes do not replicate and therefore incorporate very little label (Yaffe, 1971).

The rate of RNA accumulation, monitored by the incorporation of [^{3}H]uridine into TCA insoluble material, remained constant for the first 2 days of differentiation before falling to a minimum value by day 4 (Figure 3-2). Although the cells stopped proliferating by day 2 in Differentiation Medium, a considerable amount of RNA continued to be synthesized. Since ribosomes accumulate at a much lower rate in myotubes (Krauter et al., 1979), the fall in the rate of total RNA synthesis observed by day 3 of differentiation probably resulted from a reduction in the rate of rRNA synthesis.

On day 2 of differentiation, protein accumulation rates increased to 140% of the rate found in myoblasts, as determined by the incorporation of [^{3}H]lysine into TCA insoluble material (Figure 3-2). The increased rate of [^{3}H]lysine incorporation was not maintained, but later dropped to approximately 120% of the level found in proliferating myoblasts. High levels of protein synthesis therefore appeared to be required for the formation and maintenance of the differentiated L6-5 phenotype.

An argument can be made that the observed changes in the incorporation rates of the various isotopes resulted from alterations in the precursor pool sizes as the cells differentiated. This problem has been addressed by others

who have suggested that the intracellular pool sizes for these precursors do not change significantly following differentiation (Buckingham et al., 1977, Nadal-Ginard, 1978 and Krauter, et al., 1980). Although the serum supplement employed in the Growth Medium (10% fetal bovine serum) was significantly different from the serum used in the Differentiation Medium (2.5% donor horse serum), changes in the rates of label incorporation on subsequent days of differentiation were much more significant than the change between days 0 and 1. Therefore, although the absolute values of the rates of label incorporation may not be accurate, the trends were unmistakably a result of the differentiation process.

At the same time that the incorporation rates of the various precursors were measured, the nuclei which were syncytial were also monitored (Figure 3-2). The number of nuclei found in myotubes, expressed as a percentage of total nuclei, increased dramatically between days 2 and 3 of differentiation. This increase followed the decline in the rate of DNA synthesis, suggesting that once DNA synthesis ceased, fusion of the cells was able to proceed, forming multi-nucleated myotubes. The rate of RNA accumulation also dropped when the cells commenced fusion.

These preliminary findings provided both the morphological and biochemical evidence that the sub-clone L6-5 behaved similarly to the published observations for L6

(Richler & Yaffe, 1970, Yaffe, 1971, and Krauter et al, 1979). In addition, the conditions used to induce terminal differentiation of L6-5 myoblasts did not reduce the protein accumulation rates in myotubes. This suggested that the cells were not being subjected to starvation conditions (Moldave, David, Hutchison, Laidlaw & Fischer, 1982), a critical observation since many examples exist where the biogenesis of ribosomes is modulated by just such conditions (Warner et al., 1980). It therefore appeared that the conditions employed for the proliferation and differentiation of the L6-5 myoblasts were appropriate for the study of ribosome biogenesis and that changes in the regulation of ribosome biogenesis were the result of the differentiation program.

iii) Specific Activities of RNA

a) Poly(A) Enriched and Poly(A) Poor RNA

The RNA accumulation measurements displayed in Figure 3-2 did not discriminate among the 3 major classes of RNA found in eukaryotic cells. Experiments were designed to determine if these classes of RNA's behaved differently as a result of myogenesis. The mRNA's were separate from the other classes of RNA's by exploiting a property unique to most mRNA's: their 3' poly(A) tract. Total RNA was purified from L6-5 cells at various stages of differentiation which had been incubated in medium containing [^{3}H]uridine.

Using oligo(dT)-cellulose chromatography, 10 ug samples of RNA were separated into poly(A) enriched and poly(A) poor fractions. The total radioactivity of the 2 RNA fractions taken from various stages of differentiation were compared on the basis of the amount of input RNA (Figure 3-3).

The radioactive content of the poly(A) poor RNA fractions was similar to that of total RNA. This result was consistent with the fact that it accounted for more than 90% of the total material subjected to oligo(dT)-cellulose chromatography. After 1 day in Differentiation Medium, the L6-5 cells showed a 30% increase in the specific activity in both the total and poly(A) poor RNA fractions when compared to myoblasts. However, by day 4, the specific activity quickly dropped to approximately 40% of the level found in proliferating myoblasts. Since this RNA fraction was not pure rRNA, it cannot be unequivocally stated that the drop in the rate of RNA accumulation following differentiation was due to a reduction in the accumulation rate of rRNA.

The radioactive content of the poly(A) enriched RNA fraction, on the other hand, remained relatively constant during differentiation, suggesting that the rate of mRNA accumulation which it represents was constant. Although the poly(A) enriched RNA was only a small fraction of the total RNA measured, it was independent of the general decline in the relative rate of accumulation experienced by the bulk of the RNA.

Figure 3-3.

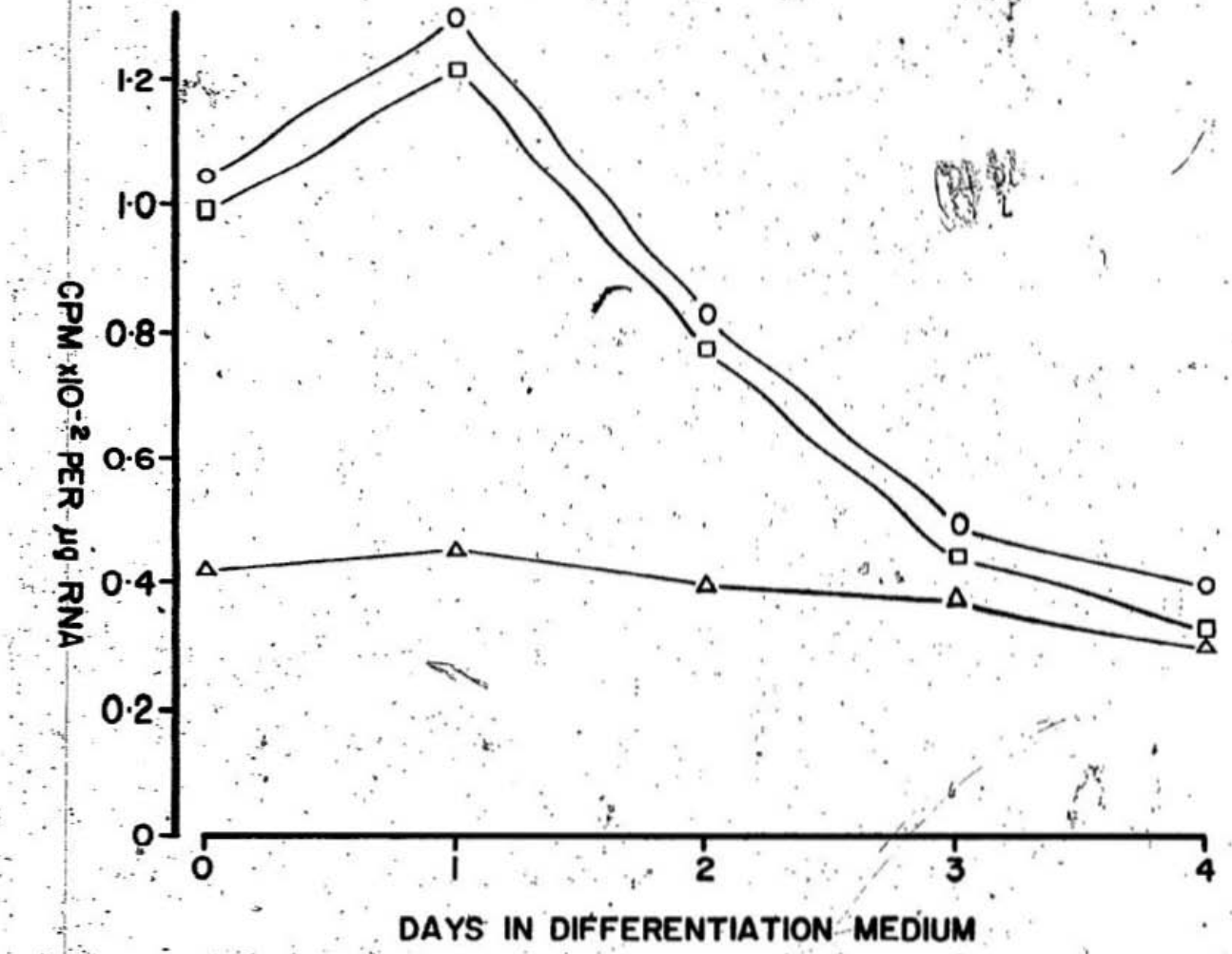
Specific Activity of RNA in Differentiating L6-5 Cells.

Total RNA was prepared from 70% confluent myoblasts and 1 through 4 day myotubes which had been incubated for 2 hours in medium containing 5 uCi per ml of [^{3}H]uridine. Ten ug aliquots of total RNA were fractionated by oligo(dT)-cellulose chromatography and the radioactive content of the fractions determined. The values are averages of duplicate determinations. Background was approximately 32 cpms.

○ Total RNA

□ poly(A) poor RNA

△ 10x poly(A) enriched RNA


1·2
1·0
0·8
0·6
0·4
0·2
0
CPM x10⁻² PER µg RNA
0
1
2
3
4
DAYS IN DIFFERENTIATION MEDIUM

b) Electrophoresis of RNA

The rRNA can be more specifically analyzed by gel electrophoresis, since the rRNA's have characteristic migration patterns in agarose gels. Total RNA was prepared from L6-5 cells at various stages of differentiation which had been incubated in medium containing 5 uCi per ml of [^{3}H] uridine for 18 hours. Aliquots of 10 ug of the RNA were subjected to electrophoresis in 1.2% agarose and transferred to Gene Screen^{tm}. After autoradiography (Figure 3-4), the autoradiograph was quantitated by scanning densitometry. The radioactive content of the 18s and 28s rRNA bands were normalized to the values obtained for proliferating myoblasts (Table 3-1).

Most of the newly labelled RNA in all samples were the 18s and 28s rRNA species. The specific activities of the 18s and 28s rRNA's dropped to 60 and 70% respectively 2 days after the induction of differentiation of the levels found in proliferating myoblasts. The levels dropped further to 17 and 35% respectively by day 4. Therefore, as the L6-5 cells differentiated, the rates of mature rRNA accumulation dropped considerably.

Figure 3-4.

Autoradiographs of Labelled RNA from Differentiating L6-5 Cells.

Total RNA was prepared from 70% confluent myoblasts and 1 through 4 day myotubes which had been incubated for 18 hours in medium containing 5 uCi per ml of [^{3}H]uridine. Ten ug aliquots of RNA were subjected to electrophoresis in 1.2% agarose, electro-blotted onto Gene Screen^{tm}, treated with Spray En^{3}Hance^{tm} and autoradiographed.

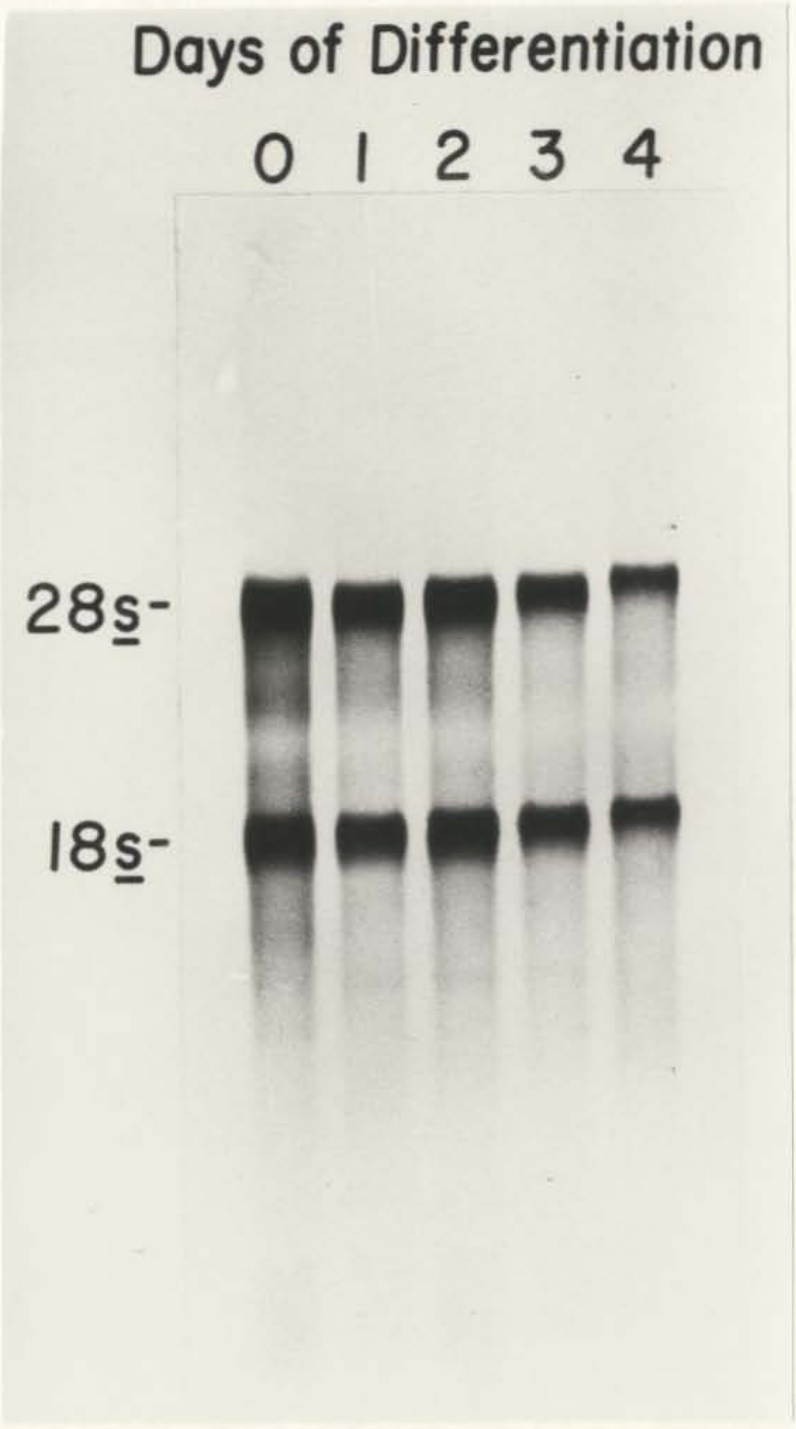

Days of Differentiation
0 1 2 3 4
28s-
18s-

Table 3-1.

Quantitation of Labelled RNA from Differentiating L6-5 Cells.

Total RNA was prepared from 70% confluent myoblasts and 1 through 4 day myotubes which had been incubated for 18 hours in medium containing 5 uCi per ml [^{3}H]uridine. Ten ug aliquots of RNA were subjected to electrophoresis in 1.2% agarose, electro-blotted onto Gene Screen^{tm}, treated with Spray En^{3}Hance^{tm} and autoradiographed (Figure 3-4). The autoradiographs were quantitated by scanning densitometry and the peak areas of the rRNA bands determined, with the myoblast values equal to unity.

	Myoblasts	Myotubes			
		1 day	2 day	3 day	4 day
rRNA 28s	1	0.60	0.63	0.29	0.17
18s	1	0.58	0.73	0.42	0.35

iv) Labelling of Ribosomes in L6-5 Myoblasts and Myotubes

The conclusion from the foregoing results was that the rate of accumulation of mature rRNA's dropped by approximately 75% upon differentiation of the L6-5 myoblasts (Figures 3-2 through 3-4). Since rRNA's and r-proteins in mature ribosomes are normally accumulated and degraded as a unit (Tsurugi et al., 1974), the r-protein accumulation rate was investigated before and after differentiation.

L6-5 cells (70% confluent myoblasts and 4 day myotubes), which had been incubated for 2 hours in medium containing [^{3}H] lysine, were lysed and centrifuged to obtain a polysomal fraction. Equal amounts of polysomes from myoblasts and myotubes were dissociated into subunits with the drug puromycin and separated on isokinetic sucrose gradients (Figure 3-5).

The radioactivity and OD_{260} profiles were similar for the myoblast and myotube preparations. The [^{3}H] lysine profile in myotubes, however, was much reduced. The total amount of radioactivity contained in the 40s and 60s peaks of the gradients indicated that there was approximately 45% as much [^{3}H] lysine in myotube as in myoblast ribosomal subunits. The rate of accumulation of r-proteins in mature ribosomes in 4 day myotubes appeared to be only 45% of the rate observed in proliferating myoblasts.

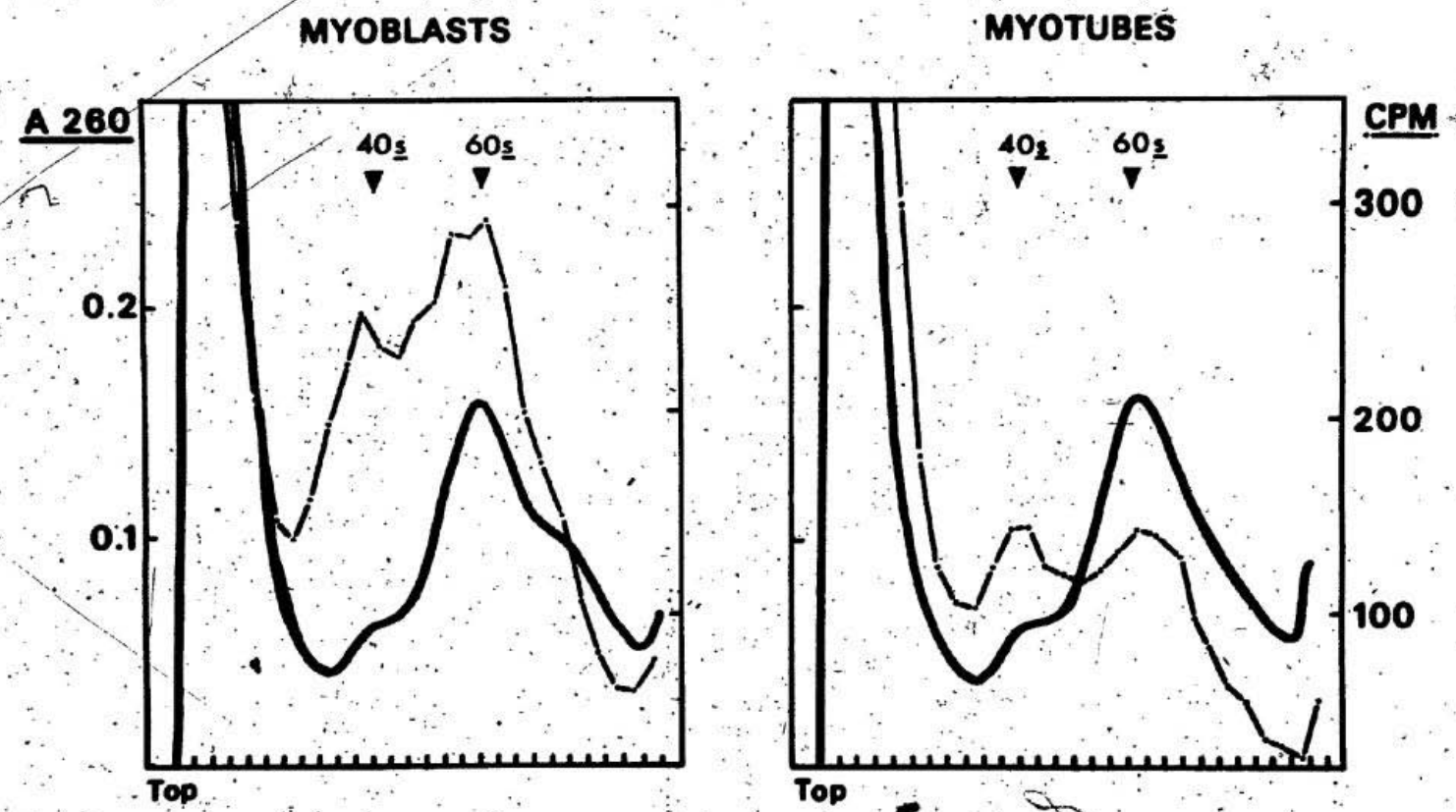
Figure 3-5.

Accumulation of Ribosomes in Myoblasts and Myotubes.

Polysomes were prepared from 70% confluent myoblasts and 4 day myotubes which had been incubated for 2 hours in medium containing 2 uCi per ml of [^{3}H]-lysine. One OD_{260} unit of polysomes was dissociated into subunits with puromycin and separated on an isokinetic 15 to 30% sucrose gradient. Profiles of optical densities and radioactivities determined from 5 ul aliquots were plotted against fraction number. Total radioactivity obtained for the 40s plus 60s ribosomal subunit peaks were 87.5k cpm and 39.7k cpm for myoblasts and myotubes respectively.

---- [^{3}H]-lysine cpm

—— Optical Density at 260 nm.


MYOBLASTS
MYOTUBES
A 260
CPM
40s
60s
40s
60s
0.2
0.1
300
200
100
Top
Top

B) Turnover of Ribosomal Proteins

Ribosomal proteins can be separated and directly characterized by employing a standardized 2 dimensional polyacrylamide gel system. The gel system of Lastick & McConkey (1976) resolves most of the individual r-proteins from a partially purified sample prepared from a total cell lysate. Since a significant proportion of newly synthesized r-proteins are in transit to the nucleolus and not yet associated with mature ribosomes (Tsurugi et al., 1983), this technique provided the opportunity to analyze the turnover of newly synthesized r-proteins in addition to those which were already associated with mature ribosomes.

To compare the turnover of individual r-proteins before and after L6-5 differentiation, the synthesis and degradation rates of new r-proteins were measured against the background of the total r-protein pool. Since mature ribosomes turn over slowly in mammalian cells (Singer & Kessler-Icekson, 1978), long term labelling of r-proteins provides a stable background against which the rate of new r-protein synthesis can be measured. L6-5 myoblasts and myotubes which had been incubated for 54 hours in medium containing [^{14}C]lysine were pulse-labelled for 30 minutes in

medium containing both [^{3}H] lysine and [^{14}C] lysine. The cells were then incubated in radioisotope-free medium containing a 10 fold excess of non-radioactive lysine for various periods of time to chase the radioactive lysine into mature protein. The r-proteins were extracted from whole cell lysates and subjected to electrophoresis in a standard 2 dimensional gel system for r-proteins. After transferring the r-proteins to nitrocellulose, the resulting Western blot was stained for protein, revealing 44 spots which could be consistently identified as specific r-proteins (Figure 3-6, McConkey et al., 1979). The radioactive content of these spots was determined and the ratios of ^{3}H:^{14}C dpm lysine were normalized to the isotope ratios for total protein. This allowed a direct comparison of the rate of new r-protein synthesis and degradation with both total r-protein and total cellular protein (Table 3-2). The results for all of the r-proteins were averaged for myoblasts and myotubes and plotted in Figure 3-7.

Prior to chase in medium containing excess lysine, the r-protein isotope ratios were similar in myoblasts and myotubes, indicating that the rates of synthesis of new r-proteins relative to the population of total r-protein were unchanged by differentiation. Furthermore, the rates of r-protein synthesis in myoblasts and myotubes, when expressed as a function of total protein, again were unchanged by the differentiation process. The isotope

Figure 3-6.

Two Dimensional Gel Electrophoresis of L6-5 Ribosomal Proteins.

Ribosomal proteins prepared from 70% confluent myoblasts were separated on a standard 2 dimensional polyacrylamide gel for r-proteins, electro-blotted to nitrocellulose and stained with amido black. The nomenclature used was based on the system employed by McConkey *et al.*, (1979). Only those r-proteins which could be reproducibly identified in myoblasts and myotubes are indicated.

Snn: Small Ribosomal Subunit Protein 'nn'

Lnn: Large Ribosomal Subunit Protein 'nn'

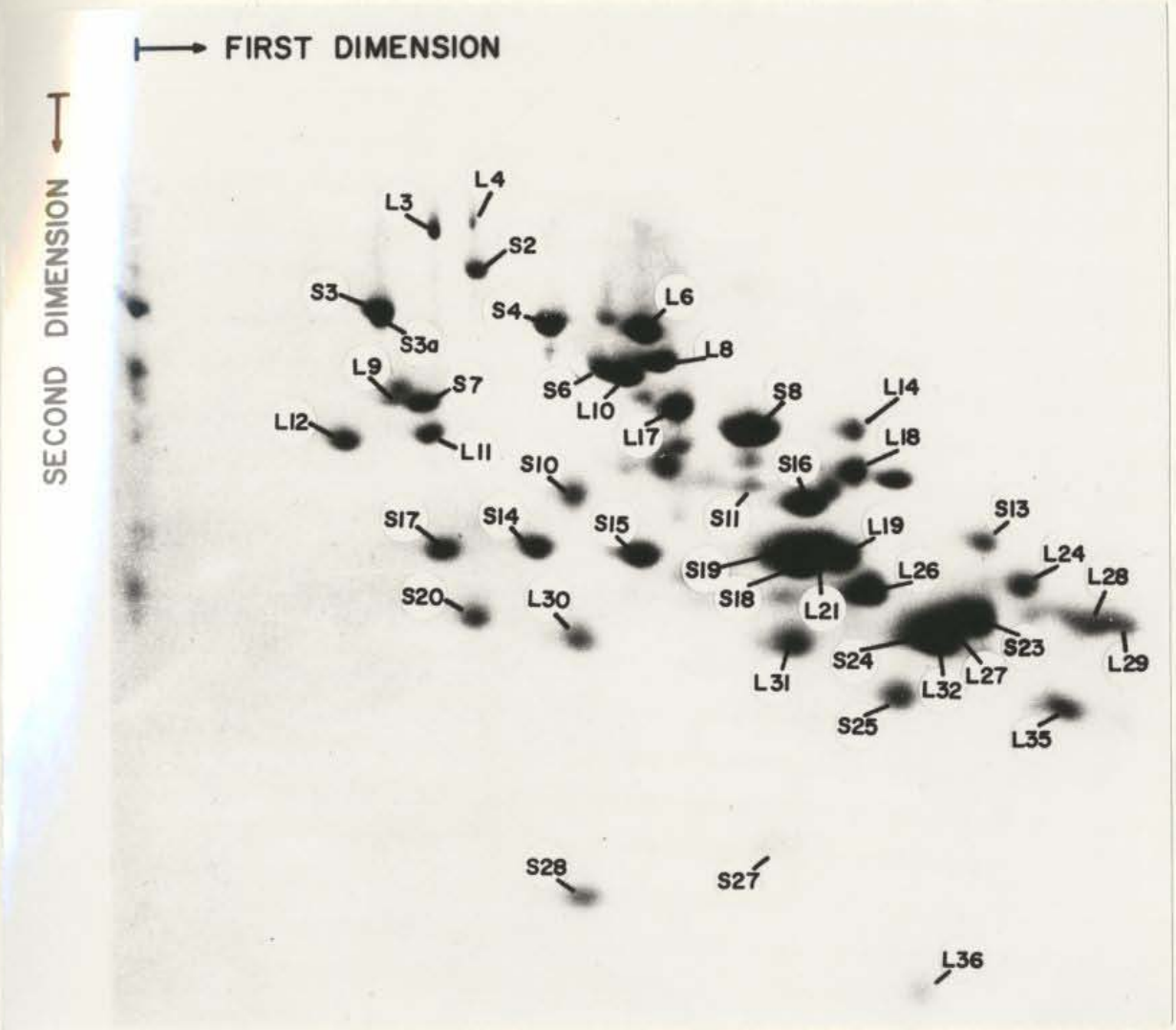

FIRST DIMENSION
SECOND DIMENSION
L4
L3
S2
S3
S4
L6
S3a
L8
L9
S7
S6
L10
S8
L14
L12
L17
L18
L11
S10
S16
S11
S17
S14
S15
L19
S13
S19
L24
L26
L28
S20
L30
S18
L21
S24
S23
L29
L31
L32
L27
S25
L35
S28
S27
L36

Table 3-2.

Turnover of Ribosomal Proteins in Myoblasts and Myotubes.

L6-5 cells (70% confluent myoblasts and 4 day myotubes) which had been incubated for 54 hours in medium containing 5 uCi per ml of [^{14}C] lysine were pulse-labelled for 30 minutes in medium containing 200 uCi per ml of [^{3}H] lysine. After 0, 0.5, 1, 4 or 24 hours of chase with medium containing 10 times the normal concentration of non-radioactive lysine, an acid soluble (0.25 M HCl) protein extract was prepared and subjected to electrophoresis in a standard 2 dimensional polyacrylamide gel system for r-proteins. The isotope content of individual r-proteins was determined and the $^{3}H:^{14}C$ ratios for the r-proteins were normalized to the ratio for total proteins. The ratios represent averages of 2 determinations. Asterisks indicate r-proteins for which recombinant DNA probes were available. Table 3-2a contains the normalized ratios for the 40s ribosomal subunit r-proteins and Table 3-2b contains the normalized ratios for the 60s ribosomal subunit r-proteins. Actual cpms ranged from 50 to 500 above a background of approximately 32.

Ave: average $^{3}H:^{14}C$ ratio.

S.D.: standard deviation.

Table 3-2a.

Small Subunit Ribosomal Proteins.

Protein	Myoblasts					Myotubes				
	Hours of Chase									
	0	0.5	1	4	24	0	0.5	1	4	24
total	1.48	1.40	1.47	1.50	1.49	1.23	1.12	1.02	1.14	1.15
2	1.05	1.08	1.07	1.05	1.03	1.31	0.27	0.28	0.23	0.17
3	1.05	1.27	0.96	1.00	1.14	1.11	0.23	0.31	0.29	0.21
3a	1.23	1.18	0.92	1.06	1.09	1.07	0.28	0.27	0.23	0.19
4	0.98	1.18	0.96	1.08	1.00	1.18	0.24	0.29	0.25	0.20
6	1.05	1.24	1.21	1.16	1.13	1.07	0.25	0.31	0.26	0.22
7	1.02	0.96	0.89	0.80	0.96	1.16	0.28	0.34	0.29	0.22
8	1.02	0.86	0.91	0.82	0.90	1.12	0.28	0.30	0.31	0.20
10	1.03	1.06	0.98	0.96	0.91	1.20	0.39	0.39	0.35	0.26
11	1.04	1.15	0.91	0.99	0.92	1.17	0.32	0.35	0.29	0.21
13	1.08	1.01	0.87	0.96	0.89	1.14	0.27	0.30	0.26	0.22
14	0.92	0.89	0.93	0.88	0.96	1.06	0.23	0.33	0.26	0.22
15	0.85	0.89	0.92	0.97	0.91	1.05	0.27	0.27	0.25	0.20
16	0.89	1.08	0.99	0.94	0.94	1.17	0.23	0.25	0.25	0.20

Table 3-2a. (continued)

Protein	Myoblasts					Myotubes				
	Hours of Chase									
	0	0.5	1	4	24	0	0.5	1	4	24
17	0.92	0.96	0.88	0.90	0.97	1.02	0.28	0.29	0.26	0.22
18	0.95	.093	0.97	0.99	0.96	1.13	0.26	0.27	0.26	0.20
19	1.02	0.86	0.91	0.82	0.90	1.12	0.28	0.30	0.31	0.21
20	0.92	0.99	0.92	0.84	0.92	1.11	0.26	0.29	0.24	0.20
23	0.96	1.01	1.00	0.94	0.85	1.29	0.25	0.35	0.32	0.16
24	0.93	0.93	0.97	0.92	0.95	1.11	0.27	0.27	0.26	0.20
25	0.93	0.99	0.95	0.93	1.02	1.08	0.28	0.29	0.25	0.22
27	0.78	0.93	0.81	0.91	0.73	1.15	0.20	0.20	0.27	0.24
28	0.85	0.92	0.90	0.97	1.04	1.24	0.28	0.27	0.23	0.21
Ave.	0.97	1.02	0.95	0.96	0.96	1.14	0.27	0.30	0.27	0.21
S.D.	0.01	0.12	0.08	0.09	0.09	0.08	0.04	0.04	0.03	0.02

Table 3-2b.

Large Subunit Ribosomal Proteins.

Protein	Myoblasts					Myotubes				
	Hours of Chase									
	0	0.5	1	4	24	0	0.5	1	4	24
total	1.48	1.40	1.47	1.50	1.49	1.23	1.12	1.02	1.14	1.15
3	0.88	0.89	0.96	0.93	0.97	1.05	0.24	0.26	0.27	0.21
4	0.90	1.07	0.92	0.97	0.93	1.13	0.25	0.29	0.26	0.27
6/7	0.98	1.05	1.04	1.01	1.02	1.06	0.26	0.34	0.29	0.24
8	0.95	1.00	0.90	0.90	0.93	1.20	0.24	0.29	0.24	0.18
9	0.95	1.08	0.87	0.93	0.95	1.07	0.25	0.26	0.22	0.17
10*	1.00	1.00	0.93	0.92	0.96	1.08	0.24	0.30	0.26	0.18
11	0.98	0.98	0.94	0.94	0.92	1.11	0.27	0.30	0.24	0.24
12	0.95	1.01	0.97	0.95	0.93	1.05	0.24	0.28	0.25	0.20
14	0.91	0.97	0.97	0.86	0.96	1.06	0.30	0.38	0.29	0.19
17	1.05	0.97	0.97	1.01	0.90	1.08	0.30	0.34	0.24	0.22
18*	0.96	1.05	0.95	0.92	0.86	1.15	0.20	0.30	0.24	0.21
19	0.95	0.98	1.04	1.00	1.06	1.07	0.25	0.33	0.34	0.20
21/23	1.09	1.13	0.96	1.00	0.87	1.02	0.29	0.34	0.31	0.19

Table 3-2b. (continued)

Protein	Myoblasts					Myotubes				
	Hours of chase									
	0	0.5	1	4	24	0	0.5	1	4	24
24	0.89	1.06	0.99	1.00	0.86	1.16	0.29	0.28	0.27	0.20
26	0.93	0.97	1.03	0.93	1.01	0.98	0.20	0.32	0.26	0.19
27/27a	0.93	1.02	0.96	0.95	0.97	1.08	0.18	0.28	0.25	0.18
28	0.88	0.93	0.94	0.90	1.07	0.92	0.29	0.38	0.18	0.20
29	0.93	0.93	1.00	1.07	0.99	1.20	0.30	0.32	0.28	0.24
30*	0.99	1.08	0.81	0.92	0.99	1.10	0.24	0.35	0.30	0.23
31	0.99	1.09	1.06	0.95	1.05	1.02	0.29	0.35	0.29	0.19
32*	1.06	1.12	1.11	0.93	0.95	1.10	0.25	0.39	0.25	0.13
35	0.94	1.17	1.02	1.04	0.89	1.24	0.26	0.30	0.27	0.24
36	0.92	1.03	1.01	0.87	0.87	1.07	0.25	0.30	0.27	0.21
Ave.	0.95	1.03	0.97	0.95	0.95	1.09	0.26	0.32	0.26	0.20
S.D.	0.06	0.07	0.07	0.05	0.06	0.07	0.03	0.04	0.03	0.03

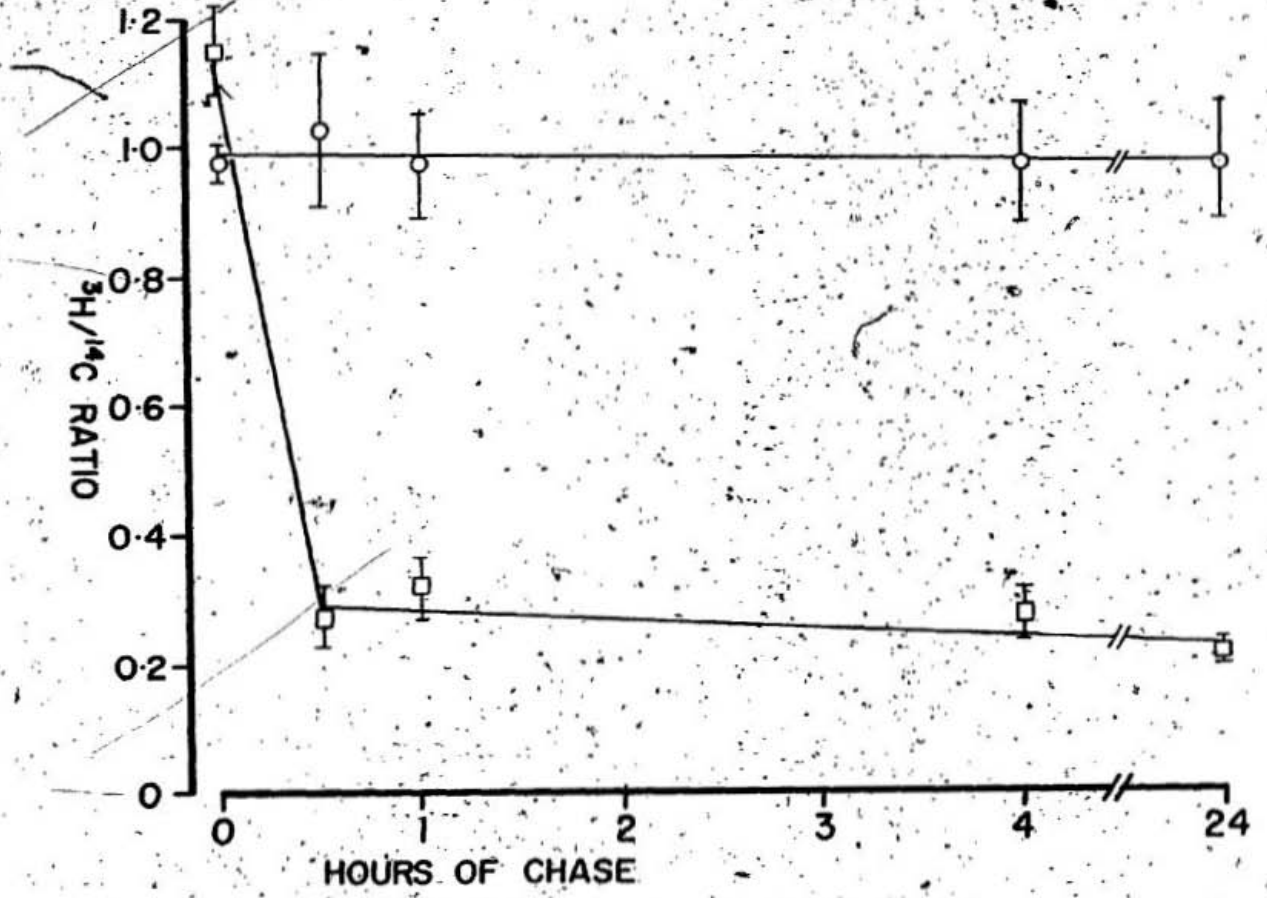
Figure 3-7.

Turnover of Ribosomal Proteins in Myoblasts and Myotubes.

The normalized $^{3}H:^{14}C$ ratios determined for individual r-proteins (Table 3-2.) were averaged and plotted against hours of chase with medium containing 10 fold excess of non-radioactive lysine. Error bars indicate standard deviations of the average.

○ Myoblasts

□ Myotubes


1·2
1·0
0·8
0·6
0·4
0·2
0
$^3H/^{14}C$ RATIO
0
1
2
3
4
24
HOURS OF CHASE

ratios remained constant during the chase period in myoblasts, suggesting that the r-proteins synthesized during the 30 minute pulse with [^{3}H] lysine were stable relative to total r-proteins for at least 24 hours. The stability of r-proteins for such a length of time can be explained by their incorporation into mature ribosomes (Warner et al., 1980). No detectable wastage or turnover of the newly synthesized r-proteins was detected relative to the total r-protein pool.

The r-proteins synthesized during the 30 minute pulse of [^{3}H] lysine in myotubes, however, were degraded largely within the first 30 minutes of chase in medium containing excess lysine. The remaining r-proteins in the myotubes (20 to 30% of the initial amount) were stable relative to total r-proteins for at least 24 hours, implying that they were incorporated into mature ribosomes.

The production of r-proteins and rRNA, which was coordinated in myoblasts (where no detectable degradation of r-proteins occurred), was uncoupled in myotubes (where approximately 75% of the newly synthesized r-proteins were degraded). In addition, the isotope ratios for each of the r-proteins after the 30 minute pulse with [^{3}H] lysine were similar, suggesting that these proteins were synthesized stoichiometrically. The stoichiometry was maintained in myotubes despite the fact that approximately 75% of the r-proteins were degraded. The drop in the rate of

incorporation of r-proteins into ribosomes also corresponded closely with the drop in the rate of rRNA production observed earlier (Table 3-1).

C) Ribosomal Protein mRNA's

The results of the r-protein turnover studies above suggest that synthesis of rRNA and r-proteins, which was coordinated in myoblasts became uncoupled in myotubes. The r-proteins in myotubes were synthesized in excess over the available rRNA and were quickly degraded. To investigate possible mechanisms for the apparent uncoupling of rRNA and r-protein production, the metabolism of r-protein synthesis must be understood. It has already been established that the rate of r-protein synthesis relative to that of total protein did not change significantly as a result of differentiation (Table 3-2). The rate of r-protein synthesis can be controlled by mechanisms including regulating the efficiency of translation of a pool of rp-mRNA's or controlling the concentration of translatable rp-mRNA's in the cell cytosol. Furthermore, the cytoplasmic levels of rp-mRNA's are affected by factors such as mRNA half lives and transcription rates. Analysis of these factors should reveal whether the uncoupling of rRNA and r-protein synthesis originates at this level.

i) Messenger-RNA Levels in Subcellular Fractions

To determine whether the various sub-cellular pools of rp-mRNA's were altered by the process of differentiation, the distribution of the rp-mRNA's within the cell was investigated. The rp-mRNA's were directly quantitated from the nuclear, post-polysomal and polysomal fractions of L6-5 cells by hybrid-selection (Lichter et al., 1982). L6-5 cells (70% confluent myoblasts and 4 day myotubes) were incubated in medium containing [^{3}H]uridine for 54 hours to label the RNA to constant specific activity. The RNA prepared from these fractions was enriched for poly(A) containing mRNA's by oligo(dT)-cellulose chromatography. The poly(A) enriched RNA was then hybrid-selected with excess amounts of plasmids pS16, pL10, pL18, pL30 and pL32, which were collectively bound to DPT-paper filters. The rp-cDNA plasmids were used collectively to generate sufficient signal over background in the hybridization reaction. Since the RNA was labelled to constant specific activity, the radioactivity bound to the DPT-paper filters represented the fraction of rp-mRNA in the total poly(A) enriched RNA in the hybridization reaction (Table 3-3). These values, however, were an underestimation of the total amount of the specific rp-mRNA's in the hybridization solution since the rp-cDNA plasmids were not full length copies.

Table 3-3.

Levels of Ribosomal Protein mRNA's in Subcellular Fractions of Myoblasts and Myotubes.

Poly(A) enriched RNA prepared from nuclear, post-polysomal and polysomal fractions of 70% confluent myoblasts and 4 day myotubes which had been incubated in medium containing 100 uCi per ml of [^{3}H]uridine to constant specific activity were hybrid-selected with recombinant probes pS16, pL10, pL18, pL30 and pL32 combined on DPT-paper filter. A separate DPT-paper filter contained the plasmid vehicle pMB9 and served as a control. The RNA which remained hybridized to the DPT-paper filters after RNase A treatment and stringent washing was eluted with water at 95^{o}C and counted. 'MB' and 'MT' indicate results from myoblasts and myotubes respectively with the control value of 44 cpms due to pMB9 subtracted. The results are averages of 2 determinations.

Table 3-3. (continued)

Source		Input cpm	Bound cpm	Bound/Input Percent
Nuclear	MB	478k	104	0.022
	MT	276k	53	0.019
Post-polysomal	MB	205k	42	0.020
	MT	74.7k	18	0.024
Polysomal	MB	3360k	1940	0.065
	MT	2600k	1680	0.065
Total	MB	4041k	2085	0.052
	MT	2948k	1750	0.059

The 5 combined rp-cDNA plasmids bound similar amounts of [^{3}H]uridine labelled RNA in the 3 respective myoblast and myotube sub-cellular fractions. The results suggest that the rate of r-protein translation in myotubes was maintained at myoblast levels because the relative polysomal levels of the rp-mRNA's were similar. In addition, no significant alteration of rp-mRNA pool sizes or net pool shifts appeared to occur to maintain the level of translatable rp-mRNA's in polysomes. Similar nuclear levels of rp-mRNA's in myoblasts and myotubes suggest that their transcription rates may also be similar.

ii) Levels of Polysomal Ribosomal Protein mRNA's

The use of the rp-cDNA's collectively on DPT-paper filters may disguise variations in the levels of individual rp-mRNA's. The poly(A) enriched polysomal mRNA's were therefore subjected to Northern blot analysis with individual (^{32}P)-labelled nick-translated rp-cDNA's.

Poly(A) enriched RNA was prepared from polysomal fractions of 70% confluent myoblasts and 4 day myotubes and 10 ug aliquots of the RNA were subjected to electrophoresis in 1.2% agarose. After transferring the RNA to DPT-paper, these Northern blots were probed with (^{32}P)-labelled nick-translated plasmids pS16, pL10, pL18, pL30 and pL32 for the respective rp-mRNA's and autoradiographed (Figure 3-8). The autoradiographs were quantitated by scanning densitometry

Figure 3-8.

Northern Blot Analysis of Specific Polysomal RNA's from Myoblasts and Myotubes.

Ten ug samples of poly(A) enriched polysomal RNA each from 70% confluent myoblasts and 4 day myotubes were subjected to electrophoresis in 1.2% agarose and electro-blotted onto DPT-paper. The Northern blots were probed with [alpha-^{32}P]dCTP nick-translated plasmids pS16, pL10, pL18, pL30, pL32, pMHC and pH4 and subjected to autoradiography. The L32 Northern blot includes the homologous control plasmid pL32 in the left lane (labelled p24).

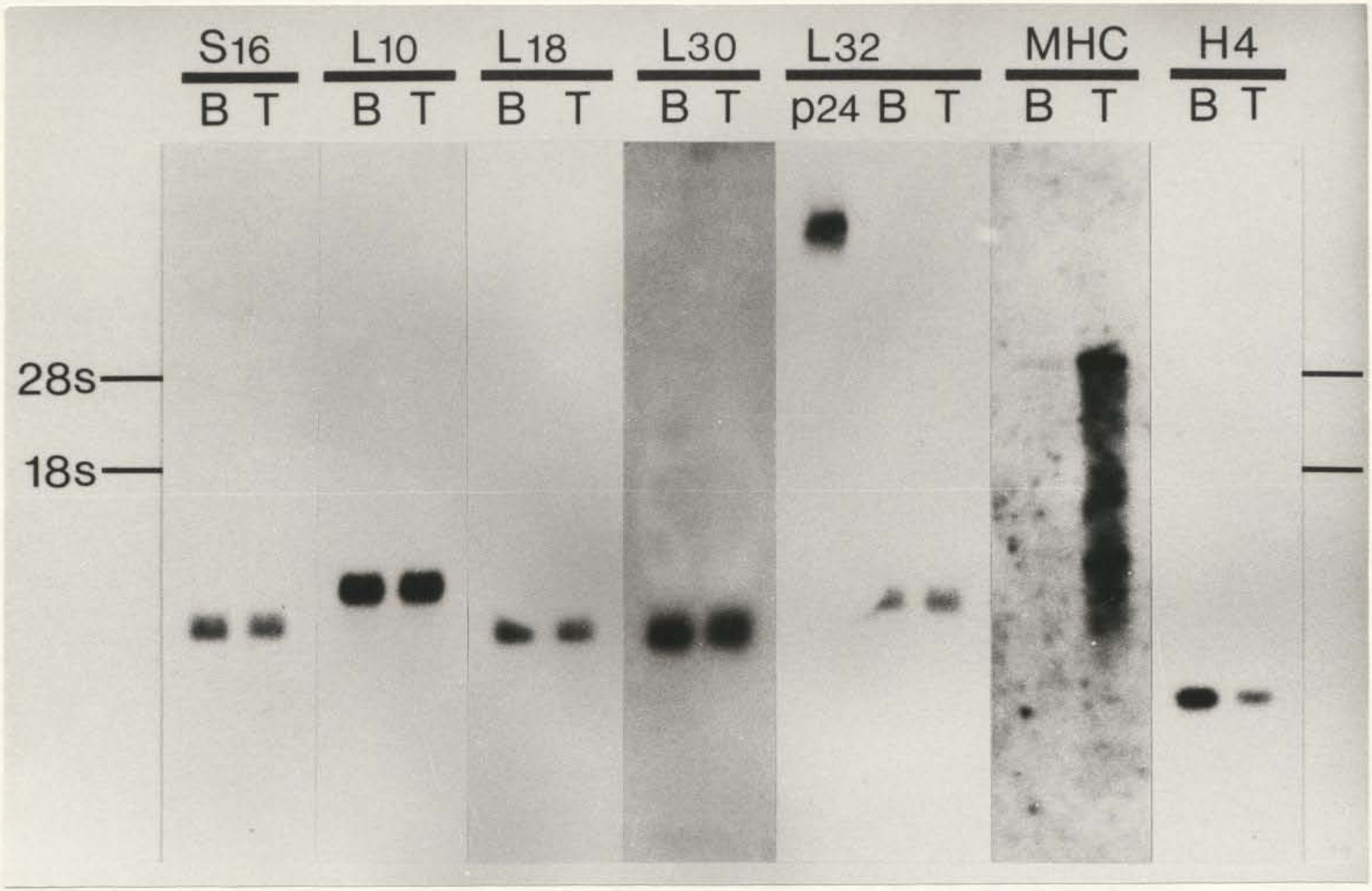

S16
L10
L18
L30
L32
MHC
H4
B T
B T
B T
B T
p24 B T
B T
B T
28s
18s

Table 3-4.

Quantitation of Specific Polysomal mRNA Levels in Myoblasts and Myotubes.

Ten ug of poly(A) enriched polysomal RNA each from 70% confluent myoblasts and 4 day myotubes were subjected to electrophoresis in 1.2% agarose and electro-blotted onto DPT-paper. The blots were probed with [alpha-^{32}P]dCTP nick translated plasmids pS16, pL10, pL18, pL30, pL32, pMHC and pH4 and autoradiographed (Figure 3-8). The Northern blots were quantitated by scanning densitometry and the peak areas of the hybridized bands determined with myoblast values equal to unity. The results are averages of 2 determinations.

	Myotubes/Myoblasts Percent
Ribosomal Protein	
S16	94
L10	80
L18	86
L30	98
L32	118
Myosin Heavy Chain	present only in Myotubes
Histone H4	22

and the relative amounts of hybridizable material in myoblasts and myotubes were determined (Table 3-4).

The levels of the rp-mRNA's in polysomes, as a fraction of total poly(A) enriched polysomal RNA, were similar in myoblasts and myotubes. These data complement both the r-protein data (Table 3-2) and the hybrid-selection data (Table 3-3) since the maintained levels of individual rp-mRNA's accounted for the maintained level of synthesis of the individual r-protein in myotubes. Because the pool sizes and distribution of the rp-mRNA's were similar in myoblasts and myotubes, the uncoupling of the coordinate expression of rRNA and r-proteins in myotubes appeared not to be due to a translational mechanism. The hypothesis generated from these results was that the uncoupling of rRNA and r-protein synthesis in myotubes occurred at the level of transcription.

To compare the data for rp-mRNA's with other mRNA's whose production were affected by myogenesis, Northern blots of poly(A) enriched polysomal RNA were probed with pMHC and pH4.

Myosin heavy chain is a muscle specific protein which is expressed only in differentiated L6 cells (Benoff & Nadal-Ginard, 1979). An almost undetectable amount of MHC-mRNA was observed in the myoblast RNA sample, while an abundance was found in the myotube sample (Figure 3-8). The heterogeneous profile of the myotube MHC-mRNA observed

resulted from the severe over-exposure of the autoradiograph to allow what little MHC-mRNA in the myoblast RNA fraction to become apparent. As a result, degradation products of the MHC-mRNA in the myotube RNA preparation were exaggerated as the main MHC-mRNA:(^{32}P)-cDNA hybrid over-exposed the autoradiograph.

The other probe used was that for histone H4-mRNA's. Since the expression of histone genes is linked to cell proliferation, their expression should occur only in dividing myoblasts (Hentschel & Birnstiel, 1982). Probing myotube polysomal RNA samples with the histone H4 genomic clone, pH4, revealed that the levels of the H4-mRNA dropped by approximately 5 fold after differentiation (Figure 3-8 and Table 3-4), confirming its connection with cell proliferation.

The induction of MHC-mRNA and the repression of H4-mRNA in L6-5 cells after the myoblasts had been exposed to Differentiation Medium for several days indicated that the cells have differentiated into myotubes. These results therefore provide the biochemical complement to the morphological evidence (Figure 3-1) of the terminal differentiation process of L6-5 cells

D) Turnover of Ribosomal Protein mRNA's

The similar levels of rp-mRNA's in the polysomal fractions of myoblasts and myotubes may be a result of

similar transcription rates of the r-protein genes. However, the half lives of the rp-mRNA's may play a significant role in maintaining rp-mRNA levels and r-protein synthesis rates in myoblasts and myotubes and therefore merit investigation. Two general strategies were employed to measure rp-mRNA turnover in L6-5 cells. The first was to determine the decay rates of rp-mRNA's in vivo after the inhibition of transcription with the drug Actinomycin D. The second was to measure their transcription rates in an in-vitro nuclear transcription assay. The latter method also allowed the direct comparison of the relative transcription rates of the rp-mRNA's and rRNA in myoblasts and myotubes.

i) Ribosomal Protein mRNA Half Lives

The drug Actinomycin D inhibits the synthesis of RNA in eukaryotes (Wilson & Hoagland, 1967). Once transcription is inhibited, the rate of decay of RNA can then be assessed by directly measuring the RNA content of the cell at various times after the drug has been administered.

Experiments were first performed to determine the amount of Actinomycin D exposure required for the complete inhibition of RNA synthesis. L6-5 myoblasts were incubated for 2 hours in medium containing [^{3}H] uridine or [^{3}H] lysine and various concentrations of Actinomycin D. Total TCA insoluble radioactivity was determined from whole cell

lysates (Figure 3-9).

Complete inhibition of RNA synthesis in myoblasts was obtained at a concentration of 2 ug per ml of Actinomycin D. This concentration was also sufficient to completely inhibit RNA synthesis in myotubes (Figure 3-9). Although protein synthesis was inhibited by 15% at concentrations above 0.5 ug per ml of Actinomycin D, it did not change with Actinomycin D concentrations of up to 10 ug per ml.

To ensure that a specific sub-class of RNA was not transcribed in the presence of Actinomycin D, total RNA was prepared from 70% confluent myoblasts previously incubated for 2 hours in medium containing 50 uCi per ml of [^{3}H]uridine and various concentrations of Actinomycin D. Equal aliquots (10 ug) of RNA were subjected to electrophoresis in 1.2% agarose, electro-blotted and subjected to autoradiography (Figure 3-10). The autoradiographs confirmed that negligible amounts of RNA were synthesized by L6-5 cells when exposed to concentrations of 3 ug per ml of Actinomycin D. It was noted that at a concentration of 0.03 ug per ml of Actinomycin D, the transcription of rRNA was specifically inhibited.

The rates of decay of rp-mRNA's were determined in 70% confluent myoblasts and 4 day myotubes. L6-5 cells were incubated in medium containing 3 ug per ml of Actinomycin D and lysed at multiples of 3 hour intervals. Equal amounts of total RNA prepared from cell lysates were subjected to

Figure 3-9.

Treatment of Myoblasts and Myotubes with Actinomycin D.

L6-5 cells (70% confluent myoblasts and 4 day myotubes) were incubated for 2 hours with medium containing 5 uCi per ml of [^{3}H]uridine or 5 uCi per ml of [^{3}H] lysine and various concentrations of Actinomycin D. Total precipitable radioactivity was determined from whole cell lysates. Duplicate samples were averaged and plotted.

△ cpm x10^{-3} [^{3}H] lysine (myoblast)

○ cpm x10^{-3} [^{3}H]uridine (myoblast)

□ cpm x10^{-3} [^{3}H]uridine (myotubes)

(data point at 2 ug per ml Actinomycin D)

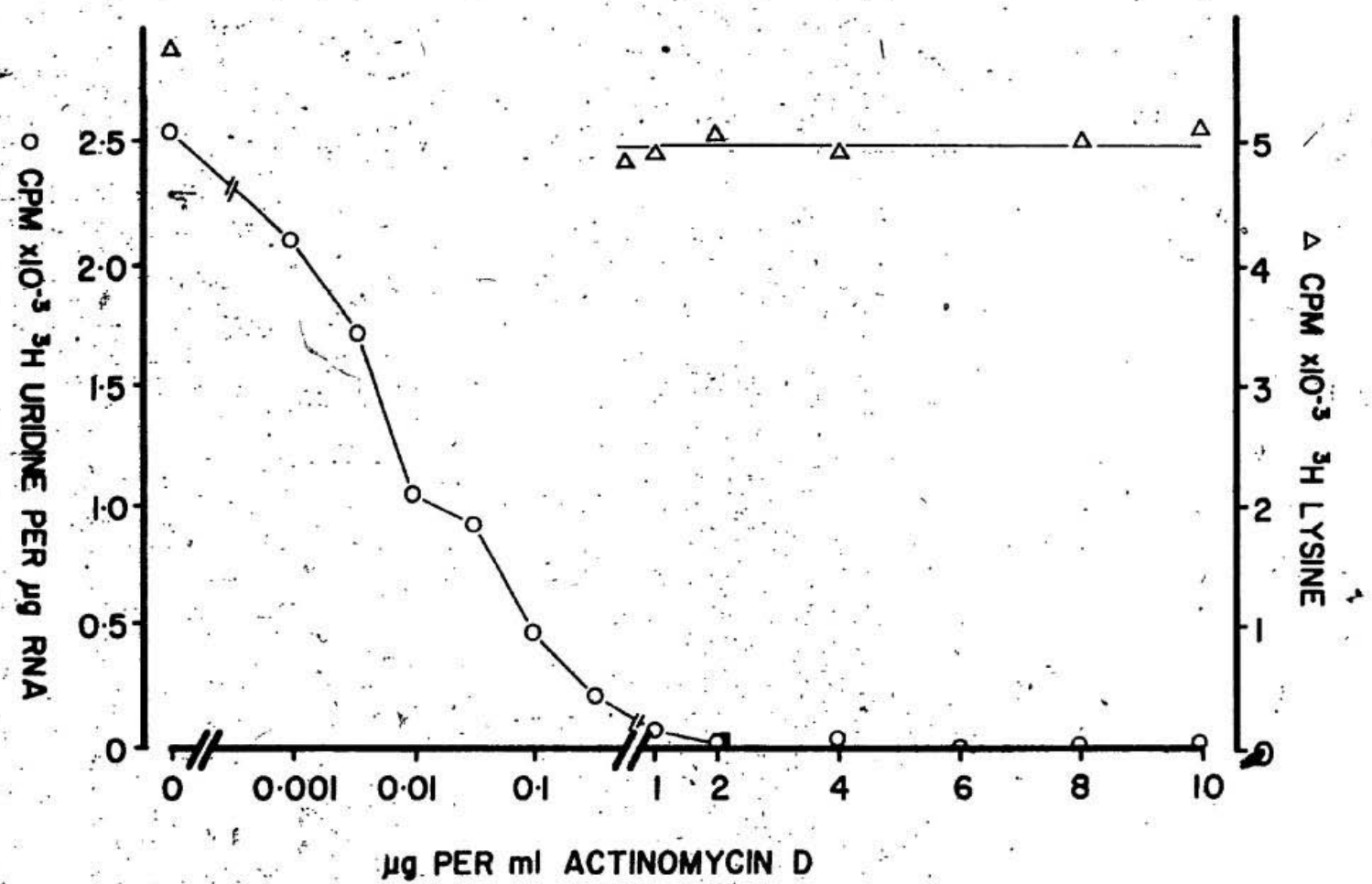
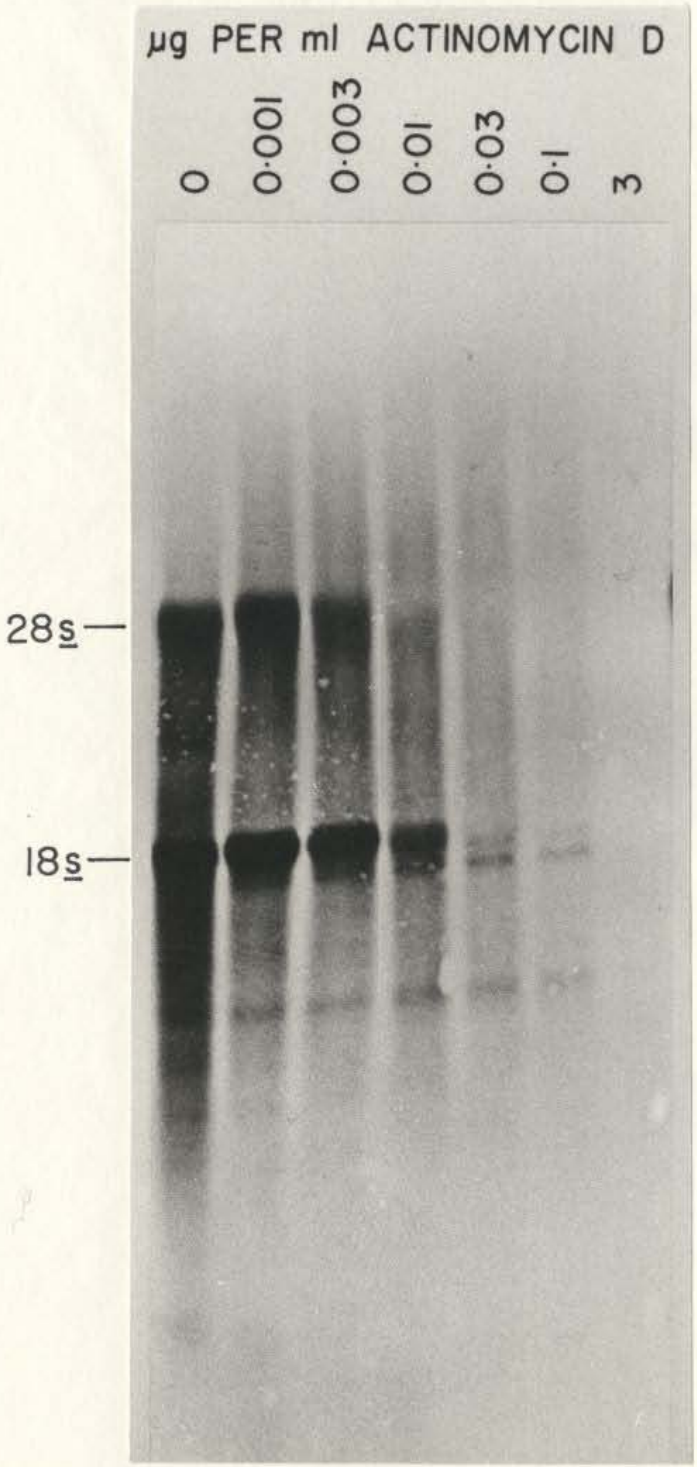

o CPM xIO-3 3H URIDINE PER µg RNA
Δ CPM xIO-3 3H LYSINE
2·5
2·0
1·5
1·0
0·5
0
5
4
3
2
1
0
0
0·001
0·01
0·1
1
2
4
6
8
10
µg PER ml ACTINOMYCIN D

Figure 3-10.

Incorporation of Uridine during Treatment of Myoblasts and Myotubes with Actinomycin D.

Ten ug samples of total RNA, prepared from 70% confluent myoblasts which had been incubated for 2 hours in medium containing 50 uCi per ml of [^{3}H]uridine and various concentrations of Actinomycin D, were subjected to electrophoresis in 1.2% agarose. After the RNA was electro-blotted onto Gene Screen^{tm}, it was treated with Spray En^{3}Hance^{tm} and autoradiographed.


µg PER ml ACTINOMYCIN D
0
0·001
0·003
0·01
0·03
0·1
3
28s
18s

electrophoresis in 1.2% agarose and electro-blotted onto Gene Screen^{tm}. The Northern blots, probed with (^{32}P)-labelled nick-translated plasmids pS16, pL18 and pL32, were autoradiographed and quantitated by scanning densitometry. The slopes of the mRNA decay rates were determined by least squares analysis (Figures 3-11a and 3-11b, Bulmer, 1979).

The apparent half lives of the 3 rp-mRNA's varied from 9.5 to 12.4 hours, with standard deviations of approximately 1 (Table 3-5). The average half lives of the rp-mRNA's were 11 and 10.6 hours in myoblasts and myotubes respectively. No significant differences were therefore apparent in the rp-mRNA half lives between myoblasts and myotubes.

To ensure that the half life studies were measuring specific decay rates of the rp-mRNA's, the half lives of MHC-mRNA and H4-mRNA were also determined. The half life of MHC-mRNA, which was only measurable in myotubes was approximately 67 hours. In a separate series of experiments, the half life of histone H4-mRNA in myoblasts was determined to be approximately 40 minutes (see Appendix A). These results indicated that the decay rates of the mRNA's in the presence of Actinomycin D were message specific and not due to non-specific degradation.

Figure 3-11.

Northern Blot Analysis of RNA from Actinomycin D Treated Myoblasts and Myotubes.

Total RNA was prepared from 70% confluent myoblasts and 4 day myotubes which had been incubated in medium containing 3 ug per ml of Actinomycin D for various periods of time. Aliquots of RNA (25 ug) were subjected to electrophoresis in 1.2% agarose and electro-blotted onto Gene Screen^{tm}. The Northern blot was probed with (^{32}P)-labelled nick-translated plasmids pS16, pL18, pL32 and pMHC and autoradiographed. The autoradiographs were quantitated by scanning densitometry and the areas determined for the hybridized bands were normalized to the values determined for the untreated samples. The MHC-mRNA was not detected in significant quantities in the myoblast samples. Northern blots of myoblasts RNA samples are displayed in Figure 3-11a and of myotube RNA samples in Figure 3-11b.

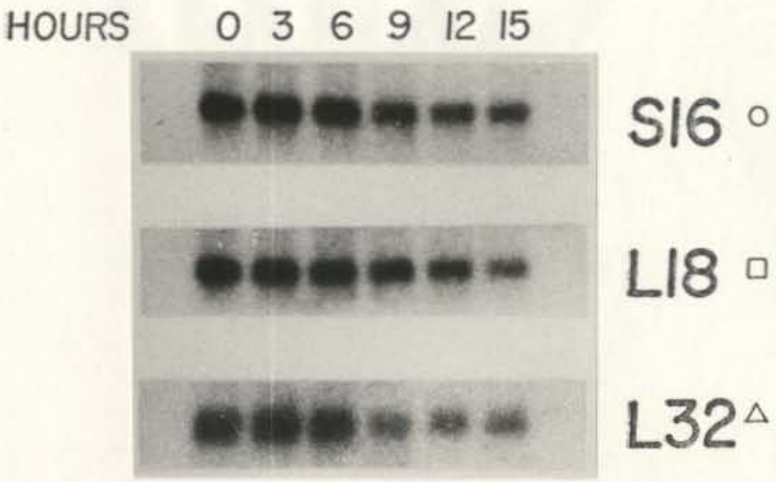
O rp-mRNA S16

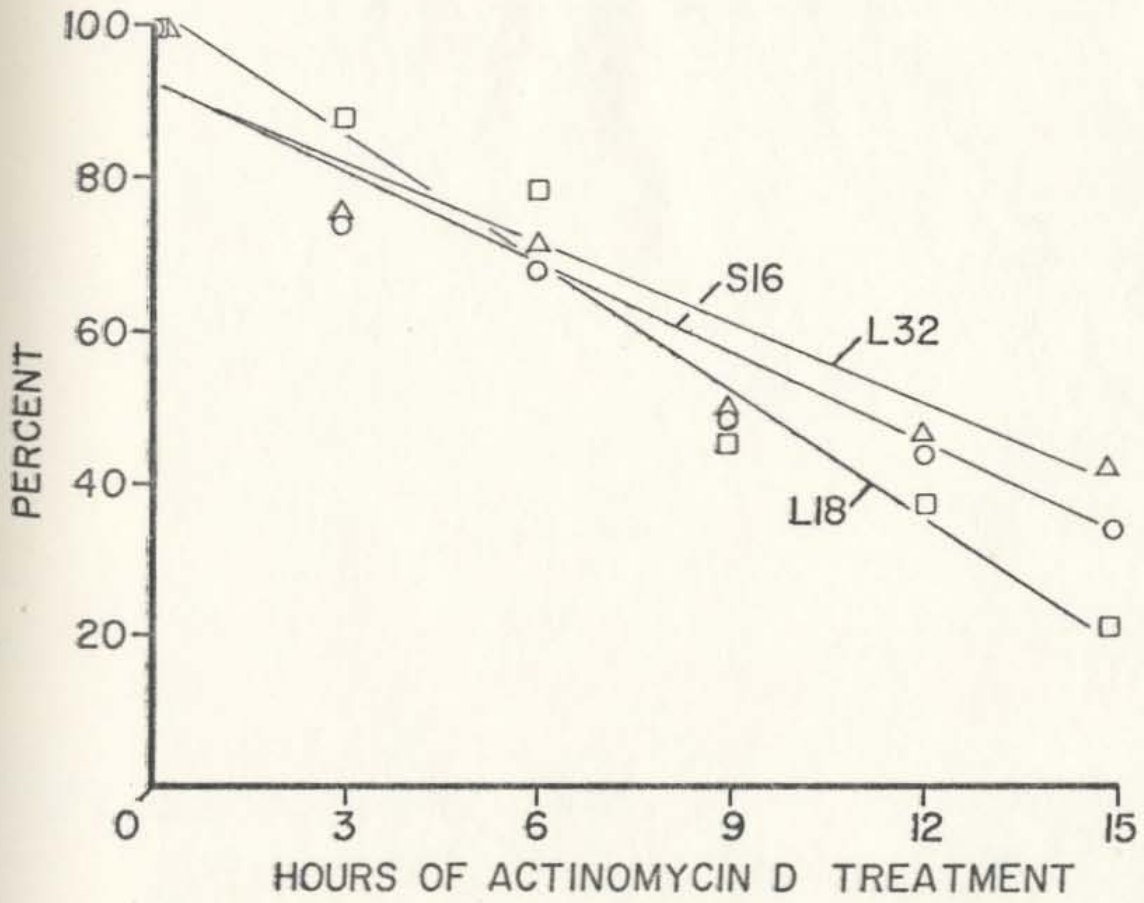
□ rp-mRNA L18

Δ rp-mRNA L32

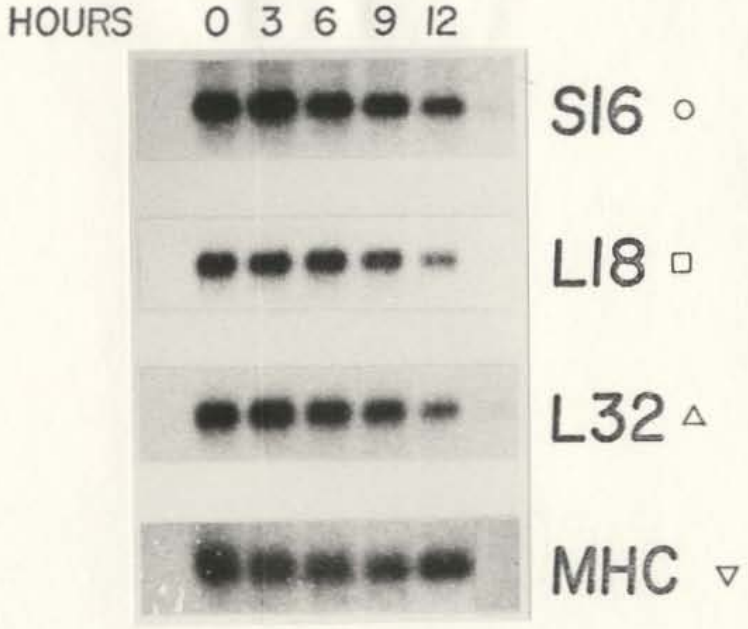
▽ MHC-mRNA

MYOBLASTS


HOURS 0 3 6 9 12 15
S16 ○
L18 □
L32 △


100
80
60
40
20
PERCENT
S16
L32
L18
0
3
6
9
12
15
HOURS OF ACTINOMYCIN D TREATMENT

MYOTUBES


HOURS 0 3 6 9 12
S16 ○
L18 □
L32 △
MHC ▽

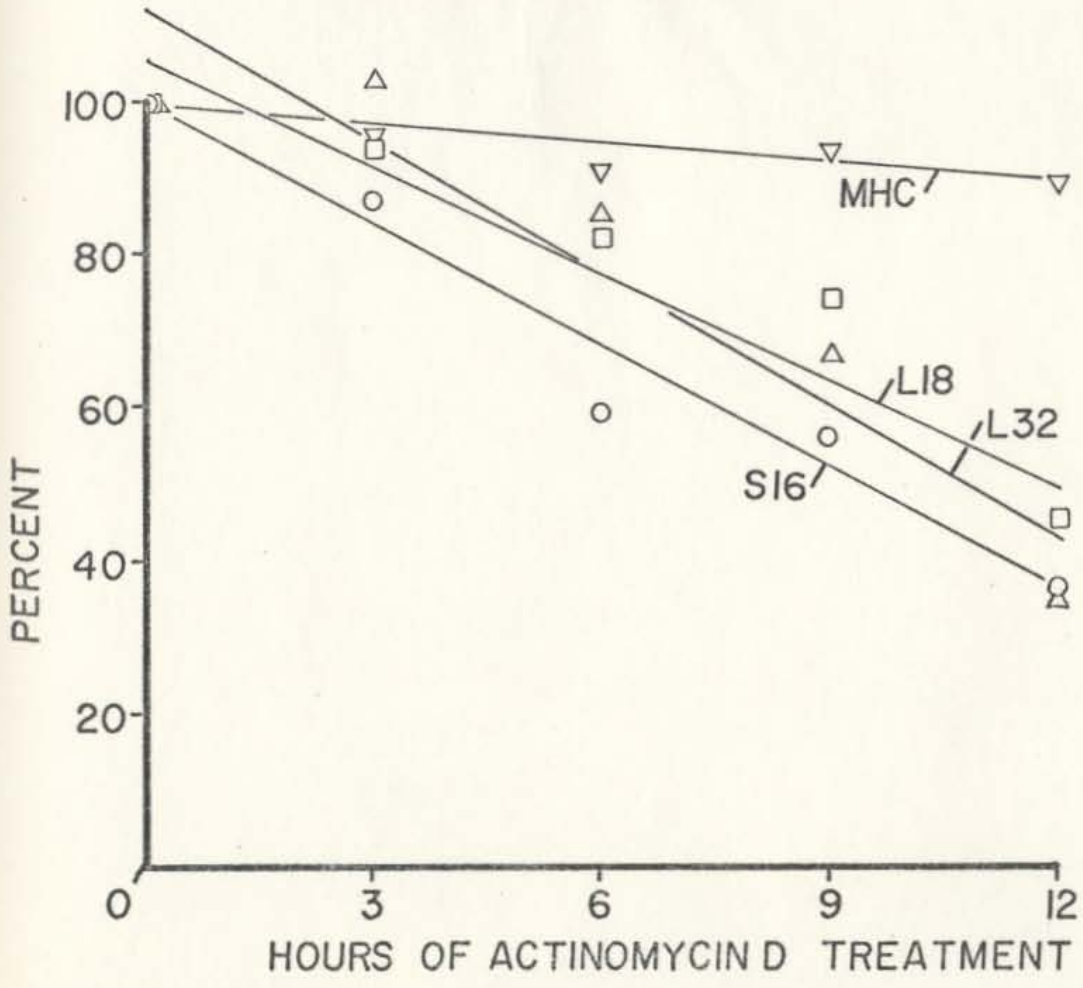

100
80
60
40
20
0
PERCENT
MHC
L18
L32
S16
3
6
9
12
HOURS OF ACTINOMYCIN D TREATMENT

Table 3-5.

Half Lives of Specific mRNA's in Myoblasts and Myotubes.

Total RNA was prepared from 70% myoblasts and 4 day myotubes which had been incubated in medium containing 3 ug per ml of Actinomycin D for various periods of time. Aliquots of 25 ug of RNA were subjected to electrophoresis in 1.2% agarose, electroblotted onto Gene Screen^{tm}, probed with (^{32}P)-labelled nick-translated plasmids pS16, pL18, pL32 and pMHC and autoradiographed. The autoradiographs were quantitated by scanning densitometry and the relative peak areas of the hybridized bands were plotted, with the zero time points equal to unity (Figures 3-11a. and 3-11b). The slopes of the decay rates were determined by least squares analysis and the results from two determinations were averaged.

Table 3-5. (continued)

	Half Lives in Hours			
Ribosomal Protein	Myoblasts	S.D.[a]	Myotubes	S.D.
S16	11.1	0.4	9.5	0.6
L18	12.3	0.5	12.4	1.2
L32	9.6	0.6	10.0	1.2
Myosin Heavy Chain	b	-	67	2.3
Histone H4[c]	0.65	nd	nd	-

a) S.D.: Standard Deviation.

b) not detectable

c) see Appendix A

nd: not determined

ii) Nuclear Transcription

Analysis of the rRNA accumulation and r-protein synthesis rates in L6-5 cells revealed that production of these ribosomal components became uncoupled after terminal differentiation. Results which indicated similar rp-mRNA levels and half lives in myoblast and myotubes supported the belief that the uncoupling occurred at the transcriptional level. To test this hypothesis, a cell-free nuclear transcription assay was employed to measure the rates of transcription of rp-mRNA and rRNA.

The reaction time course of the nuclear transcription assay was determined with nuclei purified from 70% confluent myoblasts and 4 day myotubes. The nuclei were incubated with a nucleotide triphosphate mixture including [alpha-^{32}P]UTP for various periods of time (Figure 3-12). Initial transcription rates were higher than those observed over the long term, thus the initial time points were not plotted. The transcription rates, however, were linear after the first 20 minutes. This phenomenon has been noted in other nuclear transcription systems and may be due to polymerases which have bound to promoter regions but were not transcribing when the nuclei were prepared. However, it does not appear to affect relative transcription rate measurements (Marzluff & Huang, 1984). Subsequent assays were carried out for 60 minutes.

Figure 3-12.

Time Course of Transcription in Myoblast and Myotube Nuclei.

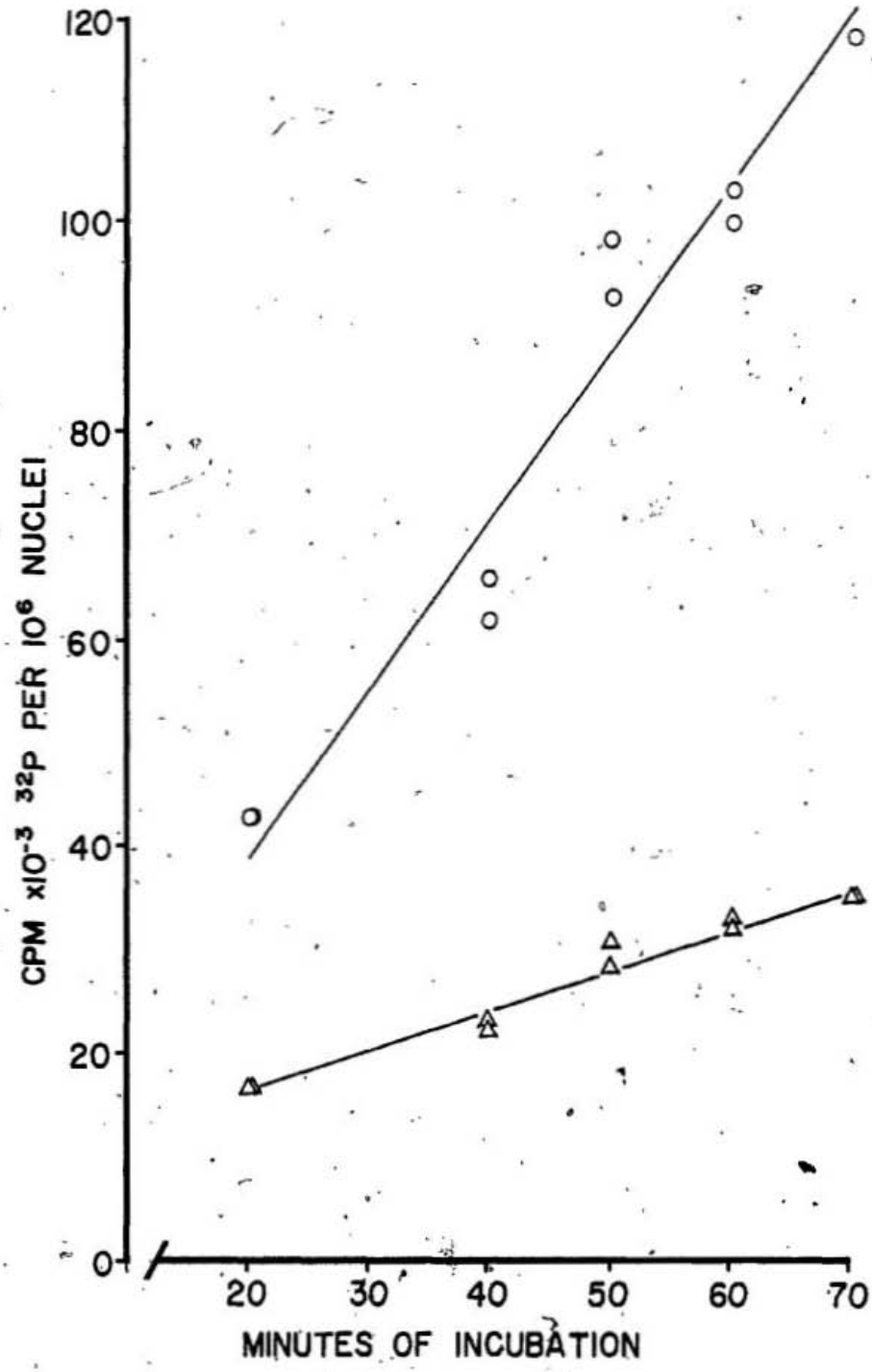
Nuclei prepared from 70% confluent myoblasts and 4 day myotubes were incubated with nucleotide triphosphates including [alpha-^{32}P]UTP at 25°C. At various time points, the reactions were terminated and total TCA insoluble radioactivity per 10^6 nuclei determined.

O Myoblasts

Δ Myotubes

Slopes: Myoblasts 1.62 kcpm/10^6 nuclei minute

Myotubes 0.39 kcpm/10^6 nuclei minute


120
100
80
60
40
20
0
CPM x10⁻³ ³²P PER 10⁶ NUCLEI
20
30
40
50
60
70
MINUTES OF INCUBATION

Incorporation of (^{32}p) into RNA was determined on the basis of the number of nuclei. The slopes of the incorporation rates therefore indicated the relative rates of overall transcription (Figure 3-12). The transcription rate in myotubes was found to be approximately 24% of the rate found in myoblasts.

The (^{32}p)-labelled RNA was purified from the transcription assay system and hybrid-selected with genomic clones for rp-mRNA L32 and for the leader sequence of the 18s and 28s rRNA's and pBR322. To establish that the hybridization conditions were RNA dependent, 2 concentrations of each of the genomic clones were used for hybrid-selection. Hybridization of the (^{32}p)-labelled RNA with the genomic clones and subsequent washing of the hybrid-selected material was performed using stringent conditions (Marzluff & Huang, 1984). After quantitating the (^{32}p)-labelled RNA bound to each clone, the pBR322 control values were subtracted from the appropriate experimental points (Table 3-6).

The actual amount of rp-mRNA L32 detected was approximately 0.006% of the total amount applied in the hybridization. This figure falls within the range commonly cited for mammalian rp-mRNA's (Meyuhas, 1984).

The values determined for the rRNA precursors, on the other hand, were much lower than would be suggested by the abundance of the 45s rRNA processing products *in vivo*

Table 3-6.

In Vitro Nuclear Transcription in L6-5 Cells.

Nuclei prepared from 70% confluent myoblasts and 4 day myotubes were incubated with nucleotide triphosphates including [alpha-^{32}P]UTP at 25^{o}C for 60 minutes. RNA purified from the transcription reaction was hybrid-selected with individual recombinant DNA clones, including pBR322 as a blank control, bound to Gene Screen^{tm}. After stringent washing, the remaining hybridized (^{32}P)-labelled RNA was counted, the background hybridization of 52 cpms due to pBR322 subtracted and the bound myotube/myoblast ratios normalized to the amount of input radioactivity in the myoblast nuclear transcription assay. 'MB' and 'MT' indicate results of assays with nuclei from myoblasts and myotubes respectively.

Table 3-6. (continued)

Probe type	ug	Nuclei Source	Input cpm	Bound cpm	Bound (MT/MB) average percent
pgL32	7	MB	700k	41.3	
	3.5	MB	700k	47.0	74%
	7	MT	910k	39.1	
	3.5	MT	910k	46.5	
p-pre18s	7	MB	7300	82.2	
	3.5	MB	7300	96.7	22%
	7	MT	7400	18.7	
	3.5	MT	7400	20.9	
p-pre28s	7	MB	7300	546	
	3.5	MB	7300	531	24%
	7	MT	7400	129	
	3.5	MT	7400	137	

(Dabev et al., 1978). However, since r-proteins and RNA methylation, both of which are required for proper 45s rRNA processing, are deficient in the in vitro transcription system (Marzluff, 1984), significant non-specific processing of the rRNA fragments may occur. The 2k bp p-pre18s does not contain any sequences found in mature rRNA's while the 2.3k bp p-pre28s contains small portions of the 3' end of the mature 18s and the 5' end of the mature 28s rRNA's in addition to the 5.8s rRNA. Therefore, some rRNA sequences complementary to p-pre28s may be more stable than those complementary to p-pre18s after 1 hour incubation in vitro and may account for the higher bound counts observed with the former recombinant plasmid.

The relative rate of transcription of rp-mRNA L32 in myotubes was approximately 74% of the rate observed in myoblasts. The transcription rate of rRNA, on the other hand, dropped by 76% in myotubes. These determinations were independent of plasmid concentration. The data reveal that these 2 sets of genes were differentially expressed in myoblasts and myotubes.

Mechanisms such as changes in translational efficiency, sub-cellular populations and half lives of the rp-mRNA's appear not to be significant factors in the observed uncoupling of the rate of rRNA and r-protein synthesis after terminal differentiation of the L6-5 myoblast. The remaining factor which could account for the uncoupling is

differential transcription of the rRNA and the rp-mRNA's. The above experiments demonstrate that, in myotubes, the rate of rRNA transcription was reduced by a much greater extent than the rate of rp-mRNA transcription when compared to myoblasts. Thus, upon terminal differentiation of L6-5 cells, coordinate transcription of rRNA and r-proteins seen in the myoblast becomes uncoupled.

CHAPTER FOUR

Discussion

The ribosome performs an instrumental function in the expression of genes. The organelle translates the genetically encoded information of inheritance into proteins. For the ribosome to perform effectively, it must itself be regulated, both in its translational activity and its biogenesis. The regulation of ribosome biogenesis, however, is complicated by the fact that its over 70 components are products of genes transcribed by three distinct RNA polymerases. The coordinate production of these components under various physiological conditions is an important subject for investigation.

A cell system which undergoes marked physiological changes *in vitro* and also significantly alters its ribosome accumulation rates is the L6 myoblast. Upon terminal differentiation of L6 cells, the rate of ribosome accumulation decreases four to five fold (Krauter *et al.*, 1979). L6-5 myogenesis therefore provides a system in which the coordinate regulation of the synthesis of the ribosomal components can be studied.

Differentiation of L6-5 myoblasts, a sub-clone of L6, is induced by a change in cell culture conditions. The shift from mitogen rich Growth Medium to mitogen poor Differentiation Medium activates the myogenesis program

(Nadal-Ginard, 1978, Linkhart, Clegg & Hauska, 1981). The myoblasts undergo at least one or two rounds of cell division before committing themselves to differentiation (Figure 3-2). Although differentiation can be induced without intervening rounds of DNA synthesis (Nadal-Ginard, 1978), the myoblasts are 'conditioning' the medium during this time to allow differentiation to proceed (Doering & Fischman, 1977). The conditioning process probably involves the degradation of specific mitogenic factors in the medium which inhibit the initiation of the differentiation process (Linkhart et al., 1981). However, once the mitogens have been depleted, the decision to proceed with myogenesis remains a stochastic one and the cells do not differentiate synchronously.

Ribosome Biogenesis in L6-5 Cells

Differentiation of L6-5 myoblasts resulted in the decrease in rRNA accumulation rates (Figures 3-2, 3-3 and 3-4 and Table 3-1). Since mRNA and protein accumulation rates were relatively unaffected, the reduced rRNA accumulation rates were interpreted to be the result of the myogenesis program. The levels of mature ribosomes in myotubes, however, were still near those of myoblasts, suggesting that the rate of ribosome accumulation was modulated to maintain their cytoplasmic levels (Figures 3-2 and Krauter et al, 1979). Since the myotube cell mass is increasing at a

much slower rate than that of the myoblast cell, it follows that a significantly lower rate of rRNA synthesis would maintain the cytoplasmic pool of ribosomes in myotubes.

The rate of r-protein synthesis, on the other hand, was not significantly affected by the differentiation process (Figure 3-7 and Table 3-2). The r-proteins in myotubes were synthesized stoichiometrically and at rates similar to those observed in myoblasts, although they were largely degraded. It therefore appeared that their production was being regulated as it would have been in myoblasts. The regulatory mechanisms invoked by the differentiation program, which reduced rRNA and ribosome accumulation rates did not affect the production of r-proteins.

One of two general situations may exist in L6-5 myotubes which could account for the uncoupling of rRNA and r-protein production. The first is the possibility that rRNA and rp-mRNA's are coordinately synthesized but that the coordination is subverted at a post-transcriptional level. If the rates of rRNA and rp-mRNA's synthesis were coordinately reduced in myotubes, abnormally high r-protein translation rates may result from 1) increased half lives of the rp-mRNA's, which would counteract their lower accumulation rates, 2) increased translational efficiency of a decreased level of rp-mRNA's, perhaps by the presence of a specific initiation factor, or 3) an increased proportion of the total cellular rp-mRNA's appearing in the polysomal

(translating) fraction at the expense of other cellular fractions.

The second situation which may exist in L6-5 myotubes is that production of rp-mRNA's and rRNA is uncoupled at the transcriptional level. The uncoupling may either result from an L6-5 specific defect in the regulatory mechanisms coordinating the two groups of ribosomal components or may be a general feature of rat myotubes.

To determine how post-transcriptional events affect r-protein synthesis in L6-5 cells, the levels and turnover of rp-mRNA's in myoblasts and myotubes were investigated. The rp-mRNA levels in polysomes of myoblasts and myotubes were shown to be similar and reflected the similar rates of r-protein synthesis (Figures 3-7 and 3-8 and Tables 3-2 through 3-4). Since the accumulation rates of nascent r-proteins corresponded with the rp-mRNA levels, efficiencies of rp-mRNA translation were also similar in the two phenotypes. It is possible, however, that fine-tuning of r-protein synthesis to maintain their precise stoichiometry is achieved by minor shifts between the polysomal and post-polysomal rp-mRNA pools (Fruscolini et al., 1984). Nevertheless, the rate of r-protein synthesis in differentiating L6-5 cells was not modified by translational regulatory mechanisms.

The half lives of the rp-mRNA's were also determined in myoblasts and myotubes (Figure 3-1 and Table 3-5). These

messages had half lives of approximately 11 hours in both phenotypes, despite the finding that mRNA's in myotubes tend to be more stable (Figure 3-11, Table 3-5 and Singer & Kessler-Icekson, 1978). The half life of rp-mRNA's therefore appeared to be an intrinsic property of the messages and not affected by the myogenesis program.

It is clear that cytoplasmic mechanisms do not play a major role in regulating r-protein synthesis in L6-5 cells. Since both the levels and half lives of the rp-mRNA's were similar in myoblasts and myotubes, the implication is that the rates of r-protein synthesis are regulated by the rates of rp-mRNA transcription.

The in vitro nuclear transcription assay provided direct evidence for the differential synthesis of rRNA and rp-mRNA's in myotubes when compared with myoblasts (Table 3-6). Although the relative transcription rates for both rRNA and rp-mRNA genes decreased upon terminal differentiation, the drop in rRNA synthesis was more pronounced. The relative drop in the rate of rRNA transcription in vitro between myoblasts and myotubes when compared to that of rp-mRNA L32 correlates with the drop in rRNA accumulation rates observed in vivo (Figures 3-3 and 3-4 and Table 3-1). The conclusion from these observations is that the regulation of r-protein production, which was coordinated with that of rRNA in myoblasts was uncoupled at the transcriptional level in myotubes.

The uncoupling of rRNA and rp-mRNA synthesis in myotubes can be examined for other specific rp-mRNA genes. This can be achieved with the analysis of the *in vitro* transcription assay with a number of genomic clones for other rat r-proteins. These clones may be obtained by screening rat genomic libraries with the available mouse rp-cDNA clones, since the latter have close homologies with rat rp-mRNA's (Monk *et al.*, 1981). Analysis using the nuclear transcription assay with these genomic clones may also reveal the degree of coordinate transcription of the rp-mRNA genes in myoblasts and myotubes.

Uncoupling of rRNA and r-protein production in mammalian cells is an unusual observation (Warner *et al.*, 1980). It has been noted that in a variety of cell lines, the degree of control over ribosome biogenesis was inversely correlated with the degree of transformation (Stanners *et al.*, 1979). While non-transformed cells modulate their ribosome content according to their physiological state, transformed cells do not.

The possibility that L6-5 is partially transformed may explain the observed uncoupling of rRNA and r-protein production in myotubes. The regulation of ribosomal precursor synthesis in myotubes appears to be split: rRNA transcription was modulated as in non-transformed cells while rp-mRNA transcription remained constant as suggested for transformed cells. The expression by L6 of other

characteristics common to transformed cells, such as anchorage independent proliferation, sensitivity to cytochalasin B and secretion of plasminogen activator have been noted by other authors (Bignami, Dogliotti, Benigni, Branca, Tato & Alema, 1982), and lends credence to this possibility.

To resolve whether the rRNA and rp-mRNA uncoupling phenomenon is specific to L6-5 myotubes, comparative studies with other rat-derived myoblast cell lines, such as L8, M42 and M48 (Richler & Yaffe, 1970) and primary cultures can be undertaken. Northern blot analysis and in vitro nuclear transcription assays may reveal whether rRNA and rp-mRNA are transcriptionally uncoupled in the myotube phenotypes of these cell lines. The results may indicate that L6-5 is deficient in its ability to regulate ribosome biogenesis.

In constructing a testable model for the regulation of ribosome biogenesis in L6-5 cells, much can be learned by examining the mechanisms of ribosome regulation in prokaryotes. The levels and accumulation rates of ribosomes in Escherichia coli, for example, are tightly coupled with its growth rate. In addition, the rates of rRNA and r-protein synthesis are closely coordinated (for reviews, see Lindahl & Zengel, 1982 and Nomura, Gourse & Baughman, 1984).

Ribosomal RNA

In Escherichia coli, rRNA is transcribed from seven rRNA genes, each of which contains perhaps four promoters (Morgan, 1982 and Boros, Kiss, Sain, Somlyai & Venetianer, 1983). One of the promoters appears to be constitutive while another may be the site of transcriptional modulation. All seven genes are continuously expressed and regulation of their transcription is achieved by modulating their initiation and pre-termination rates. Regulation of rRNA transcription may be by cellular levels of guanosine-tetraphosphate (Gallant, 1979) and by feed-back inhibition by non-translating ribosomes (Jinks-Robertson, Gourse & Nomura, 1983).

The rodent rRNA genes, on the other hand, appear to contain one promoter and perhaps enhancer sequences further upstream of the rRNA genes' start site (Wood, Bowman & Thompson, 1984). Several factors are required for the accurate initiation of rRNA gene transcription and appear to be the limiting components in rRNA synthesis (Mishima, Financsek, Kominami & Muramatsu, 1982). In addition to these initiation factors, a 100k dalton nucleolar protein has been identified in CHO cells as a regulatory element (Bouche, Caizergues-Ferrer, Bugler & Amalric, 1984). This protein appears to bind the RNA polymerase I initiation complex before being processed into several components, allowing transcription to proceed.

The possibility exists that these two regulatory features operate in L6-5 cells. Upon terminal differentiation, the supply of RNA polymerase I initiation factors or an analog of the 100k dalton protein may be severely limited, causing a large decrease in the rate of rRNA accumulation. Alternatively, an inhibitory factor induced by myogenesis may be present in myotube nucleoli which interferes with the formation of the RNA polymerase I initiation complex.

The regulatory features of rRNA transcription can be investigated with an in vitro rRNA gene transcription system reconstituted from various nuclear fractions (Miller & Sollner-Webb, 1982 and Manley, Fire, Samuels & Sharp, 1983). The nuclear fractions, separated by chromatography, variously contain RNA polymerase I and a variety of initiation factors, some of which are species specific. The reconstituted transcription system can also employ nuclear fractions mixed from myoblasts and myotubes in various combinations. These mixing experiments may reveal a myoblast-specific factor which can activate rRNA gene transcription in myotubes in vitro. Further purification of the nuclear fractions may lead to the identification and characterization of the regulatory factors.

As an alternative to a reconstituted transcription system, the RNA polymerase I initiation complex can be analyzed in myoblast and myotube nuclei (Miesfeld & Arnheim,

1984). These analyses can be qualitative, where the composition of the complex can be investigated by gel electrophoresis, or quantitative, where the number of initiation complexes per haploid genome and the factor which limits complex formation can be determined. These studies may reveal a phenotype-specific factor responsible for regulating rRNA gene initiation. If sufficient amounts of the specific factor can be separated, then antibodies raised against the factor may allow investigation into the regulation of its production, turnover and relationship to differentiation.

Another approach to identifying the regulatory components of rRNA gene expression in myotubes may be by chromatin digestion studies with DNase I (McGinnis et al., 1983). Alterations in the conformation of chromatin has been associated with changes in gene expression. DNase I digestion of chromatin from myoblasts and myotubes and subsequent Southern blot analysis of the extracted DNA with rRNA genomic clones may reveal different digestion patterns (Southern, 1975). Since rRNA gene transcription rates are reduced in myotubes, the studies may show DNase I resistant sequences in myotube rRNA genes not seen in myoblasts. These would denote a less 'open' chromatin structure and would indicate the apparent site where attenuation of rRNA gene transcription occurs.

The attenuation of rRNA transcription after terminal differentiation may occur by turning off a number of rRNA genes, with the remaining rRNA genes being transcribed normally. Such a situation exists in Xenopus and other systems (Miller & Sollner-Webb, 1982). It was hypothesized that the promoter region was either 'open' and could bind RNA polymerase I and associated initiation factors or 'closed' and not able to allow transcription of the rRNA gene (Reeder, Roan & Dunaway, 1983). The rRNA gene could be held 'open' by the association of a specific protein which allows the subsequent binding of the initiation complex. If this factor was much less abundant in myotubes, it could account for the attenuated rRNA gene transcription rates observed. The rRNA genes which are being transcribed can be enumerated by measuring the number of RNA polymerase I initiation complexes formed in vivo or in vitro or by electron microscopy of nucleolar chromatin (Miller & Sollner-Webb, 1982).

The regulation of rRNA gene transcription in L6-5 is under control of the 'myogenesis program' in myotubes. The rates of their transcription determine the rate of ribosome accumulation in both myoblasts and myotubes. The mechanism of regulation, however, remains unknown but may be effected by the amount of a specific RNA polymerase I initiation factor. A variety of experimental approaches can be taken to elucidate these regulatory mechanisms and to

ascertain how they may be related to differentiation.

Regulation of Ribosomal Protein Synthesis

In Escherichia coli, the production of r-proteins is tightly coupled with that of rRNA (Gausing et al., 1980). Under most growth conditions, the rate of r-protein synthesis is modulated autogenously (Nomura et al., 1980). Specific r-proteins, usually one per operon, bind to the mRNA in which they are encoded and inhibit their translation. The r-proteins involved with autogenous regulation have a higher affinity for rRNA's than for their own rp-mRNA's. This ensures that only the r-proteins synthesized in excess of requirements inhibit r-protein production, thus coordinating their synthesis with that of rRNA. Since most rp-mRNA's in Escherichia coli are polycistronic, the mechanism provides an efficient method for the regulation of rp-mRNA translation.

Translational regulation of r-protein production has been observed in some eukaryotes. For example, yeast cells which have been transformed with a plasmid containing extra copies of the r-protein L3 gene have elevated levels of rp-mRNA L3 (Pearson, Fried & Warner, 1982). The rate of its translation, however, remains coordinate with the other rp-mRNA's, implying that r-protein synthesis is translationally regulated.

Another example of translational regulation is found in the developing Xenopus embryo. During early stages of embryogenesis, rp-mRNA is transcribed and held in a non-translatable state until rRNA gene transcription begins (Pierandrei-Amaldi, Campioni, Beccari, Bozzoni & Amaldi, 1982). When rRNA synthesis commences, the rp-mRNA's translation rates increase coordinately with the appearance of rRNA.

These systems appear to be exceptions since numerous examples have been described where rRNA and r-protein production have been uncoupled (Warner et al., 1980). In any case, autogenous regulation of r-protein synthesis has yet to be demonstrated in eukaryotes.

The synthesis of r-proteins in L6-5 cells is not autogenously regulated after differentiation. The r-proteins were synthesized in large excess over rRNA in myotubes and then degraded. Although autogenous regulation may occur in myoblasts, it was considered unlikely since it was not detected in myotubes. This supposition can be tested by inhibiting rRNA transcription in myoblasts with low levels of Actinomycin D, which would allow near normal transcription of RNA polymerase II genes including those for r-proteins (Wilson & Hoagland, 1967). If r-protein synthesis was autogenously regulated in myoblasts, then the rates of rp-mRNA translation would be expected to decrease since there is little new rRNA capable of incorporating

nascent r-proteins into ribosomes. This type of experiment has been performed with many mammalian systems and has generally failed to demonstrate direct feed-back regulation on r-protein synthesis (Warner et al., 1980).

Further investigations into the coordinate expression of r-proteins with rRNA genes depend upon whether the uncoupling observed in myotubes is specific to the L6 cell line. If coordinate repression of rRNA and r-protein expression is observed in other rat-derived myoblast lines, then L6 cells may prove to be a valuable regulatory mutant in ribosome production.

A series of comparative studies between L6-5 and other rat and other rodent-derived myoblast cell lines can be pursued. These investigations can include DNase I sensitivity studies of the r-protein genes, particularly the 5' non-coding regions, and the degree of r-protein gene methylation. Both of these parameters have been correlated with gene expression (Manes & Menzel, 1981 and McGinnis et al., 1983). Differences in the digestion patterns of the DNA between L6-5 myoblasts and myotubes and other myogenic cell lines may reveal the location of the putative mutant's effect.

The r-protein gene initiation complex may be investigated by 'Foot Print' analysis (Smith, Jackson & Brown, 1984). Reconstituted initiation complexes using genomic clones of r-protein genes and nuclear extracts can

be digested with DNase I. The DNA sequence which is protected from digestion by the complex can be subjected to Southern blot analysis. Not only would this technique indicate the site of r-protein gene initiation but, with the analysis of a number of r-protein genomic clones, may indicate consensus sequences involved with the regulation of the stoichiometry of r-protein gene expression.

Reconstituted initiation complexes can also employ nuclear fractions from myotubes of various cell lines and may reveal a feature not observed in L6-5 myotubes. This may include the depletion of an r-protein gene-specific RNA polymerase II initiation factor (c.f. Dynan & Tjian, 1983) or the presence of a factor which specifically inhibits r-protein gene transcription.

The description of initiation or inhibitory factors of rRNA and r-protein gene transcription in rodent myotubes may lead to a model for their coordinate repression. The factor may be pleiotropic, affecting the initiation of both RNA polymerases I and II dependent transcription, or may be two distinct gene products, each responsible to a specific polymerase and each independently regulated by myogenesis. Since RNA polymerases I and II are quite different (Chambon, 1975), the latter possibility appears more likely. In the case of L6-5, it appears that the RNA polymerase II associated factor may have become independent of myogenic control.

Comparative studies between L6 and other rat myogenic cell lines may reveal, however, that the uncoupling of rRNA and r-protein expression is a feature common to all rat myotubes, If this was to be the case, then one is faced with the tactical problem of why the rat myotube synthesizes r-proteins in excess of requirements and then degrades them. It may be that coordinate regulation of r-protein with rRNA synthesis is not a priority in myotubes since it consists of less than 10% of the total translational activity (Meyuhas, 1984). On the other hand, it is possible that coordinate synthesis of rRNA and r-proteins is achieved after the cells have been differentiated for a prolonged period of time, even though the overall rates of protein translation remains undiminished (Yaffe, 1969 and Schubert, Tarikas, Humphreys, Heinemann & Patrick, 1973). Such a possibility can be addressed by analyzing rRNA and r-protein synthesis patterns in other L6-derived clones such as L6E9, which can exist as myotubes in culture for extended periods of time (Schubert et al., 1973 and Nadal-Ginard, 1978). Alternatively, L6-5 myotubes may be further studied if their life expectancy can be enhanced by medium supplements such as cortisol (de la Haba, Cooper & Elting, 1968).

5s Ribosomal RNA

One of the components of the ribosome, the 5s rRNA, has received little attention in these studies. The 5s rRNA, which is transcribed by RNA polymerase III, is present in ribosomes in stoichiometric amounts (Warner et al., 1980). It therefore follows that its production should be coordinate with the other components of the ribosome. In lower eukaryotes, such as Xenopus, synthesis of 5s rRNA is positively regulated by TF IIIA, a transcription factor which binds both the 5s rRNA gene and its transcript (Sakonju et al., 1981). Since the maturing ribosome competes with TF IIIA for 5s rRNA, the production of 5s rRNA is coordinated with that of other ribosomal components. Whether this type of regulation exists for 5s rRNA synthesis in mammalian systems is not known.

Differentiation of L6-5 cells results in the marked reduction in the synthesis of rRNA but not r-proteins. As a result, the analysis of 5s rRNA gene expression during myogenesis may reveal whether its transcription is coordinately reduced with that of rRNA or is maintained at the levels of r-protein genes. Using the Xenopus system as a model, the L6-5 cell line may prove to be an excellent system in which to study the expression of 5s rRNA genes in mammalian cells.

Coordinate production of Ribosomal Components: A Model

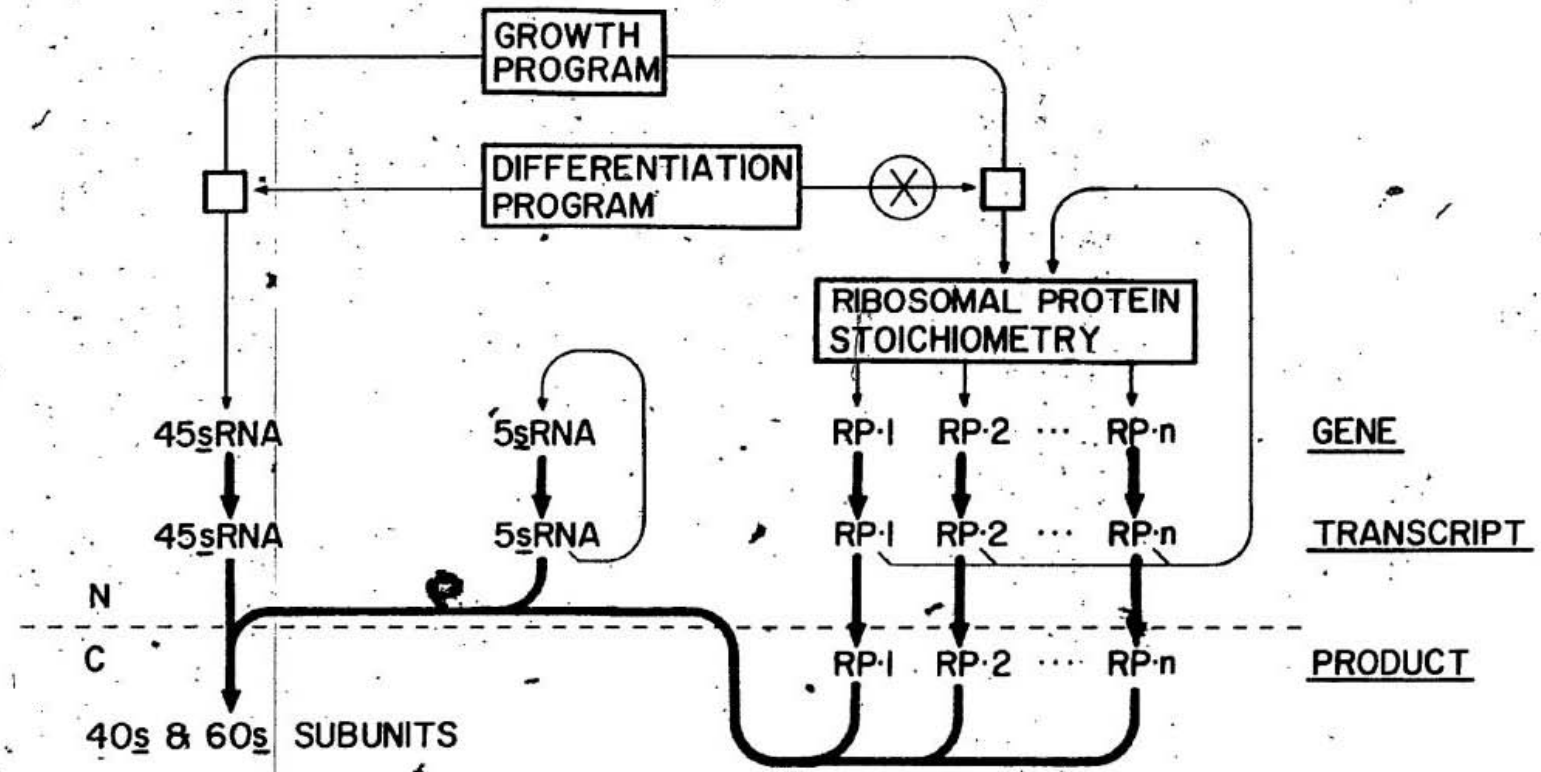
The regulatory systems which coordinate rRNA and rp-mRNA production and maintain the stoichiometry of the rp-mRNA's raise interesting questions about the hierarchy of ribosomal gene regulation. Cumulative evidence in the literature suggests that ribosome biogenesis has at least three levels of regulation, each one nested in the next (Warner et al., 1980). The regulation of overall levels and turnover of ribosomes, including their rates of production and degradation, is in response to the physiological state of the cell (Figure 4-1). This 'Growth Program' may be modulated by environmental factors (such as nutrition) or genetic factors (such as differentiation) and serves as the main regulatory level of ribosome production in proliferating cells.

The coupled synthesis of rRNA and r-proteins constitutes the second level of regulation of ribosome biogenesis and is subservient to the Growth Program. Many eukaryotic systems have demonstrated that rRNA and r-protein production is coordinate (Warner et al., 1980) and these systems can usually maintain their coordination under stress (Tsurugi et al., 1972, Faliks & Meyuhas, 1982 and Geyer et al., 1982). The mechanism responsible for the coordination of rRNA and r-protein production must therefore be able to react to the changes in the overall ribosome accumulation

Figure 4-1.

Model of Coordinate Regulation of Ribosome Biogenesis in L6-5 Cells.

The model presented portrays potential regulation sites of rRNA and r-protein transcription and how they may by coordinated in L6-5 cells. The boxes indicate factors or systems where regulation occurs. The thin lines indicate the pathways of regulation and the thick lines indicate the pathways of the gene products. The dashed line represents the boundary between the nucleus (N) and the cytoplasm (C). The encircled 'X' indicates the site where coordinate regulation between rRNA and rp-mRNA synthesis is lost after terminal differentiation.


GROWTH PROGRAM
DIFFERENTIATION PROGRAM
RIBOSOMAL PROTEIN STOICHIOMETRY
45sRNA
5sRNA
RP·1
RP·2
RP·n
GENE
45sRNA
5sRNA
RP·1
RP·2
RP·n
TRANSCRIPT
N
C
RP·1
RP·2
RP·n
PRODUCT
40s & 60s SUBUNITS

rate determined by the Growth Program.

The regulation of the stoichiometry of r-protein synthesis is subservient to the coordinate regulation of rRNA and r-protein production. This is because the balanced synthesis of the various r-proteins is always maintained regardless of their overall rates of production. The stoichiometry may be achieved by a feed-back mechanism which senses the cellular levels of rp-mRNA's and adjusts the rates of their transcription or their translatable pool sizes accordingly. The regulatory mechanism does not appear to involve the r-proteins themselves since these can be synthesized in vast excess (but still stoichiometrically) under certain conditions (Warner et al., 1980). Whether the feed-back mechanism crosses the nuclear membrane is not clear.

In L6-5 myoblasts, these three regulatory levels appear to be functional since neither rRNA nor r-proteins are produced in excess of requirements. In myotubes, however, the rate of r-protein synthesis is no longer coupled with that of rRNA and has therefore become insensitive to the regulatory mechanisms involved. Both r-proteins and their mRNA's are synthesized near levels found in myoblast. This suggests that the rate of rp-mRNA transcription is still modulated by the 'Growth Program' (Figure 4-1), unfettered by the 'Differentiation Program'. Therefore, the second regulatory level in L6-5 myotubes appears to be deficient.

Gene Expression and Differentiation

The L6-5 system has demonstrated marked changes in the expression of three sets of genes: the repression of rRNA and histone genes and the induction of myosin heavy chain genes. The temporal regulation of these genes, however, is different. Histone genes are repressed within 24 hours after the initiation of differentiation, before the accumulation rate of DNA drops (Figure A-1). The repression of rRNA synthesis, on the other hand, occurs after the decrease in the rate of DNA synthesis (Figure 3-2), as does the induction of MHC-mRNA (Benoff & Nadal-Ginard, 1979). These observations suggest that several major steps are involved in the terminal differentiation process of L6-5 cells.

Analysis of the precise time course of altered gene expression in differentiating myoblasts may reveal whether the terminal differentiation program proceeds through several steps or occurs catastrophically (Merlie et al., 1977). Studies with a recently isolated mutant of L6 which is temperature sensitive for differentiation indicate that the mutant can transiently induce myotube-specific genes at the non-permissive temperature (Nguyen et al., 1983). The mutant retained the ability to proliferate after attempts were made to differentiate the cells at the non-permissive temperature. Investigations into rRNA gene transcription in this mutant may reveal whether it is repressed coordinately

with the activation of the myotube-specific genes, or if it is repressed after commitment is made to terminal differentiation.

Other investigations into the temporal relationship between rRNA repression and differentiation may be pursued with L6-5 cells which have been synchronized with, for example, aphidicolin (Huberman, 1981). After the myoblasts have been synchronized, they may be induced to differentiate quantitatively with the aid of medium supplemented factors such as somatomedins (Ewton & Florini, 1981). The expression of specific genes can then be studied with a high degree of temporal precision by a variety of methods, such as Northern blot analysis or in vitro nuclear transcription assays. A time course of differentiation may then be constructed, showing the order of repression and induction of genes during myogenesis.

The problem of gene regulation during differentiation remains a complex one. A number of fundamental changes occur in the myoblast when it alters its phenotype to a myotube. The general metabolism shifts from an anabolic state, where the cell mass is increasing rapidly, to a catabolic state, where cell growth has virtually ceased (Nadal-Ginard, 1978). In addition to the repression of growth related genes such as histones, a number of genes specific to the differentiated phenotype, such as myosin heavy chain are induced. While these phenotypic changes are

occurring, however, the cell continues to maintain its housekeeping functions, such as its translational ability. Thus in the face of large variations in the expression of some genes, other genes are regulated so that their products are maintained.

The regulation of ribosome biogenesis as a representative set of such housekeeping genes in eukaryotes has been revealed to be much more complex than in prokaryotes. The molecular mechanisms of these regulatory systems should prove to be very interesting since they cross the two rRNA's, mRNA and protein boundaries. In addition, the regulation of ribosome biogenesis is a very old problem since ribosomes are ubiquitous and have existed since the beginning of life (Crothers, 1982). Therefore, the elucidation of the precise regulatory mechanisms involved in ribosome biogenesis continues to be a central quest in biology.

Conclusions

The purpose of this study was to investigate the coordinate regulation of ribosome production in L6-5 myoblasts and how the coordination was affected by myogenesis. Initial studies confirmed that the L6-5 subclone behaved similarly to the L6 parent cell line in terms of its morphological and biochemical differentiation characteristics. Determination of the relative accumulation

rates of r-protein and rRNA in myoblasts and myotubes indicated that their synthesis, which was coordinate in myoblasts became uncoupled in myotubes. It was observed that the accumulation rates of rRNA and whole ribosomes in myotubes was approximately 20% of the rate in myoblasts while the rate of r-protein synthesis remained essentially unchanged.

Translational mechanisms could account for uncoupling of r-protein and rRNA production in myotubes. The levels and distribution of the rp-mRNA's were therefore measured in myoblasts and myotubes. The results indicated that the levels and distributions of rp-mRNA's were unaffected by differentiation. It therefore appeared that r-protein synthesis rates were governed by cytoplasmic levels of the rp-mRNA's.

To determine the level at which r-protein production was uncoupled from that of rRNA in myotubes, the turnover rates of rp-mRNA's were examined. The half lives of the rp-mRNA's, measured by monitoring their decay rates in the absence of RNA synthesis, were found to be approximately 11 hours in both myoblast and myotubes. These results indicated that r-protein production was not regulated at the post-transcriptional level in L6-5 cells.

To test the possibility that rRNA and r-protein synthesis was uncoupled at the transcriptional level in myotubes, the transcription rates of rp-mRNA L32 and rRNA

were measured. It was determined that the rp-mRNA was transcribed at a rate over three fold higher relative to that of rRNA when compared to the situation in myoblasts. These results revealed that, upon terminal differentiation of the L6-5 myoblast, the production of rRNA and r-proteins became uncoupled at the transcriptional level.

The finding that L6-5 is deficient in its ability to coordinately regulate rRNA and r-protein synthesis in the myotube phenotype may provide a comparative system with other rat myogenic cell lines for the study of ribosome regulation. Analysis of the differences between L6-5 and other rat derived myogenic cell lines may reveal the molecular basis for coordinate regulation of rRNA and r-protein production in mammalian cells.

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APPENDIX A

Characterization of Histone H4 mRNA's in L6-5 Cells

A) Introduction

Upon differentiation, L6-5 myoblasts cease the synthesis of DNA and repress the expression of growth related genes (Figure 3-2, Richler & Yaffe, 1970 and Zevin-Sonkin & Yaffe, 1980). A set of genes whose expression is closely linked to DNA replication and with the cell cycle is the histone gene family (Wu & Bonner, 1981 and Plumb, Stein & Stein, 1983). Since differentiated L6-5 cells have retired from the cell cycle and express the myotube phenotype (Nadal-Ginard, 1978), these cells should not synthesize histones. This is in contrast to genes which are induced upon terminal differentiation, such as those coding for myosin heavy chain (Benoff & Nadal-Ginard, 1979). The study of a family of growth related genes such as histones in L6-5 provides information on gene expression complementary to that of r-proteins and myosin heavy chain.

The histone H4 gene family was chosen for investigation since it is highly conserved and the best characterized of the histone gene families (Stein, Plumb, Stein, Marashi, Sierra & Buambach, 1984). Experiments were designed to study the relationship of H4 gene expression with L6-5

differentiation. In addition, the various H4-mRNA sub-species were examined for the presence of a poly(A)-containing message, the appearance of which is rare in mammalian systems (Hentschel & Birnsteil, 1981).

B) Material and Methods

In addition to the methods outlined in Chapter Two, the following procedures were employed.

i) Acrylamide Gel Electrophoresis of RNA

RNA samples were subjected to electrophoresis in a high resolution 6% acrylamide gel system (Lichter et al., 1982). The gel buffer contained 50 mM Tris borate (pH 8.3), 1 mM EDTA, 8.3 M urea, acrylamide (6%, w/v) and N,N'-methylene-bis-acrylamide (0.2%, w/v). After degassing the gel buffer, ammonium persulphate (0.007 volumes of a freshly prepared stock solution (10%, w/v)) was added and the solution poured into a 30 cm x 14 cm x 0.5 cm slab gel form (32 cm Protean, Biorad) with a slot-forming comb in place and allowed to polymerize. After the gel system was assembled and the reservoir filled with buffer containing 50 mM Tris HCl (pH 8.3) and 1 mM EDTA, the gel was heated by pre-running at 15 W for 1 hour. During the pre-running of the gel, ethanol precipitated RNA samples (50 ug of total or poly(A) poor RNA or 5 ug of poly(A) enriched RNA) were dissolved in a 10 ul solution containing 5 mM EDTA, 8.3 M urea and xylene cyanol (0.01%, w/v) and heated to boiling for 2 minutes. Following

electrophoresis of the RNA samples towards the anode at 200 V for 18 hours, the gel was washed in 200 ml of a solution containing 50 mM NaOH for 20 minutes, in 2 changes of 200 ml of a solution containing 200 mM KH_2PO_4 (pH 5.0) for 20 minutes and in 2 changes of 100 ml of a solution containing 25 mM KH_2PO_4 (pH 5.0) for 20 minutes. The gel was then electro-blotted onto freshly prepared DPT-paper and hybridized (Chapter 2, Section 'K').

ii) Oligo(dT)-Cellulose Chromatography

Oligo(dT)-cellulose chromatography of RNA was performed as outlined in Chapter 2, Section 'G-iii' with the following additions. The poly(A) poor RNA fractions were subjected to oligo(dT)-cellulose chromatography twice to remove residual oligo(dT)-cellulose binding material before analysis. Where indicated, a high stringency wash of 5 ml of a solution containing 10 mM Tris HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA and SDS (1%, w/v) was interposed between the Binding Buffer and Elution Buffer steps to ensure removal of non-specifically bound RNA from the column (Bantle et al., 1976).

To demonstrate the specificity of the poly(A) enriched RNA binding to the oligo(dT)-cellulose, poly(A) enriched RNA was hybridized with excess poly(U) before being subjected to oligo(dT)-cellulose chromatography. Equal amounts (5 ug) of poly(U) and poly(A) enriched RNA were combined in 1 ml of a

solution containing 10 mM Tris HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA and SDS (1%, w/v). The solution was heated to 65°C for 1 minute and allowed to cool to room temperature over a period of 1 hour. NaCl (0.1 volume of a 5 M stock solution) was then added and the sample was subjected directly to oligo(dT)-cellulose chromatography, omitting the pre-heating step.

C) Results

Preliminary evidence of histone gene expression in L6-5 cells revealed that the level of H4-mRNA was lower in mature myotubes than in proliferating myoblasts (Figure 3-8 and Table 3-4). To characterize the relationship of histone gene expression and L6-5 differentiation, the levels of H4-mRNA's were investigated in more detail. Polysomal RNA samples (50 ug each), prepared from 50% confluent myoblasts and developing myotubes were subjected to electrophoresis in 6% acrylamide and electro-blotted onto DPT-paper. The Northern blot was probed for H4-mRNA sequences with (^{32}P)-labelled nick-translated pH4 (Figure A-1).

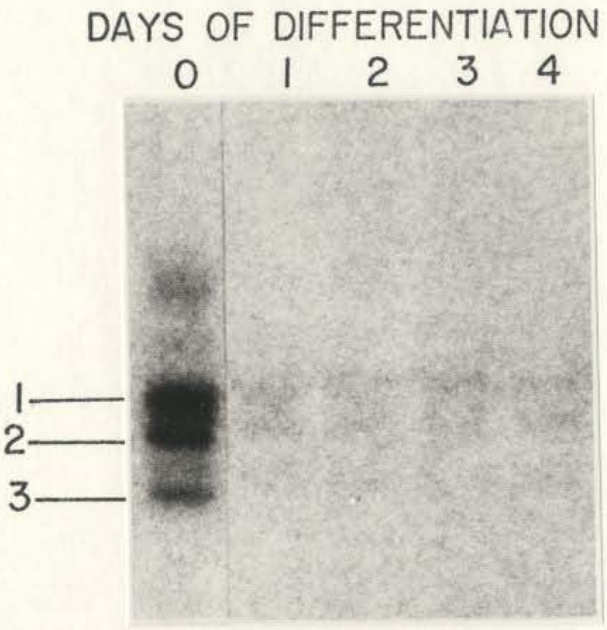
Autoradiography of the Northern blot showed at least three sub-species of H4-mRNA, labelled '1', '2' and '3'. Quantitation of H4-mRNA's revealed that the levels of H4-mRNA's dropped by more than 95% in myotubes from the levels found in proliferating myoblasts. In addition, the three sub-species appeared to be coordinately reduced. Upon

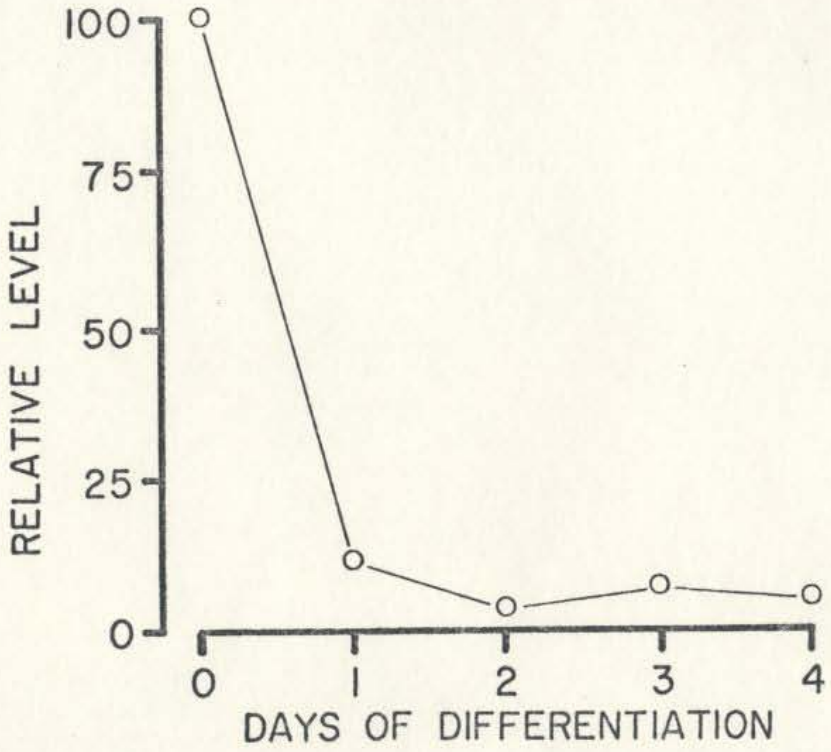
Figure A-1.

Levels of H4-mRNA's During L6-5 Differentiation.

Total polysomal RNA was prepared from 50% confluent myoblasts and 1 through 4 day myotubes. Aliquots of 50 ug of RNA were subjected to electrophoresis in 6% acrylamide, electro-blotted onto DPT-paper and probed for H4-mRNA sequences with (^{32}P)-labelled nick-translated pH4. The Northern blot was quantitated by scanning densitometry.

O Total polysomal H4-mRNA


DAYS OF DIFFERENTIATION
0 1 2 3 4
1
2
3


100
75
50
25
0
RELATIVE LEVEL
0 1 2 3 4
DAYS OF DIFFERENTIATION

differentiation of L6-5 myoblasts, therefore, the H4-mRNA levels were quickly repressed.

As noted earlier, a portion of the H4-mRNA population appeared to contain a poly(A) rich sequences (Figure 3-8). Electrophoresis of RNA in a high resolution acrylamide gel, capable of resolving low molecular weight RNA's (Lichter et al., 1982), demonstrated that the poly(A)-containing H4-mRNA was a specific sub-species of H4-mRNA.

Total polysomal RNA from myoblasts was fractionated by oligo(dT)-cellulose chromatography into poly(A) enriched and poly(A) poor fractions. Aliquots of 50 ug of total or poly(A) poor RNA or 5 ug of poly(A) enriched RNA were subjected to electrophoresis in 6% acrylamide, electro-blotted onto DPT-paper and probed for H4-mRNA sequences with (^{32}P)-labelled nick-translated pH4 (Figure A-2).

Autoradiography of the Northern blot of the H4-mRNA's revealed three major sub-species of H4-mRNA in both total and poly(A) poor RNA fractions (Figure A-2, lanes "a'" and "a"). The poly(A) enriched RNA fraction contained a single sub-species of H4-mRNA which co-migrated with the largest of the three sub-species seen in the total RNA preparation (Figure A-2, lane "b"). Quantitation of the autoradiographs by scanning densitometry revealed that the poly(A)-containing H4-mRNA was approximately 20% of the H4-mRNA subspecies 1 and approximately 8% of the total H4-mRNA population (Table A-1). In the multiple repetitions of the

Figure A-2.

Oligo(dT)-Cellulose Chromatography of H4-mRNA.

Total polysomal RNA was prepared from L6-5 myoblasts and subjected to oligo(dT)-cellulose chromatography. The RNA samples were subjected to electrophoresis in 6% acrylamide electro-blotted onto DPT-paper and probed for H4-mRNA sequences with (^{32}P)-labelled nick-translated pH4.

Lane a') Total polysomal RNA.

Lane a) Poly(A) poor RNA fraction.

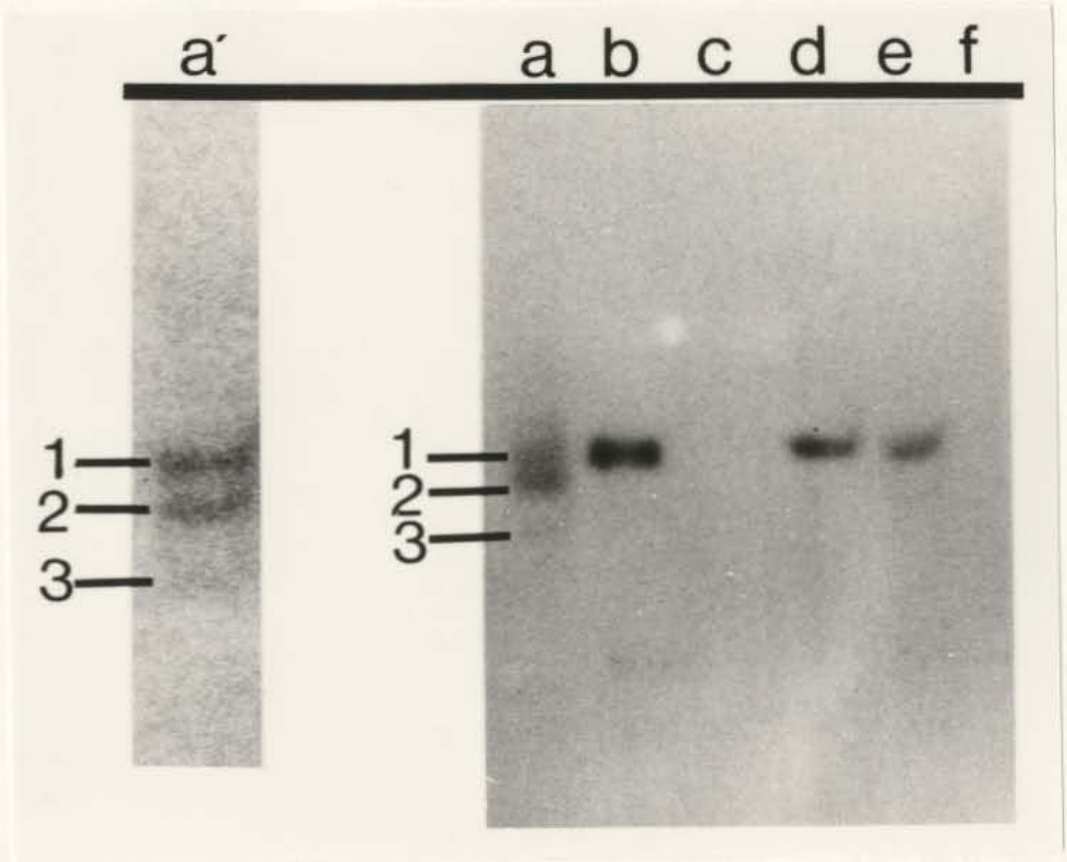
Lane b) Poly(A) enriched RNA fraction.

Lane c) Binding Buffer and stringent wash eluates of poly(A) enriched RNA bound to oligo(dT)-cellulose.

Lane d) Elution Buffer eluate of Poly(A) enriched RNA bound to oligo(dT)-cellulose after stringent wash.

Lane e) Binding Buffer eluate of poly(U) pre-hybridized poly(A) enriched RNA applied to oligo(dT)-cellulose.

Lane f) Elution Buffer eluate of poly(U) pre-hybridized poly(A) enriched RNA bound to oligo(dT)-cellulose.


a´
a b c d e f
1
2
3
1
2
3

experiment (more than 15), no evidence of heterogeneity in the poly(A) containing H4-mRNA was detected.

To confirm that the interaction between the presumptive poly(A)-containing H4-mRNA and the oligo(dT)-cellulose was specific (ie. due to the poly(A) tract in the mRNA interacting with the oligo(dT) tracts on the cellulose), additional examinations were conducted with poly(A) enriched RNA. The first study employed a wash of the poly(A) enriched RNA bound to the oligo(dT)-cellulose with a solution containing one-fifth of the salt concentration (0.1 M NaCl) of the Binding Buffer. This stringent wash was designed to remove non-specifically and poorly bound material from the column (Bantle et al., 1976). No H4-mRNA was detected in either the Binding Buffer or the stringent wash eluates (Figure A-2, lane "c") while all of the poly(A)-containing H4-mRNA originally bound to the column was recovered in the Elution Buffer eluate (Figure A-2, lane "d").

In separate experiments, the poly(A) enriched RNA was allowed to hybridize with an excess of poly(U) before being subjected to oligo(dT)-cellulose chromatography. It was expected that, since the poly(U) would hybridize with the poly(A) tracts in the poly(A)-containing RNA, the RNA would be unable to bind to the column via their poly(A) tracts. After the poly(U) pre-hybridized RNA sample had been chromatographed on oligo(dT)-cellulose, the Binding Buffer

Table A-1.

Relative Amounts of H4-mRNA Sub-species.

Autoradiographs of Northern blots of total and poly(A) poor RNA prepared from 70% confluent myoblasts, probed for H4-mRNA sequences (Figure A-2), were quantitated by scanning densitometry. The relative proportions of the sub-species were calculated and tabulated and the percent of poly(A)-containing H4-mRNA determined by difference.

RNA Fraction	Relative amount of H4-mRNA sub-species Subspecies (Percent of total per lane)		
	1	2	3
Total	42%	43%	15%
Poly(A) poor	34%	43%	10%
Poly(A) enriched →	8%		

and Elution Buffer eluates were subjected to Northern blot analysis. The results revealed that the poly(A)-containing H4-mRNA hybridized with poly(U) did not bind to the oligo(dT)-cellulose column but was recovered in the Binding Buffer eluate (Figure A-2, lanes "e" and "f"). It therefore appeared that the interaction between the putative poly(A)-containing H4-mRNA and the oligo(dT)-cellulose column was specific. It should be noted that the capacity of the oligo(dT)-cellulose column was approximately 100 times greater than the total amount of material applied to the column ensuring that competition between the excess poly(U) and the mRNA:poly(U) hybrids for the oligo(dT) tracts would not occur.

The H4-mRNA's were further characterized as to their rates of decay once their synthesis had been inhibited with the drug Actinomycin D. Total and poly(A) enriched polysomal RNA was prepared from myoblasts which had been incubated for various periods of time in medium containing 3 ug per ml of Actinomycin D. Equal aliquots of RNA (50 ug of total or 5 ug of poly(A) enriched) were subjected to electrophoresis in 6% acrylamide and electro-blotted onto DPT-paper. After hybridization with (^{32}P)-labelled nick-translated pH4, the blot was autoradiographed and quantitated by scanning densitometry (Figure A-3).

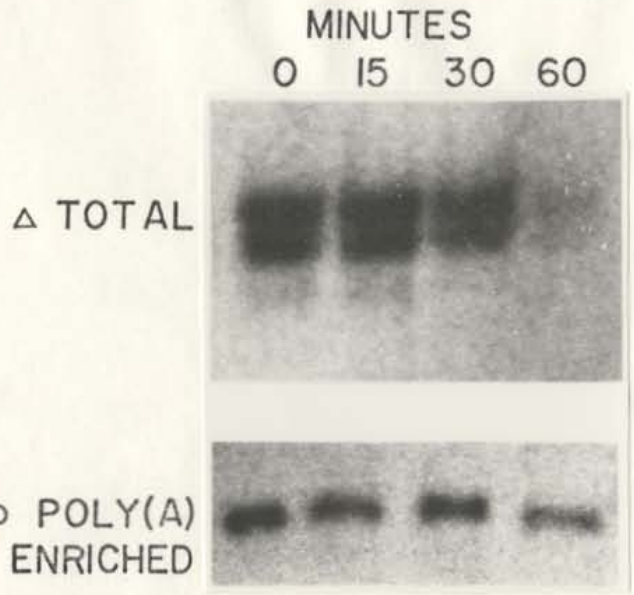
Figure A-3.

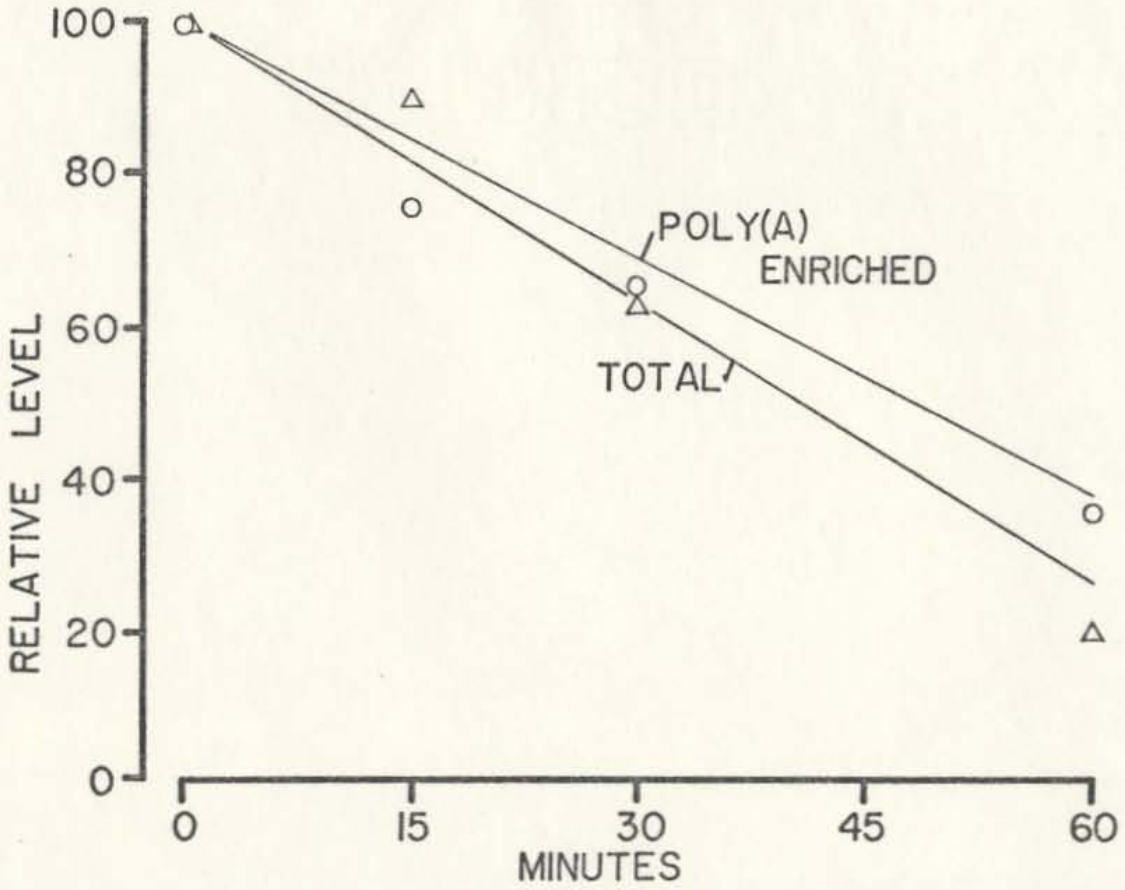
Half Lives of H4-mRNA's

Total and poly(A) enriched polysomal RNA was prepared from L6-5 myoblasts which had been incubated in medium containing 3 ug per ml of Actinomycin D for various periods of time. Aliquots of RNA (50 ug of total or 5 ug of poly(A) enriched) were subjected to electrophoresis in 6% acrylamide, electro-blotted onto DPT-paper and probed for H4-mRNA sequences with (^{32}P)-labelled nick-translated pH4. The Northern blots were quantitated by scanning densitometry and the decay rates of the specific mRNA's determined by least squares analysis (Bulmer, 1979). The half lives for total and poly(A)-containing histone H4-mRNA's were determined to be 40 and 47 (S.D. 5) minutes respectively.

Δ Total polysomal H4-mRNA

O Poly(A) enriched polysomal H4-mRNA


MINUTES
0 15 30 60
△ TOTAL
○ POLY(A)
ENRICHED


100
80
60
40
20
0
RELATIVE LEVEL
POLY(A)
ENRICHED
TOTAL
0
15
30
45
60
MINUTES

The rates of disappearance of the three sub-species of H4-mRNA from the polysomal fraction appeared to be similar. The H4-mRNA was not detected in other cytoplasmic fractions thus the disappearance rate of the H4-mRNA from polysomal fractions was interpreted as being due to turnover, with half lives of approximately 40 and 47 minutes for total and poly(A)-containing H4-mRNA's respectively.

D) Discussion

Differentiation of L6-5 myoblasts results in the induction of myotube-specific genes and the repression of growth related genes (Zevin-Sonkin & Yaffe, 1980). The investigation of growth related gene expression, however, has been limited by the number of candidates available for study (Hastings & Emerson, 1981). Histone genes are perhaps the best described growth related genes (Hentschel & Birnstiel, 1980) and are the subject of this work.

The expression of histone genes is tightly coupled with DNA replication (Stein et al., 1984). Inhibition of DNA synthesis results in the rapid reduction in histone mRNA levels and transcription rates. Other studies of histone gene expression revealed that histone gene transcription was limited to the portion of the cell cycle devoted to DNA synthesis (Alterman et al., 1984). L6-5 myoblasts provide a system in which DNA replication is halted as part of the differentiation process. Thus the coupling of histone gene

expression with DNA synthesis can be investigated during L6-5 myogenesis.

Northern blot analysis of RNA samples prepared from myoblasts revealed three distinct H4-mRNA sub-species (Figure A-1). The occurrence of multiple histone mRNA's has been previously documented in HeLa cells, where at least seven different H4-mRNA sub-species have been identified as unique gene products (Lichter et al., 1982). It is likely that the number of sub-species observed in L6-5 cells is more numerous, with the three major sub-species each perhaps containing several distinct H4-mRNA's. Electrophoresis of H4-mRNA's under a variety of conditions may further resolve the sub-species found in L6-5 cells. Characterization of the individual H4-mRNA sub-species is possible by two dimensional gel electrophoresis after RNase T1 treatment. These studies may indicate the number of unique sequences found in L6-5 (Lichter et al., 1982).

Upon differentiation of L6-5 myoblasts, the polysomal levels of the three H4-mRNA sub-species were rapidly and coordinately reduced. These results are consistent with the hypothesis that histone gene expression is coupled to DNA replication, which is also reduced after differentiation (Figure 3-2). The expression of histone genes is therefore a marker for the proliferating phenotype of L6-5.

The repression of histone gene expression upon terminal differentiation may not be under the direct control of the

'myogenesis program'. The rapid and coordinate disappearance of histone mRNA's also occurs upon inhibition of DNA synthesis with such agents as hydroxyurea, cytosine arabinoside and aphidicolin (Stein et al., 1984). Analysis of these results suggests that DNA replication is required for the maintenance of histone gene expression. Once DNA synthesis is halted, the histone messages are actively degraded. With such tight coupling of DNA and histone synthesis, a separate regulatory mechanism under control of the myogenesis program would not be necessary. Similar studies with the inhibition of DNA synthesis can also be performed with L6-5 myoblasts and may demonstrate the coupling of histone gene expression with DNA replication in the absence of myogenesis.

During the course of these studies, it was noted that a portion of the histone H4-mRNA's appeared to contain poly(A). Histone mRNA's are often cited as examples of eukaryotic mRNA's which are not polyadenylated (Kedes, 1979). Minor amounts of heterogeneously polyadenylated histone mRNA's representing all classes of histone messages have been detected in HeLa cells but have not been characterized (Borun, Ajiro, Zweidle, Dolby & Stevens, 1977). Polyadenylated histone mRNA's are also found in lower eukaryotes, such as Tetrahymena (Bannon, Calzone, Bowen, Allis & Gorovsky, 1983), yeast (Fahrner, Yarger & Hereford, 1980) and the maternally supplied messages in

Xenopus oocytes (Ruderman & Pardue, 1978). The discovery of a single sub-species of mammalian H4-mRNA in rat myoblasts which contains poly(A) is unique and merits further investigation.

The association of the putative poly(A)-containing H4-mRNA with the oligo(dT)-cellulose was specific. The mRNA species remained bound to the oligo(dT)-cellulose column even with stringent washing of the column. In addition, its interaction with the oligo(dT)-cellulose column was completely blocked by pre-hybridization of the mRNA with poly(U). These observations are consistent with the H4-mRNA containing a poly(A) tract (Bantle et al., 1976).

The poly(A)-containing H4-mRNA migrated as a tight band on a high resolution acrylamide gel (Figure A-2), suggesting that the poly(A) tract may be genetically encoded. If the poly(A) tract was added to the H4-mRNA as a post-transcriptional modification as is usual for most mRNA's (Corden, et al., 1980), then the polyadenylated message, which would be heterogeneous in length, would be expected to migrate as a diffuse band in the gel. The polyadenylated rp-mRNA's, for example, could not be detected in this gel system.

The synthesis of the poly(A)-containing H4-mRNA can be studied in the presence of cordycepin, a nucleotide analog which inhibits polyadenylation. (Nevins, 1983). Transcription of the poly(A)-containing H4-mRNA under these

conditions would strengthen the argument that the poly(A) tract is encoded.

Sequence data to date have not reported any mammalian histone genes containing encoded poly(A) sequences (Perry, 1981). To determine the location of the poly(A) tract in the H4-mRNA sub-species, it must be cloned and sequenced. The cloning should be performed in a manner which preserves the 3' end of the message since it may be unique and not polyadenylated.

The poly(A)-containing H4-mRNA composed approximately 8% of the total H4-mRNA in myoblast preparations. Upon differentiation, the poly(A)-containing H4-mRNA level dropped by approximately 78% (Figure 3-8), significantly less than the drop observed for total H4-mRNA of over 95% (Figure A-1). These results may suggest that the regulation of the poly(A)-containing H4-mRNA is 'leaky' and not as tightly coupled to DNA replication as the other H4-mRNA sub-species. The rate of poly(A)-containing H4-mRNA synthesis relative to total H4-mRNA upon drug-induced inhibition of DNA synthesis may indicate the degree of coordination of their expression. Alternatively, the poly(A)-containing H4-mRNA sub-species may serve a special function during myotube development (Liew, Man & Morris, 1980).

The half lives of the H4-mRNA's were short (40 and 47 minutes for total and poly(A)-containing messages

respectively) when compared with other mRNA's measured in this system, such as rp-mRNA's (11 hours) and MHC-mRNA (67 hours) (Table 3-5). Similar half life results have been described for HeLa cell histone mRNA's (Heintz, Sive & Roeder, 1983).

The rapid and coordinate decay of the histone mRNA's after inhibition of their transcription was in keeping with its close association with DNA synthesis. The rate of production of histones and their mRNA's follows the rate of DNA synthesis during the cell cycle (Hereford, Osley, Ludwig & McLaughlin, 1981 and Heintz et al., 1983). A high turnover rate of histone mRNA's would ensure little lag in their clearance from the translational machinery once DNA synthesis and histone mRNA transcription ceased at the end of S phase of the cell cycle or upon differentiation.

These results describe the preliminary characterization of histone H4 expression in L6-5 cells. The two L6-5 phenotypes exhibit marked differences in the levels of H4-mRNA's, which was in keeping with the cells' physiological states. These studies can be extended in L6-5 by examining the transcription of histone genes in vitro, which may demonstrate the coupling of histone gene transcription with DNA replication. In addition, transcription initiation complex analyses may reveal the mechanisms by which histone genes are repressed in myotubes. These may involve cell cycle specific initiation factors or transient alterations

in chromatin structure during DNA replication. The specific analysis of the poly(A)-containing H4-mRNA gene may be of special interest in this regard since it appears to be expressed to a greater extent in myotubes than the other H4-mRNA species. The characterization of these cell proliferation specific genes in L6-5 provides complementary data on gene expression to genes induced by the differentiation process.

E) Conclusions

Upon terminal differentiation of L6-5 cells, DNA replication ceased and the expression of histones genes was almost eliminated. Characterization of the H4-mRNA's revealed three sub-species which were coordinately regulated. A portion of one sub-species probably contained poly(A), although it was indistinguishable from the other H4-mRNA sub-species in terms of half lives. Its expression in myotubes, however, was more significant than the other H4-mRNA sub-species. These results begin to characterize the behavior of histone genes in L6-5 cells and include the first description of a unique sub-species of mammalian histone mRNA's which contain poly(A).