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The role of c-myc in mouse myoblast differentiation

Kohtz, Jhumku Dutt, Ph.D.

City University of New York, 1991

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

The Role of C-myc in Mouse Myoblast Differentiation

by

Jhumku Dutt Kohtz

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1991

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Supervisory Committee

The City University of New York

Abstract

The Role of C-myc in Mouse Myoblast Differentiation

by

Jhumku D. Kohtz

Advisor: Professor Edward M. Johnson

C2 mouse myoblasts are a rapidly growing immortalized cell line capable of differentiating into multinucleated myotubes and expressing muscle-specific markers. It is known that serum-rich medium maintains their undifferentiated state; shortly after its replacement serum poor medium, cells cease proliferating, and biochemical and morphological changes associated with differentiation occur. In this thesis, it is found that upon induction of differentiation, c-myc mRNA steady state levels decrease transiently; this decrease occurs at the transcriptional level. Although the function of c-myc has yet to be delineated, it has been implicated in controlling

differentiation in a variety of systems. This thesis addresses whether the decrease in c-myc expression is an obligatory event in myogenesis. One approach used is to constituitively express c-myc in C2 myoblasts, and test the effects on differentiation. Both the C2 line and a C2 subclone, C25, are used. It is found that c-myc over-expression (8-fold) in a transfected C25 clone (C5M15) can inhibit differentiation, as indicated by a failure of this cell line to accumulate myosin heavy chain, and troponin T. At lower levels (2-5 fold), however, c-myc transfection allows isolation of myoblast clones which express higher levels of differentiation markers than the parental C25 line. These results imply concentration dependant effects of the c-myc gene during myoblast differentiation. Because C25 is a myod1 negative, myogenin positive (dominantly acting, myogenic phenotype inducing genes, Davis et. al., (1988), Wright et. al., (1989)) myoblast line, questions as to how myogenesis occurs in the presence and absence of myod1 are also addressed. Using a previously established, myod1 positive, myoblast line (C2C12,

Blau et. al., 1985) the differentiation properties of the two myoblast lines are compared.

Aknowledgements

I would like to thank Dr. Edward Johnson, my thesis advisor for his help and guidance throughout this work. In addition, I'd like to thank all the investigators willing to provide advice, plasmids, and antibodies, enabling the execution of many the experiments: Dr. R. Chizzonite, Dr. R. Gubits, Dr. A. Lassar, Dr. H. Arnold, Dr. B. Schacter, and Dr. S. Kohtz.

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Introduction

The c-myc gene has been cloned from a variety of species, including humans and salmon trout (Van Beneden et. al., 1986). Many of its coding sequences have been conserved over 200 million years of evolution, implying that it has a biologically significant role in the cell (Sarid et. al., 1987). The v-myc gene was one of the first viral oncogenes shown to exist in cellular DNA (Sheiness et. al., 1978, Vennstrom et. al., 1982). Since their discovery, the cellular homologues of viral oncogenes such as c-myc have proven to function in roles as diverse as signal transduction and gene regulation. Four major classes of oncogenes have emerged: tyrosine kinases, growth-factors and receptors, cytoplasmic protein kinases, and nuclear or DNA-binding proteins (see Alitalo et. al., 1987). The c-myc gene falls into the last category. Because of recent experiments, two additional classes should be included: recessively acting oncogenes (rb-1, Seemayer and Cavenee, 1989), and steriod hormone receptors, capable of binding DNA (erb-A, reviewed by Vennstrom

and Damm, 1989). Many of these genes, like c-myc, were discovered as the regions within retroviruses responsible for transforming activity (see Coffin, RNA Tumor Viruses). However, other mechanisms such as insertional activation caused by adjacent retroviral integration revealed some, (int-1, for review see Hunter, 1984, and Oncogene Handbook, 1989). Most recently, tumors with associated chromosomal deletions have allowed the isolation of recessively-acting oncogenes; inactivation of these genes cause cellular transformation (Hansen and Cavenee, 1987, and Seemayer and Cavenee, 1989).

The normal cellular roles for oncogenes from every class are being extensively researched. Many have been sequenced, cloned in eukaryotic expression vectors, and mutagenized, in order to study their regulation, function and protein structure. Although c-myc has been one of the most intensively studied oncogenes, its normal function in the cell remains a mystery. Belonging to the class of nuclear oncogenes, it is known to, at least, non-specifically bind DNA (Hann and Eisenman, 1984, Watt et. al., 1985).

Experiments employing eukaryotic origins of replication imply that it may have a possible function in DNA synthesis (Klein et. al., 1987, Iguchi-Ariga et. al., 1987). Because of its fluctuation during cellular proliferation and differentiation, it is also thought to have a controlling force in these processes (for review see Kelly and Siebenlist, 1987). As yet, there is no unifying principle in regard to c-myc regulation or function. It is widely accepted that inappropriately expressed c-myc can affect cellular growth and differentiation (see Cole, 1986, for review). Differences exist according to the various systems in which the gene has been studied. For instance, mouse erythroleukemia cells transiently down regulate c-myc gene expression upon differentiation induction with DMSO (Lachman and Skoultchi, 1984). Such down regulation is obligatory for differentiation to occur, as demonstrated by inapropriate expression of the c-myc gene during this process (Coppola and Cole, 1986).

Other experiments with BC3H1 myoblasts and C2C12 myoblasts indicate that differentiation of these cells

is only partially or not at all inhibited by inappropriately expressed c-myc (Schneider et. al., 1987, Payne et. al., 1987). However, activated ras or fos are both capable of inhibiting myogenesis (Payne et. al., op. cit, Lassar et. al. 1989). Myogenesis provides an ideal system to study the role of c-myc gene regulation, not only due to the speed with which myoblasts can progress to myotubes, but also the well characterized array of differentiation markers induced during this progression. At the onset of the work presented in this thesis, none of the above studies had been reported, and the effects of c-myc on differentiation, both in the myogenic and erythroid lineages were a mystery. The role of c-myc regulation during myogenesis is still a controversial issue. The major aim of this work has been to determine how c-myc over-expression can affect myogenesis of cells in culture. The delineation of this role has become especially important in light of the recent homology between the myc family of genes and myod1, myogenin, and myf5 (myogenic phenotype-inducing genes, Davis et. al., 1988, Wright et. al., 1989, Braun et al a., 1989).

Historical

In 1908, Ellerman and Bang demonstrated that leukemia in chickens was transmissable with cell-free extracts, which were later proven to contain an RNA tumor virus. This finding was largely ignored because leukemias were not considered true cancers. It was not until 1919, when Peyton Rous discovered avian sarcoma virus that an infectious agent of cancer was accepted. Since then a wide spectrum of species have been demonstrated to transmit retroviruses, including man (see RNA Tumor Viruses, Coffin, eds).

Studies of avian systems revealed the first tumor viruses, and logically the first oncogenes, src and myc. MC29, an acute leukemia virus containing myc, was isolated in Bulgaria by Ivanov et. al. in 1964. Subsequently, other type C retroviruses were also demonstrated to contain myc (see Figure 1, taken from Enrietto et. al., 1987), OK10, CMII, and MH2. These caused a variety of cancers including myelocytomatosis, endotheliomas, and liver and kidney carcinomas. In addition to avian retroviruses, myc

has also been identified in isolates of FeLV and MuLV, (Braun et. al., 1987) and (Selten et. al., 1984), respectively.

In vivo (chickens), the myc-containing retroviruses induce tumors of hematopoetic tissue. In vitro (cultured cells), they transform cloned fibroblasts as well as macrophages and hematopoetic cells. MC29 can transform fibroblasts of avian or mammalian origin (Langlois et. al., 1967, Graf et. al., 1977, Quade 1979) and cells of epitheloid morphology (Graf and Beug, 1978). The MH2 virus contains two oncogenes myc and mil; mutagenesis studies demonstrate that the myc containing region is necessary for transformation of primary quail fibroblasts (Pachl et. al., 1983). Transformation allows fibroblasts to grow in soft agar, lose actin cables and increase growth rate (Royer-Pokora et. al., 1978). Although bone marrow cells infected in vitro resemble the immature myeloid cells observed in tumors in vivo (Beug et. al. (1979) fully differentiated macrophages

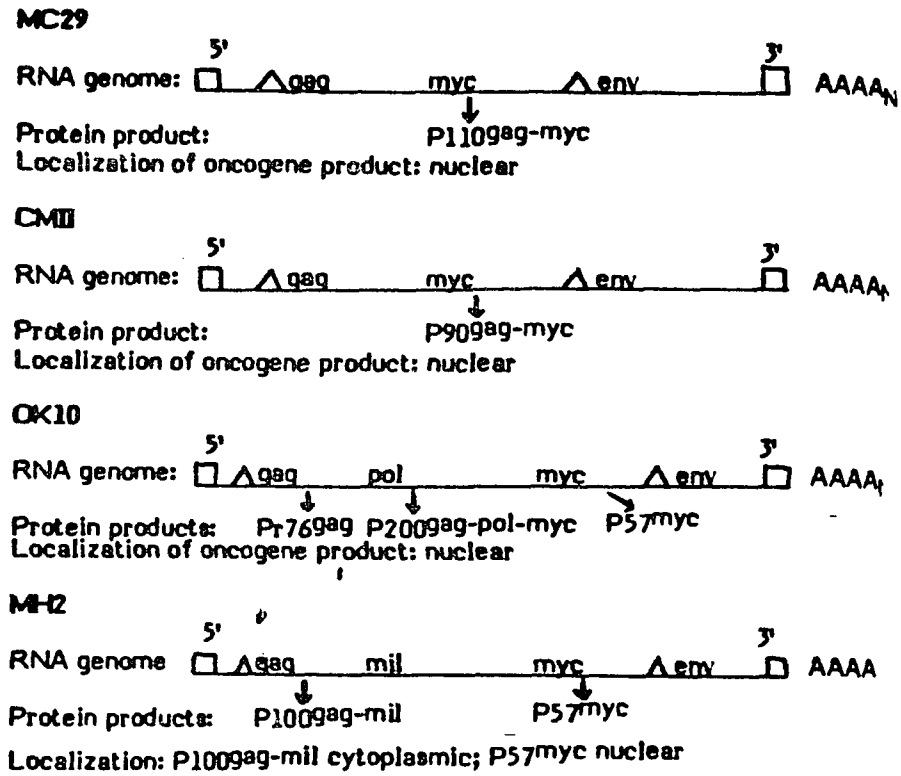

MC29
5'
RNA genome:
gag
myc
env
3'
AAAA
Protein product:
P110gag-myc
Localization of oncogene product: nuclear
CMII
5'
RNA genome:
gag
myc
env
3'
AAAA
Protein product:
P90gag-myc
Localization of oncogene product: nuclear
OK10
5'
RNA genome:
gag
pol
myc
env
3'
AAAA
Protein products:
Pr76gag
P200gag-pol-myc
P57myc
Localization of oncogene product: nuclear
MH2
5'
RNA genome
gag
mil
myc
env
3'
AAAA
Protein products:
P100gag-mil
P57myc
Localization: P100gag-mil cytoplasmic; P57myc nuclear

Figure 1

Figure 1: Map of Known Retroviruses Containing V-myc: Four avian retroviruses contain the v-myc gene: MC29, CMII, OK10, and MH2. All four have deletions within their gag and env genes. MH2 has an addition oncogene called mil. MC29 and CMII encode gag-myc fusion proteins, whereas, OK10 and MH2 encode $p57^{c\text{-}myc}$. Both types of c-myc proteins localize to the nucleus. This figure was taken from (Enrietto, 1987)

are also a target for infection (Durbam and Boettiger 1981). These cells increase expression of F_C receptor, growth rate, and phagocytic activity (Beug, 1979). Thus, myc-containing retroviruses can transform (as defined by Royer-Pokora, op. cit.) both fibroblasts and macrophages, regardless of their stage of differentiation.

Duesberg et. al. (1977) and Bister et. al. (1977) identified the nucleotide sequence and the protein products of the myc oncogene, respectively. The cellular homologue of v-myc, c-myc, was demonstrated to exist in cellular DNA by Vennstrom et. al. (1982). Nucleotide sequence analysis of v-myc, c-myc, and the MC29 virus revealed that v-myc contained only the second two exons of c-myc. The MC29 virus is a defective retrovirus, requiring a helper virus for replication (Langlois and Beard (1967), Hanafusa et. al. (1977)). Figure 1 shows the structure of the viral genomes of myc containing retroviruses. In the MC29 virus, the myc gene has replaced all of the pol gene (responsible for replication of the virus) and part of the gag and env

genes. The v-myc gene is expressed as a gag-myc fusion protein, as determined by in vitro translation (Mellon et. al., 1978). The MH2 virus is also defective needing a helper virus for replication. It differs from MC29 by containing an additional oncogene, mil. MH2 expresses myc as a 57 kd protein from a subgenomic message (Jansen et. al., 1983). The CMII virus, like MC29, expresses a gag-myc fusion protein, p90, whereas OK10 expresses myc both as a gag-pol-myc fusion protein and a 57kd protein from a subgenomic RNA. All v-myc containing proteins localize to the nucleus.

The oncogenic properties of v-myc were originally delineated by spontaneouly arising MC29 mutants selected on the basis of varying v-myc protein molecular weights (Ramsay, 1980). These non-conditional mutants lost their ability to transform macrophages but not fibroblasts in vitro. They were later shown to be unable to cause tumors in chickens (Enrietto et. al., 1983). In frame deletions within v-myc existed in these mutants removing a large central region of the protein. A conditional mutant

of MH2 has a similiar phenotype at the non-permissive temperature (Palmieri, 1986). Site-directed mutagenesis of MC29 further characterized the v-myc gene (Heaney et. al., 1986). In these studies mutated v-myc containing pMC29 plasmids were co-transfected with helper virus genomic plasmid DNA defective in transformation, but replication competent. This allowed the generation of recombinant MC29 viruses containing gag and myc mutations. Figure 2 delineates the regions on v-myc important for transformation of bone marrow, chick or quail embryo cells. Mutants bearing deletions within gag or the middle region of myc retain their ability to transform chicken embryo cells in vivo. Deletion of as few as 11 residues in this region of myc decreases macrophage transformation. Deletion within the amino or carboxy terminal indicates that these regions are responsible for transformation but not nuclear localization. In addition, deletion of myc from MH2 demonstrates that v-myc, not mil is responsible for transformation of primary quail fibroblasts (Pachl et. al., 1983). These studies clearly show that v-myc is an oncogene responsible for the transformation ability of MC29 and

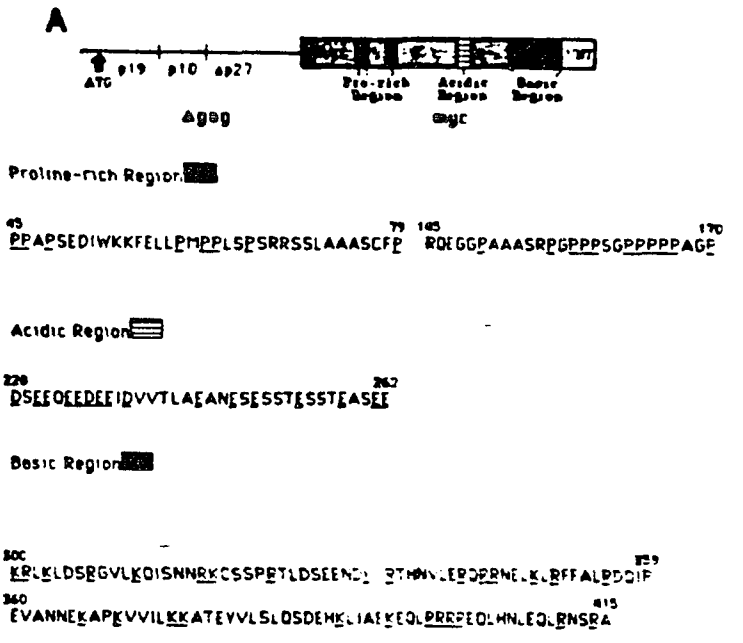

A
ATG
p19
p10
ap27
Pro-rich Region
Acidic Region
Basic Region
Δgag
Δmyc
Proline-rich Region
Acidic Region
Basic Region

Figure 2

Figure 2: Functional Regions of the V-myc Protein: Schematic diagram of the v-myc protein (gag region has been excluded). Functional regions as proven by site-directed mutagenesis are indicated by the numbers 1-4. (taken from Heaney et. al., 1986).

MH2.

Although v-myc has been cloned, sequenced, mutagenized, and antibodies made, its precise biochemical function is still unknown. It is known that v-myc is a phosphoprotein containing both phoshoserine and phosphothreonine (Ramsay et. al., 1982). In addition, antibodies generated to myc reveal its nuclear localization (Donner et. al., 1982) as does subcellular fractionation (Abrams et. al., 1982). Experiments demonstrating its DNA binding activity and association with chromatin (Bunte et. al., 1982) imply, but do not prove, that v-myc plays a role in DNA replication or gene expression.

C-myc Gene Structure

The cellular homologue of v-myc, c-myc, was discovered by Sheiness et. al. in 1982. The c-myc gene is composed of three exons and 2 introns. Originally exon 2 and 3 were thought to be the only coding exons; later, it was demonstrated that a small portion of exon 1 can be coding (Hann et. al., 1988). The chicken c-myc gene was cloned by Vennstrom et. al.

(1982) and sequenced by Watson et. al. (1983). Exon 1 was discovered as a non-coding region during sequencing of the 5' region of the chicken gene (Shih et. al., 1984). Exon 3 was revealed by studies of a translocation (Hamlyn and Rabbitts, 1983). Comparisons with v-myc show that varying portions of intron 1 and all of exons 2 and 3 were transduced by the viruses described above (Bister and Jansen, 1986). Nucleotide sequence analysis reveals seven amino acid changes between the v-myc and c-myc proteins. The major differences occur in their mRNAs. The v-myc gene lacks the long 5' and 3' untranslated regions characteristic of the c-myc gene (Hann et. al., 1985). This conservation between c- and v-myc extends to avian and mammalian myc genes, which are 70-75% homologous to each other (Bernard et. al., 1983, for review se DePinho et. al., 1987).

The c-myc transcription unit contains more than one promoter. The two major promoters, P1 and P2, can be used at different ratios depending on cell type. Differential promoter usage was first described using S1 nuclease analysis of mRNAs derived from normal

somatic and spermatogenic cells (Stewart et. al., 1984). Chromosomal translocation 5' of the myc gene can increase the ratio of usage of P1:P2 by decreasing P2 usage in murine plasmacytomas (Yang et. al., 1985). In addition, translocation can activate cryptic promoters within the first intron, resulting in heterogeneously initiated bipolar transcription (Calabi and Neuberger, 1985, Nishikura, 1987). One of these promoters, termed P3, can generate an mRNA with a longer half life (Piechaczyk et. al., 1985), as shown for both murine plasmacytomas and Burkitt's lymphomas (Eick et. al., 1985). The human myc gene contains a fourth promoter PO, 500 bp upstream of P1 and P2, with 2 open reading frames (Bentley and Groudine, 1986). This P0 mRNA translates into a 12.5 kd protein. It is expressed in the B-cell lymphoma line Manca on the non-translocated allele where P1 and P2 are repressed. Utilization of four different promoters allows numerous ways of regulation, at both transcriptional and post-transcriptional levels, by producing RNA's differing in stability or secondary structure.

Because of its rapid regulation during proliferation and differentiation, amplification and translocation, the chromatin structure of the c-myc gene has been studied in detail. Studies of a single gene in different contexts often reveal how some of these events may affect chromatin structure. Siebenlist et. al. (1984) delineate the DNAase I hypersensitive site in the human myc gene (Roman numerals in Figure 3). Hypersensitive sites have often been associated with regions of regulatory protein binding to DNA. Site I is located within a sequence well conserved between mouse and human, near a putative repressor binding site. III^1 and III^2 are located directly upstream of the major myc promoters P1 and P2. Usage of P2 correlates with site III^2 intensity, whereas P1 usage correlates with III^1 (Taub et. al., 1979). Site II^1 is just 5' of a putative NF1 (nuclear factor 1) binding site. This site is also very close to a potential origin of chromosomal replication in HeLa cells (Vassilev and Johnson, 1989). Site IV appears cell-type specific whereas V is associated with the cryptic promoters within intron 1. Translocation can increase the DNAase I

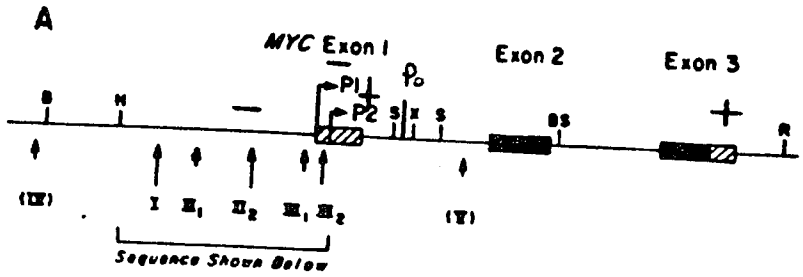

A
MYC Exon 1
Exon 2
Exon 3
P1
P2
Po
B
H
S
X
S
BS
R
(IX)
I
II1
II2
III1 III2
(V)
Sequence Shown Below

Figure 3

Figure 3: Genomic Map of C-myc. DNASE I hypersensitive sites (Roman numerals) and negatively and positively acting sequences are indicated (+ or -). (modified from Siebenlist et. al. 1986)

hypersensitivity of sites III^1 and III^2, suggesting that disruption of a repressor binding site may be responsible (Siebenlist, op. cit.). Farhrlander et. al. (1985) found that mouse plasmacytoma translocations contain hypersensitive sites within the translocated locus not present in the germline. However, hypersensitive sites can remain unaffected by translocation, unless the translocation occurs near the 5' end resulting in their loss, (Dyson and Rabbitts, 1985). Therefore, the effects of translocation on hypersensitive sites remains controversial, and seems to differ between cell types.

Comparisons of DNAase hypersensitive (DH) sites between transcriptionally active and silent loci reveal that these sites are associated with active loci (Farhrlander et. al., ibid). This contrasts with a report by Dyson et. al. (1985) in which the inactive allele of a Burkitt's line Ramos maintains a HP site 2.5 kb from the promoter. This study also finds that all DH sites except this 2.5 kb upstream site disappear during HL60 differentiation, when myc changes from a transcriptionally active to an inactive

locus. These results imply that this region is involved in negative regulation. This region, however, is not identified as a negative regulatory element by others (Chung et. al., 1986). These experiments are discussed in detail below. It is possible that the latter assay could miss detection of this region, if it functions cooperatively with another region or is cell-type specific.

Regions of the c-myc gene binding putative negative or positive trans-acting factors have been mapped using c-myc/CAT indicator gene competition experiments (Chung et. al., 1986). These results are incorporated into Figure 3. Such competition experiments were first described by Scholer and Gruss (1984). Co-transfection of an upstream region linked to CAT with an excess of a potential regulatory sequence can test whether this sequence competes for a trans-acting factor. Any sequence that binds positively acting factors would reduce CAT activity by competition off the indicator CAT construct; conversely, sequences that bind negatively acting factors would increase CAT activity. Three regions of

c-myc contain sequences that compete for negatively acting factors: 400-600 bp upstream of P1, in the 1st exon and the 5' half of the 1st intron. This 3rd site might be responsible for the transcription elongation block seen in retinoic acid induced differentiation of HL60 cells (Bentley and Groudine, 1986). In addition, these negative regulatory regions are associated with Burkitt lymphoma and murine plasmacytoma breakpoints, providing a possible explanation of the mechanism of activation of c-myc in these cases.

Conventional myc-CAT experiments use constructs in which upstream deletions of myc are linked to CAT. These are transfected into different cell types (BJAB, COS, and HeLa) and assayed for CAT activity, S1 nuclease protection or run-on transcription. These experiments reveal a 46 bp region in the 3' end of exon 1 which acts a positive regulatory region affecting P2 activity. This region only functions in sense orientation 3' of a nearby promoter. Another such experiment inserted the region 428-1188 bp 5' of P1 into pSV2CAT and transfected into BJAB and Cos, revealing a negative regulatory region (Remmers et.

al., 1986). This region is termed a dehancer (see Figure 3) because it can influence expression from the SV40 promoter in either orientation as far as 3.2 kb 5' or 1.7 kb 3' (on the circular plasmid).

In addition to the regions discussed above, Kakkis and Calame (1987) have identified a plasmacytoma-specific factor which binds the myc promoter region. Using an electrophoretic gel mobility shift assay, they compared DNA-protein complexes formed with extracts from B-cells in an early developmental stage and plasmacytomas. This protein binding site is 290 bp 5' of P1. The specificity of this protein to plasmacytomas in which the normal myc gene is repressed, suggests that it may play a role in c-myc repression. Therefore, in addition to NF-1 binding, there may be a cell-specific repressor which controls c-myc expression. Such a protein would be a good candidate for a recessively acting oncogene similiar to rb-1 (Seemayer and Cavenee, 1989). As with the retinoblastoma gene, deletion of the myc binding repressor may transform cells.

In addition to DH sites and negative and positive regulatory sequence mapping, c-myc gene structure has been studied in terms of topo II sites (Riou et. al., 1986), methylation state (Dunnick et. al., 1985), S1 nuclease sensitivity (Gross and Pitot, 1985), and protein binding by electrophoretic gel mobility shift assays. The methylation state of a gene often reflects its transcriptional activity (see for review). C-myc in both undifferentiated and differentiated HL60 is hypomethylated in 5' CCGG sequence but extensively methylated in 3' exon and 3' flanking sequences (Gross and Pitot, op. cit). Although methylation patterns do not change during HL60 down regulation, translocation of the c-myc gene in murine plasmacytomas results in demethylation of the 3' end of the translocated locus (Dunnick et. al., 1985). The non-translocated locus remains highly methylated refecting its transcriptional inactivity. This implies that chromosomal translocation is important in determining methylation of the c-myc gene. In addition, a fetal liver derived hepatocellular carcinoma line reveals that myc and EGF are hypomethylated compared to normal human liver

(Kaneko et. al., 1987). Therefore, methylation pattern of the c-myc gene can reflect its transcriptional state, hypomethylation associated with activity, hypermethylation with inactivity. However, as the HL60 differentiation system demonstrates, methylation patterns of the c-myc gene also remain invariant during transcriptional changes.

S1 nuclease sensitivity can also indicate the transcriptional state of the chromatin surrounding a gene. Four S1 nuclease sensitive sites have been determined for myc in HL60 cells in the undifferentiated state (Gross and Pitot, 1985). Three of these remain after differentiation induction. The 4th site corresponds to myc transcriptional activity.

The location of topoisomerase II (topo II) sites within a gene can often indicate its transcriptional state (Gellert, 1981). In order to determine cleavage sites within the myc gene, W4r7 cells from small cell lung carcinoma cells with 45-50 copies of myc were treated with mAMSA, 9-hydroxyellipticin and VM26 (Riou et. al., 1986). These anti-tumor drugs induce dsDNA

beaks by stabilizing topo II-DNA complexes. A subsequent Southern blot analysis then can reveal a putative topo II binding site. Major cleavage sites are located in the 5' end of exon 1, close to DH sites III_1, and III_2.

Nucleosomal particles obtained by micrococcal nuclease digestion can also assess the transcriptional state of chromatin (see for review). Nucleosomes which contain actively transcribing genes are relaxed and more prone to micrococcal nuclease digestion. Non-transcribed genes are contained in more compact nucleosome structures and cannot be released in this manner. The down regulation of the c-myc gene during HL60 differentiation is reflected by a change in nucleosome structure from relaxed to compact (Chou et. al., 1986).

The compact or relaxed state of the nucleosomes surrounding a gene can also be assessed by brief micrococcal digestion and subsequent affinity for Hg^{++} (Chen and Allfrey, 1987). The unfolded nucleosomes of transcriptionally active genes contain sulfhydryl

groups of H3, which are accessible for binding to Hg^{++}. Conversely, compactly beaded nucleosomes of transcriptionally inactive genes do not bind H3 sulfhydryl groups. Thus, Hg^{++} affinity chromatography allows separation of transcriptionally active nucleosomes from inactive nucleosomes. Using this method, Chen and Allfrey (1987) determined that rapid, reversible changes in the c-myc and c-fos associated nucleosomes accompany the activation of these genes during growth factor stimulation of fibroblasts. Nucleosome structure in both cases correlates with the transcriptional activity of these genes; growth factor stimulated cells yield c-myc and fos gene regions containing unfolded nucleosomes, whereas unstimulated cells yield such regions containing compact nucleosomes.

In summary, chromatin and gene structure of the c-myc gene have been studied in great detail. Experiments in this field reveal a complex array of modifications that are possible and perhaps necessary for c-myc gene regulation. Four different promoters can be utilized depending on cell type, stage of

differentiation, growth factor stimulation or location in the genome. Some of these factors also determine the position of DNAseI hypersensitive sites, TopoII or S1 nuclease sensitive sites, methlyation patterns, DNA-binding by regulatory proteins, or nucleosome structure surrounding the gene. The myc gene regulatory elements have also been mapped in detail. Positively-acting sequences influencing P1 and P2 usage and an upstream "dehancer" have been identified. In addition, three negatively-acting and two positively-acting regions mediated by trans-acting factors have been identified. These studies reveal an intricate network of interactions within the nucleus responsible for the regulation of this gene. They also support the assumption that c-myc can function as a regulatory protein, profoundly affecting cellular phenotype.

RNA Structure and Transcriptional Regulation of C-myc

As mentioned above, the c-myc gene can utilize 4 promoters termed PO, P1, P2, and P3. The mRNA's generated from these promoters can vary in size, polyadenylation, folding potential, stability, and

translation efficiency. Evidence that exon 1 contains a degradation signal stems from analysis of mRNA's generated by P3 (Rabbitts et. al., 1985). Such aberrant c-myc mRNA's have longer half lives (50 minutes) than normal (15 minutes). More direct evidence comes from transfection of exon 1 deleted myc expression vectors into COS cells (Butnick et. al., 1985). These exon 1 lacking myc mRNA's are more stable than those containing exon 1. Although these RNA leaders have different folding potentials, they are translated with the same efficiency (Butnick et. al., op.cit.). This conflicts with a previous report indicating that RNA's containing 448 vs 83 nucleotides of the 5'untranslated region have different in vitro translation efficiencies (Darveau et. al., 1985). This difference can be attributed to the difference between in vitro and in vivo systems. These experiments indicate that exon 1 contains signals important for RNA degradation and translation efficiency. Exon 1 may mediate degradation through another mechanism (as proposed in a theoretical model by Gariglio et. al., (1987). This model proposes that a hairpin (ds RNA structure) formation between exon 1

and 2 could induce cleavage and subsequent removal of the 5' end and cap site.

Of the 4 promoters P1 and P2 are the most commonly utilized. P2 promotes 2 major RNA's of 2.4 and 2.2 kb (Swartwout et. al., 1987). The 2.4 kb RNA is less stable ($T_{1/2}$=12 min) with a long poly A. The 2.2 kb RNA is more stable with ($T_{1/2}$=1 hour) and no poly A. These 2 RNA's have been found in HeLa cells, normal human bone marrow, and mouse fetal liver cells. Thus, the variable polyadenylation of c-myc can also function in post-transcriptional regulation. In addition, a consensus AU stretch in the 3' region may signal degradation (Shaw and Kamen, 1988), indicating that both 5' and 3' regions determine stability. The role of this region in degradation can directly be addressed by mutagenesis or contruction of hybrid molecules. This type of experiment identified the AU rich consensus responsible for destabilizing GM-CSF (granulocyte macrophage, colony stimulatory factor) RNA's (Shaw and Kamen, 1988). In addition, Schuler and Cole (1988) identified a tumor activated, RNA stabilizing factor which can bind to the GM-CSF 3'

instability regions, using 3' linkage to the neomycin resistance gene. This RNA-binding, trans-acting factor, however, did not bind myc and fos 3' instability regions. These experiments demonstrate that post-transcriptional regulation can be both cell-type and gene specific.

Any or all of the mechanisms described above may be responsible for post-transcriptional regulation of the myc gene. Message stability accounts for regulation of myc in the following cases : treatment of cells with β and α interferon (Knight et. al., 1985, and Dani et. al., 1985), cycloheximide induction (Linial et. al., 1985) and superinduction (Endo and Nadal-Ginard, 1986), Xenopus development (Taylor et. al., 1986), F9 cell differentiation (Dean et. al., 1986), and partially for MEL cell differentiation (Mechti et. al., 1986) and treatment of WEHI 231 cells with αIg or phorbol esters (Levine et. al., 1986). Although it is widely accepted that growth factor modulation of myc occurs at the transcriptional level, Blanchard et. al. (1985) find that Chinese hamster lung fibroblasts increase myc message stability in

response to growth factors. Therefore, post-transcriptional regulation occurs in a variety of systems, both normal and neoplastic, and provides another level at which myc can be controlled.

Transcriptional regulation of the c-myc gene has also been studied in several different systems. Down regulation of the c-myc gene at this level occurs during TNFα, DMSO or vitamin D3 induced differentiation of HL60 cells (Kronke et. al., 1983, Pachl et. al. 1983, High et. al., 1987), rat L6 myoblast differentiation (Endo and Nadal-Ginard, 1986), and MEL cell differentiation (Mechti et. al., 1986); PDGF addition to 3T3 cells causes transcriptional stimulation (Greenberg and Ziff, 1986), whereas TNF or gamma interferon added to HeLa cells causes transcriptional down regulation (Yarden and Kimchi, 1986). The majority of these experiments use the nuclear "run-on" assay, which studies transcription in isolated nuclei (Marzluff et. al., 1973). Superinduction by anisomycin (a protein synthesis inhibitor) occurs at the transcriptional level (Greenberg et. al., 1986), while cycloheximide

superinduction is post-transcriptional. These experiments imply that both a transcriptional repressor and RNA degradation protein (s) can act to down regulate c-myc mRNA levels.

Transcription from P2 can be mediated by both RNA polymerases II and III (Chung et. al., 1987). Although pol II and III commonly use overlapping sequences (Bock et. al. 1987, Carbon et. al. 1987, Krol et. al., 1987), the c-myc promoter is the first to support transcription by both enzymes from a single initiation site. The preceding experiments demonstrate that pol III transcription is blocked at the 3' end of exon 1, whereas pol II pauses and then reads through this block. Both in vitro transcription experiments using whole cell HeLa extracts, pol II or III rich HeLa cell extracts, partially purified components, or in vivo experiments using Xenopus laevis oocytes support their conclusion. This results in a disproportionate level of exon 1 transcription relative to the rest of the gene. Previous reports by Mechti et. al. (1986) indicate that there is a transcriptional elongation block during DMSO or

retinoic acid induced differentiation of HL60 cells. This block occurs in the exon 1/intron 1 boundary resulting in the persistence of exon 1 transcription, even after the disappearance of exon 2 and 3 containing transcripts. It has not been proven whether the persistent exon 1 containing transcript in differentiated cells is a result of pol III activity, since pol II has been identified as pausing at this boundary in differentiated MEL cells. However, as Chung et. al., (1987) point out, run-on analysis indicating discontinuous transcription due to pol II may be caused by pol III "leaking" in this assay. Thus, the superimposed activity of pol III could affect pol II in a negative manner by interfering with its transcription initiation or elongation during differentiation.

By studying the effects of proflavine on c-myc transcription, Eick and Bornkamm (1986) assessed the role of RNA secondary structure in transcription elongation block. Proflavine prevents RNA processing and transcriptional termination by interfering with secondary structure. A 5 minute pulse of proflavine

in HL60 cells abolishes the signal given by exon 1. These expereiments predict 2 alternative RNA conformations around the PVU II site (position 2850) in the 1st exon. Both conformations are structurally and energetically similiar to those in the leader of SV40 VP1 RNA. In addition, mutations in the above region of exon 1 found in Burkitt's lines are associated with altered transcription (Cesarman et. al., 1987). These mutations may affect transcriptional termination sites located in the 3' of exon 1, therefore removing the block and resulting in myc activation. Thus, exon 1 seems to have a crucial role in c-myc transcription by mediating binding of pol II and/or pol III. In addition, this binding may be influenced by RNA secondary structure in this region.

Another mechanism which may regulate c-myc gene expression is anti-sense transcription (Nepveu and Marcu, et. al., 1986). Transcription of the coding and noncoding strands of the c-myc have been demonstrated to be regulated independently (Kindy et. al., 1987). Anti-sense transcription occurs mainly in

3 noncontiguous regions: upstream of exon 1, within intron 1, intron 2 and exon 3. Transcriptional changes associated with growth state transitions occur on the coding but not the noncoding strand. In contrast, transcription of both strands decreases during DMSO induced MEL differentiation. In both cases, the ratios of the levels of transcription from coding and non-coding strands fluctuate (Nepveu and Marcu, op. cit.). The MEL cell results suggest that regulation through anti-sense transcription may be more prominent during differentiation.

The role of anti-sense transcription by c-myc is still unclear. In E.coli, anti-sense transcripts have been shown to regulate plasmid DNA replication (reviewed by Green, 1986). In addition, anti-sense ompF RNA transcripts generated from a separate locus inhibits ompF gene expression (Mizuno, 1984). In eukaryotic cells, transfection of anti-sense RNA producing constructs into cells in culture cause specific decreases in expression of the complementary gene (Izant, 1984, McGarry, 1986). These experiments indicate that anti-sense RNA can interfere with gene

expression or translation. In addition, closely spaced actively transcribing regions may influence each other in a negative manner (Cullen, 1984). Therefore, c-myc anti-sense transcription could conceivably regulate gene expression at several different levels. Whether this mechanism plays a key role in c-myc down regulation still remains to be determined.

In summary, regulation of c-myc at both the transcriptional and post-transcriptional levels has been studied in great detail. RNA stability is determined by both 5' and 3' regions. The large, untranslated 1st exon is highly conserved between species (Battey, 1983, Bernard, 1983), playing a role in both types of regulation. Exon 1 containing RNA's are more unstable than those lacking. In addition, the 3' end of exon 1 can form stem-loop structures causing premature termination or pausing during transcription. The c-myc promoter P2 is unique in mediating transcription by both pol II and pol III. The latter is thought to influence the activity of the former. Finally, anti-sense transcription just

upstream of exon 1 and in other regions of the gene may also control myc gene expression.

Protein Structure

The c-myc protein is a member of a larger family of oncogenes containing, at present, three well-defined members : c-myc, N-myc, and L-myc. The less well-defined members are R-myc, L-myc , p-myc (Figure 4, taken from review (Astrin, Oncogene Handbook). The well defined members share a 2 intron/ 3 exon structural organization, containing highly conserved amino acids common to all members of the family (DePinho et. al., 1987). The cross-species conservation of c-myc and its shared domains within the myc gene family imply an important physiological role(s) for these set of genes. Proteins homologous to c-myc, but not within the myc gene family are discussed in detail below.

The c-myc gene produces 2 phosphoproteins, human c-myc proteins having apparent molecular weights of 62kd and 66kd, and mouse 64kd and 66kd (Ramsay et. al.

Figure 4

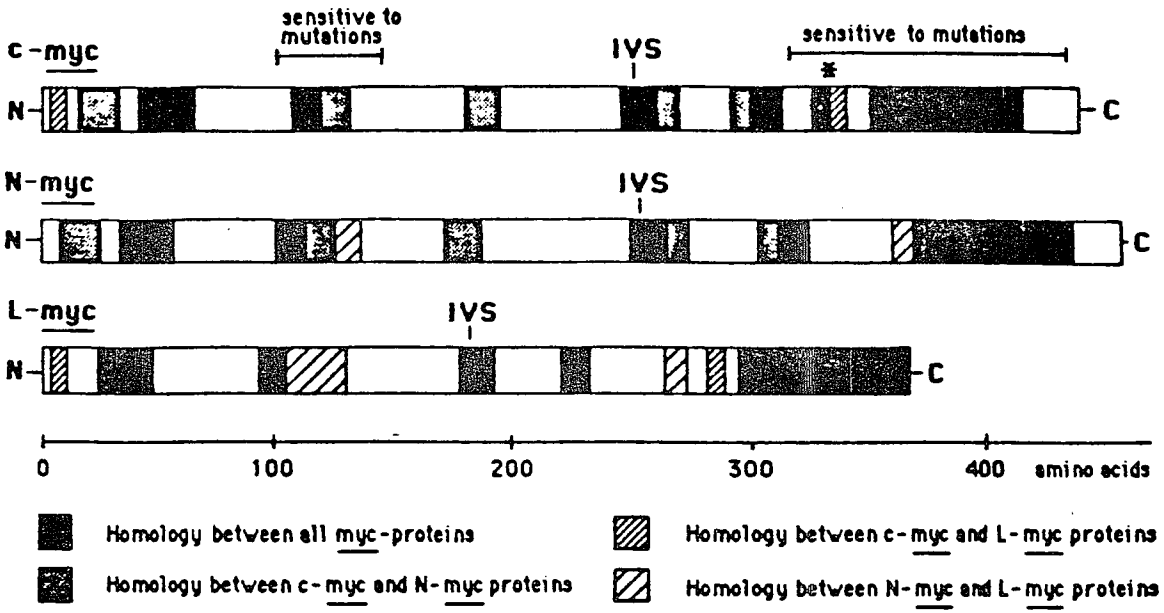


Fig. 3. Structural comparison of the human *myc*-proteins. The length of the proteins as well as segments of over 70% amino-acid homologies between the different *myc*-proteins are indicated. Amino-acid sequences, which are essential for the transforming activity of the *myc* proteins as expressed in cotransformation assays together with mutated *ras*-proteins, are marked above c-*myc*. These regions are therefore sensitive for in vitro mutagenesis. The asterisk indicates the approximate location of the major signal amino acid sequences for nuclear localization of the c-*myc* protein. IVS = location of the intervening sequence in the corresponding gene. The figures have been adapted from the publications of Stanton et al. [219] and Alt et al. [280] and redrawn to the same scale.

Figure 4: The MYC Gene Family. A structural comparison of three members of the cellular c-myc gene family. Segments of over 70% amino acid homologies between members are indicated. Regions necessary for co-transformation ability with ras proteins are indicated above the c-myc gene. * approximate location of the major signal amino acid sequences necessary for nuclear localization of the c-myc protein. IVS = location of the intervening sequence in the corresponding gene. This figure was taken from Oncogene Handbook (1989).

1984). The smaller protein is made in larger quantities than the larger. C-myc has been demonstrated to be a casien kinase II substrate both in vivo and in vitro (Luscher et. al., 1989). In early experiments, it was found that the majority of v-myc and c-myc proteins are associated with the nuclear matrix. About 60-90% of c-myc proteins were found to remain in DNA and RNA depleted nuclei after low and high salt extraction (Hann and Eisenman, 1984). They also found that less than 10% of the myc protein extractable with salt or detergent can bind single stranded or double stranded DNA. Others have also shown that $pp62^{myc}$ can bind DNA (Watt et. al., 1985, Persson and Leder, 1984). A subsequent analysis of the association of c-myc with the nuclear matrix has revealed that this association is dependant on the temperature of extraction (Evan and Hancock, 1985). The nuclear matrix is defined as a residual insoluble structure remaining after nuclei are treated with nuclease, and extracted with high salt in the presence of detergents. In the above experiments, $pp62^{myc}$ can be extracted from nuclei by mild salt concentrations (below 200 mM), from non-nuclease treated nuclei at

37°C, or nuclease treated nuclei at 4°C. Therefore, weak ionic interactions are probably responsible for retention of c-myc in the nucleus (Evan and Hancock, 1985), and not a true association with the nuclear matrix. This would be more congruent with the short half life of c-myc and its possible role as a regulatory protein.

The finding that c-myc is not a real matrix associated protein was followed by the demonstration that it co-localizes with sn- RNP's (small nuclear ribosomal binding proteins, Sullivan et. al., (1986). Since snRNP's most likely function in hnRNA processing, either splicing or polyadenylation (Krainer and Maniatis, 1985, Berget and Robberson, 1986), immunofluorescent co-localization of c-myc with snRNP's may indicate some involvement of c-myc in these processes. However, direct evidence of binding of c-myc to snRNP's or snRNP associated hnRNA by immunoprecipitation still remains to be obtained. Since both N-myc and c-myc nonspecifically bind DNA, experiments determining whether specific interactions

may require the presence of other factors still remain to be performed.

More recently, the 3' region of exon 1 has been shown to contain a non-AUG translational initiation site (Hann et. al., 1988). This site generates the larger $pp66^{myc}$ observed in lesser quantities than $pp62^{myc}$ (termed p67 and p69, respectively, in this paper). That the two proteins are highly related was demonstrated by staphyloccocus V8 protease or trypsin mapping and hybrid arrest in vitro translation (Hann and Eisenmann, 1984). Site-specific mutations of c-myc exon 1 pinpoint the translation initiation start site of the larger protein to a CTG codon located 14 codons upstream of the ATG start site in exon 2. They also demonstrate that Burkitt's lines with translocations or mutations within exon 1 only express p62 and not p66. If $pp66^{myc}$ regulates the activity of $pp62^{myc}$, its lack of expression in Burkitt's lymphoma cells may explain how myc can be oncogenic without being overexpressed in these cells. Before such an interaction can be tested, however, sequence specific DNA binding of the c-myc protein must be demonstrated.

Therefore, a great deal of protein chemistry still needs to be done with the c-myc proteins, including dimerization, and DNA binding studies, to determine how pp66 expression could influence pp62 activity.

Functional domains: Mutagenesis experiments have delineated the functional domains on the c-myc gene in terms of their importance in co-transformation with ras and immortalization. Stone et. al. (1986) constructed pSV-c-myc plasmids containing various deletions and mutations within the protein coding regions. These experiments divide the protein into 4 regions, as shown in Figure 5. Region I is required for co-transformation of rat embryo fibroblasts with ras, even though small segments can be deleted. Regions II and IV are the most essential for co-transformation, where small deletions or point mutations alter activity. Nuclear localization determinants are also located in these two regions. Region III is important for rat 1a immortalization, but not co-transformation. Region IV is important for both types of transformation. These regions

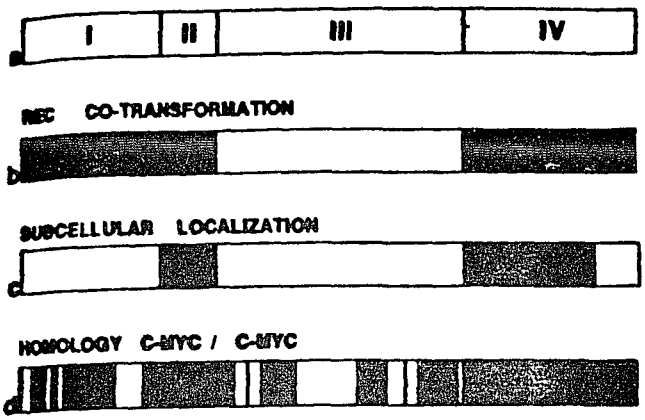


FIG. 9. Functional regions of the human c-*myc* protein and regions of conserved amino acids among c-*myc* proteins of chickens, mice, and humans. (a) Regions I through IV are defined in the text and in Fig. 2. (b) The darker shading indicates regions essential for activity; lighter shading indicates the region that only tolerates insertions and partial deletions; the clear area indicates the region that is not essential for activity (see text for details). (c) The shaded areas indicate regions important for normal subcellular distribution, with the darker shading representing the area determined by immunofluorescence studies and the lighter shading representing additional areas determined by fractionation and immunoprecipitation studies. (d) Blackened areas indicate regions of amino acid identity among normal c-*myc* proteins from the three species (8).

Figure 5

Figure 5: The Functional and Conserved Domains on the Human C-myc Protein. a. Regions I-IV are defined in the text. b. Darker shading indicates regions essential for activity; lighter indicates tolerance for partial deletions and mutations; clear regions not essential for activity. c. shaded regions necessary for subcellular localization. d. black areas indicate amino acid identity among normal c-myc proteins from chickens, mice and humans. (Figure taken from Stone et. al., 1987).

correspond to those identified on v-myc by Heaney et. al.(1986). The most conserved sites in the protein occur within regions II and IV required for the maintenance of myc in nuclei after NP40 treatment.

Sarid et. al. (1987) performed a detailed mutagenesis study of exon 2, mapping its conserved regions according to co-transformation ability with EJ-ras. There are 3 regions in exon 2 conserved between mouse, chicken, and fish, over 400 million years of evolution. These are 20 amino acids long, separated by areas of low or no homology. Deletion of the third region does not alter co-transformation with ras. This means that all three regions are not necessarily involved in transformation. This result is important to those searching for a normal cellular role for c-myc. If a region conserved 400 million years is not involved in transformation, it may participate in such a normal role, indicating this role to be essential to the organism.

Homologous proteins and putative DNA binding domains: Protein homologies can often indicate

structure-function relationships between proteins, thereby delineating the most important functional regions. This has proven true for the myc family of genes. Recently, several genes important in development have been shown to be homologous to c-, N-, and L-myc. Site-directed mutagenesis (described above) has indicated that the basic C-terminal region and N-terminal region constitute the transforming domains. These sequences are also the ones found to have the most homology to other proteins. There are two groups of DNA binding proteins homologous to the c-myc protein in its C-terminal region. The first contains the helix-loop-helix motif described by Murre et. al. (1989a). Table 11 was complied from a paper by Murre et. al. (1989b), listing proteins known to contain the helix-loop-helix motif. The most relevant to this work are the dominantly-acting, myogenic phenotype inducing genes: myod1, myogenin, and myf5. The second group of DNA binding proteins homologous to c-myc contains a leucine zipper at the carboxy terminus. These are the products of the jun, fos, GCN4, and C/EBP, myogenin genes (see Landschulz et. al. 1988). Some of the proteins, like c-myc and

Table 11.
Helix-loop-helix proteins.

Mammalian genes:	
c-myc	Alt et. al. (1986), unknown function
N-myc	"
L-myc	"
E12	Murre et. al. (1989), binds kE2 DNA motif
E47	"
myod1	Davis et. al. (1987), induces myogenic phenotype
myogenin	Wright et. al. (1989), induces myogenic phenotype
myf5	Braun et. al. (1989), induces myogenic phenotype
Drosophila genes:	
achaete scute	Villares and Cabrera (1987), helps specify formation of central and peripheral nervous system of Drosophila complex (AS-C)
daughter-less	Caudy et. al. (1988), similiar function to AS-C
twist	Thisse et. al. (1988)
enhancer of split	Klaemat et. al. (1989)

myogenin, contain both motifs. More recently, homo- and heterodimer formation between helix-loop-helix proteins have also been demonstrated (Murre et. al., 1989b).

The mouse c-myc helix-loop-helix (HLH) region lies adjacent to a leucine zipper in the carboxy terminal region of the protein, spanning residues 346-401. Its hypothetical loop would consist of pro-glu-leu-glu- asn-asp-glu. Since loops are found at the surface of proteins (Lesczynski and Rose, 1986), the HLH model predicts that these loops may be responsible for interacting with DNA either in conjunction with the hydrophilic residues of the helices or the conserved basic stretch amino terminal to helix I, or alone. The helices contain a 4-3 heptad repeat of hydrophobic residues similiar to that found in the nuclear lamins A and C, which form coiled-coil interactions. However, unlike the lamins, each helix spans less than 28 amino acids. Since Lau et. al., (1984) determined that a minimum of 28 amino acids is required to form a stable, two-stranded α-helical coiled-coil, it is not known whether these

helices are able to form such stabilizing interactions. Like the lamins, the region spanned by the proposed helices is important for dimerization of E12 and E47 (kappa E2 enhancer binding proteins, Murre et. al., 1989a). These two proteins do, in fact, bind DNA as a dimer. Whether c-myc binds DNA as a dimer, or possibly as a tetramer still remains to be determined. Additional evidence of the functional importance of the helix-loop-helix region stems from mutagenesis experiments with both c-myc and myoD1. The c-myc protein loses its transformation ability when this region is altered (Stone et. al., 1987). The HLH containing region in myoD1, a nuclear protein which can induce a myogenic phenotype in non-myogenic cells, encodes a truncated protein which by itself is able to induce myogenesis (Tapscott et. al., 1988). Deletion analysis on myod1 reveals that the HLH region is necessary to bind the muscle creatine kinase enhancer (E-box) (Lassar et. al., 1989b).

The c-myc protein also contains a leucine zipper at its carboxy terminus. This DNA binding motif was originally described by Landschulz et. al. (1988) for

C/EBP (CAAT box enhancer binding protein). Breifly, leucines appear at every 7th position over a stretch of 22-30 amino acids. They extend from an unusually long α helix. The c-myc protein contains 4 leucine repeats yielding six hypothetical turns of the helix, beginning 32 amino acids from the carboxy terminus. The leucine zipper model predicts that dimerization of proteins containing this motif occurs by interdigitation of the leucine side chains of one helix with a matching helix in a non-covalent linkage. Recently, the leucine zipper has been demonstrated to be important in heterodimer formation between the c-fos and c-jun proteins (Kouzarides and Ziff, 1989). Logically, the same questions have to be addressed for c-myc. Does c-myc utilize its leucine zipper motif to form heterodimers with other proteins? If so, which proteins, what determines this capacity, and are these heterodimers functional?

Some fundamental concepts emerge from the DNA binding motifs described above. DNA binding domains on HLH proteins contain regions capable of forming amphipathic α helices. These are stabilized by the

non-covalent interactions of 4-3 heptad repeats of hydrophobic residues. The inter- or intra-chain linkages cause dimerization of these proteins, allowing adjacent basic residues to interact with DNA. The conservation of the hydrophobic character and positioning of the residues within these helices among several transforming and differentiation-inducing, DNA-binding proteins, emphasizes the biological significance of these domains. Unlike many of the other proteins listed in Table 11, the c-myc gene product has only been demonstrated to bind DNA non-specifically; its exact function is still unknown. The existence of these motifs within the the c-myc protein only implies, but does not prove the importance of c-myc in differentiating cells.

Possible role in proliferation: Since the initial experiments by Kelly et. al. (1983) demonstrating its induction by PDGF stimulation of quiescent fibroblasts, the c-myc gene has been thought to play an integral role in cellular proliferation. The subsequent finding that c-fos activation precedes c-myc indicated that myc induction may be part of a

cascade response to growth factors (Muller et. al., 1984). The hypothesis that c-myc acts as a competence factor was further substantiated by microinjecting c-myc into quiescent 3T3 cells, in which it induced DNA synthesis (Kaczmarek, 1985). More recently, a direct role for c-myc in replication has been reported based on the ability of anti-myc antibodies to inhibit pARS65 replication (Iguchi-Ariga et. al., 1987). The pARS65 is a plasmid capable of autonomous replication in human and mouse cells (Iguchi-Ariga, 1988, 1989). In addition, c-myc has been demonstrated to enhance the replication of SV40-origin containing plasmids (Classon et. al., 1987). These experiments done both in vivo and in vitro suggest that c-myc is actively involved in replication. Whether it is part of the actual replication machinery, or indirectly involved, still remains to be determined.

In addition to PDGF, other growth factors and inhibitors have been demonstrated to regulate c-myc (see Cole, 1987 for review). Most mediators which induce proliferation also induce or increase c-myc levels. However, this is not the case for A431 cells

inhibited from growing (myc levels unchanged, [Bravo et. al., 1985]), and cardiac hypertrophy (myc increases even though no proliferation occurs, [Starksen et. al., 1986]). These exceptions emphasize the importance of determining the regulation of c-myc in various systems before assuming what it does in any one system. They indicate that signals given to the cell by myc may be replaced or overcome by other genes in the cell. Although most systems show a positive correlation between c-myc and proliferation, c-myc may be playing an altogether different role in some systems, a role yet to be investigated. Table 3 compiles some of the effects of c-myc transfection on cell growth that have been reported in recent years.

Possible role in differentiation: The c-myc gene has been implicated in the development of several different naturally occurring tumors (reviewed by Astrin, 1989, Alitalo et. al. 1987). However, how it does so remains a mystery. Unlike the ras gene which was found to contain a single point mutation responsible for tumorigenicity (Reddy et. al., 1982), there are no consensus point mutations differentiating

c- and v-myc. It is widely accepted that over expression of the myc gene by amplification, translocation, or insertional activation (see Alitalo et. al. 1987, for review), probably causes c-myc-induced tumors. One of the ways of investigating how c-myc can induce tumors has been by studying its normal cellular role. Many of these studies utilize cell lines which activate or suppress c-myc expression in response to external stimuli, namely modulators of proliferation and differentiation. Down regulation of the c-myc gene during differentiation is generally thought to be a result of the cessation of proliferation. Table 5 lists some of the reports of c-myc regulation in differentiating cell lines. Most of the lineages including myeloid, erythroid, monocytic, and myogenic down regulate c-myc expression upon differentiation induction. The exceptions are CLL (chronic lymphocytic leukemia) and lens epithelium which upregulate c-myc upon differentiation induction in vitro. At different times during Xenopus development, c-myc is both down and upregulated, resulting in high expression in adult skin and muscle (King et. al.,

Table 5. Regulation of C-myc During Differentiation

Cell Line	C-myc Regulation	Inducing Agent	References
myogenic:			
mouse C110	decrease	mitogen removal	Sejersen et. al. (1985)
rat L6-differentiation defective	no change	"	
L6E9	decrease	"	Endo and Nadal-Ginard (1986)
non-myogenic:			
MEL	dec/inc/dec	DMSO	Lachman and Skoultchi (1984)
"	decrease	"	Faletto et. al. (1985)
MEL differentiation defective	dec/inc, remains at a high level	DMSO, TPA, or dexamethasone	Sasaki et. al. (1987)
myeloid leukemia	decrease	DMSO	Gonda and Metcalf (1984)
HL60-monocytic pathway	decrease	D3	Grosso and Pitot (1985)
"	decrease	D3	Watanbe et. al. (1985)
"	decrease	interferon gamma, or interferon gamma + D3	Matsui et. al. (1985)
HL60-myeloid pathway	decrease	retinoic acid	Yen et. al. (1986)
HL60 differentiation defective line 1E3	dec/inc	PDBu (phorbol dibutyrate)	Ely et al (1987)
	decrease, stays off	PDBu + DMSO (cells differentiate)	
human monocytic cells THP-1	no change	TNFα	Lee et al (1987)
bone marrow cells	decrease	CSF	Resnitsky et. al. (1986)
CLL	increase	PMA	Larsson et al. (1987)
K562	decrease	araC (1-β-D-arabinofuranosylcytosine)	Tonini et. al. (1987)
hu BFU-E	decrease		Umemura et. al. (1986)
human medullary thyroid carcinoma	decrease	phorbol esters	deBustros et. al. ()
F9 (Teratocarcinoma)	decrease	retinoic acid dibutyrylcAMP	Dony et. al. (1985)
mouse primary keratinocytes	no change	Ca^{2+}	Dotto et. al. (1986)
	decrease	TPA	
In Vivo			
chick heart development	decrease from embryonic day 13		Schneider et. al. (1986)
Xenopus development			King et. al. (1986)
-early cleavage stages of embryogenesis	decrease		
-early neurula stage	increase		
-adult skin	high		
-adult muscle	high		

1986). Another exception includes primary mouse keratinocytes, which fail to exhibit any changes in c-myc regulation during Ca^{+} induced differentiation. These results suggest that if c-myc plays an important role in differentiating cells, this role may vary with cell lineage.

One of the major questions about the decrease in c-myc mRNA during differentiation is whether this event is obligatory for differentiation to proceed. If the answer to this question is yes, then it would indicate that c-myc not only functions in some aspect of proliferation, but can suppress the expression or action of genes important to differentiation. Transfection and inappropriate expression of c-myc demonstrate its ability to inhibit differentiation in several differentiating cell lines (see Table 4). These include MEL cells, HL60 cells, and myogenic BC3H1 (partially) and L6 cells. Deregulated c-myc expression in BC3H1 myoblasts only inhibits the expression of differentiation markers in the presence of growth factors, but not upon their removal. In vivo, deregulated c-myc can inhibit B-cell development

Table 4. Effect of C-myc Transfection on Differentiation

Cell Line	C-myc Construct	Effect on Differentiation	References
MEL	pSVmyc1 (SV40 promoted, mouse c-myc exon 2 and 3)	inhibits	Coppola and Cole (1986)
MEL	human c-myc, exon 2 and 3	inhibits	Dmitrovsky et. al. (1986)
MEL	mouse c-myc, exons 1, 2 and 3	inhibits	Prochownik and Kukowska (1986)
transgenic mouse	Eμ enhancer -mouse c-myc exons 2 and 3	inhibits, increased pre-B cells, early stages over-represented	Langdon et. al. (1986)
3T3	human c-myc	increases expression of dihydroxyvitamin D3 receptor	Manolagas et. al. (1987)
BC3H1, myoblast	pSVmyc1	partial inhibition of creatine kinase and acetylcholine receptor	Schneider et. al. (1987)
L6, rat myoblast	pMTc-myc, metallothionein promoted human c-myc exons 2 and 3	inhibits	Denis et. al. (1987)
18 day chick embryo	v-myc retroviral vector	increases stem cell proliferation	Thompson et. al. (1987)
HL60	anti-sense myc-gpt	decreases cellular proliferation, triggers differntiation	Yokoyama and Imamoto (1987)
chick embryonic day 2 and 3	MC29 virus (v-myc)	skin and muscle hypertrophy, rhabdomyosarcoma of the heart, interfers with cardiac development	Saule et. al. (1987)
C2C12, mouse myoblast	pSVmyc1	no inhibition of differentiation by c-myc, but by activated ras	Payne et. al. (1987)

in E_{μ} - c-myc transgenic mice. In addition, v-myc can interfere with chick cardiac development and cause skin and muscle hypertrophy. Constructs expressing anti-sense c-myc can trigger HL60 differentiation. In addition, the v-myc containing retrovirus, MC29, has been demonstrated to inhibit quail myogenesis, in culture (Falcone et. al., 1985). These reports indicate that inappropriate expression of c-myc in a variety of systems can inhibit differentiation.

Table 3 . Effect of c-myc on cell growth

Cells	Transfection effects	References
FR3T3	Growth in soft agar in the presence of EGF or PDGF + TGF ß	Roberts et. al. (1985)
rat-1	decreased rate of glycolysis, and increased sensitivity after TGF ß exposure	Racker et. al. (1985)
SCLC	large cell morphology, shorter doubling times, higher cloning efficiency	Johnson et. al. (1986)
fibroblasts, established	anchorage independant, tumor formation in nude mice	Baumbach et. al. (1986)
quail fibroblasts	transformation, human c-myc	Martin et. al. (1986)
primary rat embryo fibroblasts	failure to be immortilized by human c-myc exon 2 and 3 linked to a metallothionien promoter	Nicolaiew and Dautry (1986)
C3H/10T1/2	more responsive to TGF ß	Leof et. al. (1987)
HL60	anti-sense oligonucleotides to c-myc inhibit proliferation	Heiksila et. al. (1987)
chick embryo neuroretinal cells	viral infection with MH2 or MC29 (v-myc containing viruses) does not transform; trasnfromation only in cooperation with v-mil	Bechade et. al. (1987)
chicken heart mesenchymal cells	heparin treated cells are hypersensitive to FGF and EGF (v-myc infection)	Balk et. al. (1985)
IL3 or IL2 dependant cells	loss of dependance on IL3 or IL2 (v-myc infection)	Rapp et. al. (1985)

Statement of Thesis Problem:

The C2 myoblast line was chosen to study the role c-myc may play during myogenic differentiation. These cells grow rapidly in serum rich medium, but cease proliferating and induce muscle specific genes upon serum deprivation (Yaffe and Saxel, 1977). In vitro differentiating lines HL60, MEL, or 10T1/2, require agents such as PMA, DMSO, or 5-azacytidine (Huberman et. al., 1982, Lachman and Skoltchi, 1984, and Taylor and Jones, 1979), respectively, for differentiation induction. However, it has been demonstrated that fibroblast growth factor (FGF) is responsible for inhibition of myogenesis in culture (Compton et. al., 1986). The requirement that growth factors such as (FGF) be removed from the medium for myogenic differentiation in culture implies that genes controlling proliferation, in response to growth factors, must be turned off during this process (Linkart et. al.(1981), Compton et. al., op. cit.). The c-myc gene has been closely linked to differentiation and DNA replication. In addition, it is rapidly induced by a variety of growth factors.

The purpose of these experiments was to determine if changes in c-myc mRNA occur during myoblast differentiation, and test to whether such changes are obligatory for muscle specific gene expression.

RESULTS

C2 myoblasts activate muscle specific markers, myosin heavy chain and desmin upon differentiation induction: C2 myoblasts (C2M) grow in serum-rich medium, 20% fetal calf serum in DMEM + 1% chick embryo extract (CEE-DMEM). Figure 6A is a phase contrast photograph of a single myoblast. These myoblasts have characteristically visible nucleoli and short doubling times. Confluent myoblasts are shown in Figure 6B. When their growth medium is changed to a low- serum medium (4% horse serum in DMEM [HS-DMEM]), these rapidly growing cells cease proliferation, induce muscle-specific markers, and eventually form multinucleated myotubes (Figure 6C). In this thesis, the term "differentiation" includes the cessation of proliferation and induction of muscle specific markers, but not fusion, unless otherwise noted. Many of the muscle specific proteins that are induced during differentiation induction are components of the contractile apparatus. Myosin heavy chain (MHC), one of the contractile proteins activated during this process, is often used as a marker for the

C2M mouse myoblasts

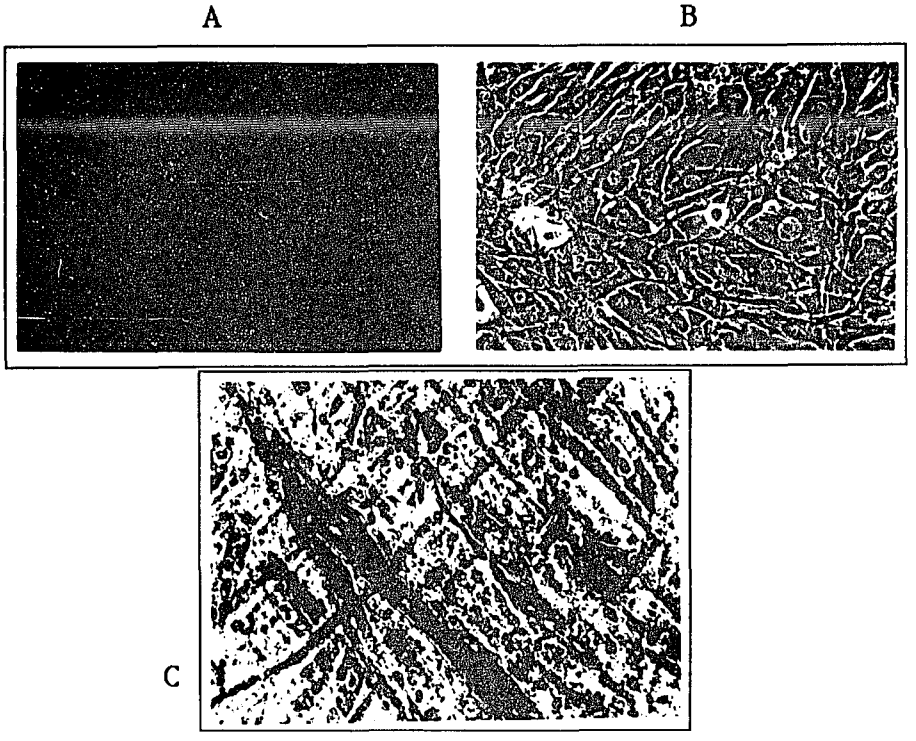


Figure 6

Figure 6: Different Stages of C2M Myoblasts Grown in Culture. A. Phase contrast microscopy of C2M myoblasts at low density. These cells were grown in DMEM containing 20% fetal calf serum and 0.5% chick embryo extract. B. Phase contrast microscopy of C2M myoblasts at confluence. These cells were also grown in DMEM containing 20% fetal calf serum and 0.5% chick embryo extract. C. Immunoperoxidase staining of C2M myotubes with mAb MF20 (anti-myosin heavy chain). Differentiation was induced by incubating confluent C2M myoblasts in DMEM containing 4% horse serum for 6 days, when myotubes were apparent.

differentiated phenotype (see Stockadale and Miller, 1987, for review). In addition, the intermediate filament, desmin, has also been shown to be specific for the myogenic lineage (Danto et. al. 1984, see Steinert 1988, for review). Figure 7 is a western blot of whole cell extracts derived from C2 myoblasts at different times after differentiation induction. Hours after differentiation induction are indicated above each lane (120 hours, 96 hours, 24 hours, 6 hours, and 0 [undifferentiated]). The blot was probed with an anti-myosin heavy chain monoclonal antibody (MF-20, Bader et. al. 1982) and an anti-desmin monoclonal antibody (D3, Danto et. al. 1984,). Myosin heavy chain can be detected at 24 hours after induction, peaks at 96 hours, and decreases afterwards. This subsequent decrease may be due to a decrease in the viability of these cells by this time. Desmin is detectable in both myoblasts and myotubes, although higher in the latter.

Fusion deficient lines differ from C2 myoblasts in c-myc regulation: In order to test whether c-myc has a role in differentiating myoblasts,

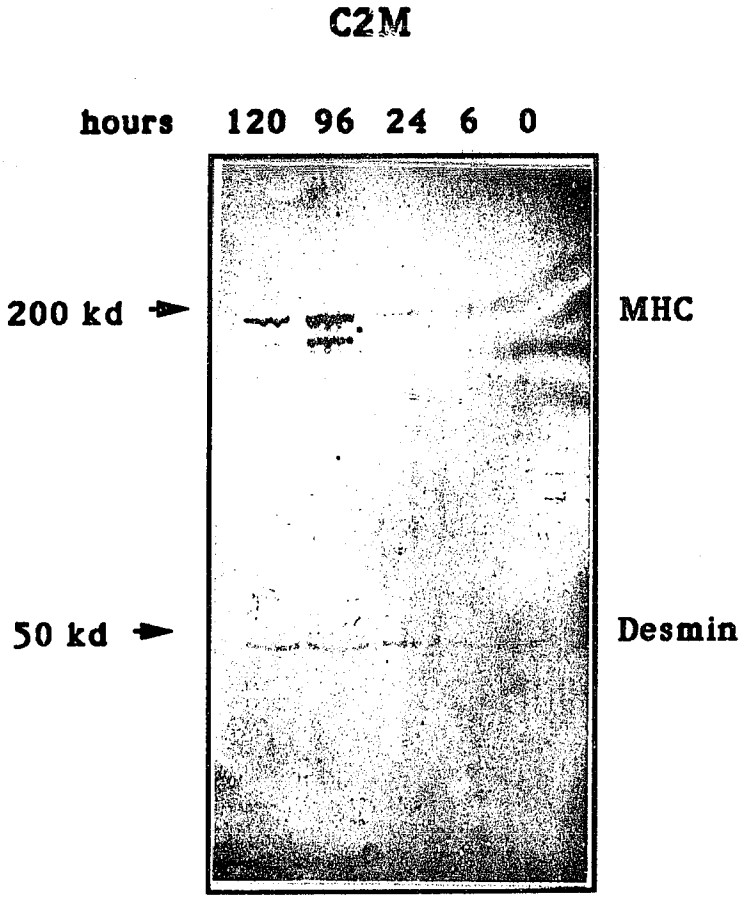


Figure 7

Figure 7: Muscle-Specific Proteins, Myosin Heavy Chain and Desmin, Increase During C2M Myoblast Differentiation. Whole cell extracts (approximately 10^6 cells/lane) were loaded on a 5-15% polyacrylamide gradient gel, blotted onto nitrocellulose, and probed with MF20 (mAb anti-myosin heavy chain), and D3 (mAb anti-desmin). Western blots were then incubated with peroxidase conjugated, goat anti-mouse second antibody, and developed with 1-4 chloronapthol, as described in the methods. The cells were harvested at 0, 6, 24, 96, and 120 hours after differentiation induction, as indicated above each lane. Equivalent protein amounts were loaded as assessed by Coomassie staining prior to blotting. High molecular weight standards (Sigma) were run alongside and Coomassie stained to determine if myosin heavy chain migrated at its expected 200 kd, and desmin at 50 kd. Arrows point to myosin heavy chain and desmin bands visualized in this manner.

non-differentiating, fusion deficient clones were derived from the C2 myoblast line. It was thought that if c-myc has a role, then it may differ among differentiating and non-differentiating variants. Since C2M is a heterogeneous population of cells, selection for such clones is possible without chemical treatment. The C2 cells are allowed to differentiate and fuse in differentiation medium. At 6 days after differentiation induction, the myotubes are fed with cold differentiation medium. This causes the majority of differentiated cells to detach from the plate in a sheet-like layer. The few remaining, adherent cells are fed with growth medium (20% FCS + .5% CEE in DMEM) and cloned in cloning cylinders. Two of these clones (termed FD-2 and FD-6) fail to fuse or express MHC upon induction. Dot-blot analysis of c-myc steady state mRNA levels of these two lines compared to C2 cells indicate that c-myc is still detectable in these lines at 52 hours after induction, whereas C2 levels had decreased below detection levels (Figure 8). Hours after differentiation induction are indicated on the left. Two amounts of RNA are included, 30 μg and 6 μg, for each time point, as indicated on the bottom.

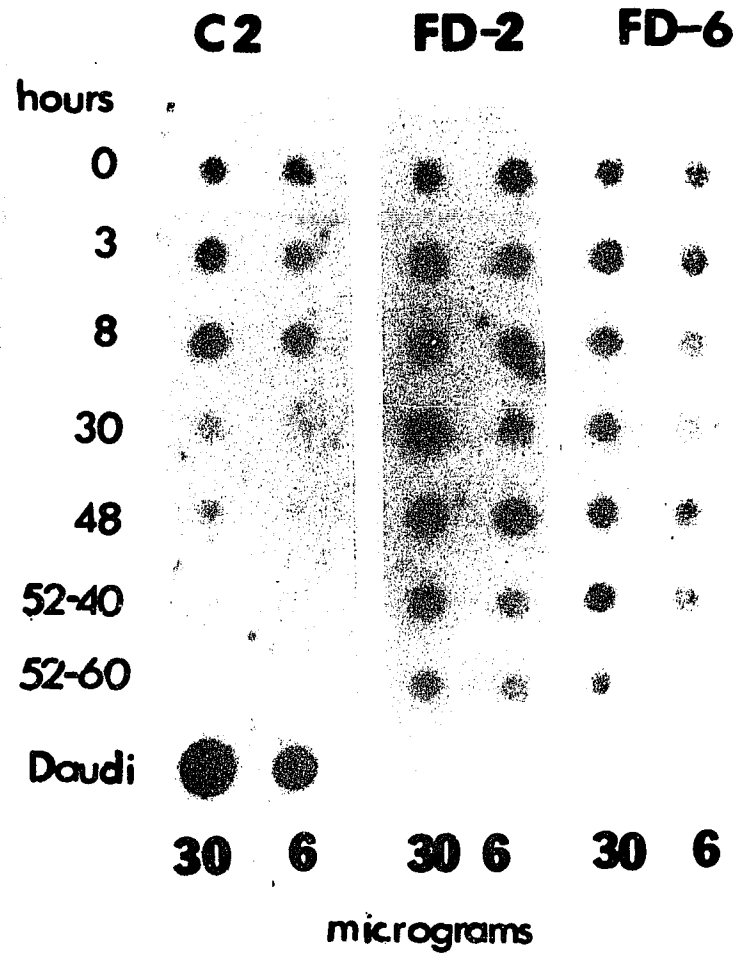

C2
FD-2
FD-6
hours
0
3
8
30
48
52-40
52-60
Daudi
30
6
30
6
30
6
micrograms

Figure 8

Figure 8: Differentiation Induced Fusion Deficient Lines Regulate C-myc mRNA's Differently From Parental C2M Myoblasts. RNA's derived from C2M myoblasts, FD-2, and FD-6 (fusion deficient lines derived from C2M) at different times after differentiation induction were bound to nitrocellulose in a dot blot apparatus (Schleicher and Schuell). Hours after differentiation induction are indicated on the left (0, 3, 8, 48, and 52). Different amounts for each time point (30 and 6 μg) were also loaded. Daudi RNA loaded in the last row was included for a positive control. The blot was probed with a nick-translated, Hind III fragment of pMcmyc54. In order to determine whether the density at which cells were placed in differentiation medium had any role in c-myc expression, RNA was isolated 52 hours after induction, from cells which were induced to differentiate at 40% or 60% confluence (52-40, 52-60).

Differentiated points taken from cells induced to differentiate at 40% confluence and 60% confluence (52-40, 52-60) are also included, as indicated on the left. Also, RNA derived from Daudi cells, which contain a translocated c-myc gene, is also included as a positive control. Northern analysis of both c-myc and histone H3 in C2M and one of these non-differentiating clones, FD-2, indicates that both lines cease proliferating in response to differentiation induction (Figure 9). Hours after differentiation induction are indicated above each lane :52 hours, 30 hours, 8 hours, 3 hours and 0 (uninduced). Daudi RNA is included for a positive control. The migration of the 28S and 18S ribosomal RNAs are marked to the right of the gel. Levels of c-myc mRNA decrease dramatically 8 hours after differentiation induction in C2 cells. In contrast, FD-2 cells still have detectable c-myc mRNA 52 hours after induction. These results suggest that the decrease in c-myc observed during myoblast differentiation is not just associated with the cessation of proliferation, but may have a regulatory role.

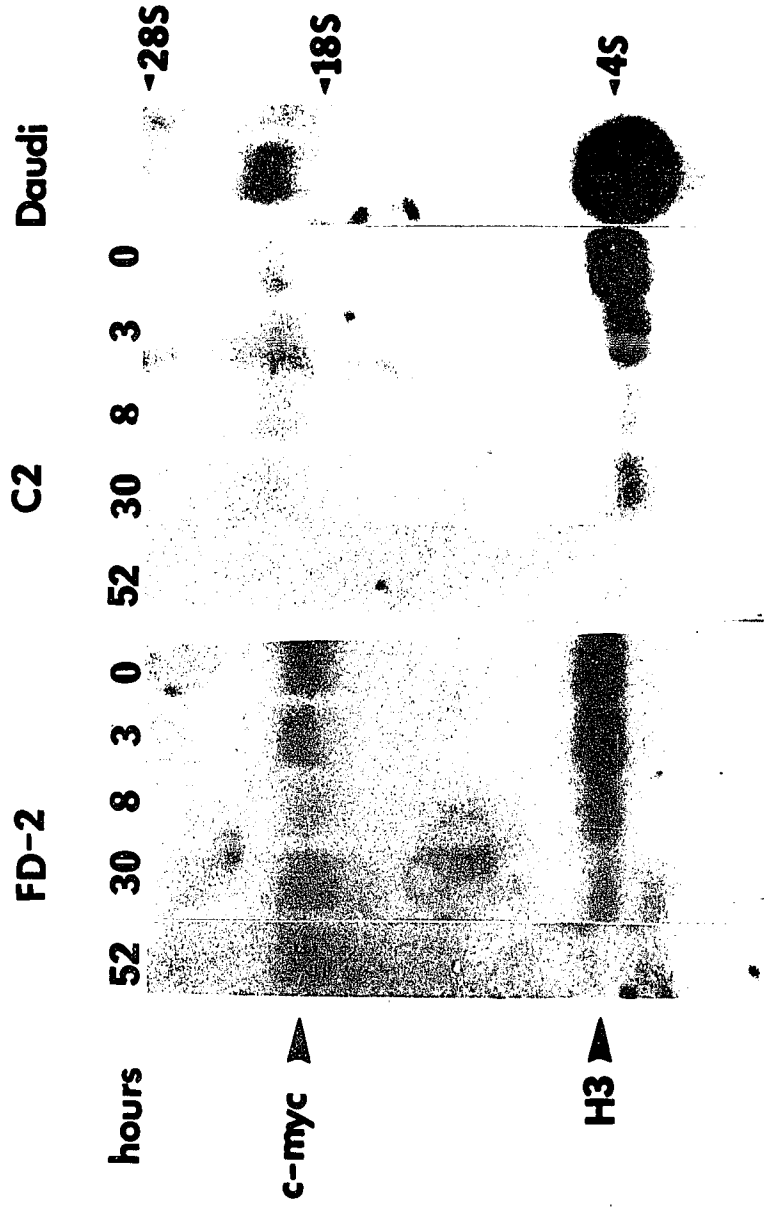


Figure 9

Figure 9: Northern Analysis of Fusion Deficient vs. C2M C-myc mRNA's Indicates That C2M Cells Down Regulate C-myc mRNA, whereas, FD-2 Does Not. RNA's were isolated from different times after differentiation induction of C2M and FD-2 myoblasts: 0, 8 hours, 30 hours, 48 hours and 52 hours. Northern analysis of RNA's (25 μg/ lane)was as described in the Methods. Migration of 28S and 18S ribosomal RNA's, as visualized by acridine orange staining of gels after transfer, is indicated on the right. Blots were probed with the Hind III fragment of pMc-myc54 (mouse c-myc cDNA), and the EcoR1 fragment of pHh5b (human histone H3). The migration of each of these mRNA species is indicated on the left.

Transcriptional regulation of c-myc: Nuclear transcription assays are often used to determine whether regulation of a gene occurs at the transcriptional or post-transcriptional level. Such assays performed with undifferentiated and differentiation induced C2M nuclei indicate that a decrease in c-myc transcription largely accounts for the decrease in steady-state mRNA levels observed for this gene. Transcription assays are performed according to Greenberg and Ziff (1986) with minor modifications (Marzluff and Huang, 1987), as described in the Methods section. Nuclei isolated at different times after differentiation induction: 72 hours, 12 hours, and 0 (uninduced), are incubated with labelled ribonucleotides and hybridized to linearized plasmids, which have been bound to nitrocellulose or Gene Screen Plus (see Figure 10). A plasmid containing 28S sequences (p1-19) is included as a positive control. These experiments indicate that c-myc, N-myc, and H-c-ras are transcribed in undifferentiated C2 myoblasts. Transcription of c-myc and N-myc decreases after differentiation induction. Transcription of Hc-ras, however, remains constant. The p1-19 plasmid

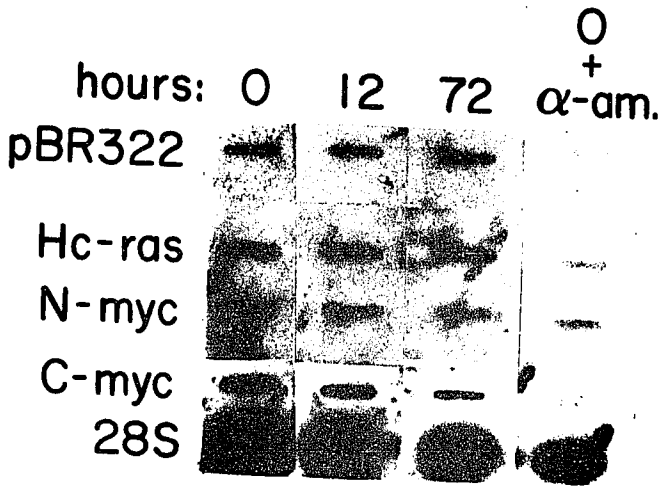


Figure 10

Figure 10: Transcriptional Activity of C-myc, Hc-ras, and N-myc Genes During C2M Myoblast Differentiation. Nuclei were isolated from C2M myoblasts at 0, 12, and 72 hours after differentiation induction. Transcription reactions were performed as described in the Methods. Labelled RNA's were hybridized to linearized probes bound to Gene Screen Plus, as indicated on the left. Each slot contained 10 micrograms of the indicated plasmid DNA: pBR322, Hc-ras, N-myc, C-myc, and p1-19 (28S). Alpha amanitin (1 μg/ml) was added to inhibit RNA polymerase I and III activity. Hybridization to the 28S ribosomal probe provided a positive control. Hybridization to pBR322 indicates the level of background.

(28S) is also included to show that transcription of ribosomal RNAs remains constant, also. Hybridization to pBR322 indicates the level of background hybridization. Inclusion of α-amanitin at 1 μg/ml in uninduced nuclei abolishes transcription from c-myc, N-myc and H-c-ras, but not significantly of 28S, suggesting that RNA polymerase II activity is mediating the transcription of these genes. In another transcription experiment (Figure 11), nuclei are isolated from differentiating C2M myoblasts at 0, 12, 24 and 48 hours after induction. Transcription of c-myc decreases between 0 and 24 hours (see 12 hour point), and increases to original levels by 48 hours. The latter experiment conflicts with the results shown in Figure 10. A possible explanation for this discrepancy is that the C2M cell line is used for Figure 10, whereas, the C25 cell line is used for Figure 11. The C2M line has fibroblast contamination, or biochemically non-differentiating myoblasts. The regulation of the c-myc gene in fibroblasts may differ in fibroblasts and myoblasts. Evidence for this comes from northern analysis of c-myc mRNA's in clonal, purely myogenic cell lines such as C25 and C2C12 (see

Figure 32, probe #4). In this experiment, c-myc levels decrease, but are reinduced to twice original levels. Therefore, the results obtained for C25 in Figure 11, i.e. the transcriptional down regulation and reinduction, are a more accurate reflection of c-myc regulation during myogenic differentiation, than the results shown in Figure 10, which probably reflect growth factor-dependent transcription of c-myc in fibroblasts, as reported by Greenberg and Ziff (1987).

Transfection and analysis of pSVmyc transfected C2M: In order to address whether c-myc overexpression can directly inhibit myoblast differentiation, C2M is co-transfected with pSV2neo and a vector containing c-myc linked to the SV40 promoter (pSVmyc1, Land and Weinberg). After G418 selection, several clones expressing elevated levels of the transfected c-myc gene are obtained. Having demonstrated that these clones contain high levels of c-myc mRNA, their myosin heavy chain accumulation is quantitated to determine the effect of deregulated c-myc on differentiation. Figure 12 shows the levels of c-myc mRNA in different pSVmyc1 transfected C2M clones. The following clones are included: 27, 19, 16, 4, 3 1, and 6, as indicated

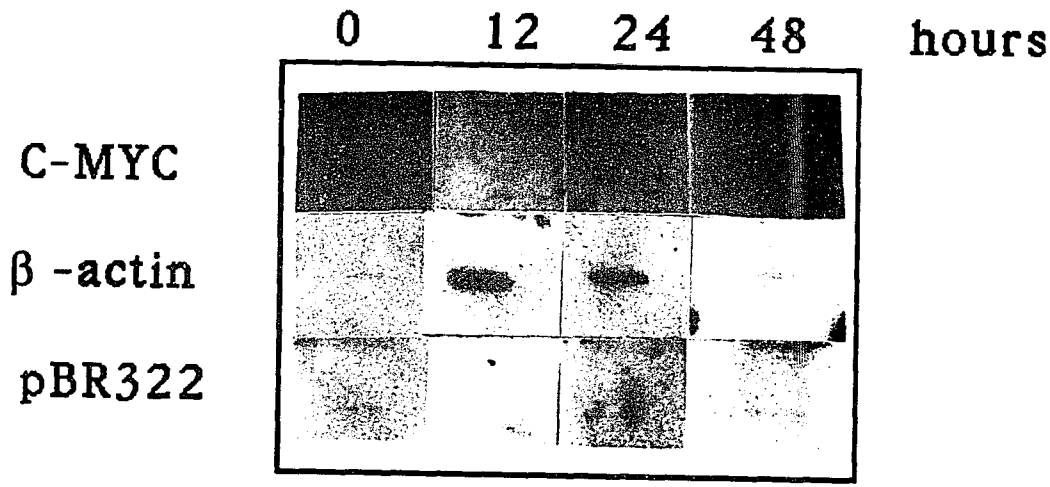


Figure 11

Figure 11: A Transient Drop in C-myc Transcription Occurs upon C25 Differentiation Induction. Nuclei were isolated from C25 cells at 0, 12, 24 and 48 hours after differentiation induction, as indicated above each lane; transcription reactions were performed as described in the Methods. Labelled RNA's were hybridized to linearized DNA probes bound to nitrocellulose, as indicated on the left. The Hind III fragment of pMc-myc54 contained all three exons of c-myc was used. The Eco-R1 fragment of the beta-actin plasmid, 3' untranslated region, was bound in the middle slots. The bottom slot contained 10 micrograms of linearized pBR322 to indicate nonspecific binding of the labelled RNA transcripts to DNA.

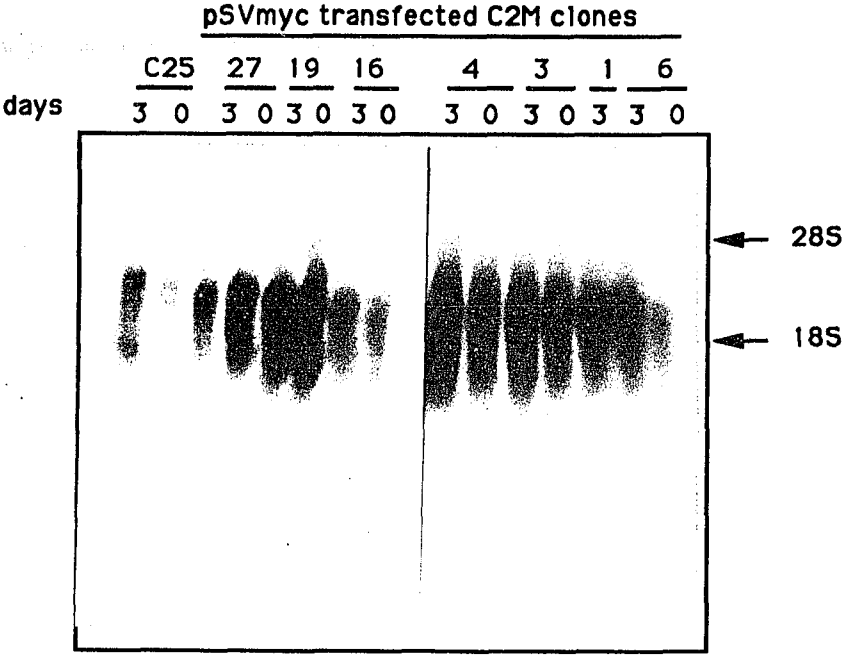

pSVmyc transfected C2M clones
C25
27
19
16
4
3
1
6
days
3 0 3 0 3 0 3 0 3 0 3 0 3 3 0
28S
18S

Figure 12

Figure 12: Levels of C-myc mRNA in pSVmyc1 Transfected, C2M Myoblasts. RNA's (10 μg) from differentiation induced (3) and uninduced (0), pSVmyc1 transfected C2M myoblast clones, 27, 19, 16, 4, 3, 1, and 6, and pSV2neo (control) were loaded onto 1.5% formaldehye gels, and transferred to Gene Screen Plus. Blots were probed with a riboprobe generated from pSP64myc (as described in the Methods).

above each lane. Messenger RNAs from both uninduced (0) and 3 days after differentiation induction (3) are included for most of the clones. Clones 19, 4 and 3 express significantly higher levels of c-myc mRNA than a non-transfected subclone (C25) shown in the left most lane. Migration of the 28S and 18S ribosomal RNAs are marked on the left. In order to insure that the same amount of RNAs (10μg) is loaded in each lane, RNAs are first stained with acridine orange in a separate gel. In addition, 19 and 3 either retain or induce c-myc mRNA levels after differentiation induction. Figure 13 is a western blot of these lines indicating their relative levels of myosin heavy chain. The monoclonal antibody, MF20, specific for sarcomeric myosin heavy chain (Bader et. al.). is used as an indicator for the onset of differentiation. Densitometric scanning of these blots yields the values in Table 10. The highest c-myc expressing clone MN19 fails to activate myosin heavy chain upon differentiation induction. In a time course experiment (Figure 14), whole cell extracts derived from two transfected clones (MN19 and MN16) and the parental C2M, are run on SDS-PAGE, blotted to

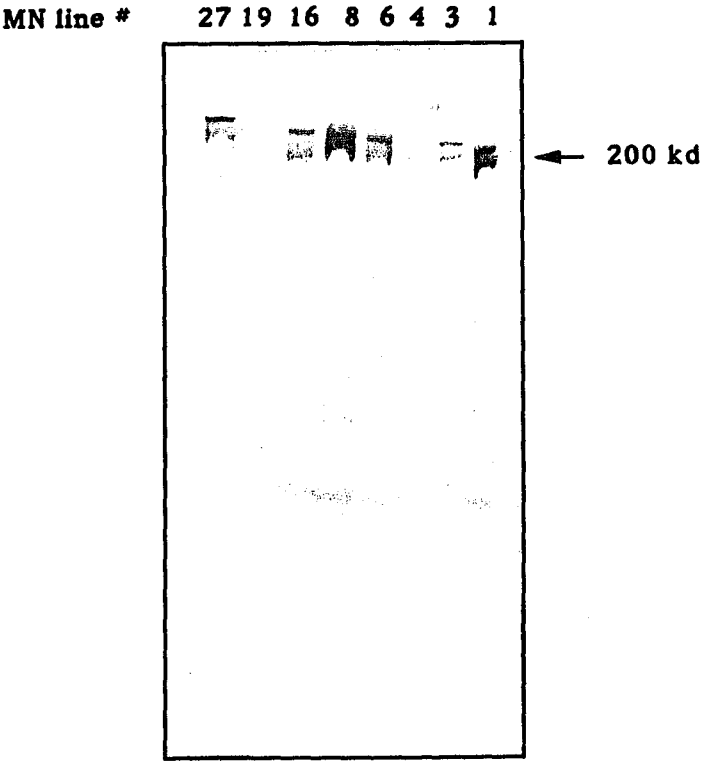


Figure 13

Figure 13: Myosin Heavy Chain Accumulation in pSVmyc1 Transfected C2M Myoblasts. Whole cell extracts were obtained from G418 resistant C2M myoblast clones transfected with pSVmyc1 and pSV2neo (MN lines: 1, 3, 4, 6, 8, 16, 19, and 27), at 96 hours after differentiation induction. Clone numbers are indicated above each lane. Extracts (approximately 10^6 cells/lane) were loaded onto 7.5% polyacrylamide gels, transferred to nitrocellulose, and probed with MF20 (mAb anti-myosin heavy chain). Incubation with alkaline phosphatase conjugated, goat anti-mouse second antibody and development with a Vector substrate kit allowed visualization of MHC bands, which migrated at 200 kd (arrow on the right). Equivalent protein amounts were loaded as assessed by Coomassie staining prior to blotting. High molecular weight standards were run alongside, and stained with Coomassie Blue (not shown).

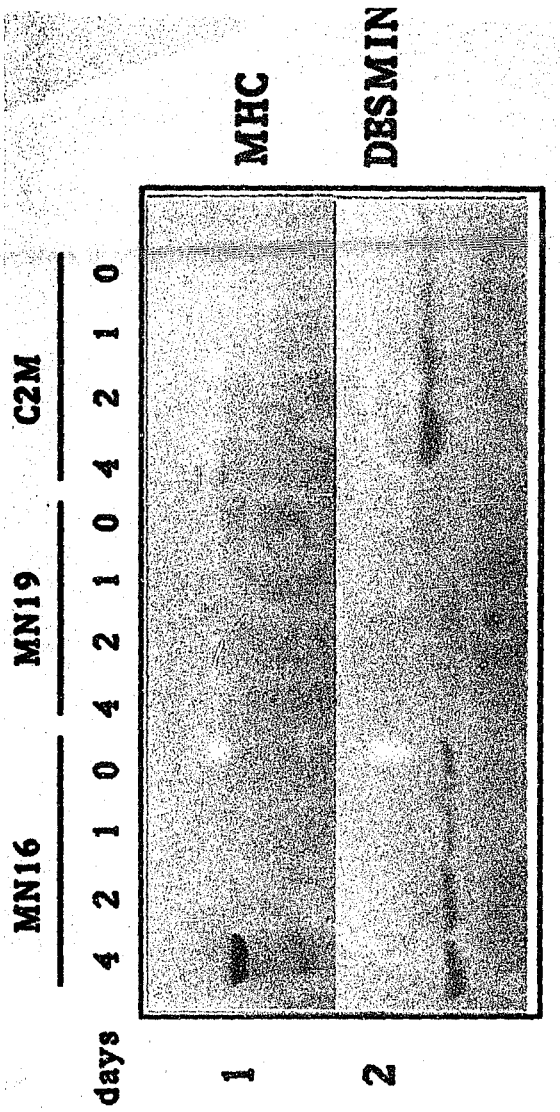


Figure 14

Figure 14: Comparison of Myosin Heavy Chain and Desmin Accumulation Upon Differentiation Induction of MN19 and MN16 and C2M. Whole cell extracts were isolated from each cell line at 0, 1, 2, and 4 days after differentiation induction, as indicated above each lane. Western blot 1 was probed with MF20 (mAb anti-myosin heavy chain), whereas western blot 2 was probed with D3 (mAb anti-desmin). Approximately 10^6 cells were loaded in each lane.

nitrocellulose, and probed with either 1. MF20 (ant-myosin heavy chain antibody), or 2. D3 (anti-desmin antibody). The number of days after differentiation induction are indicated above each lane: 0 (uninduced), 1 day, 2 days, and 4 days. Both myosin heavy chain and desmin are inhibited in clone MN19. In these experiments, it is also found that the parental C2M line expresses low levels of MHC, compared to some of its transfected subclones. Figure 15 shows photographs of phase microscopy of parental C2M (A), clone MN19 (B), and clone MN16 (D), after five days in differentiation medium. In addition, pSV2neo alone transfected C2M clones show a wide variation in their abilities to induce differentiation markers (not shown). Although some had the appearance of MN16 as seen in Figure 15 (D), non-elongating, myosin negative clones, similiar to MN19 were also recovered. Figure 15 (C) shows one such pSV2neo transfected clone, which is differentiation defective. From these experiments, it is concluded that C2M is a heterogeneous line, made up of both differentiation competent and defective lines. Therefore, although there is a close association between high levels

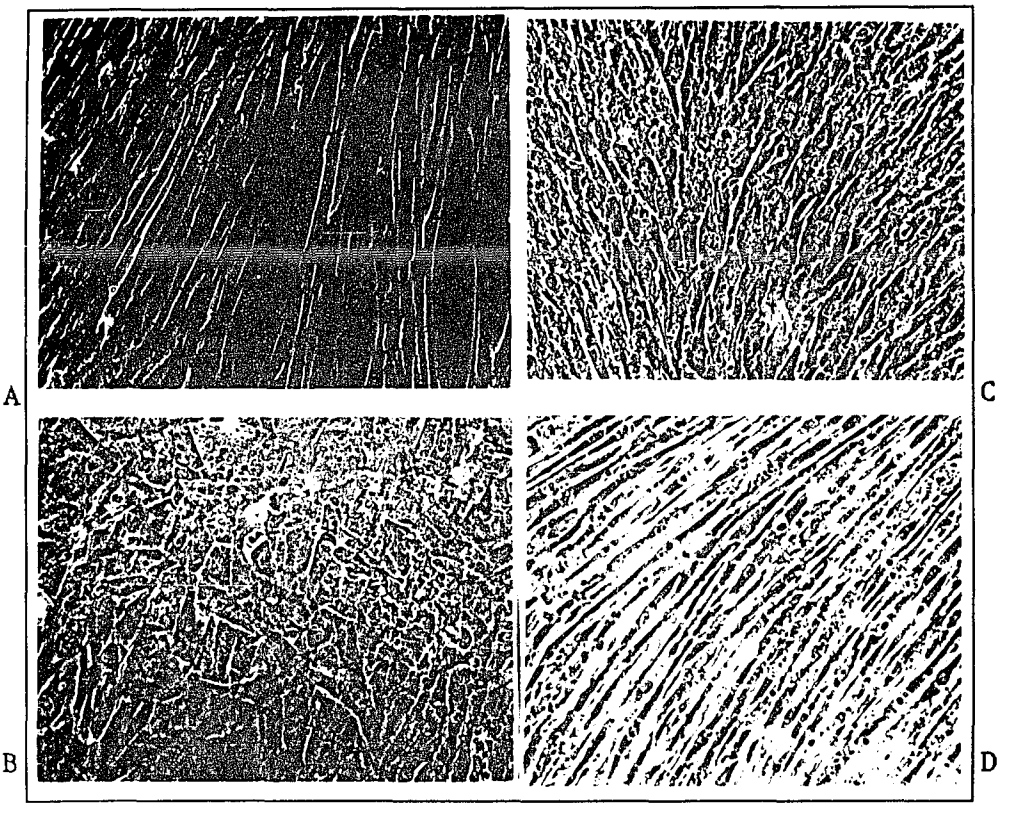


Figure 15

Figure 15: Highest Level C-myc Expressing Clone, MN19, is Similiar in Appearance to FD-2, Upon Differentiation Induction. Phase contrast microscopy of cells induced to differentiate for 4 days: A. Parental C2M myoblasts. B. MN19, pSVmyc1 transfected C2M myoblast clone. C. FD-2, fusion deficient clone, obtained by selection against myotube formation. D. MN16, pSVmyc1 transfected C2M myoblast clone.

of c-myc expression and differentiation inhibition (as seen by clone MN19), it can not be concluded from experiments performed with C2M that c-myc causes this inhibition.

Concentration dependant differentiation of pSVmyc transfected C25 myoblasts. Subcloning C2M yields a number a clones with varying myogenic potentials. For transfection experiments, it is important to choose a parental clone which has a stable phenotype. Since myosin heavy chain is widely used as an indicator of myogenic differentiation, subclones are judged to be "stable" based on their expression of this marker, upon differentiation induction. Myoblast fusion is not used, because it is found to be a less stable phenotype during prolonged culturing. One such C2M subclone, C25, after differentiation induction, yields only MHC positive subclones, even after several passages. Therefore, the C25 clone is chosen for subsequent transfection experiments.

Transfection of c-myc into C25 cells is performed to determine the ability of this gene to

inhibit differentiation, as with the C2M cell line. The SV40 promoted c-myc construct (pSVmyc1, see Appendix for map) is again utilized to overexpress the c-myc protein in these cells. Co-transfection with pSV2neo and selection with G418 yields several, stably transfected, G418 resistant myoblast colonies. Control transfections with pSV2neo alone are also included. In addition, the p53 gene (p11-4, SV40 promoted p53 cDNA) is also transfected to see if it has any effect on differentiation. Figure 16 shows the results of a typical transfection experiment. The C25 line is transfected with 1. pSV2neo (135 colonies), 2. pSVmyc1 and pSV2neo (65 colonies), 3. p11-4 and pSV2neo (11 colonies), or 4. pSVmyc1 and pSV2neo (11 colonies), as indicated above each set of photographs. The pSVmyc1 transfected clones in 4. are on a 100 mm plate, for a backgroun control, whereas clones from 1. are on a 150 mm plate. When the majority of colonies are larger than 2 mm in diameter (about 20 days after G418 selection is begun), the medium is switched to HS-DMEM without G418 for 6 days. Colonies are fed on the third day after switching. On the 6th day after differentiation

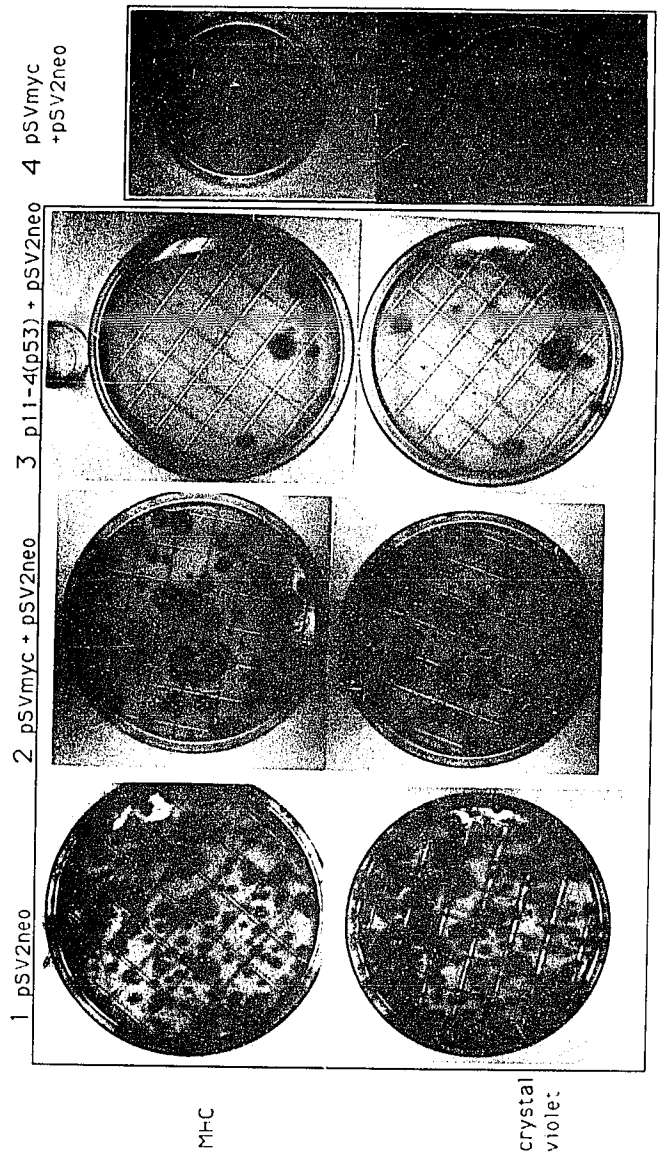

1 pSV2neo
2 pSVmyc + pSV2neo
3 p11-4(p53) + pSV2neo
4 pSVmyc +pSV2neo
MHC
crystal violet

Figure 16

Figure 16: Myosin Positive Colonies Resulting From pSVmyc Transfection Into C25 Myoblasts. C25 myoblasts were transfected with (1) pSV2neo, (2) pSVmyc1 and pSV2neo, (3) p53 (p11-4) and pSV2neo, (4) pSV2neo (no first antibody), selected in G418, and allowed to grow to at least 3 mm in diameter. Medium was then changed to HS-DMEM (differentiation medium) for 8 days. The colonies were fixed in acetone/formaldehyde/ethanol, and incubated with (A) anti-myosin heavy chain mAb MF20, goat anti-mouse, peroxidase conjugated second antibody, and developed with DAB. After phase photography of these plates, colonies were stained with (B) crystal violet to visualize the percentage of myosin positive colonies for each transfection.

induction, cells are fixed (see Methods) and stained for myosin heavy chain (Figure 16(A)). The bottom set of plates (B) are the same plates from (A), stained with crystal violet. Figure 16 (4A and 4B, no first antibody) are included as controls for background staining obtained with second antibody. Colonies do not stain when anti-myosin antibody (MF20) is excluded. Crystal violet staining is included to compare myosin positive versus the total number of colonies. Transfection with pSVmyc/pSV2neo yields a 2-fold reduction in the number of colonies obtained, compared to that of pSV2neo alone. Transfection with p11-4/pSV2neo results in over 10-fold reduction in the number of colonies, conforming to this genes possible role as an inhibitor of replication. From this colony assay, and several repetitions of this assay, no differences between control (pSV2neo alone) and c-myc transfected plates (pSVmyc/pSV2neo) can be observed. If c-myc transfection inhibits differentiation of C25 myoblasts, it either does so at a frequency of less than 1 out of 356 (total number screened in this assay) C25 clones, or high level expression required for inhibition is toxic to the cell. Evidence that

c-myc overexpression can be toxic in transfected cells has already been reported (Wurm et. al., 1986). Although these results suggest that C25 may be refractory to c-myc mediated differentiation inhibition, such differentiation defective clones may grow at a slower rate because of c-myc toxicity, thereby escaping detection by this assay.

In order to be certain that c-myc is being expressed at elevated levels compared to controls, several clones are picked for further analysis. In addition, a variety of different sized, G418 resistant clones are picked, in order to screen clones with reduced growth capacities. Twenty-two individual clones are picked from c-myc transfection. They are called C5M lines (1-22). Thirteen pSV2neo transfected (pSV 1-17) are also picked. After passaging these clones from 24-well plates to 25 cm^2 flasks, these cells were plated in 100 mm dishes for comparison of their differentiation capabilities. Figures 17 and 18 are Western blot analyses of whole cell extracts from uninduced pSVmyc transfected C25 myoblasts (1-19, number above each lane denotes each clone),

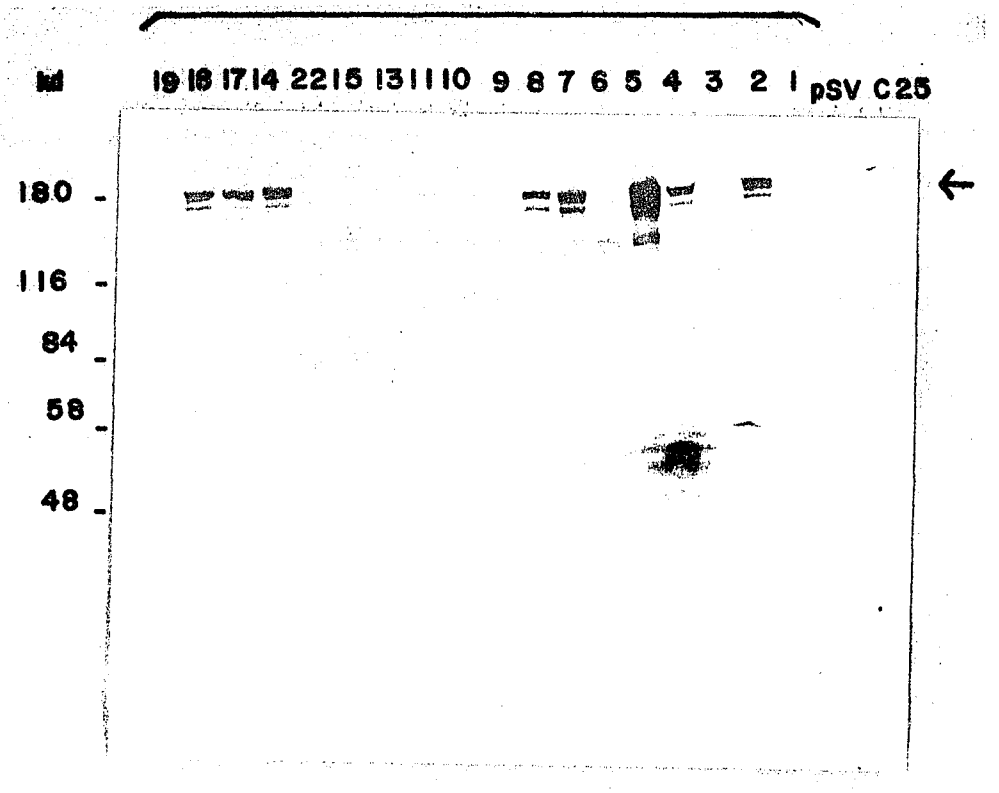


Figure 17

Figure 17: Myosin Heavy Chain, in Uninduced, pSVmyc Transfected C25 Myoblasts. Western blot analysis of whole cell extracts derived from G418 resistant, pSVmyc/pSV2neo transfected C25 myoblast clones (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 22, 14, 17, 18, 19), or controls, pSV2neo clone (pSV), or C25 (parental C25 myoblasts), as labelled above each lane. These extracts were derived from cells grown in mitogen rich medium (DMEM/CEE/FCS). 7.5 % SDS polyacrylamide gels were run. The gel was transferred to nitrocellulose, and probed with MF20. The blot was incubated with alkaline phosphatase conjugated second antibody, (see Methods). Markers (SIGMA), were run alongside in order to assess the proper migration of MHC, as indicated by the arrow.

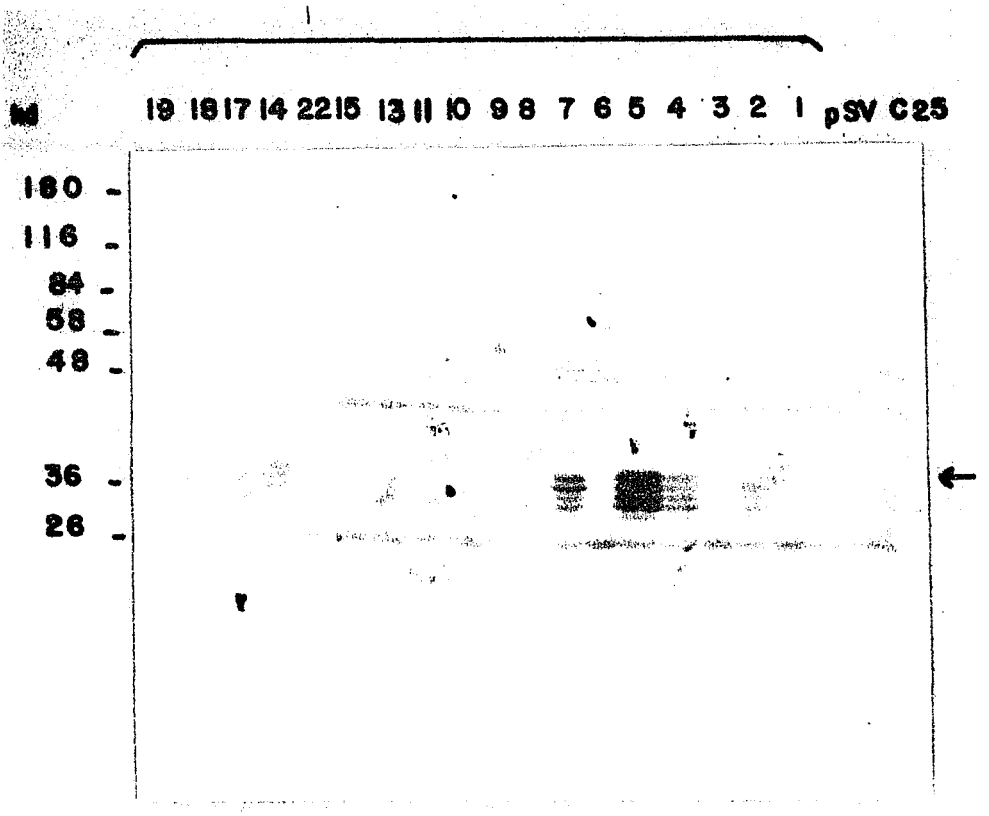

M
19 18 17 14 22 15 13 11 10 9 8 7 6 5 4 3 2 1 pSV C25
180
116
84
58
48
36
26

Figure 18

Figure 18: Troponin T, in Uninduced, pSVmyc Transfected C25 Myoblasts. The same extracts as in Figure 17, run on a 10% SDS polyacryamide gel, probed with monoclonal antibody JLT-12.

pSV2neo control (pSV), and parental C25. Figure 17 is probed with monoclonal antibody MF20 (anti-myosin heavy chain antibody [MHC]). Migration of molecular weight markers (Sigma) are marked on the left. The arrow points to the MHC band; this is the expected region of migration for this protein (200 kd). As can be seen, there is variation in the degree of MHC accumulation among these lines. Figure 18 contains the same samples, probed with monoclonal antibody JLT-12 (anti-troponin T (TnT), fast isoform specific). Four fast isoforms (1f, 2, 3f, and 4f) can be distinguished in these blots; the migration of these forms is also as expected (see arrow, 36 kd). Again, there is variation in accumulation of TnT in uninduced transfected clones. Figures 19 and 20 are Western blot analyses of differentiation induced pSVmyc transfected clones (1-22, as marked above each lane), and control pSV2neo (pSV). The blot in Figure 19 is probed with MF20, whereas the blot in Figure 20 is probed with JLT-12. These blots indicate that, although there is variation among pSVmyc transfected lines, C5M15 (lane marked 15) is the only pSVmyc transfected clone that is inhibited from expressing

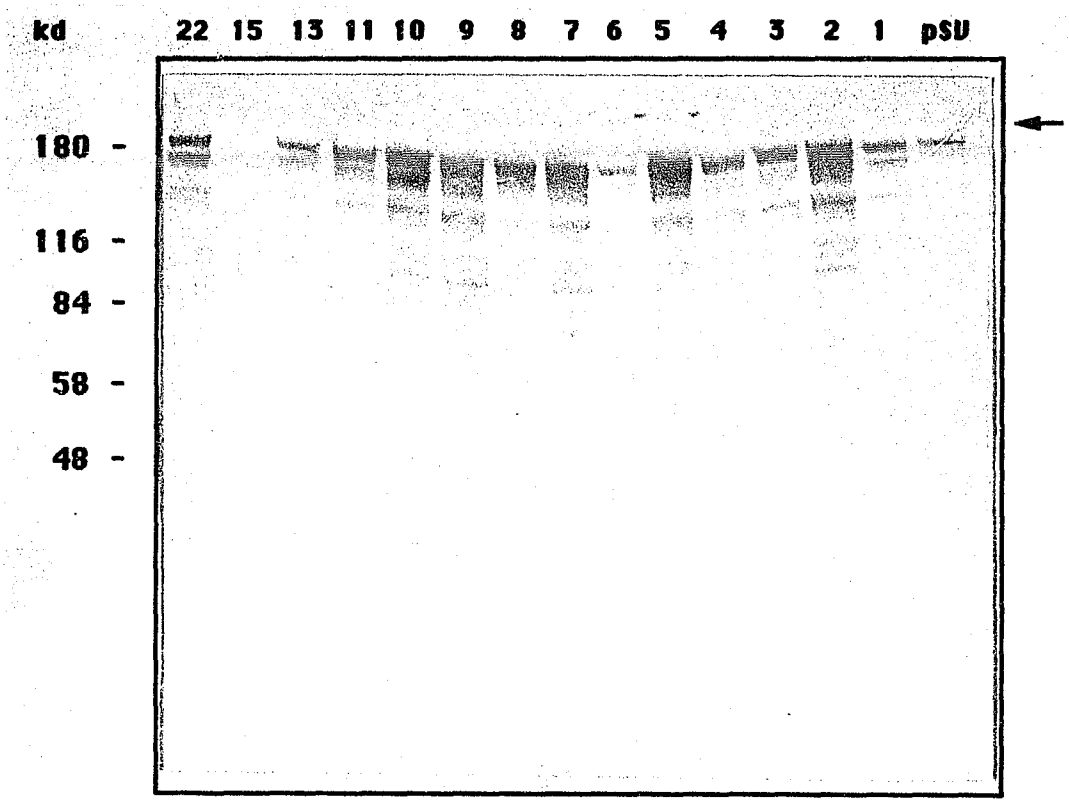


Figure 19

Figure 19: Myosin Heavy Chain Accumulation in Differentiation Induced, pSVmyc Transfected C25 Myoblasts. Western blot analysis of whole cell extracts derived from G418 resistant, pSVmyc/pSV2neo transfected clones (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 22), or control pSV2neo clone (pSV), as labelled above each lane. Extracts were derived from differentiation induced cells, 4 days after induction, and loaded onto 7.5% polyacrylamide gels. Approximately 10^6 cells were loaded in each lane. Blots were probed with MF20, an anti-myosin heavy chain monoclonal antibody, and alkaline phosphatase conjugated second antibody, as described in the Methods. Arrows indicate that MHC migrates in the expected region, slightly above the 180 kd marker.

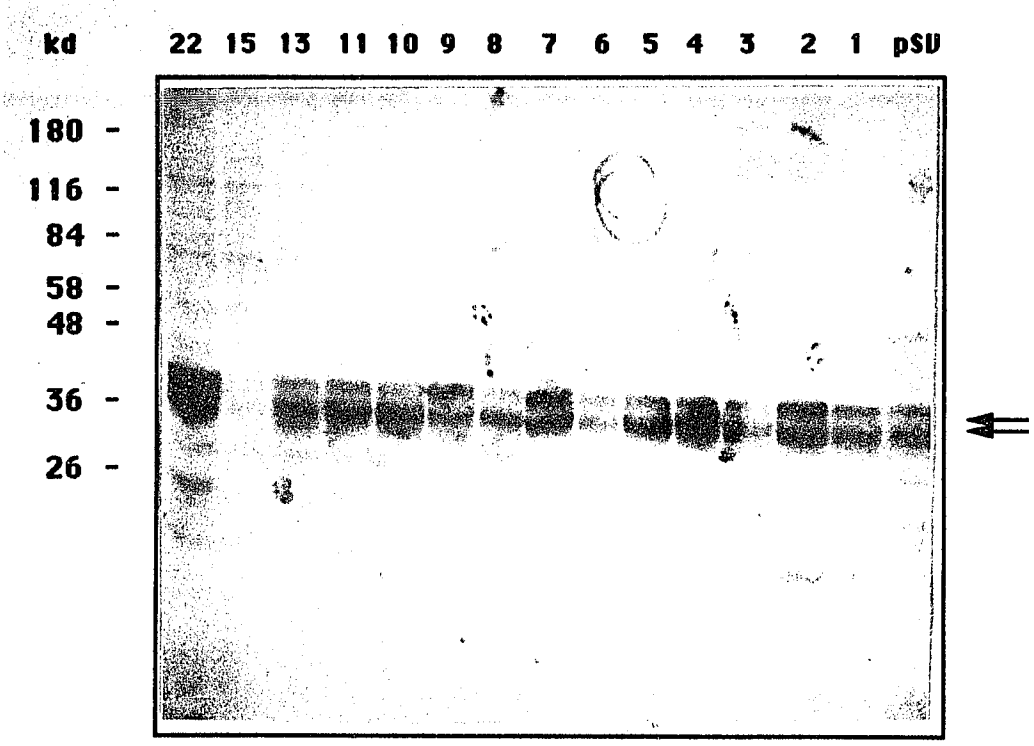


Figure 20

Figure 20: Troponin T Accumulation in Differentiation Induced, pSVmyc Transfected C25 Myoblasts. Western blot analysis of whole cell extracts derived from G418 resistant, pSVmyc/pSV2neo transfected clones (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 22), or control pSV2neo clone (pSV), as labelled above each lane. Extracts were derived from differentiation induced cells, 4 days after induction, and loaded onto 10 % polyacryamide gels. Approximately 10^6 cells were loaded in each lane. Blots were probed with JLT-12, an anti-TnT monoclonal antibody, and alkaline phosphatase conjugated second antibody, as described in the Methods. Arrows indicate that the fast TnT isoforms (1f, 2f, 3f, and 4f) migrate in the expected region, near the 36 kd marker.

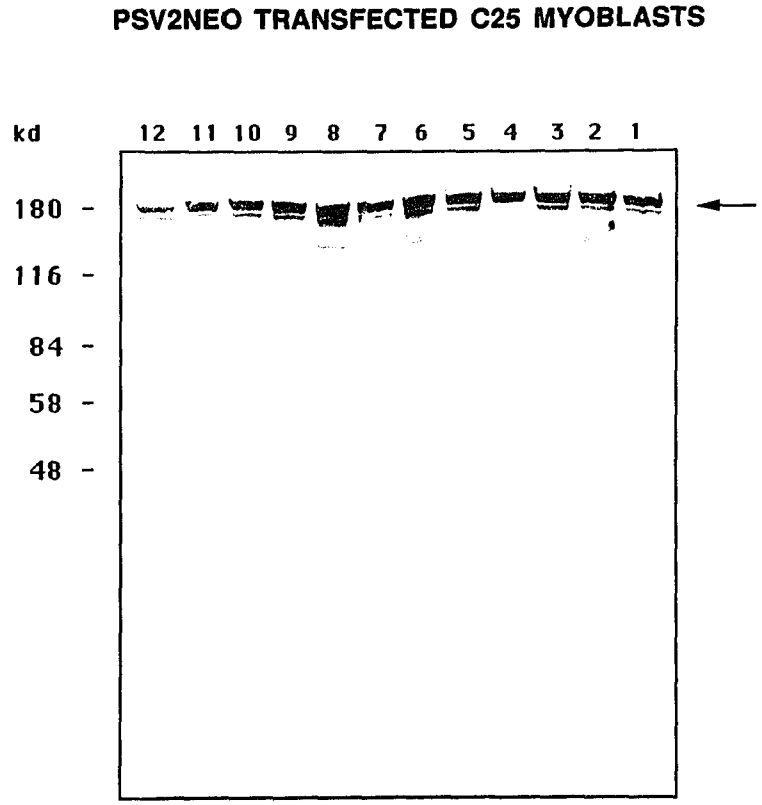

PSV2NEO TRANSFECTED C25 MYOBLASTS
kd
12 11 10 9 8 7 6 5 4 3 2 1
180 -
116 -
84 -
58 -
48 -

Figure 21

Figure 21: Myosin Heavy Chain Accumulation in pSV2neo Transfected C25 Myoblast Clones. Western blot analysis of C25 myoblasts clones 1-13, transfected with pSV2neo alone. The blot was probed with MF20, and developed using alkaline-phosphatase conjugated second antibody. The clone number is indicated above each lane.

differentiation markers, MHC and TnT. For additional controls, whole cell extracts from differentiation induced, pSV2neo transfected C25 clones (1-13) are probed with MF20 in Figure 21. This blot indicates that there is very little variation in the levels of MHC accumulation between pSV2neo transfected clones. In addition, none of the clones are inhibited from accumulating MHC. Figure 22 presents the data from uninduced, and differentiation induced pSVmyc transfected blots, probed with MHC, TnT, and desmin, assembled in one figure. For a loading control, whole cell extracts from differentiation induced cells are also probed with anti-desmin polyclonal antibody (Figure 22, 2C). Although clone C5M15 fails to induce MHC and fast TnT, it accumulates desmin (see 2C, lane marked 15). Results from densitometric scanning of these immunoblots are presented in Table 8.

Although a differentiation inhibited c-myc transfected C25 myoblast is obtained, it is still necessary to determine the levels at which the transfected c-myc gene is expressed. Analysis of mRNAs derived from 8 different transfected clones allows comparison of the levels of c-myc expression

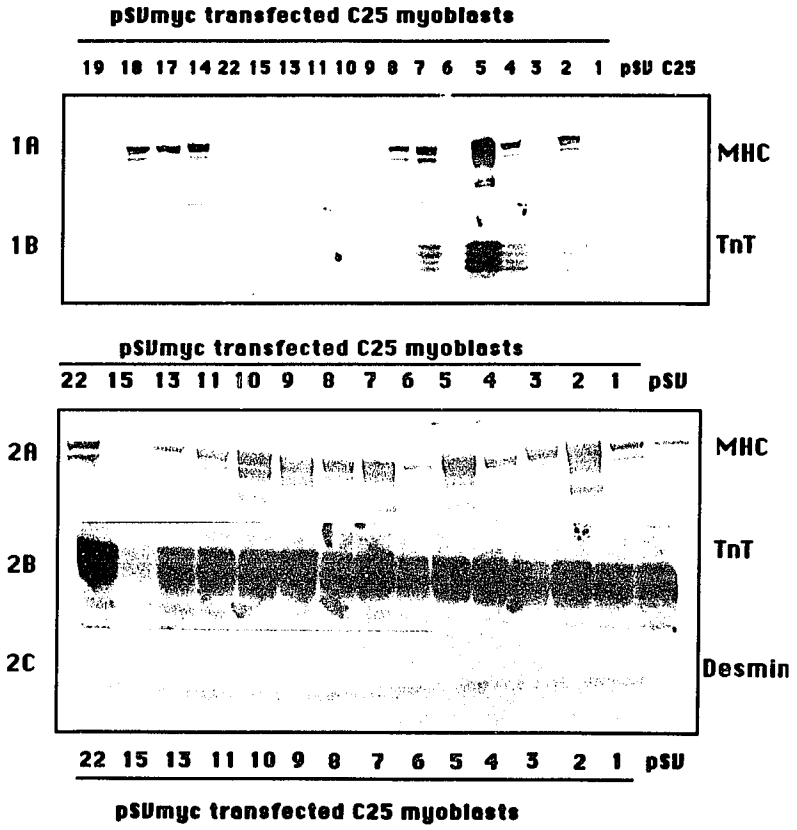

pSUmyc transfected C25 myoblasts
19 18 17 14 22 15 13 11 10 9 8 7 6 5 4 3 2 1 pSU C25
1A
MHC
1B
TnT
pSUmyc transfected C25 myoblasts
22 15 13 11 10 9 8 7 6 5 4 3 2 1 pSU
2A
MHC
2B
TnT
2C
Desmin
22 15 13 11 10 9 8 7 6 5 4 3 2 1 pSU
pSUmyc transfected C25 myoblasts

Figure 22

Figure 22: Myosin Heavy Chain, Troponin T, and Desmin Accumulation in pSVmyc Transfected C25 Myoblasts. Western blot analysis of whole cell extracts derived from G418 resistant, pSVmyc/pSV2neo transfected C25 myoblast clones (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 22, 14, 17, 18, 19), or controls, pSV2neo clone (pSV), or C25 (parental C25 myoblasts), as labelled above each lane. SDS polyacrylamide gels were 7.5% for blots 1A and 2A, and 10% for 1B, 2B, and 2C. Blots 1A (MHC, probed with MF20), and 1B (TnT, probed with JLT-12) contain extracts from cells grown in mitogen rich medium. Blots 2A (MHC, probed with MF20), 2B (TnT, probed with JLT-12), and 2C (Desmin, probed with a rabbit polyclonal) contain extracts from cells induced to differentiate for 4 days. Blots 1A, 1B, 2A and 2B were incubated with alkaline phosphatase conjugated second antibody, whereas, 2C was incubated with a peroxidase conjugated second antibody (see Methods).

among these lines (see Figure 23). Ten μg of RNA from each clone, in addition to the parental C25 line, are loaded in every lane. The number of each clone is indicated both above and below the lanes. Figure 23 (1) is probed with a c-myc riboprobe generated from an exon 3 fragment (Bgl 1) of pSVmyc1 (pSP64-myc, see Appendix for map). This vector was kindly provided by Dr. Ruth Gubits. Hybridization to 28S is indicated in blot (2), probed with plasmid, p1-19 (Tiemeier et. al.). Densitometric scanning (see Table 9) of these blots indicates that C5M15 expresses over 8 fold higher c-myc mRNA levels than the parental C25, or pSV control, the second highest expressing clone is C5M4 (6 fold), and the third highest, C5M6 (4 fold).

Whether c-myc mRNA levels correspond to c-myc protein levels accumulated in these clones is also addressed. Purified preparations of the monoclonal antibody 1F7, generated against c-myc peptide (amino acid residues 307-322) was kindly provided by Dr. R. Chizzonite (Miyamoto et. al., 1985). Figure 24 shows that by Western blot anaylsis, this antibody reacts with a 58 kd doublet in insect cell extracts infected

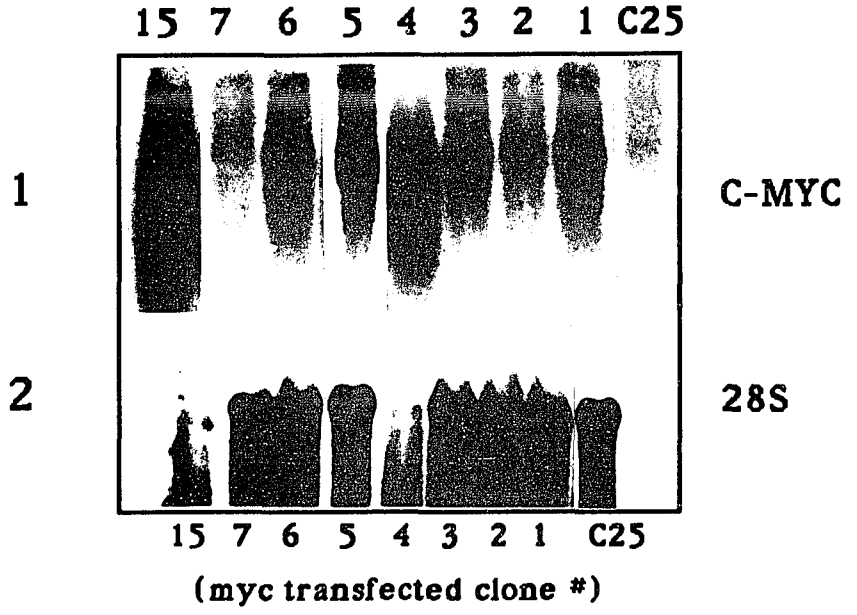

15 7 6 5 4 3 2 1 C25
1
C-MYC
2
28S
15 7 6 5 4 3 2 1 C25
(myc transfected clone #)

Figure 23

Figure 23: Steady State C-myc mRNA Levels in pSVmyc Transfected C25 Myoblasts. Northern blot analysis of RNA isolated from G418 resistant, pSVmyc/pSV2neo transfected C25 myoblast clones (1, 2, 3, 4, 5, 6, 7, 15), and control C25 myoblasts (parental). RNA was isolated from cells grown in mitogen rich medium; 10 micrograms of RNA from each clone was loaded per lane, onto 1.5% formaldehyde gels, transferred to Gene Screen Plus, and probed with 1. c-myc, exon 3 (riboprobe generated from the Bgl 1 fragment of pSP6myc, as described in the Methods), or 2. 28S (nick translated probe generated from plasmid p1-19). Blot 1 was autoradigraphed for 24 hours, whereas blot 2 for 1 hour, on Kodak XAR film.

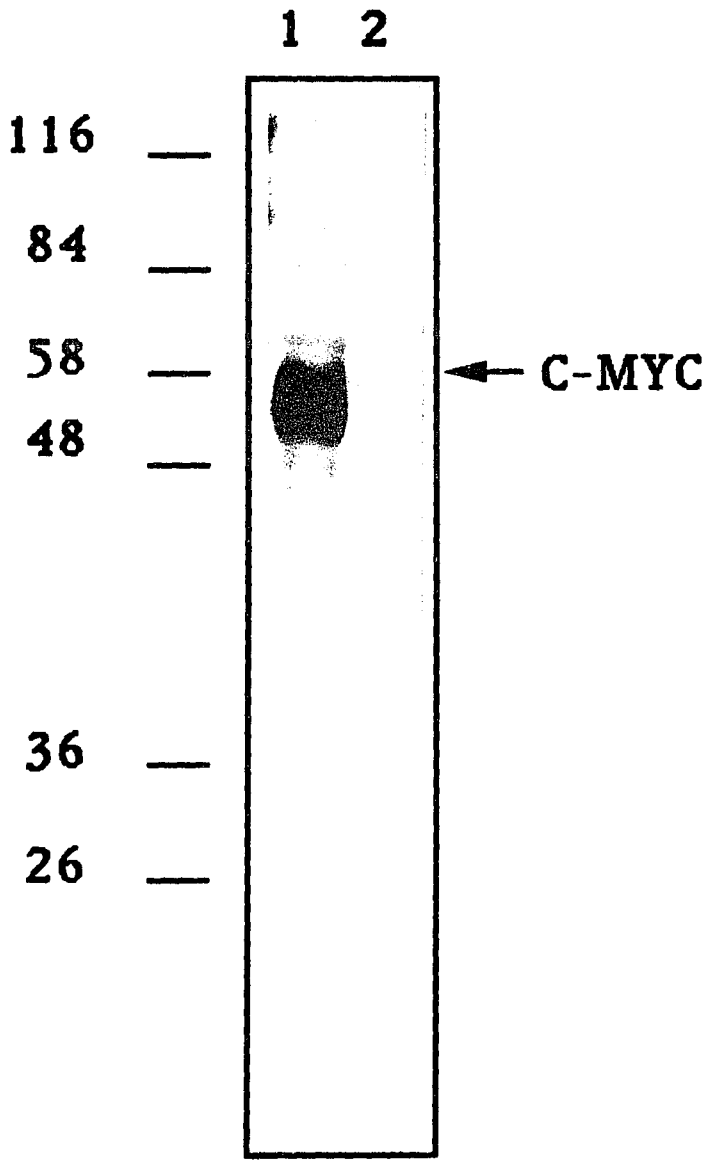


Figure 24

Figure 24: Monoclonal Antibody 1F7 Reacts With a 58 kd Protein in Insect Cells Infected With a C-myc Containing Baculovirus Vector. A. Western blot analysis of whole cell extracts from 1. insect cells infected with a baculovirus vector containing c-myc, 2. insect cells infected with the parental baculovirus vector, lacking c-myc sequences. The blot was then probed with purified 1F7 monoclonal antibody, at a concentration of 5 micrograms/ml, and incubated with alkaline phosphatase conjugated second antibody, as described in the Methods. Low molecular weight markers are shown at the left. The arrow indicates that recombinant, human c-myc migrates as a doublet near the 58 kd marker.

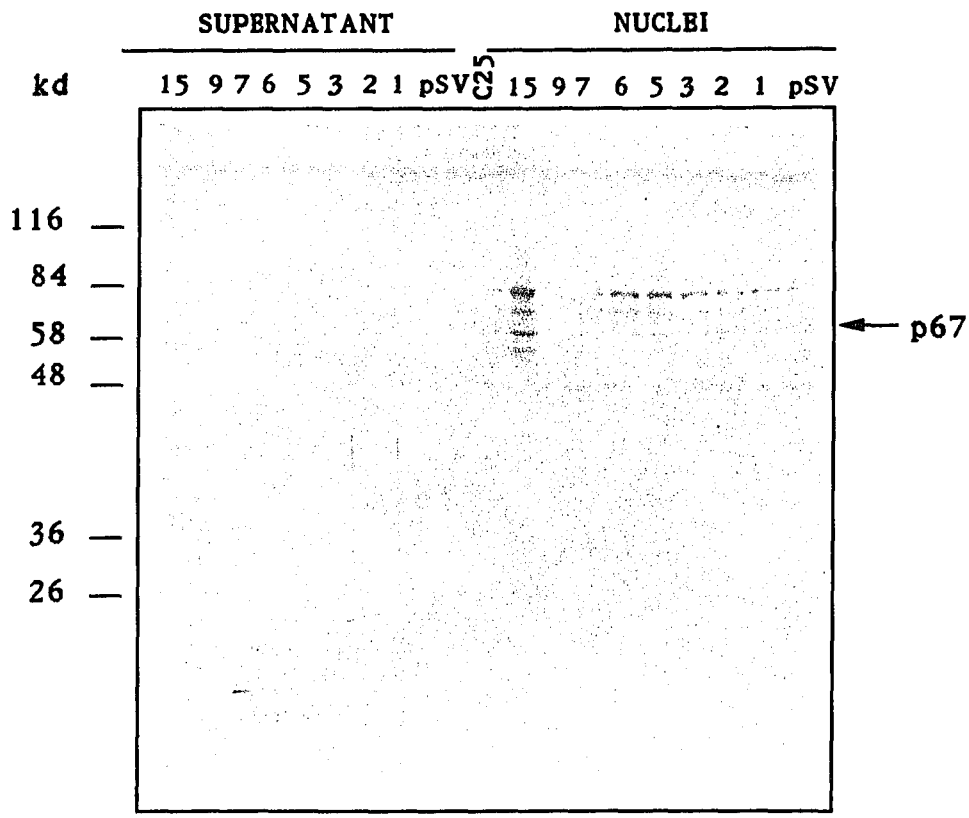

SUPERNATANT
NUCLEI
kd
15 9 7 6 5 3 2 1 pSV
C25
15 9 7 6 5 3 2 1 pSV
116
84
58
48
36
26
p67

Figure 25

Figure 25: C-myc Protein Accumulation in pSVmyc Transfected C25 Myoblasts. Western blot analysis of nuclear and cytoplasmic extracts derived from pSVmyc/pSV2neo transfected C25 myoblast clones 1, 2, 3, 5, 6, 7, 9, 15, and control pSV2neo (pSV) and C25 (parental) cells, as labelled above each lane. Extracts were derived from cells grown in mitogen rich medium; an equivalent of 10^7 nuclei/lane was loaded on 10% polyacrylamide gels (described in detail in the Methods). The arrow points to the c-myc bands, migrating between the 58 kd and 84 kd markers. The blot was probed as described in the legend to Figure 25.

gene (lane 1), but not in cells infected with baculovirus lacking this gene with a baculovirus vector containing the human c-myc (lane 2). These insect cell extracts were also kindly provided by Dr. Chizzonite. Figure 25 is a western blot analysis of low salt nuclear and cytosol (supernatant) extracts derived from clones (1-15), parental C25, and a pSV2neo control line (pSV), as indicated above each lane. Three major bands migrating between 58 and 84 kd are revealed in these blots. The arrow points to the expected migration of $p67^{c\text{-}myc}$. Although the higher migrating form of c-myc (84 kd) has been observed in other mouse extracts (Evans and Hancock, 1985), its significance is unknown. It is evident from this blot that these proteins are present in the nucleus, since none are detected in the supernatant extracts. Nuclei are isolated according to the protocol by Evan and Hancock (1985). Transfected clone, C5M15, expresses the highest c-myc protein levels (see lane marked 15), indicating that 8-fold normal levels of transfected c-myc mRNA levels result in at least 2-fold normal levels of c-myc protein levels.

An inverse correlation between c-myc expression and differentiation marker induction (TnT and MHC) is evident for the highest level c-myc expressing, c-myc transfected clone, C5M15: c-myc mRNA (8-fold normal), c-myc protein (2-fold normal), MHC (3.3-fold less than normal, not detected above background), TnT (2-fold less than normal, not detected above background). Desmin accumulation remains unaffected. The second and third highest c-myc expressing clones, C5M4 and C5M6, express reduced, but detectable levels of MHC. Phase microscopy of parental (C25) and C5M15 cells which have been induced to differentiate for 6 days in HS-DMEM is shown in Figure 26. Because C25 is a non-fusing, myogenic line, it is difficult to visually define differences by appearance alone. In order to address the mechanism of c-myc mediated differentiation inhibition, the C5M15 line is probed for myogenin expression. Figure 27 shows that neither uninduced (U) or differentiation induced (D) C5M15 myoblasts (15) express myogenin (1). Parental (C25, U and D) and pSV2neo (pSV) control mRNA's express myogenin at very high levels. For comparison, one fourth as much (1.5 μg) thigh muscle mRNA (M) is loaded in the last lane. An acridine orange

C25

C5M15

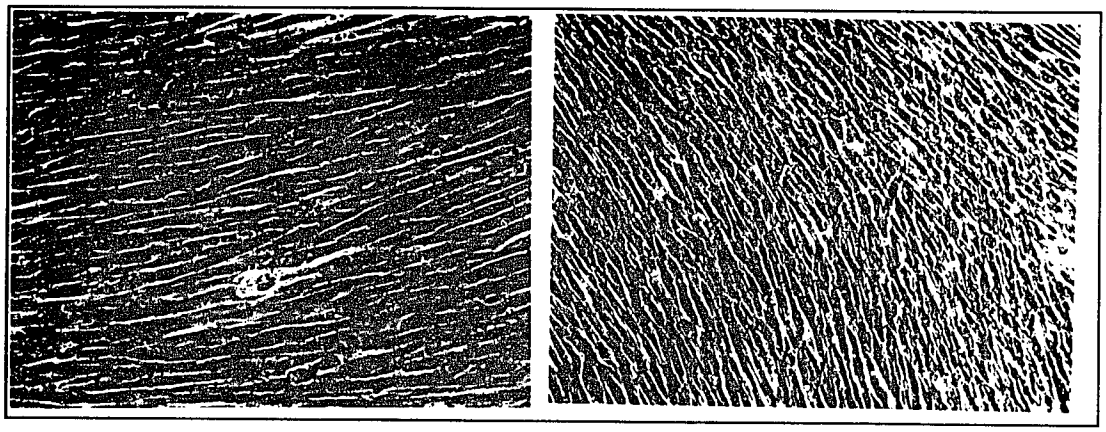


Figure 26

Figure 26: Phase Contrast Microscopy of Differentiation Defective, C-myc Expressing Clone, C5M15. Parental C25, or pSVmyc/pSV2neo transfected clone, C5M15, were grown to confluence, and induced to differentiate for 5 days in differentiation medium (after which C5M15 started to die). Cells were photographed on a Nikon inverted, microcope at a magnification of , using a polaroid camera.

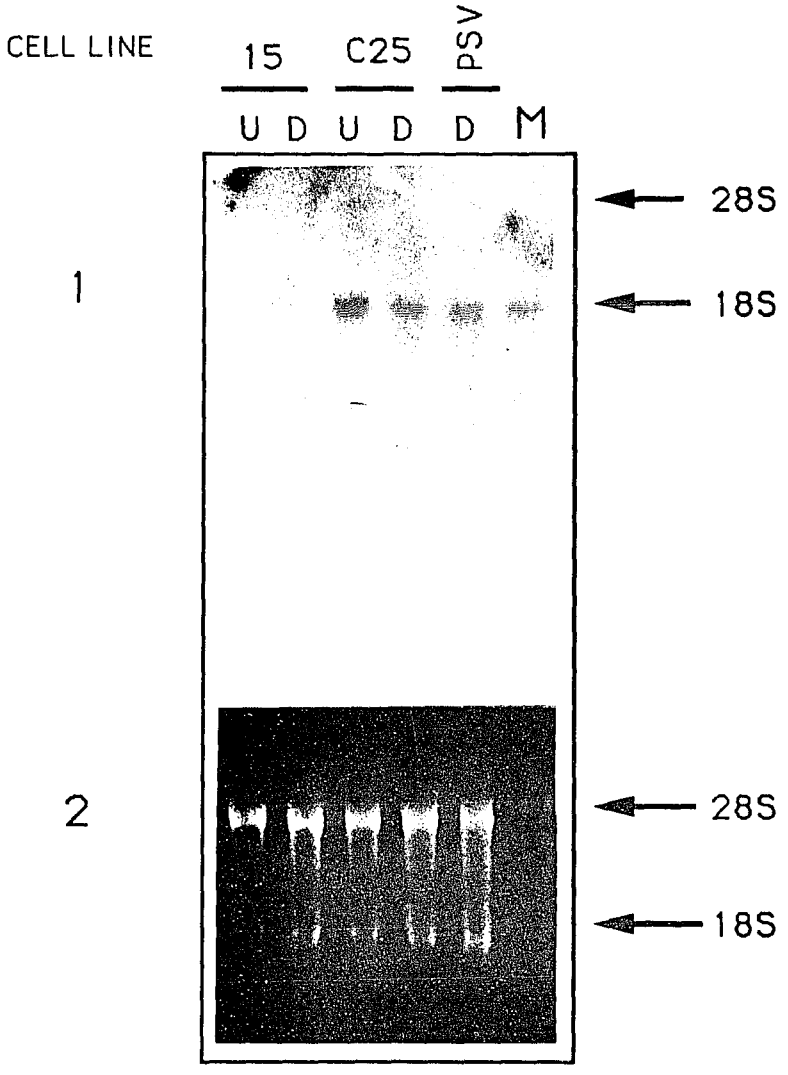


Figure 27

Figure 27: Myogenin Gene Expression is Inhibited in C-myc Transfected Clone C5M15: 6 μg of RNA's isolated from C5M15 (15), C25 before (U) and after differentiation induction (D), pSV2neo control transfected (pSV, D), and 1.5 μg from thigh muscle (M) were loaded on a 1.5% formaldehyde gel, transferred to Gene Screen Plus, and probed with a nick translated, myogenin cDNA probe (1). RNA's were stained with acridine orange on a separate gel (2), as shown below.

stained, formaldehyde gel is shown below (2) to verify the amounts and integrity of the mRNA's. The migration of 28S and 18S ribosomal RNA's are marked on the right. As expected, C5M15 proves negative for MHC staining by both immunofluorescence, and peroxidase labelling (not shown).

Characterization of C25, a myogenic subclone of C2M: The origins of our C2 cells were, at the very least, indirect. By the time we had received the C2M line, it had passed three laboratories, Dr. Yaffe (Weissman Institute, Israel, where it was generated), Dr. Blau (Stanford), and Dr. L. Rubin (Rockefeller, from whom we obtained the line). It had lost much of its myogenic potential, as evident by its reduced MHC accumulation and fusion capabilities (see MHC western blot, Figure 7, and Figure 15). It is well accepted that myogenic lines lose their capacity for differentiation if kept too long in culture. Other groups have found it necessary to subclone C2 to generate a more myogenic cell line (C2C12, Blau et. al., 1983). However, at the beginning of these experiments, C2C12 was not available. By subcloning

C2M in this laboratory, a variety of clones are obtained. One of the myogenic clones, C25, was chosen to perform transfection experiments, as presented in the previous section. Selection of C25 over other subclones was not only based on the basis of its intense staining for myosin heavy chain in immunofluorescence (see Figure 28), but also its ability to maintain a myogenic phenotype, even after prolonged culture. None of the clones (0/25 screened by visualization) derived from C2M, fuse to form multinucleated myotubes, as those seen in primary myogenic cultures or C2C12. Later in these studies, C2C12, the clonal myogenic line derived by Dr. H. Blau, was obtained from ATCC, and used to compare the C25 line, both biochemically and phenotypically.

Comparison of C25 and C2C12 cell lines. Biochemical and phenotypic differences between the two myogenic cell lines, C25 and C2C12 are compared. Whole cell extracts from the two lines are isolated in order to compare the accumulation of myosin heavy chain, fast and slow, troponin T fast isoforms, smooth muscle actin, and desmin. Figure 29 presents 6 western blots

Figure 28

Figure 28: Myosin Heavy Chain Immunofluorescene of Differentiation Induced C25 Myoblasts. C25 cells were grown in HS-DMEM for four days, fixed in methanol, -20°C, incubated with MF20, and rhodamine conjugated second antibody. Cells were photographes using a Nikon Diaphot system, at magnification 125X.

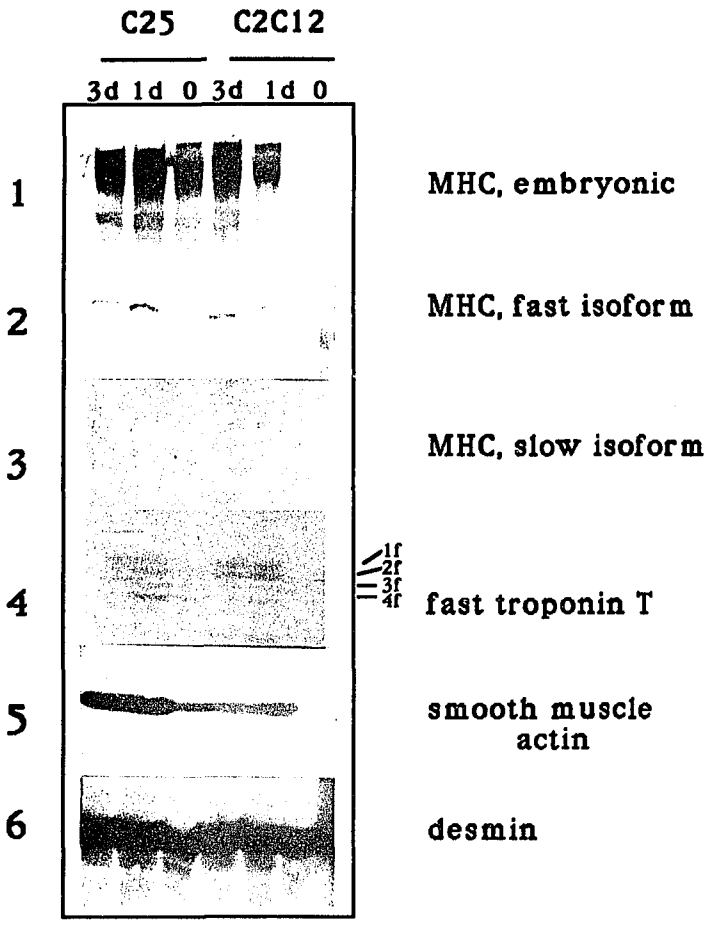

C25
C2C12
3d 1d 0 3d 1d 0
1
2
3
4
5
6
MHC, embryonic
MHC, fast isoform
MHC, slow isoform
1f
2f
3f
4f
fast troponin T
smooth muscle actin
desmin

Figure 29

Figure 29: Myoblast Cell Lines, C2C12 and C25, Differ in Their Muscle Protein Accumulation During Differentiation. Whole cell extracts were isolated from C25 or C2C12 cells at 0, 1 day (1d), or 3 days (3d) after differentiation induction, as indicated above each lane. The gel was transferred to nitrocellulose and probed with: 1. MF20 (anti-myosin heavy chain) 2. F59 (anti-fast isoform specific, myosin heavy chain) 3. S (anti-slow isoform specific, myosin heavy chain) 4. JLT12 (anti-troponin T) 5. (anti-smooth muscle actin), 6. polyclonal anti-desmin. Each lane contains approximately 10^6 cells/lane. Equal protein amounts were loaded in each lane as assessed by Coomassie staining.

of whole cell extracts derived from differentiating C25 and C2C12 myoblasts. Both lines accumulate fast isoforms of myosin heavy chain (2), but not slow myosin heavy chain (3). Both lines accumulate fast isoforms of troponin T (4), desmin (6), and smooth muscle actin (5). A comparison of MHC (1), and smooth muscle actin (5) in the uninduced lanes of C25 and C2C12 (lanes [0]) suggests that C25 is capable of spontaneous biochemical marker accumulation without mitogen removal, whereas C2C12 is not. In addition, a difference in fast TnT isoforms is also observed (see lane C25 (1d) vs. C2C12 (1d); C25 accumulates higher levels of fast TnT 4f. Densitometric scanning of these blots are presented in Table 6. The significance of these differences is discussed in greater detail in the Discussion Section.

Immunofluorescence analysis of differentiation induced C25 and C2C12 myoblasts reveal their major phenotypic difference. Figures 30 and 31 contain immunofluorescence photomicrographs of C25 and C2C12 myoblasts, 6 days after differentiation induction. Although C2C12 forms multinucleated myotubes (greater

than 90% of the nuclei included in myotubes), C25 elongates, but fails to form myotubes. Both cell lines stain brightly for myosin heavy chain using monoclonal antibody MF20, and an anti-mouse rhodamine conjugated second antibody.

The recent isolation of myogenic determination genes, myod1, myogenin, and myf5, allows the following question to be addressed: Are the biochemical and phenotypic differences observed between the two lines reflected by a difference in expression of these genes? Figure 32 presents 6 northern blots of differentiating C25 and C2C12 myoblasts. Isolation of RNAs from both lines at 0 (uninduced), 1 day, and 3 days after differentiation induction are isolated in order to compare expression of myod1 [1], myogenin [2], myf5 [3], c-myc [4], alpha-cardiac actin [5], and 28S ribosomal RNA [6]. Expression of myod1 RNAs differs greatly between the two lines as shown in Figure 32 [1]. The C2C12 line increases expression of myod1, upon differentiation induction, whereas, C25 myod1 levels are undectectable. Both lines are induced to express myogenin, another muscle

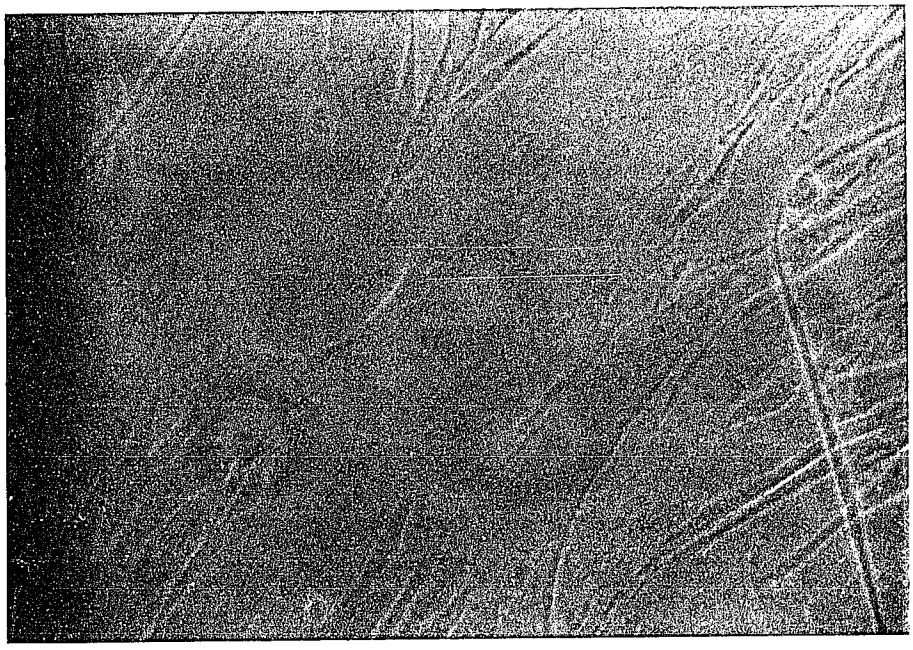
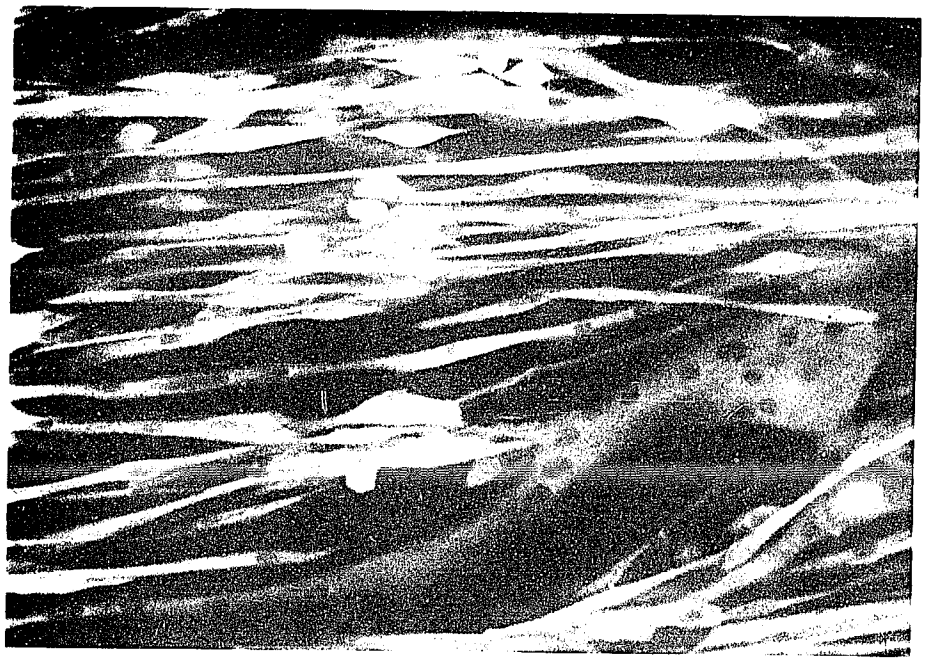


Figure 30 (A)



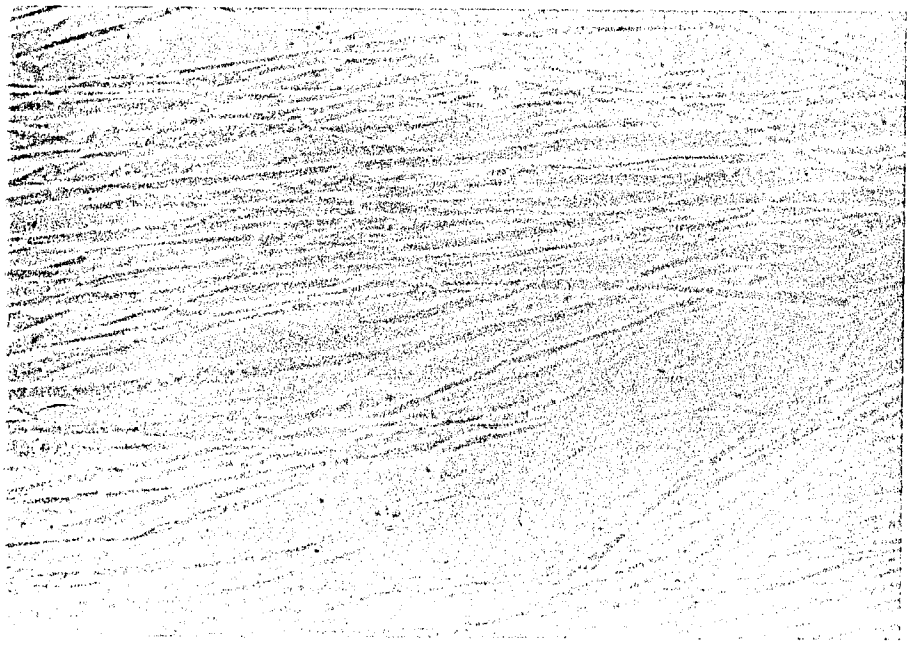


Figure 30 (B)

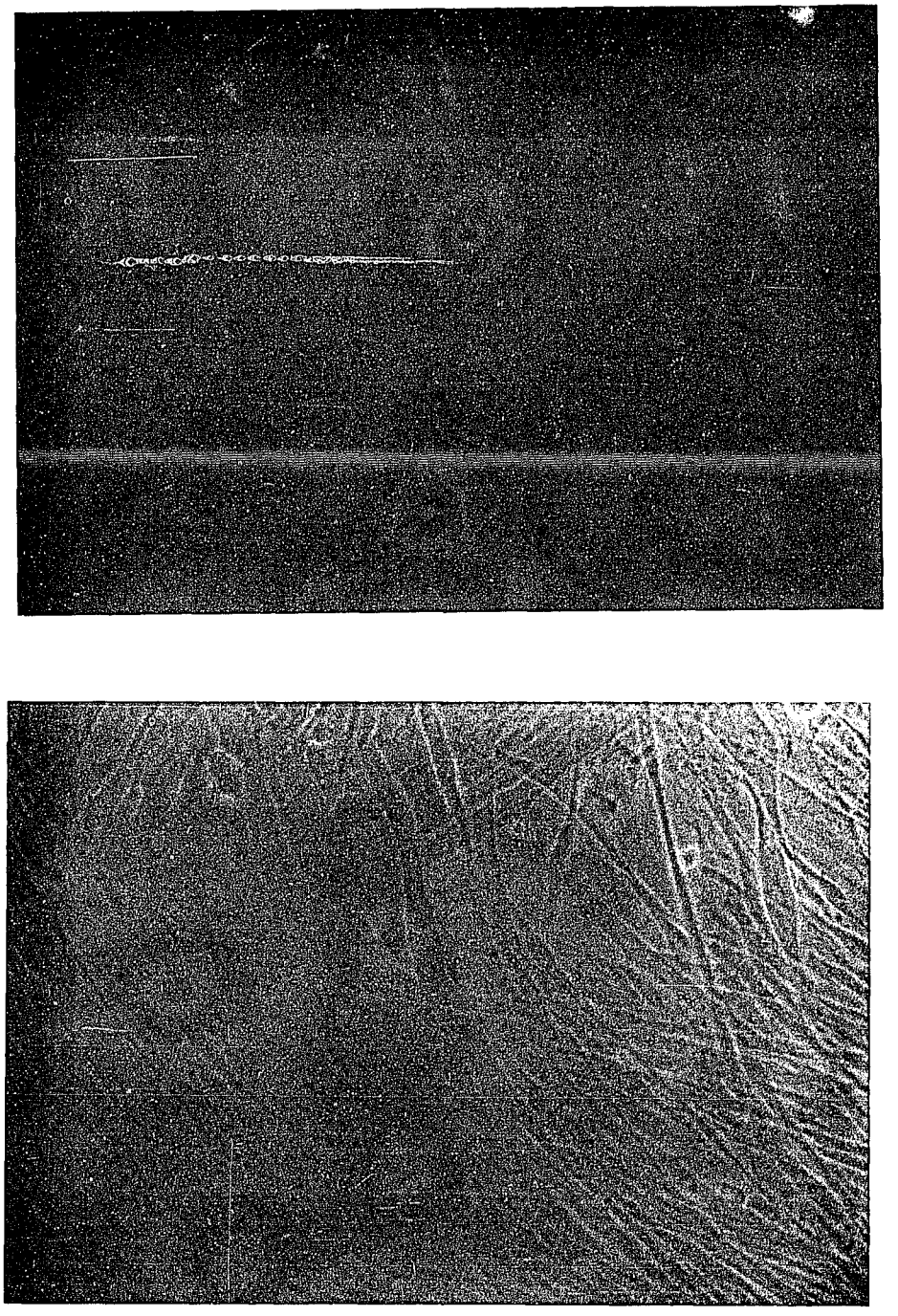


Figure 30 (C)

Figure 30: Myoblast Cell Line, C2C12 Fuses to Form Multinucleated Myotubes Upon Differentiation Induction. A and B: C2C12 myotubes formed after 4 days in differentiation medium I. Phase contrast microscopy II. Myosin heavy chain immunofluorescence (mAb MF20), visualized with rhodamine conjugated, goat anti-mouse antibody. C:
control, background immunofluorescence due to second antibody alone (incubation without MF20)

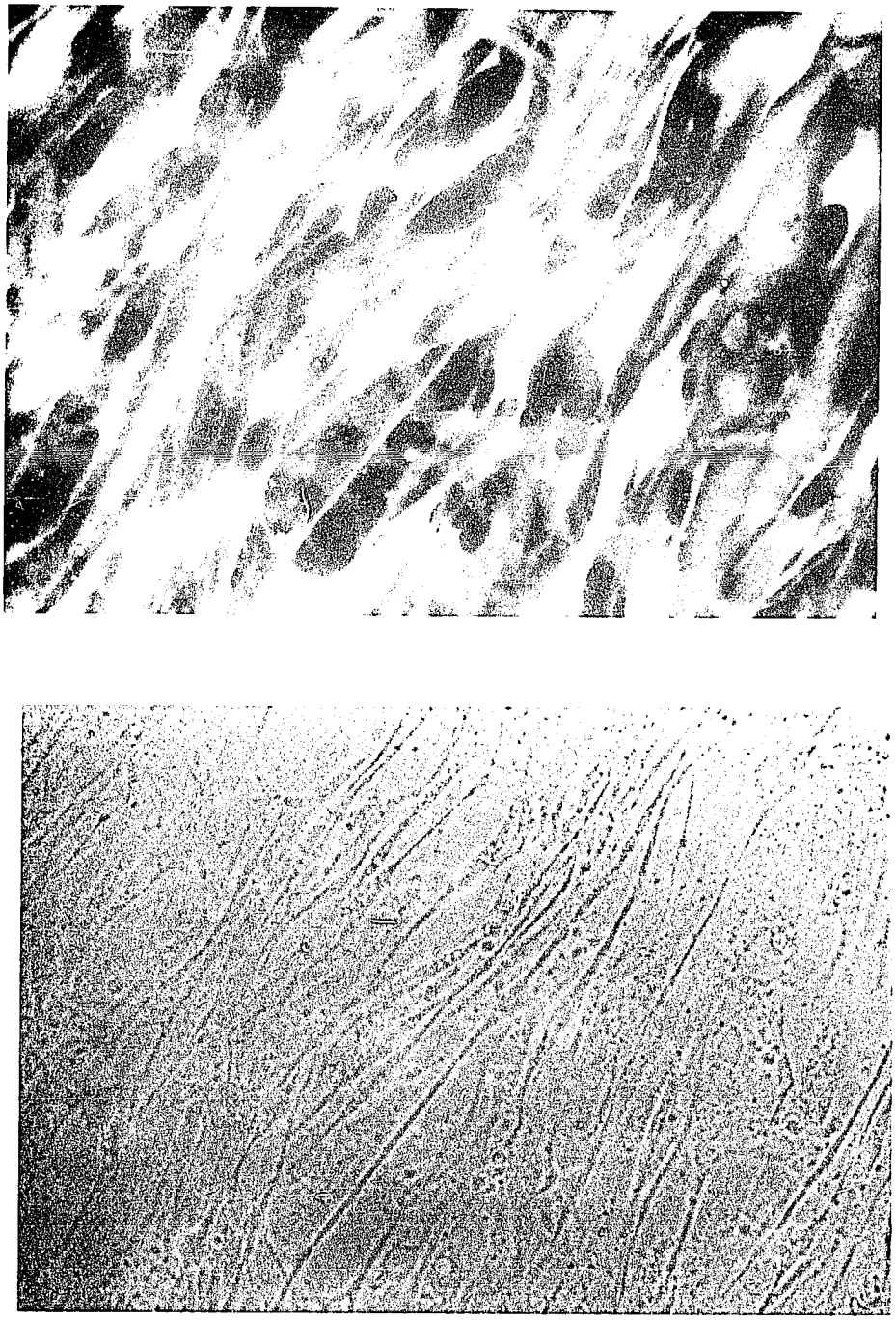


Figure 31

Figure 31: Myoblast Cell Line, C25 Remains Mononucleated After Differentiation Induction. C25 cells after 4 days in differentiation medium. I. Phase contrast microscopy. II. Myosin heavy chain immunofluorescence (mAb MF20), visualized with rhodamine conjugated, goat anti-mouse antibody.

determination gene, C2C12 expressing higher levels than C25 (Figure 32 [2], compare all three C25 lanes with C2C12 lanes). After extended exposure to film (10 days), low levels of myf5 are detected in C25, but not C2C12 (compare C25 [2], (0) and (1d) lanes with all C2C12 lanes). Both C25 and C2C12 express high levels of myogenin, C2C12 expressing higher levels than C25. The kinetics of this expression differs between the two lines: myogenin is expressed at low, but detectable levels in uninduced C25 but not in uninduced C2C12 (see [2], lanes [0]), again suggesting an increased level of spontaneous differentiation in C25 cells. Densitometric scanning of these blots are presented in Table 7. The two lines differ in their regulation of alpha-cardiac actin [5] expression, decreasing in differentiating C25's, and increasing, then decreasing in C2C12 cells. Because these two cell lines express different complements of the three myogenic determination genes (myod1, myogenin, and myf5), they provide a model system in which phenotypic and biochemical changes during myogenesis in culture can be compared.

The lack of myod1 expression in C25, and the

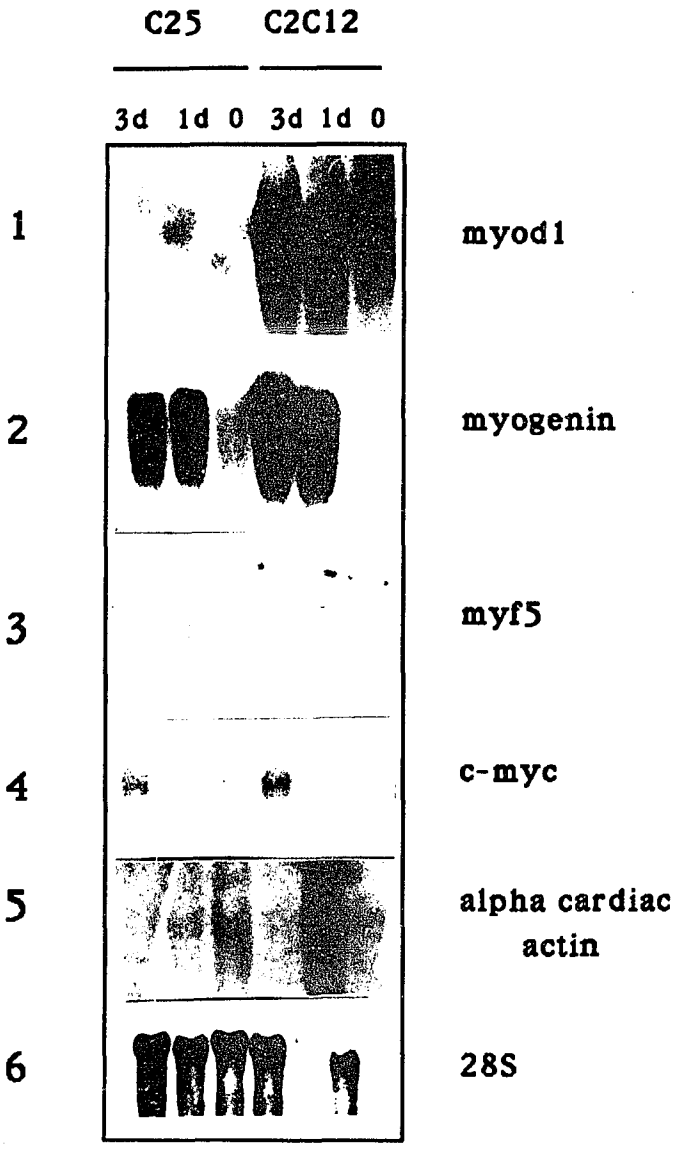

C25
C2C12
3d 1d 0 3d 1d 0
1
myod1
2
myogenin
3
myf5
4
c-myc
5
alpha cardiac actin
6
28S

Figure 32

Figure 32 Myoblast Cell lines, C2C12 and C25 Differ in Their Regulation and Expression of Myod1, Myogenin, Myf5, and Alpha Cardiac Actin. RNA was isolated using guanadine isothiocyanate from C25 or C2C12 cells at 0, 1 day (1d), 3 days (3 d), after differentiation induction. RNA was transferred to Gene Screen Plus and probed with nick translated DNA probes: 1. myod1 2. myogenin 3. myf5 5. cardiac actin 6. 28S (p1-19), and riboprobe: 4. c-myc (Bgl 1 fragment of pSP654myc). Each lane was loaded with 10 micrograms of total RNA, and checked for integrity by hybridization to 28S (6).

failure of this line to form multinucleated myotubes, implies that there may be a close association between myod1 expression and fusion. Additional evidence for this association is obtained from two pSVmyc transfected C25 myoblast clones. Although high (8-fold normal) levels of c-myc are demonstrated to inhibit accumulation of differentiation markers, some transfected clones expressing lower c-myc levels actually exhibit an enhanced myogenic phenotype. Densitometric scanning of Western blots probed with MHC indicates that C5M5 accumulates 4.3-fold higher levels of MHC than controls, C5M7, 2-fold, and C5M2, 1.4-fold, in uninduced cells. This enhancement is more significant in uninduced cells in comparison to differentiation induced cells. Figure 33 contains phase photographs of two of these clones, C5M7 and C5M2, and the parental C25. After 3 days in HS-DMEM, C5M7, and to a lesser extent, C5M2, form multinucleated myotubes. The parental C25 fails to do so, even after 6 days in this medium. Figure 34 contains two Northern blots of RNA's isolated from pSVmyc transfected C25 clones (1-9), uninduced and differentiation induced (5d). The number of each C5M

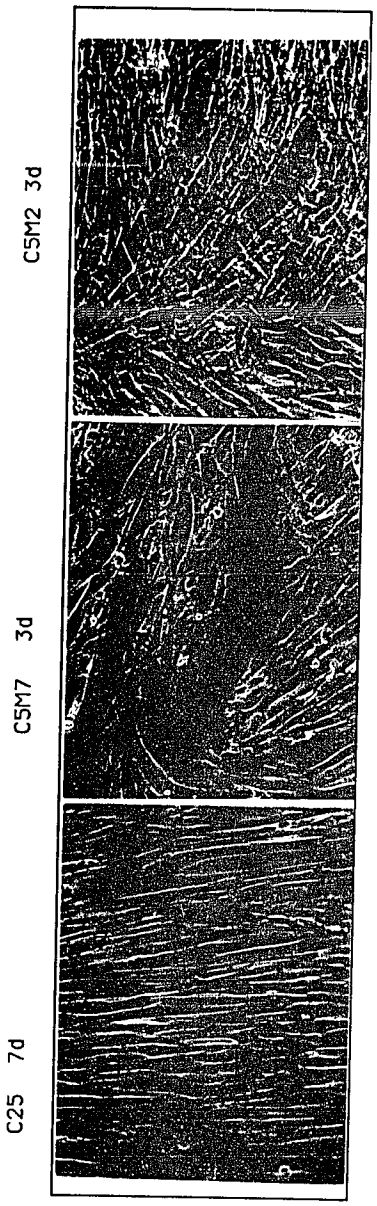


Figure 33

Figure 33: Phase Contrast Microscopy of Fusion Capable, Low Level C-myc Expressing Clones, C5M7 and C5M2. Parental C25 myoblasts were grown to confluence, and induced to differentiate for 7 days in differentiation medium. pSVmyc transfected clones C5M7 and C5M2 were allowed to differentiate for 3 days, at which time multinucleated myotubes were present. Cells were photographed on a Nikon inverted, microcope at a magnification of, using a polaroid camera.

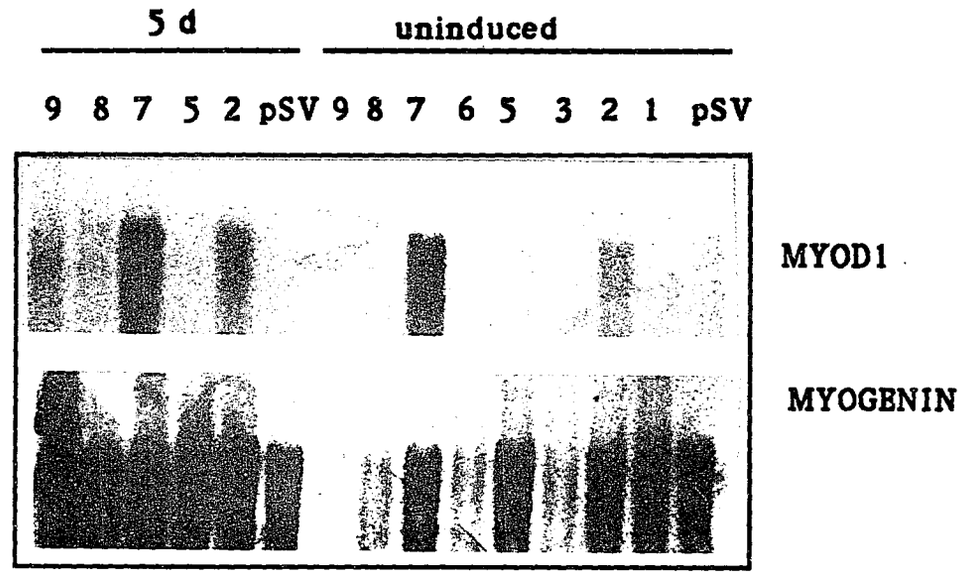


Figure 34

Figure 34: Recovery of Fusion in Two C25 c-myc Transfected Clones is Associated with Expression of Myod1: Northern analysis of 10 μg of RNA from uninduced, pSVmyc transfected C25 myoblasts clones (1, 2, 3, 5, 6, 7, 8, 9), and pSV2neo control (pSV), and differentiation induced (5 days in HS-DMEM) pSVmyc transfected C25 myoblast clones (2, 5, 7, 8, 9). and pSV2neo control (pSV). The top blot was probed with a nick translated plasmid containing a full length myod1 cDNA. The same samples were run in a separate gel, and probed with a plasmid containing myogenin cDNA (bottom blot).

clone is indicated above each lane, pSV indicates pSV2neo control. The top blot is probed with myod1, the bottom blot is probed with myogenin. The two fusogenic clones, C5M7 and C5M2 express myod1, whereas parental C25 (shown in Figure 34 [1]), pSV2neo (pSV) and other c-myc transfected clones do not. The more fusogenic line, C5M7, expresses myod1 at higher levels than C5M2, which is less fusogenic. Since C5M7 does not express significantly higher levels of c-myc mRNA than the parental C25, myod1 activation in this line is probably not due to the direct effects of c-myc. However, since none of the 17 isolated, pSV2neo alone transfected C25 lines fuse to form multinucleated myotubes, these fusing, variant clones may not be a result of clonal variation, either. The fusing variant clones express levels of c-myc most closely to that of the parental line (C5M7 [1.3-fold], C5M2 [1.9-fold]). Therefore, expression of transfected c-myc, at levels approaching those normally expressed, may allow isolation of fusing variants of C25. These results along with the comparisons of C25 (myod1 negative, non-fusing) and C2C12 (myod1 positive,

fusogenic) imply that myod1 expression may be a prerequisite for fusion of myoblasts in culture.

In order to address whether myod1 expression is sufficient to confer fusogenic capabilities on C25 cells, these cells are transfected with a plasmid containing the LTR driven myod1 cDNA (pEMCIIs, see Appendix for map). Figure 35 depicts the results from such an experiment. The C25 line is co-transfected with myod1 and pSV2neo (2) or pEMScribe (parental plasmid, minus myod1 insert) and pSV2neo (1) and colonies selected in G418 containing medium. Colonies are allowed to reach 3 mm in diameter, and induced to differentiate for 8 days in HS-DMEM, without G418. Colonies are then stained for MHC positivity using MF20 and a peroxidase conjugated second antibody. In two separate experiments of this type, over 67 C25, myod1 transfected clones were screened for fusogenic ability. Although all myod1 and control transfected colonies are positive for MHC, none fuse to form multinucleated myotubes. These experiments suggest that myod1 may be obligatory for fusion, but that its expression alone is not sufficient to confer a fusogenic phenotype on C25 myoblasts. However,

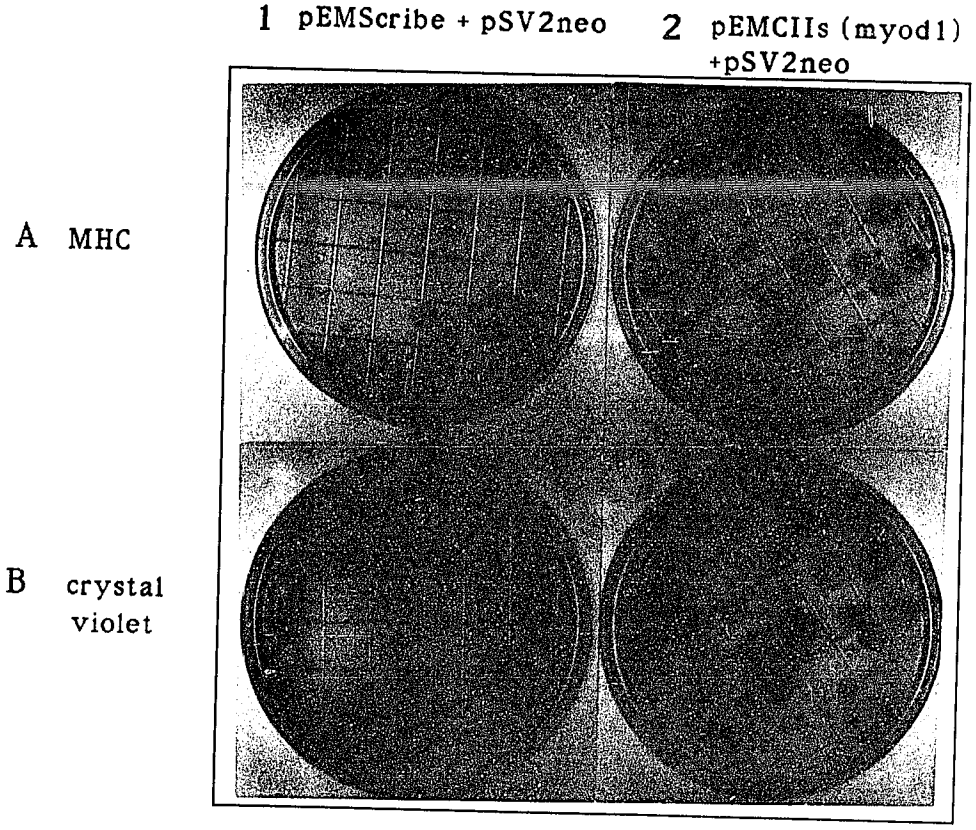

1 pEMScribe + pSV2neo
2 pEMCIIs (myod1) +pSV2neo
A MHC
B crystal violet

Figure 35

Figure 35: Transfection of Myod1 Into C25 Myoblasts Implies That Myod1 May be Obligatory, But Not Sufficient For Fusion: C25 myoblasts were co-transfected in 150 mm plates with 1. control, pEMScribe (expression vector without myod1) and pSV2neo, or 2. pEMCIIs (myod1) and pSV2neo, and selected for G418 resistant colonies. Colonies were allowed to grow to 3mm in diameter, before replacement of medium with HS-DMEM for 8 days, and stained for A. MF20 positive clones. B. The same plates were then stained with crystal violet. Photography was performed using a poraloid camera.

individual clones would have to be isolated in order to insure that 1. myod1 is being expressed at high levels, and 2. those clones growth retarded by myod1 expression are also assayed for fusion. Although the latter is a possibility, fewer numbers of resistant colonies are not obtained with myod1 transfections in comparison with control transfections, which would be expected if this were true.

It is demonstrated that C25 can be inhibited from accumulating both MHC and TnT if transfected c-myc levels reach high enough levels. However, the low frequency of this event (1/25 isolated clones), and the failure of the colony assay to detect such differentiation inhibited clones implies that such high level expression of c-myc may be toxic. Toxicity of high level c-myc expression in transfected cells has previously been reported (Wurm et. al 1986). The C2C12 line differs both phenotypically and biochemically from C25, as described above. In addition to the characteristics described, it is found that C2C12 is more susceptible to c-myc mediated differentiation inhibition than C25. Figure 36

contains G418 resistant colonies resulting from transfection of C2C12 cells with (1) pSVmyc and pSV2neo, or (2) and (3) pSV2neo alone. After selection in G418, colonies are allowed to grow to 2 mm in diameter, changed to HS-DMEM for 8 days, and stained with MF20 (A) and subsequently with crystal violet (B). Panel (3) shows background staining obtained in the absence of incubation with MF20. Two plates of pSVmyc transfected cells are shown, because the efficiency of transfection was low for these experiments. The plate on the right contains three clones negative for MHC, but visible by crystal violet staining, the plate on the left contains one such clone. Therefore, out of 15 colonies tested, 4 are inhibited from MHC induction. Although pSV2neo control colonies varied in their intensity of peroxidase staining, none are completely inhibited from MF20 positivity. These results suggest that there is at least a 5-fold difference in susceptibility of c-myc mediated differentiation inhibition between the two lines. Other explanations of this phenomenon include the possibility that the

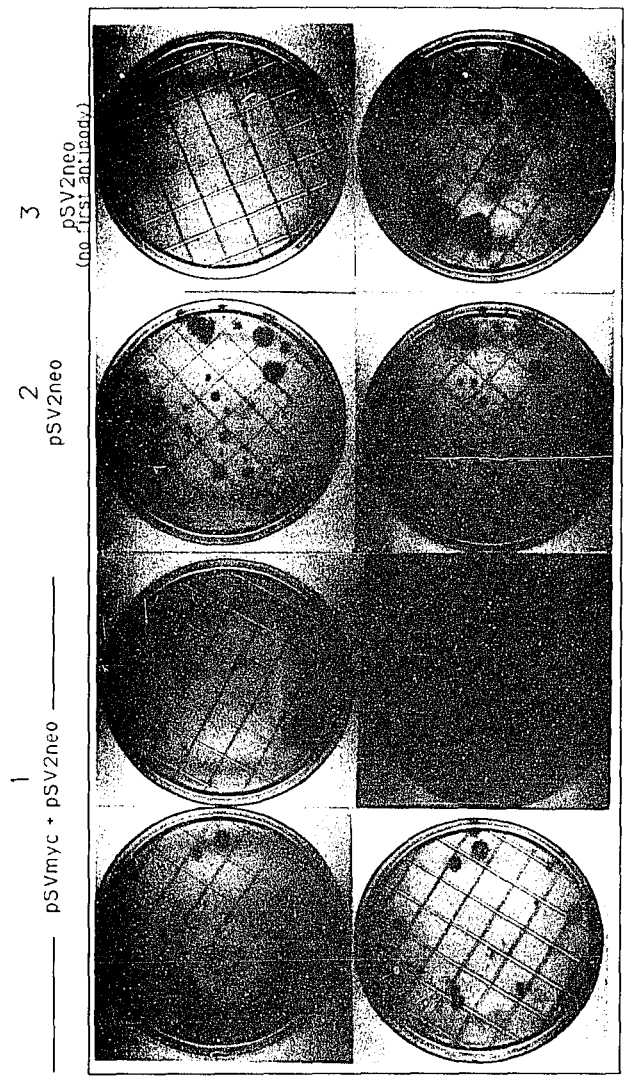

1
pSVmyc + pSV2neo
2
pSV2neo
3
pSV2neo
(no first antibody)
A MHC
B Crystal violet

Figure 36

Figure 36: Myoblast Cell Line, C2C12 is More Susceptible to C-myc Mediated Differentiation Inhibition than C25. C2C12 cells are transfected with (1) pSVmyc1 and pSV2neo, (2) and (3) pSV2neo alone, selected in G418 containing growth medium, and induced to differentiate for 8 days in HS-DMEM. Four out of 15 pSVmyc transfected clones are inhibited from expressing myosin heavy chain, as seen by comparison of MF20 positive versus crystal violet positive colonies.

SV40 promoter is more active in C2C12 than C25, thereby generating a higher frequency of high-level c-myc transfectants. Again, individual clones would have to be isolated and characterized to further investigate the reasons for this result. **Co-culturing of C25 and FD-2 results in multinucleated myotube formation:** Several different myoblast phenotypes are encountered during the course of this work. The C2C12 line biochemically differentiates, and fuses to form large, multinucelated myotubes. The C25 line biochemically differentiates, but fails to form such myotubes. Other lines derived from the same source (C2), FD-2 and FD-6, are unable to biochemically differentiate or fuse. The C2C12 and C25 lines are subclones of C2, derived without selection. However, FD-2 and 6 are selected on the basis that they do not fuse or elongate, upon changing the medium to HS-DMEM (see Methods section). In order to test if any of these lines have complementable defects, co-culture experiments are performed. It is found that co-culturing two non-fusing lines, C25 and FD-2, at a ratio of 1:10 is optimal for myotube formation (Figure 37). Figure 37 contains duplicate phase contrast

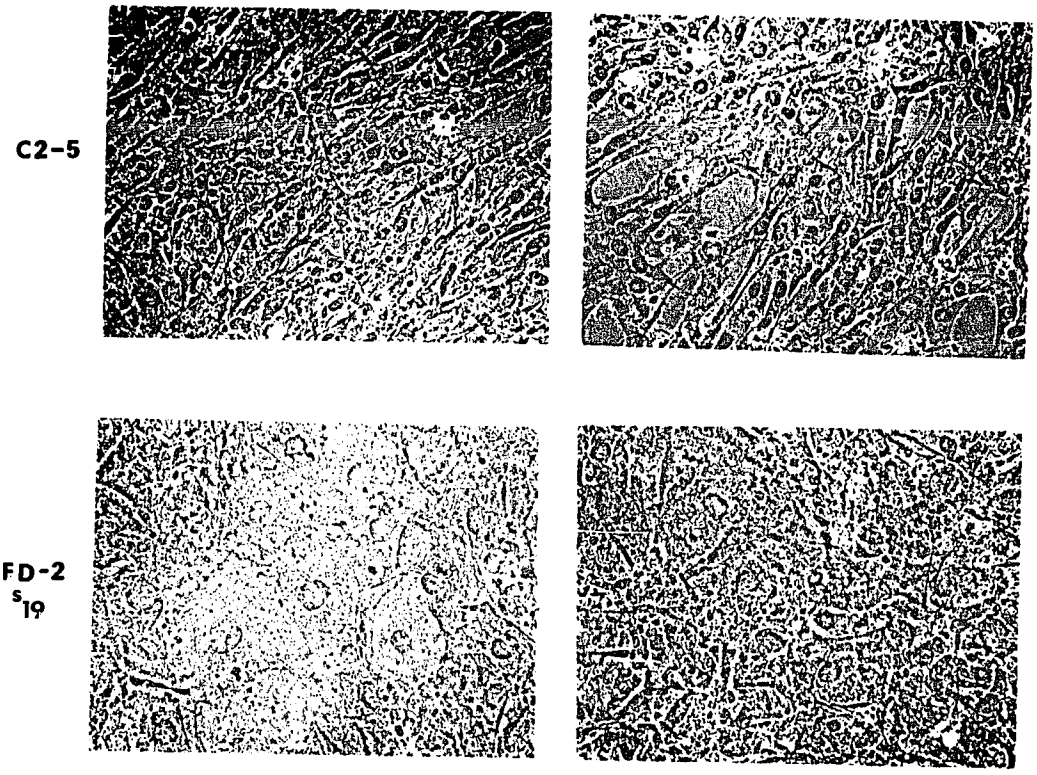


Figure 37

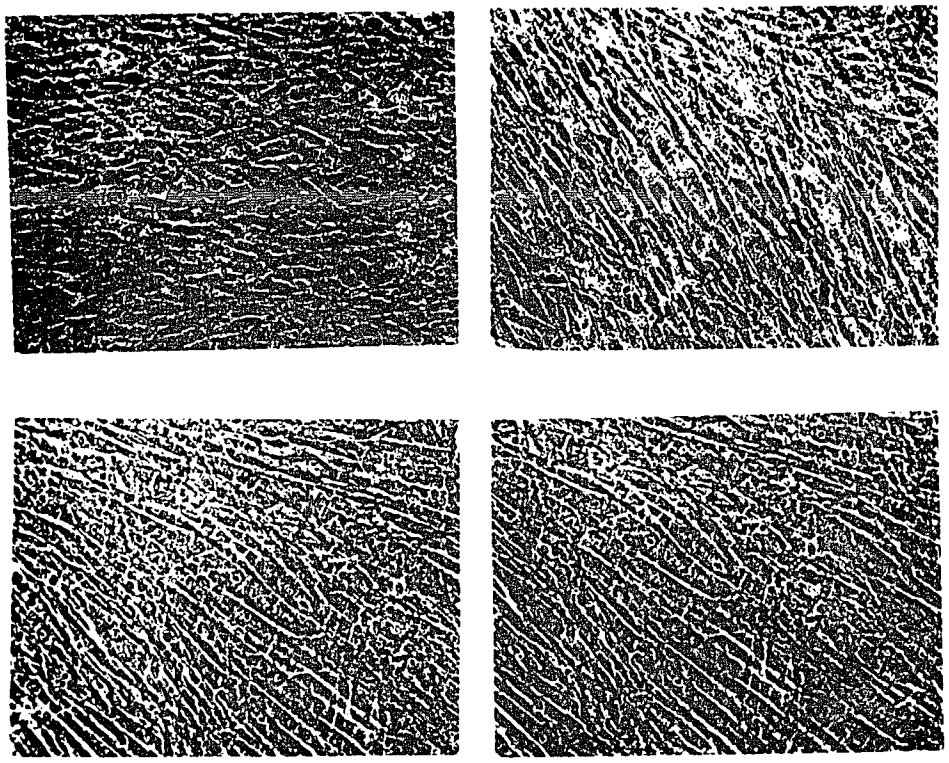


Figure 37

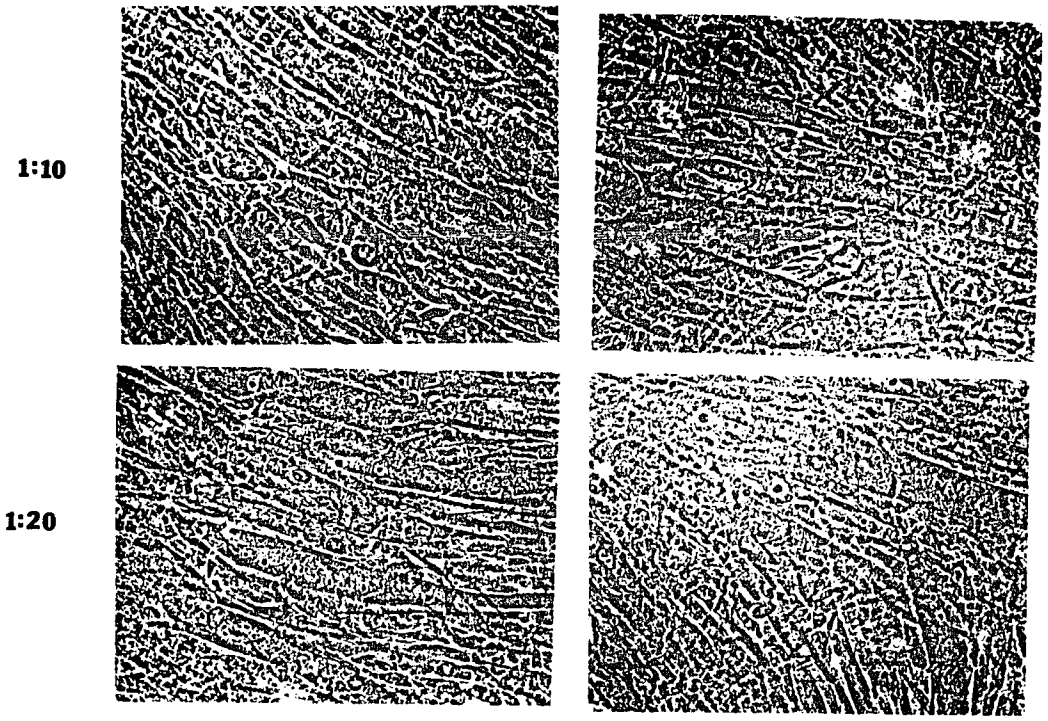


Figure 37

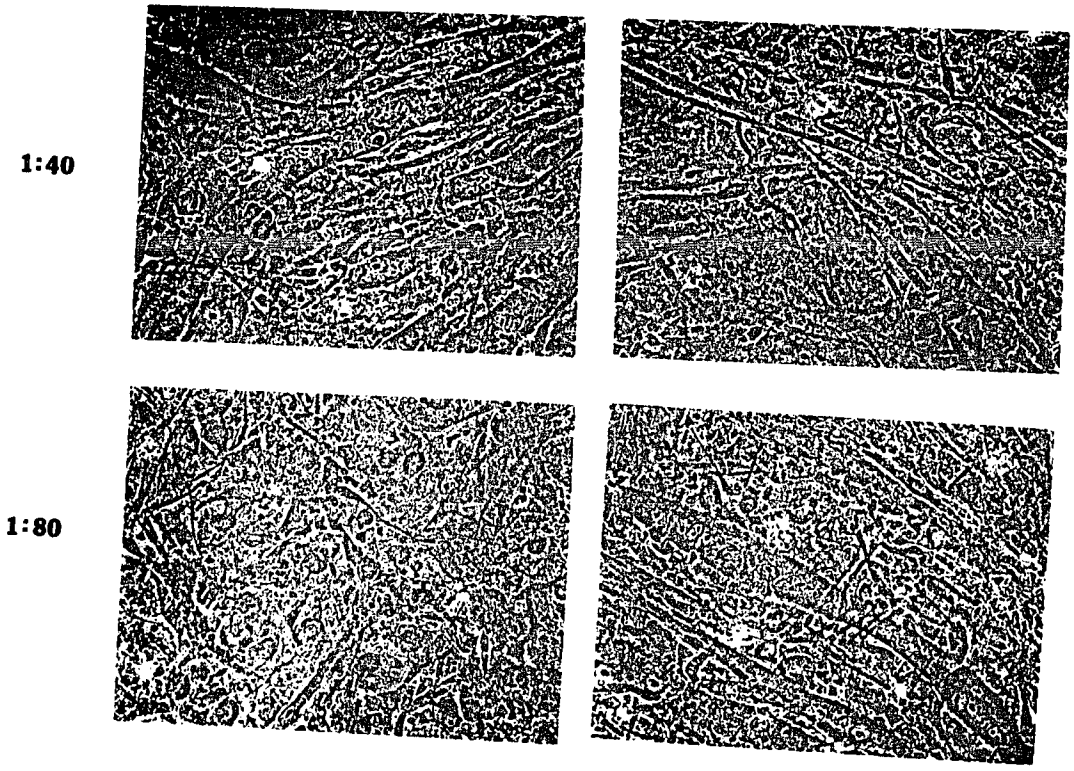


Figure 37

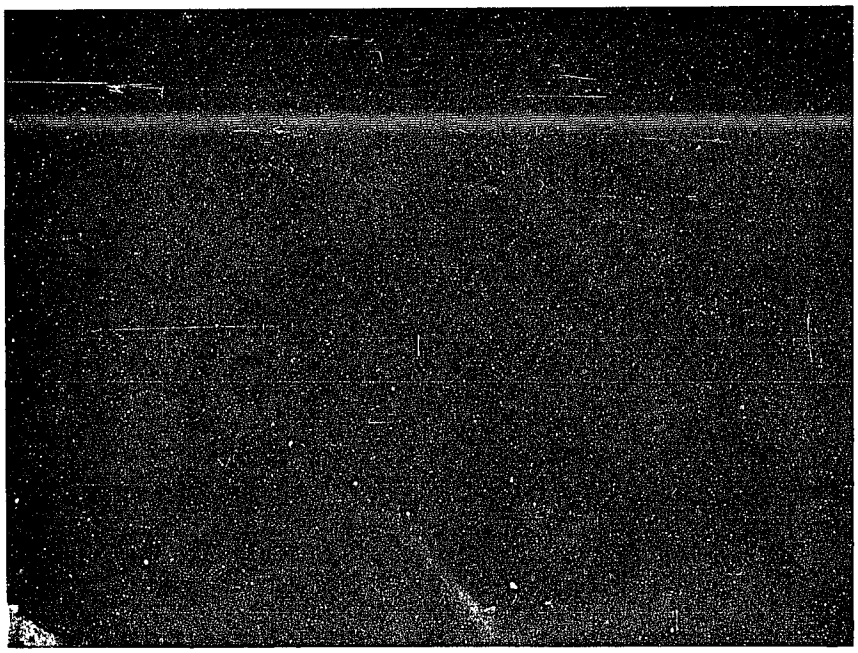


Figure 37

Figure 37: Recovery of Fusion by Co-culturing C25 and FD-2. Phase contrast microscopy of C25 and FD-2, and differing ratios of C25:FD-2 (320X). Cells were grown in CEE-DMEM, then induced to differentiate in HS-DMEM for 3 days. Cultures were photographed using a Nikon, Diaphot system at 320X magnification. Two different fields of each culture are shown (marked on the left). C25 cells cultured alone do not show any appearance of fusion at day 3. FD-2 cells cultured alone also do not show any appearance of fusion. Co-culturing C25 and FD-2 cells at varying ratios, and inducing them to differentiate for 3 days results in fusion at certain ratios. C25:FD-2 1:1, 1:5, 1:10, 1:20, 1:40, 1:80. The last photograph is immunofluorescence microscopy of co-cultures of C25:FD-2 at a 1:10 ratio, 4 days after differentiation induction, using MF20 and rhodamine conjugated, goat anti-mouse IgG.

photographs of C25 cultured alone, FD-2 cultured alone, and co-cultures of C25:FD-2 cells at different ratios: 1:1, 1:5, 1:10, 1:20, 1:40, and 1:80. All photographs are taken of cells 3 days after medium is changed to HS-DMEM. At 1:10 ratios, 80% of all nuclei are in large myotubes, 1:5 (50%), and 1:20 (45%), 1:1 (0), and alone (0). The reverse ratios, where C25 outnumbers FD-2 (10:1, 20:1, etc.), fails to produce myotube formation. Neither line fuses three days after differentiation induction. At this time, C25 is beginning to elongate, whereas, FD-2 remains flat and fibroblast-like. Co-culturing at 1:10 yields the most optimal fusion index (80%). These myotubes are wide, and multinucleated, containing an average of 20-50 nuclei. If dilutions of C25:FD-2 are continued to 1:320, clusters of fused cells appear in a background of unfused myoblasts (not shown). These experiments suggest that C25 can induce fusion of FD-2, but not vice versa. C25 participates in the formation of the myotubes directly, through cell to cell contact, and not through a diffusable factor; culture supernatants from C25 cannot induce FD-2 myotube formation. Immunofluorescence of fusion optimized co-cultured

cells, using anti-MHC is also shown in Figure 37. In addition, two Western blots probed with anti-MHC or anti-TnT (Figure 38) suggest that co-culturing does not enhance muscle specific marker accumulation, compared to C25 myoblasts cultured alone. In addition, FD-2 is unlike C25 in that it does not accumulate detectable levels of muscle-specific markers upon differentiation induction. These experiments suggest that FD-2 is capable of fusion upon co-culturing with another fusion deficient line, C25. However, increased fusion does not necessarily result in increased muscle-specific marker accumulation. By uncoupling fusion and muscle-specific protein accumulation, this culture system may allow analysis of genes which specifically control fusion.

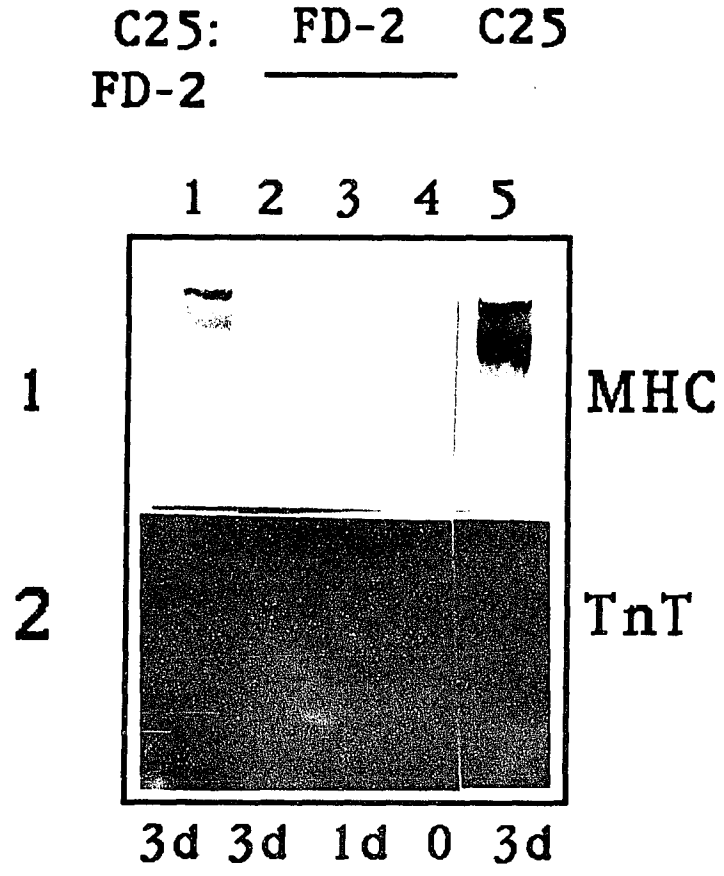


Figure 38

Figure 38: Increased Fusion Capabilities of Co-cultured C25 and FD-2 at Their Optimal Fusion Ratio (1:10) Does Not Result in Increased Accumulation of Biochemical Markers, MHC and TnT. Western blot analysis of whole cell extracts derived from: lane 1. co-cultured C25:FD-2, (ratio 1:10), 2. FD-2, 3 days after differentiation induction, 3. FD-2, 1 day after differentiation induction, 4. FD-2, grown in mitogen rich medium, 5. C25, 3 days after differentiation induction. Blot number 1 was probed with MF20, an anti-myosin heavy chain monoclonal antibody. Blot number 2 was probed with JLT-12, an anti-troponin T monoclonal antibody. Both blots were incubated with alkaline phosphatase conjugated goat anti-mouse second antibody, and developed with a Vector SK-5200 kit.

DISCUSSION

In this thesis, initial experiments indicate that c-myc mRNA levels decrease upon differentiation of C2 myoblasts (C2M). Nuclear run-on transcription assays suggest that this regulation is transient and occurs at the transcriptional level. The decrease in c-myc mRNA levels observed for C2M does not occur in fusion deficient myoblasts, further supporting the hypothesis that c-myc can function as a gene preventing differentiation.

Persistant levels of c-myc mRNA in differentiation defective L6 myoblasts and C110 myoblasts have been reported by Sejersen et. al.(1985), supporting the results shown with C2 variants, FD-2 and Fd-6. In addition, results included in this thesis show that the differentiation defective line, FD-2, decreases its histone H3 mRNA levels upon differentiation induction. Histone H3 has been demonstrated to be associated with the S phase during mitosis (Zhong et. al. 1983). These results suggest that even though FD-2 ceases proliferation, it

does not induce differentiation markers. Therefore, the persistance of c-myc mRNA levels is not due to continued proliferation in differentiation medium (HS-DMEM). Differentiation defective L6 and C110 myoblasts have been shown to cease proliferation by other methods, supporting this data (Sejersen et. al., 1985). These results indicate that c-myc levels decrease during normal differentiation and fail to do so in differentiation defective cells. They imply an inverse association between c-myc and differentiation, supporting the hypothesis that a decrease in the c-myc gene is obligatory for myoblast differentiation.

Initial analysis of c-myc expression during C2M differentiation reveals that c-myc RNA levels decrease by 52 hours after replacement of medium with HS-DMEM. However, analysis of later time points (72 hours after induction) in a C2 subclone, C25, reveal that the decrease observed originally is transient. Levels of c-myc RNA in this line return to 2 times original levels in differentiated myoblasts. The results of nuclear run-on transcription assays indicate that c-myc transcription decreases by 12 hours after

induction of C2 myoblast differentiation. In addition, c-myc transcription is shown to be alpha amaintin sensitive, therefore RNA polymerase II mediated. In an independant experiment, 12, 24 and 48 hour time points reveal that c-myc transcription, decreases (12 hours), and increases (24-48 hours) upon induction. Therefore, the fluctuation of c-myc RNA levels during differentiation are a result of differences in its transcriptional regulation. In C2M, the transcription assay is not sensitive enough to detect differences in exon regulation. Therefore, it could not be determined whether myoblasts, like differentiating HL60 cells, contain a transcription arrest signal at the 3' end of exon 1.

A transient decrease of c-myc has been described for mouse erythroleukemia cells (MEL) induced to differentiate (Skoulthi and Lachman) with dimethylsulfoxide (DMSO). These cells are induced to express beta globin genes upon treatment with DMSO. Co-transfection experiments of MEL cells with pSVmyc1 and pSV2neo, and subsequent analysis of transfected clones revealed that forced expression of c-myc during

differentiation can inhibit beta globin expression (Coppola and Cole). Therefore, the transient down regulation of c-myc during differentiation of this cell line proved to be necessary for the activation of differentiation markers.

Similiar transfection experiments performed in myogenic cell lines have yielded conflicting results. Transfection of the non-fusing BC3H1 myoblast line with c-myc suggested muscle-specific marker inhibition occurs only in the presence of mitogens (Schneider et. al., 1987); upon removal of mitogen-rich medium, transfected cells activate muscle specific proteins regardless of c-myc gene expression. In addition, Olson et. al. (1987) found that activated ras genes, but not c-myc, inhibited fusion and muscle creatine kinase gene expression in transfected C2 myoblasts. The issue of clonal variation in the parental line was not addressed in these papers. From the data presented in this thesis, it is evident that the C2 line is not clonal, and that differentiation defective cell lines can easily be derived from this line. One way to avoid interference of clonal variation on

transfection results is the isolation of polyclones, as done by Olson et. al., op. cit. In these experiments, out of 11 individual ras transfected clones, one was capable of "limited differentiation" (not defined in the paper), the rest were differentiation inhibited. The results presented in the paper by Olson et. al. utilized polyclonal transfected cells (50 individual clones, pooled). Although such an approach can detect high frequency inhibition, as mediated by activated ras genes, it may not detect low frequency inhibition. Analysis of differentiation inhibited, activated ras transfected myoblasts, revealed that these cells failed to down regulate their c-myc gene, upon differentiation induction. Again, that c-myc down regulation is required for myogenic differentiation is implied, but not proven by these results. The failure to obtain differentiation inhibited clones by c-myc transfection of C2 cells by Olson et. al. may be because c-myc mediated inhibition requires very high levels of expression. This level may only be achieved at low frequencies, thereby escaping detection by polyclonal analysis.

In this thesis, initial co-transfection experiments of C2M's with pSVmyc1 (mouse c-myc exon 2 & 3 driven by the SV40 promoter) and pSV2neo, generated C2M lines expressing high levels of c-myc mRNA are obtained. The highest c-myc expressing clone, MN19, failed to accumulate myosin heavy chain upon differentiation induction. However, these early experiments had a a high background frequency of differentiation defective lines resulting from pSV2neo (alone) transfected C2M myoblasts. In order to be sure that the effects of the c-myc gene on differentiation were being determined, and not the effects of clonal variation, the C2M line was subcloned, and screened for phenotypically stable, homogeneous clones, as assayed by myosin heavy chain staining. Using such standards, the C25 subclone was chosen for future transfection experiments.

In order to increase the number of clones tested for c-myc mediated inhibition, a colony assay instead of polyclonal analysis was used, in addition to isolating individual transfected clones. In the colony assay, C25 cells are grown in mitogen rich

medium, co-transfected with a plasmid containing c-myc (pSVmyc1), and a plasmid bearing a drug resistance gene (pSV2neo). Cells are diluted 1:5 into mitogen rich medium and plated onto large 150 mm tissue culture dishes, and selected with G418. Colonies are allowed to grow to 3mm in diameter, induced to differentiate, and stained with anti-myosin heavy chain mAb (MF20) and a second antibody conjugated to peroxidase. They are subsequently stained for crystal violet to compare the percentage of total clones that are myosin positive. When the C25 line was assayed for c-myc inhibition by this method, none of the macroscopically visible colonies were negative for MHC. Since over 200 clones were tested, this assay suggests that the frequency of c-myc mediated inhibition is less than 1/200 clones. Transfection of a different subclone of C2M, C2C12, using this method, revealed that 4/15 clones are inhibited from expressing the differentiation marker, MHC. Successful inhibition of C2C12 differentiation indicated that the colony assay can determine whether c-myc transfection specifically inhibits differentiation in myoblasts. Transfection of C2C12

indicates that c-myc inhibited myoblast clones are significantly smaller than non-inhibited clones in the same plate. That deregulated c-myc expression may be toxic to these cells seems a likely explanation. Toxicity, resulting in death or growth retardation of clones expressing high levels of transfected c-myc RNA may explain the failure of this method to detect differentiation inhibited C25 clones.

Transfection of C25 with pSVmyc and pSV2neo, and subsequent isolation of individual clones (25, total), generated a single, differentiation inhibited clone (C5M15). This clone failed to accumulate MHC and TnT. At the same time, C5M15 expressed the highest c-myc mRNA of all of the transfected lines screened (8 fold normal). Desmin accumulation in this line is not inhibited. Since this protein is present in uninduced cells, these results imply that the mechanism of c-myc mediated inhibition does not involve suppression of ongoing gene expression, but, rather prevention of the activation of newly expressed genes. Transfected clones (C5M5, C5M2) expressing lower levels (2-5 fold normal) of c-myc mRNA accumulate higher MHC and TnT in

both mitogen rich and depleted medium. These results suggest that c-myc expression requires a threshold level (at least 8 fold normal) as those seen in clones C5M15 and MN19 in order to inhibit myogenic differentiation. Below this level, c-myc expression may actually increase accumulation of myogenic markers MHC and TnT. Why differentiation inhibited clones such as C5M15 are not detected as small colonies in the colony assay remains unexplained. One possibility is that differentiating C25 colonies adjacent to inhibited colonies can induce the latter to differentiate. This may be a characteristic of the C25 line, as supported by co-culture experiments with the differentiation defective line FD-2.

The differentiation inhibited clone, C5M15, expresses the highest c-myc RNA and protein, and the least myosin heavy chain and fast troponin T proteins. These results indicate that the decrease in c-myc transcription observed upon myoblast differentiation is obligatory for differentiation to occur. The transient nature of this decrease, and the failure of c-myc over expression to inhibit desmin accumulation

imply that reinduction of c-myc in committed cells does not affect progression through the differentiation pathway. That re-induction of c-myc in differentiated myotubes does not affect muscle-specific gene expression has already been shown for L6 myoblasts (Endo and Nadal-Ginard, 1987). However, these results are the first demonstration that C2 myoblasts can be inhibited from differentiation by c-myc overexpression. In previous reports, C2 (Olson et. al., 1987) and C2C12 (Payne et. al., 1987) have only been shown to be susceptible to activated ras mediated inhibition, but not c-myc. The frequency of c-myc inhibition in C25 cells is 1/25, or less, when individual colonies are isolated. Using the colony assay or polyclonal analysis, this inhibition is not detected. Whether this is due to toxic effects of high level c-myc expression or the effects of differentiation competent, adjacent cells is unknown. The C5M15 line provides a system in which to study the mechanisms of c-myc mediated inhibition. Future experiments with this cell line can address whether c-myc over-expression affects the activation or action of myogenic determination genes, such as

myogenin. In addition, transfection of C5M15 with constructs expressing such genes could address whether differentiation capabilities can be rescued by over expression of myogenic determination genes. Recent experiments with fos and ras differentiation inhibited myoblasts indicate that the mechanism of inhibition by these oncogenes occurs by suppressing the expression of the myogenic determination gene, myod1 (Lassar et. al., 1989). Therefore, c-myc may mediate differentiation inhibition of C25 cells by suppressing the activation of a myogenic determination gene. Since, the main myogenic determination gene expressed in C25 cells is myogenin (as demonstrated in this thesis), this may also be the target of action for the c-myc gene. The failure of C5M15 to express myogenin mRNA supports the hypothesis that c-myc inhibits myogenesis by preventing myogenin transcription. That such prevention requires a threshold level of c-myc is suggested by the ability of lower level c-myc expressing clones to express myogenin, and other differention markers. The successful isolation and charaterization of a c-myc inhibited myoblast clone such as C5M15 allows detailed analysis of c-myc

mediated, differentiation inhibition, and also resolves some of the puzzling results obtained with c-myc transfections of myoblasts by other investigators.

The isolation of the differentiation inhibited line C5M15 indicates that c-myc requires a threshold level of expression (at least 8-fold normal) in order to inhibit differentiation. However, there are several c-myc transfected clones (C5M5,-2, -7) which express lower levels of c-myc, and higher levels of differentiation markers (MHC and TnT) than the parental line, both before and after differentiation induction. Although these clones may be a result of clonal variation among C25 subclones, such variation is not observed for pSV2neo alone transfected clones. Thus, the effects of transfected c-myc on C25 cells may be concentration dependant.

A possible explanation of the enhanced expression of differentiation markers in low-level, c-myc transfected myoblasts stems from recent experiments performed both with leucine zipper

proteins and myogenic determination genes. Experiments with the myogenic determination gene, myod1, have demonstrated that the c-myc homologous region contained in myod1, by itself, can induce the muscle phenotype (Tapscott et. al., 1989). This region is also responsible for binding to the muscle creatine kinase promoter (Lassar et. al., 1989). In addition, myod1 has been demonstrated by this group to positively autoregulate itself. In addition to myod1, myogenin and myf5 also contain the c-myc homologous region. The functional significance of the c-myc homologous region to myod1, implies its probable importance in myogenin and myf5 function. In cells that express very high levels of c-myc, such as C5M15, c-myc may interfere with myogenin or myf5 activation, thus preventing the cells from activating muscle specific genes. Below a certain threshold of expression, however, unable to suppress myogenin expression, c-myc may interact with myogenin via the homologous region or their leucine zippers, forming heterodimers with a higher affinity for target DNA sequences than homodimers. Thus, certain muscle specific markers would increase. Support for the

latter lies in the demonstration that heterodimers formed between leucine zipper proteins, c-fos and c-jun, bind the target AP-1 binding site with greater affinity than homodimers (Halzonetis et. al., 1989, Kouzarides and Ziff, 1989). In addition, the HLH region on DNA binding proteins have also been deomstrated to mediate hetero- and homodimer formation (Murre et. al., 1989). Because myogenin and c-myc contain both an HLH region and a leucine zipper, these proteins may form heterodimers capable of enhanced DNA binding; this provides an explanation for the increased accumulation of muscle-secific markers observed for clones C5M2, 7, and 5.

Finally, the possibility that clones C5M2 and C5M7, which express higher levels of differentiation markers than the parental C25, are spontaneous variants of C25 should not be overlooked. Low level c-myc expression may increase the probability of their selection, by giving them a growth advantage, but may not have a role in this increased expression. Two reasons why this is improbable are the following: 1. Transfections with pSVmyc do not result in a larger

number of clones than with pSV2neo transfections alone. If low level c-myc expression does give C25 a growth advantage, this should be true. 2. Fusing variants of C25 have not been isolated by transfection of pSV2neo alone.

The role oncogenes, specifically c-myc, may play in differentiation has been an area of intense investigation over the past several years. A possible role for c-myc during myoblast differentiation can be studied using stable transfectants. Recently, it was discovered that the c-myc genes belong to a class of HLH (helix-loop-helix) proteins along with a long list of differentiation specific, DNA-binding proteins. Included among these proteins are the dominantly acting, muscle phenotype inducing genes, myod1, myogenin, and myf5 (class B HLH, Table 12, Murre et. al., 1989b). Questions remain as to whether heterodimer formation is possible between c-myc and myod1, myogenin or myf5, and whether or not these are functional. Since the latter two proteins both contain leucine zippers at their C-terminal regions, it is theoretically possible to obtain c-myc-myogenin or

Table 12.

Classes of helix loop helix proteins.

Class A	Class B	Class C
E12 E47 da	myoD1 AS-C	c-myc gene family
ubiquitous acting through enhancer sequences	tissue specific enhancer binding	
Heterodimers formed between A and B bind with high affinity to enhancer sequences.		C does does not form hetero-dimers with A or B capable of binding to kE2.

c-myc-myf5 hetrodimers. Since HLH may determine a dimerization region, myod1 and c-myc could form dimers through this region. The results obtained from c-myc transfection of C25, a myod1 negative myoblast, indicate that only at 8 fold normal levels can c-myc inhibit differentiation of C25 myoblasts. At intermediate (2-5 fold normal) levels, it is found that some clones exhibit an increase in differentiation markers, and an increase in their spontaneous expression. Homodimers of c-myc capable of inhibiting myogenin expression or action (DNA binding) may only form at very high concentrations of c-myc. However, at lesser concentrations, c-myc may be capable of forming functional heterodimers with myogenin via their leucine zippers. This would explain why an increase in spontaneous differentiation marker accumulation is observed in some clones expressing intermediate levels of c-myc. By stabilizing myogenin, c-myc may increase its positive autoregulation, and further amplify differentiation marker and myogenin expression, as exemplified by clone C5M5. Support for the possibility that c-myc can have such a dual role in these cells stems from

Table 11.
Helix-loop-helix proteins.

Mammalian genes:	
c-myc	Alt et. al. (1986), unknown function
N-myc	"
L-myc	"
E12	Murre et. al. (1989), binds kE2 DNA motif
E47	"
myod1	Davis et. al. (1987), induces myogenic phenotype
myogenin	Wright et. al. (1989), induces myogenic phenotype
myf5	Braun et. al. (1989), induces myogenic phenotype
Drosophila genes:	
achaete scute	Villares and Cabrera (1987), helps specify formation of central and peripheral nervous system of Drosophila complex (AS-C)
daughter-less	Caudy et. al. (1988), similiar function to AS-C
twist	Thisse et. al. (1988)
enhancer of split	Klaemat et. al. (1989)

the recent demonstration that the basic and c-myc homologous domain of myod is both necessary and sufficient to bind the muscle creatine kinase enhancer (Lassar, 1989).

Alternative homo-and hetero-dimerization provides one possible explanation of the data obtained by transfection. Future studies proving such interactions are presently being pursued by a variety of groups. However, evidence that at 8-fold normal levels, c-myc inhibits expression of myogenin (and possibly other myogenic determination genes), and not their action, stems from the failure of C5M15, but not other transfectants, to express myogenin. It has been recently demonstrated that c-fos and activated ras genes inhibit myogenesis of C2C12 cells by inhibiting myod1 expression (Lassar et. al., 1989), and not by direct action on muscle specific gene expression. Since C5M15 is the only c-myc transfected cell line which fails to accumulate muscle specific markers, and at the same time fails to express myogenin mRNA fails, future experiments should address whether transfection

of myogenin into this line can rescue c-myc mediated inhibition.

Some of the results obtained in this thesis conflicts with that found in the literature. Both L6 myoblasts and BC3H1 cells can be inhibited from differentiating upon c-myc overexpression (Payne et. al. (1987)). However, C2C12 cells have been reported to be refractory to this inhibition (Olsen et. al., 1987). In this thesis, it is shown that both C2C12 and C25 can be inhibited to differentiate by c-myc overexpression. It seems that C2C12 is more susceptible to this inhibition than C25, as evident from the number of differentiation defective clones generated by c-myc overexpression. Although the latter could be due to the relative instability of this line compared to C25, this would be apparent from the pSV2neo transfected clones. A larger pool of clones would have to be analyzed to make a more definitive conclusion. One would think that a line that eventually expresses myod1 and myogenin (C2C12) would be less susceptible to differentiation inhibition than a line that is myod negative,

expressing lower levels of myogenin, and minute levels of myf5 (C25). The increased frequency of C2C12 inhibition may be due to the ability of c-myc to inhibit myod1 expression or action relative to myogenin.

The myogenic determination genes, myod1, myogenin, and myf5 can induce a myogenic phenotype on non-myogenic cells (Davis et. al., 1988, Wright et. al., 1989, Braun et. al., 1989). In order to further delineate the roles each of these genes plays in the progression of myoblasts to myotubes, the non-fusing C2 variant, C25, is compared with the highly fusogenic C2 subclone, C2C12. One of the questions addressed is whether the phenotypic and biochemical differences observed between the two lines are reflected by differential expression of these myogenic determination genes. Phenotypically, the C25 line differs from the well documented C2C12 clone by its failure to fuse. Biochemically, it differs in its failure to express myod1, profile of fast TnT isoforms, and spontaneous expression of differentiation markers. Other lines have been

reported to lack myod1 expression (L6, [Wright et. al., 1989], and BC3H1, [Edmondson and Olson, 1989]. However, the generation of both myod1 positive (C2C12) and negative (C25) lines from the same parental line is a surprising finding. Two c-myc transfected C25 lines (C5M2 and C5M7) express myoD1 at levels detectable in northern analysis, suggesting that C25 has an intact myod1 locus. This indicates that the gene is either suppressed or its mRNA is highly unstable in the parental C25 line.

Similiarities between the two lines also exist. Both lines are derived from C3H thigh muscle, and express fast muscle isoforms, as detected by isoform specific antibodies against myosin heavy chain isoform and troponin T, cardiac actin, and smooth muscle actin. The expression of fast muscle isoforms in both lines implies that differential expression of myod1 and myogenin does not determine differences between fast and slow muscle types, in culture, but may instead delineate differences among fast muscles.

One observation supporting the hypothesis that differences among fast muscles may stem from differential expression of the myogenic determination genes is that the two lines differ in the ratios of fast troponin T isoforms. The fast TnT 1f, 2f and 3f proteins are generated from alternatively spliced forms of one gene (Medford et. al., 1984, Briggs and Schachat, 1987, and 1989, see Breitbart et. al., 1987 for review). These three isoforms contain functionally different N-terminal regions, which modulates the activation of the thin filament by calcium (Schachat et. al., 1987, Briggs and Schachat, 1989)). The TnT 4f isoform is thought to result from differential processing of 2f or 3f, however, it has not been reported to mediate a differential response to calcium. The C2C12 line predominantly accumulates TnT 1f and 2f, and low levels of 3f and 4f. Such a profile is similiar to that obtained for extracts of extensor digitorum longus probed with the same anti-fast troponin T monoclonal antibody (JLT-12, Briggs et. al., 1987). The C25 line accumulates 1f, 2f and 4f, and low levels of 3f. Tibialis anterior muscle extracts probed with JLT-12 generate profiles

similiar to C25 extracts (Briggs et. al., 1987). Although both lines originated from C2, which was derived from mouse thigh muscle, they express TnT isoform profiles typical of rabbit calf muscles. Whether this is a reflection of species differences, or a culture-induced phenomena is difficult to conclude from the available data (rabbit thigh muscle profiles are not given by Briggs et. al., 1987). Both tibialis anterior and extensor digitorum longus are fast muscles consistent with reactivity with the anti-troponin T monoclonal antibody, JLT-12. Because of the differences in myogenic determination genes expressed by the two lines, it is tempting to hypothesize that differential expression of myogenic determination genes, myod1 and myogenin, may delineate alternative splicing pathways or processing. By transfecting myod1 into the C25 line, this question could be directly addressed. Support for the hypothesis that myod1 expression is associated with predominating TnT 1f and 2f accumulation also derives from the myod1 expressing, C5M7 cell line. This cell line expresses an altered TnT profile, similiar to

C2C12, from the parental C25 line (see Figure 18, lane 7).

Although culture models simplify molecular analysis, difficulties often arise when correlating the results obtained with in vivo results. A recent report describes the differential expression of myod1 and myogenin during development. By in situ hybridization of mouse embryos, myogenin transcripts are detected earlier in the somites than myod1 (Sassoon et. al., 1989). Although both C25 and C2C12 are derived from the same adult mouse, these lines may reflect co-existing, persistant, myoblasts from different developmental stages. The differences in TnT isoform profile expressed by the two lines may result from such a difference. Since the developmental regulation of alternatively spliced TnT isoforms has already been reported in skeletal muscle (Medford et. al., see Breitbart et. al, 1987 for review), it is plausible that C25 and C2C12 are myoblasts committed to the fast lineage, but differ in their "stage" of differentiation. The hypothesis that developmental regulation of a few (so far four)

myogenic determination genes may eventually result in the generation of different muscle types is attractive, but as yet unsupported. The data obtained with C25 and C2C12 imply that myoblasts derived from the same source can express different complements of myogenic determination genes in culture. Future expriments delineating the roles of each of these genes will determine whether this is strictly a culture phenomena, or is also reflected in vivo.

The lack of myod1 expression in the C25 line suggests that it belongs to a class of myod1 negative myoblasts, which include BC3H1 (Edmonson and Olson, 1989) and L6 (Wright, unpublished) myoblasts. That myogenic cells are capable of expressing muscle specific proteins in the absence of this gene indicates myod1 is not essential in muscle gene activation. This particular step in the progression of myoblasts to myotubes can be replaced by other genes such as myogenin and myf5. The inability of C25 to fuse properly and its lack of expression of myod1 implies that myod1 may play an essential role in fusion. It is well documented that the BC3H1 line is

a non-fusing myogenic line (Olson et. al. 1983, Strauch et. al., 1986, Edmunson and Olson, 1989). Is the similiar non-fusing phenotype, between BC3H1 and C25, and the lack of myod1 expression in these two cell lines co-incidental, or causal? Support for a close association between myod1 and fusion stems from the generation of c-myc transfected clones, C5M7 and C5M2 from the parental C25 cell line. These lines express close to normal levels of transfected c-myc, express myod1 and are capable of myotube formation. The C5M7 line is more fusogenic than C5M2, is expresses higher myod1 levels. Howevr, direct transfection of myod1 into C25 cells failed to change their fusion potential, implying that myod1 may be obligatory, but not sufficient for fusion of myoblasts in culture. Future experiments involving the screening of individual clones, and generation of more variant lines, may resolve this question.

In addition to the difference in fusion capabilities and TnT isoform profiles between C25 and C2C12, a high level of spontaneous differentiation is observed for the C25 line, but not for C2C12. Here,

spontaneous differentiation is defined as the ability of myoblasts to induce muscle-specific marker accumulation, even in the presence of mitogens. In many of the experiments performed, C25 expressed MHC and TnT at low levels, even in mitogen-rich medium. These proteins were never detected in C2C12 extracts until after growth medium was replaced with HS-DMEM. This difference in "spontaneous differentiation" between the two lines could be attributed to the differential expression of myod1 and myogenin in the uninduced state. In mitogen rich medium, C25 expresses low levels of myogenin, whereas, C2C12 does not. The C2C12 line, does, however, express myod1 in mitogen rich medium. These results imply that myogenin may be able to activate muscle specific genes in the presence of mitogens; however, myod1 cannot function in this way until after mitogens are removed. In addition, the lack of myogenin expression in C2C12 cells in the presence of mitogens implies that myod1 may somehow prevent myogenin activation, until after mitogens are removed. That myod1 in myoblasts differs from myod1 in differentiated myotubes has already been demonstrated; the lack of DNA binding activity (muscle

creatine kinase) of myoblast derived myod1 compared to that of myotubes has already been reported (Lassar et. al, 1989). Further support for the close link of myod1 activity to mitogens, but not myogenin, stems from the ability of the BC3H1 line (myod1 negative, myogenin positive myoblast) to spontaneously differentiate (Olson et. al. 1983, Strauch et. al., 1986). Finally, transfection of C2C12 with c-myc indicates that it is possible to obtain c-myc inhibited clones at a much higher frequency than with C25. One possible reason for these results is that myod1 expression in the myoblast state can affect myogenin expression, thereby decreasing spontaneous differentiation and increasing the likelihood of c-myc induced differentiation inhibition.

In addition to experiments performed with c-myc, a culture system capable of delineating the roles myod1, myogenin and myf5 play during the progression of myoblasts to myotubes has been developed. The C2 derivatives C2C12 and C25 express different complements of these myogenic determination genes. These two lines are ideal for comparing myogenesis in

two cell lines expressing different complements of the myogenic determination genes, myod1 and myogenin. Major phenotypic differences between the two lines indicate that myod1 may be obligatory, but not sufficient for fusion to occur, and that the presence of the former in myoblasts may actually prevent spontaneous differentiation.

The knowledge of nuclear transcription factors, and their role in myogenesis has increased greatly since the original heterokaryon experiments implying the presence of diffusable, dominantly acting myogenic genes (Silberstein et. al., 1985). Not only have these factors been identified, but their DNA binding domains, and sequence targets have also been reported. The homology of their DNA binding domains with the c-myc family of genes supports the hypothesis that c-myc is involved in differentiation, and also implies a mechanism by which it may do so. This thesis has utilized transfection to determine that deregulated c-myc expression can inhibit myoblast differentiation, and proposes a mechanism of this inhibition.

CONCLUSIONS

1. During C2 myoblast differentiation, c-myc mRNA levels decrease transiently, increase, and then decrease again after differentiation has proceeded.

2. The decrease of c-myc mRNA observed during myoblast differentiation occurs at the transcriptional level.

3. Biochemically differentiation defective myoblasts do not decrease c-myc mRNA levels upon differentiation induction.

4. Transfection of the c-myc gene into C2 myoblasts and a homogeneous subclone (C25) inhibits the accumulation of muscle-specific markers MHC and TnT, and myogenin expression. Such inhibition requires at least 8 fold higher than normal expression of c-myc mRNA.

5. Some lower level c-myc expressing clones (2-5 fold normal levels) show increased accumulation of myogenic

markers, implying a concentration dependant effect of c-myc in these cells.

6. The C25 subclone of C2 myoblasts belongs to a class of myod1 negative, myogenin positive, myf5 positive myogenic cells. These cells are capable of biochemical differentiation, but not fusion.

7. The C25 cell line differs from the myod1 positive cell line (C2C12) phenotypically by its inability to fuse, spontaneous expression of differentiation markers, and biochemically by accumulating higher ratio of fast troponin TnT and 4f in comparison to 1f and 2f.

8. Co-culturing C25 with a differntiation defective line, FD-2, generates a mixed population of cells, capable of forming myotubes.

METHODS

Cell culture: C2 mouse myoblasts were obtained from Dr. L. Rubin, Rockefeller University; his lab had obtained a stock of these cells from Dr. H. Blau, Stanford University. These cells were originally derived from adult, C3H mouse thigh muscle (Yaffe and Saxel, 1977). Mouse C2C12 myoblasts were obtained from ATCC (American Type Tissue Collection). They are a clonal derivative of these C2 myoblasts generated by Dr. Blau (Stanford). The C25 myoblast cell line was derived for this thesis by subcloning the C2 cells obtained from Dr. L. Rubin (Rockefeller). About 15 cells were plated in a 150mm dish in conditioned medium, and allowed to form colonies. Over 25 clones were picked in cloning cylinders, and screened for myosin heavy chain accumulation (MF20, Western blotting). The C25 colony was chosen on the basis of its ability to accumulate the highest level of myosin heavy chain. All myoblast lines were grown in Dulbecco's Modified Eagle's medium (DMEM), containing 20% fetal calf serum, 1% chick embryo extract, and 4500 mg/liter glucose (DMEM/CEE). Cells were

trypsinized every three to four days and placed in fresh growth medium at 1:15 to 1:20 the original dilution. Cells were induced to differentiate when they were 70-80% confluent by washing with phosphate buffered saline, and replacing with DMEM + 4% horse serum (DMEM/HS). Differentiation induced cells were fed with this medium every three days.

In order to obtain fusion deficient lines, C2 cells were allowed to differentiate for 8 days in fusion medium, at which time medium at 4°C was added. This removed the fused layer of cells and left a few mononucleated cells which had failed to fuse. This method was derived from our observation that differentiation induced cells detach readily from the plate when fed with medium at 4°C, instead of medium that has been prewarmed to 37°C. The remaining few cells were washed in PBS, and returned to DMEM/CEE. These fusion deficient and differentiation defective cells were subcloned (plated at clonal density in conditioned medium, and individual clones picked). The individual clones generated from this procedure (FD-2 and FD-6) were screened for myosin heavy chain

(MHC) accumulation by immunoblotting. Although these lines are referred to as fusion deficient, they also fail to accumulate muscle specific markers, MHC or TnT.

Co-culture: The ability of two fusion deficient lines FD-2 and C25 to fuse upon co-culturing was demonstrated by the following: both lines were grown to 70% confluence in separate t-75cm^2 flasks, trypsinized, and counted. 10^5 cells from each line were co-incubated in 35 mm tissue culture dishes for the 1:1 ratio. The remaining ratios of C25:FD-2 were performed according to the following table (35 mm dish):

C25: FD-2 ratio	#C25 cells	#FD-2
1:1	10^5	10^5
1:5	$4x10^4$	$2x10^5$
1:10	$2x10^4$	$2x10^5$
1:20	10^4	$2x10^5$
1:40	$5x10^3$	$2x10^5$
1:80	$2.5x10^3$	$2x10^5$
1:160	$1.25x10^3$	$2x10^5$
1:320	$6.125x10^2$	$2x10^5$
control 1:0	$2x10^5$	0
control 0:1	0	$2x10^5$

These cells were allowed to incubate in growth medium overnight. The following day, the cells were washed once in phosphate buffered saline (20 mM sodium phosphate (pH 7.2), 150 mM sodium chloride, PBS), and fusion medium was added (HS medium). By 72 hours, myotubes were apparent in all plates except the controls and 1:1 incubations.

Limiting dilutions of the FD-2 cells with C2-5 (the reverse ratios) failed to produce myotube formation.

Myoblast Transfection: The calcium phosphate mediated DNA transfection protocol, as described by Graham and Van der Eb (1973), was utilized for all transfections. Myoblast lines were first titrated for sensitivity to G418. All parental C2 lines were completely killed within day 8-10 after exposure to G418 at a concentration of 1.0 mg/ml (active concentration, 425 micrograms/ml). The protocol for G418 selection was that of Southern and Berg (1982). Typically, cells were plated on 100 mm tissue culture dishes; 6 micrograms of pSVmyc1 + 1 microgram of pSV2neo supercoiled plasmid DNA was first added to 1ml HBS (140 mM NaCL, 5 mM KCL, .7 mM Na_2HPO_4, 6mM dextrose, 20 mM Hepes pH 7.05-7.1). Calcium chloride to 125 mM was then added to this mixture by adding .05 ml of 2.5M $CaCl_2$. The DNA was allowed to precipitate at room temperature for 15-20 minutes and added to 50-60% confluent cells. After 24 hours, the cells were washed 3 times in PBS, trypsinized, and replated at 1:5 dilution. Cells were allow3d to grow an

additional 24 hours, at which time, G418 was added at 1 mg/ml. Subsequently, the medium on the cells was replaced with DMEM/CEE + 1 mg/ml G418 every 4 days. Individual colonies were large enough to trypsinize in cloning cylinders made of Tygon tubing by 14 days after transfection. Thereafter, cells were maintained in 0.5 mg/ml G418, until differentiation induction. HS medium did not contain G418, as this was found to reduce MHC accumulation.

Immunofluorescence and immunoperoxidase staining of cells: For immunofluorescent microscopy, myoblasts were grown in 35 mm tissue culture dishes, and induced to differentiate in HS medium. Cells remained in 35 mm dishes throughout the procedure. They were washed 2 times with PBS, and fixed for 15 minutes in 3.75% paraformaldehyde in PBS, at room temperature. The cells were washed once with PBS and permeabilized in PBS containing .1% Triton. Cells were washed 3 times in PBS before incubation of first antibody (MF20, anti-myosin heavy chain (MHC) monoclonal antibody). First antibody was diluted 1:1000 in PBS containing 10% horse serum, and incubated in a humidified chamber

for 1 hour at 37°C. Cells were again washed 3 times with PBS. Second antibody incubations were performed in PBS + 1% BSA containing a 1:100 dilution of rhodamine conjugated, goat anti-mouse antibody (Boehringer Mannheim) for 45 minutes at 37°C. Cells were washed 3x with PBS, mounted in aqueous mounting medium (Geltol), and visualized in a Nikon fluorescent microscope, using a filter. Controls without first antibody indicated that background reactivity of the second antibody with the cells was negligible.

Immunoperoxidase staining was performed on colonies generated by transfection with the approprate plasmid(s) and selection for G418 resistance. Such colonies were allowed to grow to a size of 2-3 mm in diameter, and fixed in AFA (acetic acid, formaldehyde, ethanol), for 20 minutes at -20 C. It was found that 3.75% paraformaldehyde fixation yielded poor results for this procedure, however AFA or methanol fixation at -20°C did not inhibit peroxidase activity. After fixation with AFA, cells were washed 3 times with PBS at room temperature, and incubated in first antibody (MF20, anti-MHC) at 1:1000 dilution in PBS containing

10% horse serum for 1 hour at 37°C, in a humidified chamber. They were washed 3 times with PBS at room temperature and incubated with peroxidase conjugated, goat anti-mouse antibody (Sigma) at a 1:1000 dilution in PBS + 1% BSA, in a humidified chamber at 37°C, for 1 hour. After three washes in PBS, the peroxidase reaction was developed in PBS containing DAB (diaminobenzidine) 30mg, + .075 ml 30% H_2O_2 /100ml H_2O, for 15-20 minutes. Positive colonies appeared dark brown. Control plates without first antibody yield all clear colonies, indicating that backgroud reactivity of the second antibody was negligible. Plates were photographed using a polaroid camera, and ASA 3000-speed film. After photographing the peroxidase reaction, colonies were stained with a 0.5% crystal violet solution, and rinsed with ddH_2O to visualized the presence of any MHC negative colonies.

Immunoblotting: All antibodies used for immunoblotting are listed in Table 1. Polyacrylamide gels were run using a discontinuous buffer system (Laemmli, 1970). For blotting of myosin heavy chain, 7.5% gels were run; for c-myc, desmin, actin, and

Table 1. List of Antibodies

Monoclonal Antibodies

Name	Description	Source, Reference
MF20 mAb	myosin heavy chain, embryonic, skeletal ascites generated by injection into nude mice immunoblotting: 1:2000 immunofluorescence: 1:500 immunoperoxidase cell labelling: 1:1000	Developmental Studies, Hybridoma Bank, Bader et. al. (1982)
D3 mAb	desmin, ascites generated by injection into nude mice same dilutions as for MF20	Developmental Studies, Hybridoma Bank, Danto et. al. (1984)
JLT-12 mAb	Troponin T, fast isoform specific reacts with 1f, 2f, 3f, 4f, ascites same dilutions as for MF20	SIGMA, Lin, JJC et. al. in Monoclonal Antibodies and Functional Cell Lines, Kennett, RH, et. al. (eds), Plenum Press, pg. 119, (1984)
1A4 mAb	smooth muscle actin ascites same dilutions as for MF20	SIGMA, Skalli et. al., Cell Biol. 103:2787 (1983)
1F7 mAb	human c-myc peptide against amino acids: 305-317 purified (10 mg/ml) immunoblotting: 5 micrograms/ml	Dr. R. Chizzonite, Butnick et. al. (1985) and Miyamoto et. al. (1985)
F27 mAb	myosin heavy chain fast isoform specific culture supernatant immunoblotting: 1:50	Dr. Frank Stockdale, Stockdale and Miller, (1987)
S58 mAb	myosin heavy chain slow isoform specific culture supernatant immunoblotting: 1:50	Dr. Frank Stockdale, Stockdale and Miller, (1987)

Polyclonal Antibodies:

Name	Source
rabbit anti-desmin	SIGMA immunoblotting: 1:5000
goat anti-mouse IgG+IgM, alkaline phosphatase conjugated	Boehringer Mannheim immunoblotting: 1:7500
goat anti-mouse IgG, alkaline phosphatase conjugated	Promega Biotec immunoblotting: 1: 7500
goat anti-mouse IgG, peroxidase conjugated	Boehringer Mannheim immunoblotting: 1:1000
goat anti-mouse IgG, peroxidase conjugated	SIGMA cell labelling: 1:1000
goat anti-mouse IgG, rhodamine conjugated	Boehringer Mannheim immunofluorescence: 1:100

troponin T, 10% or 5-15% gradient gels were poured, as indicated in each figure legend. Prestained protein markers were obtained from Sigma and blotted alongside samples. Cellular extracts were made by washing cells grown in T-25cm^2 flasks with PBS and lysing in Laemmli sample buffer (1ml/flask). Equivalent protein amounts were loaded in each lane, as assessed by Coomassie blue staining. Transfer to nitrocellulose was performed according to Biorad, at 4°C, 150-200 mAmp, for 24 hours. The transfer buffer contained 20mM Tris base, 150mM glycine, and 20% methanol, prechilled to 4°C for at least 12 hours before use. Gels, nitrocellulose and 3MM blotting paper were all soaked in cold transfer buffer prior to transfer, 30 minutes, 4°C. After transfer, the nitrocellulose was fixed with 25% isopropanol + 10% acetic acid for 20 minutes and washed 2x with TBS (50 mM Tris pH7.6, 150 mM NaCl). Blots were blocked with 10% nonfat dry milk in TBS for 1 hour at room temperature. After this was poured off, the blot was incubated with first antibody diluted in TBS + 0.2% Tween 20, 10% horse serum and 2% BSA (TBS-THB) for 1 hour at room temperature or overnight at 4°C. Incubations were

performed with constant agitation. First antibody dilutions differed for each antibody: 1:2000 dilution (ascites, MF20 and D3), 5 micrograms/ml (purified, 1F7 10 mg/ml), 1:5000 (rabbit polyclonal antibody, anti-desmin), 1:10 (culture supernatant, anti-fast (F27) and anti-smooth (S58) MHC specific). Anti-c-myc antibody (1F7) was provided by Dr. R. Chizzonite as a purified 10 mg/ml preparation. This antibody was used at 5 micrograms/ml in TBS-THB containing 0.5 M NaCl (TBS.5M-THB), because no signal was obtained with TBS containing 0.15 M NaCl. MF20, and D3 hybridoma cells were purchased from the Developmental Studies Hybridoma Bank, propagated in culture, and injected into nude mice for generating ascites. Anti-fast (F27) and slow (S58) myosin heavy chain monoclonal antibodies were sent in culture supernatant form by Dr. F. Stockdale. These were used at 1:10 dilution. Other antibodies, JLT12 (anti-troponin T), 1A4 (anti-smooth muscle actin), and polyclonal anti-desmin were purchased from Sigma. After incubation with first antibody, the blots were washed in 2 changes of TBS + 0.1% Tween (TBST) for 15 minutes. Second antibody incubations were performed for 1 hour at 4 C

in TBS +.2% Tween + 2% BSA (TBST-B) with either peroxidase (1:1000) or alkaline phosphatase (1:7500) conjugated goat anti-mouse antibody (Boehringer Mannheim). Alkaline phosphatase conjugated goat anti-rabbit antibody (Promega Biotech, 1:7500) was used for blots probed with the polyclonal desmin antibody. Alkaline phosphatase was more sensitive and its color reaction did not fade, unlike the peroxidase substrate color reaction. Therefore, it was used almost exclusively in later experiments. The blots were washed 3 times in TBST, and once in TBS containing 0.5 M NaCl. Peroxidase reactions were developed in 1,4 chloronapthol, whereas alkaline phosphatase were developed using Vector Laboratories substrate kit SK-5200, following their instructions. Bands were usually visible within 2-10 minutes for alkaline phosphatase conjugated antibodies, and 10-20 minutes for peroxidase conjugated antibodies.

Isolation of nuclei for immunoblotting c-myc: Crude nuclei were obtained from cells grown in mitogen rich medium by a slight modification of the method described by Evan and Hancock (1985). Myoblasts were

grown to 60-70% confluence, washed 2 times in PBS, 4°C, and lysed in 3 ml of 20 mM Hepes pH 7.2, 5 mM KCl, 5 mM $MgCl_2$, 0.5% NP40, 0.1% deoxycholate, 1% aprotinin, 0.1mM phenylmethylsulfonylchloride. Cells were scraped on ice, homogenized in a glass dounce 10 times, and centrifuged at 1000 xg for 5 minutes, 4°C. The pellet containing crude nuclei was resuspended in 0.5 ml of 1X Laemmli buffer; 0.5 ml of 2X Laemmli buffer was added to 0.5 ml of the supernatant from this centrifugation to yield the cytoplasmic fraction. Approximately $5x10^7$ nuclei per cell line was obtained. Typically, the equivalent of 10^7 nuclei was loaded per lane for immunoblotting purposes. Nucleii were isolated to enrich for c-myc and demonstrate its expected localization in this fraction. The signal obtained for c-myc protein by immunoblotting whole cell extracts was below the detection level of this assay.

Plasmid DNA Preparation: E.coli containing plasmids were maintained as frozen stocks in glycerol at -70 C. Table 2 lists plasmids used, their sources

Table 2. Probes Relevant to This Thesis

plasmid	description	Source, Reference
pSVmyc1	Xba-BamH1 4.5 kb mouse c-myc, in the Hind III-BamH1 site of pSV2gpt	ATCC, #41029, Land and Weinberg (1983)
pSP64myc	Hind III-BamH1 4.5 kb mouse c-myc, in the Hind III-BamH1 site of pSP64	Dr. R. Gubits, unpublished
pMc-myc54	2.2 kb mouse c-myc cDNA from Balb/C, in the Pst1 site of pBR322	Dr. C. Croce Stanton et. al. (1983)
pNB-1	1 kb EcoR1-BamH1 fragment of N-myc	Dr. F. Gilbert, Schwab et. al.,(1983)
cH-ras-1	2.9 kb SacI fragment of normal ras	Oncor, inc., Pulciani et. al., (1982)
p1-19	28S gene in pBR322	Dr. Cheng-Kiang, Tiemeier et. al. (1977)
pHmcA-3'-UT-DB	bp 1-171 of human cardiac actin 3' untranslated region, isotype specific	Dr. L. Kedes, Bains et. al. (1984)
pHh5B	human histone H3 EcoR1 fragment in pBR325	Dr. N. Heintz, Heintz et. al. (1983)
pEMC11s	myod1 EcoR1 fragment in the EcoR1 site of pEMSVscribeα2, sense orientation	Dr. A. Lassar, Davis et. al. (1988)
Hmyf4	myogenin (myf4), human myogenin cDNA fragment	Dr. H. Arnold, Braun et. al. (1989)
Nmyb1815	myf5, human cDNA fragment	"
pEMSVscr-ibeα2	eukaryotic cDNA expression vector	Dr. A. Lassar, Davis et. al. (1988)
pSV2neo	eukaryotic cDNA expression vector	Dr. S. Goff, Southern and Berg (1982)
pBR322	cloning vector	Dr. S. Goff, Bolivar et. al. (1977)

and references. E.coli were streaked on ampicillin or tetracycline containing agar plates. Plasmids were isolated according to the protocol of Katz et. al., (1973). Single colonies were picked and grown in 10 ml of L-broth (10 gm bactotryptone, 5 gm yeast extract, 1 gm dextrose, and 10 gm NaCl in 1 liter of double distilled H_2O). Two milliliters of this overnight was used to inoculate 500 ml of L-broth containing the approprate antibiotic. After 6 hours of shaking at 250 rpm, 37 C, chloroamphenicol was added, and incubation was allowed to occur an additional 24 hours. Cells were kept on ice during harvesting, pelleted at 5000 rpm (Sorvall GS-3 rotor), and resuspended in TES (10 mM Tris HCL pH 7.5, 1 mM EDTA, .15 M NaCl). Cells were pelleted again and resuspended in 6 ml sucrose buffer (25% sucrose, 50 mM Tris 7.5). Lyzczyme was added (6 mg) and incubation continued on ice for 15 minutes; 2.4 ml .25M EDTA, and 9.6 ml Triton buffer (.1% Triton X100, 10 mM EDTA, 50 mM Tris pH 8) was added and the mixture incubated for 15 minutes. Bacterial DNA and debris were pelleted in a SW27 rotor at 25,000 rpm for 45 minutes. The supernatant was made 17.4 ml with TE and 17.6 gm CsCl

+ 1.8 ml of ethidium bromide (10 mg/ml) was added. Centrifugation of this mixture was performed in a Ti50 rotor, 37,000 rpm, for 48 hours. The plasmid band was visualized with a long wave UV lamp, isopropanol extracted, diluted in TE, and ethanol precipitated. Yields varied from 300 micrograms to 1 mg for a 500 ml preparation. All restriction enzyme digestions were carried out according to manufacturer's suggestions. DNA fragemnts were isolated using gene clean Bio101, according to manufacturer's suggestions.

Northern blotting: RNA was isolated according to an established protocol described by Maniatis et. al. (1982), and originally reported by Chirgwin et. al. (1979) using guanidine isothiocyanate, and subsequent pelleting through a cesium chloride cushion. RNA samples were further prepared for formaldehyde gel electrophoresis as described by Maniatis (1982). Formaldehyde gels contained 1.5% agarose, 40 mM MOPs, pH7.0 (morpholinopropanesulfonic acid), 10 mM sodium acetate, 1 mM EDTA, pH 8.0, 2.2M formaldehyde; 10 micrograms of total RNA was loaded per well, unless otherwise indicated. Transfer of RNA

from formaldehyde gels to Gene Screen Plus (GSP) was performed according to the manufacturer. Whatman 3MM paper was soaked in 10xSSC and laid on a glass plate. Gels were laid on top of this; GSP which had been prewetted in 10xSSC was placed on top of the gel, pushing out all bubbles. About 4 inches of absorbent paper was layered on top of this sandwich. Transfer was allowed to occur overnight. The RNA was UV cross-linked to the GSP for 3 minutes and baked for 2 hours at 80°C. For hybridization with nick translated probes the following protocol was utilized: blots were prehybridized at 65°C in 10% dextran sulfate, 1M NaCl, 1% SDS, 250 micrograms/ml herring sperm DNA, 500 micrograms/ml yeast RNA (Boehringer Mannheim), and 20 micrograms/ml poly A (SIGMA). The prehybridization solution was discarded and the hybridization was performed in the same formulation containing 10^6 cpm/ml of nick translated probe. Blots were washed in 2 changes of 2xSSC + 1%SDS, 65°C, and 2 changes of 0.1xSSC room temperature, and exposed to Kodak XAR film for 1-8 days. For riboprobe hybridizations, the prehybrization was performed in 50% deionized formamide, 10% dextran sulfate, 2% SDS, 1M NaCl, 250

micrograms/ml herring sperm DNA, 500 micrograms/ml yeast RNA, and 20 micrograms/ml poly A. Hybridization solutions contained 10^6 cpm/ml of probe. Blots containing riboprobes were washed in 3 changes of 0.1xSSC + 0.1%SDS at 65 C, rinsed in 0.1xSSC and exposed to Kodak XAR overnight. Nick translations were performed according to Rigby et. al. (1977). A typical reaction volume contained 250 ng of DNA (2.5 microliters), 10 microliters [alpha]^{32}PdCTP (10 microcuries), 2.5 microliters 10xNTB(0.5M Tris pH 7.8, 50 mM $MgCL_2$, 100 mM 2-mercaptoethanol, 0.1 mg/ml BSA), 0.5 microliters 1 mM deoxriboynucleoside triphosphates (dTTP, dGTP, dATP), 8 microliters H_2O; one microliters of 1 ng/ml DNASE (Worthington) was added to the reaction for 15 minutes on ice. The reaction was incubated with 1 unit E. coli DNA polymerase for 1.5 hours, 15°C. Nick translation was stopped with an equal volume 0.2 %SDS + 20 mM EDTA, and labelled DNA was purified on a spun column containing Sephadex G-50. Riboprobes were generated according to Melton et. al., (1987). The pSP6myc plasmid was provided by Dr. R. Gubits. It was linearized with Bgl1 so that only exon 3 was labelled. A typical reaction

contained 100 ng linearized plasmid (7 microliters H_2O), 10 lambda [alpha]^{32}PCTP (10 microcuries), 10 microliters 5xtranscription buffer (200 mM Tris pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine), 5x nucleotides (2.5 mM each GTP, ATP, UTP, 0.11 mM CTP), 2 microliters RNASIN (Promega Biotec), 5 microliters 0.1M DTT, 5 microliters 1 mg/ml BSA, 1 microliters (SP6 polymerase, Promega Biotec). The transcription reaction was allowed to occur at 40°C for 1 hour. Following this, the DNA template was removed by adding 2 microliters RNASIN and DNASE (Worthington) to a final concentration of 20 micrograms/ml, for 10 minutes at 37°C. Enzymes were inactivated by the addition of SDS to 0.2%; labelled RNA was purified by Sephadex G-50 spun column chromatography.

In vitro transcription: Nuclei were isolated by the method of Greenberg and Ziff (1986). Briefly, cells were grown in 150mm tissue culture plates and harvested at the appropriate time after differentiation induction. Each plate was washed 3 times in ice cold PBS, lysed in 3 ml of ice-cold NP40 buffer, and scraped with a rubber policeman. This mixture was then vortexed at medium speed 3 times,

incubated on ice for 5 minutes, and spun at 1800 rpm in a Beckman J6B clinical centrifuge for 5 minutes, 4°C. The pellet was washed with NP40 buffer and spun again at 1800 rpm, 5 minutes, 4°C. The resulting nuclear pellet was resuspended in 0.1 ml storage buffer (25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 5 mM DTT, 50 mM Tris-HCL, pH 8.0), flash frozen in liquid nitrogen, and stored in liqiud nitrogen. Transcription reactions were performed according to Marzluff and Huang (1984). For a 0.2 ml reaction volume, .05 ml of solution A (2 mM each ATP, CTP, 0.2 mM each [alpha-^{32}P]GTP, [alpha-^{32}P]UTP (1 mCi/ml), 0.1 mM S-adenosylmethionine), .04 ml of solution B (.6M KCL, 12.5 mM magnesium acetate) and .01 ml H_2O were mixed. The reaction was started by adding 0.1 ml of nuclei in storage buffer. This mixture was incubated for 30 minutes at 25°C. Reactions involving alpha-amanitin were preincubated with 100 micrograms/ml before the addition of nuclei. The reaction was stopped by the addition of 10 volumes of solution C (1 % SDS, 10 mM EDTA, pH 7.0), thereby lysing the nuclei. In order to extract the newly formed RNA, 1/10th volume of 2 M sodium acetate, pH

5.0 was added. The mixture was extracted with an equal volume of phenol-chloroform (2:1), and incubated at 55 °C for 5 minutes, then cooled on ice for 5 minutes and spun at 10,000 xg for 10 minutes at 4 C. The upper phase was recovered, ethanol precipitated, dried, washed with 70% ethanol, and dissolved in 0.5 ml of 0.3 M Nacl, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCL, pH 7.5. Unincorporated nucleotides were removed by Sephadex G50 spun column chromatography (Molecular Cloning Manual, 1982). The RNA was recovered in the void volume, counted and used directly to hybridize to DNA immobilized on Gene Screen Plus. The plasmids were cesium chloride purified, as described above, linearized with the appropriate enzyme, phenol/chloroform extracted and precipitated. These plasmids were dissolved in NH_4OH, 2 M NaCl at a final concentration of 50 micrograms/ml. After denaturing (boiling at 100°C for 5 minutes), DNA (10 micrograms) was bound to Gene Screen Plus that had been presoaked in 2xSSC and assembled in a slot blot apparatus (Schleicher and Schuell). Prehybridization was performed at 42°C overnight in 50% formamide, 10% dextran sulfate, 250 micrograms/ml denatured herring

sperm DNA, 0.05 micrograms/ml poly(A), 1 M NaCl, 2% SDS, 1 mM Tris-HCL, pH 7.5, 1 mM EDTA, pH 7.5. Hybridization to labelled RNA was performed for 20 hours with equal number of counts for each strip ($2x10^5$ cpm/ml). Strips were washed at 52 C, 2xSSC, .1%SDS, 1 mM EDTA, 10 mM Tris, pH 7.5 for 30 minutes, and .1xSSC at room temperature for 30 minutes. They were dried and exposed to Kodak XAR5 film for 3-7 days at -70 C.

APPENDIX

List of Terms and Abbreviations:

topo II	topoisomerase II
DH	DNAase hypersensitive
GM-CSF	granulocyte macrophage, colony stimulatory factor
TNF	tumor necrosis factor
PDGF	platelet derived growth factor
DMSO	dimethlysulfoxide
pol	polymerase
MEL	mouse erythroleukemia cell line
SDS-PAGE	sodium dodecyl sulfate, polyacrylamide gel electrophoresis
$pp62^{myc}$	phosphoprotein, c-myc, migrating at 62 kd during SDS-PAGE
snRNP	small nuclear ribosomal binding proteins
HLH	helix loop helix

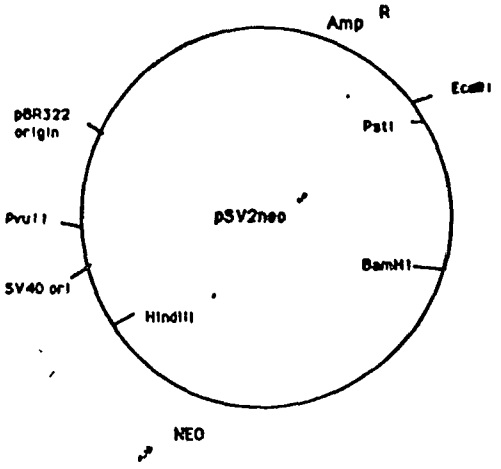
MHC	myosin heavy chain
TnT	troponin T
DMEM	Dulbecco's modified Eagle's medium
FCS	fetal calf serum
PBS	phosphate buffered saline
TBS	Tris buffered saline
mitogen rich, serum rich	Dulbecco's modified Eagle's medium, supplemented with CEE-DMEM 20% fetal calf serum and 1 % chick embryo extract
mitogen poor, serum poor	Dulbecco's modified Eagle's medium, supplemented with HS-DMEM 4% horse serum

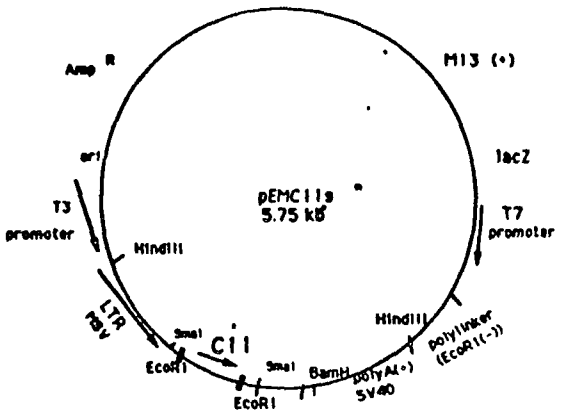
*Differentiation: Unless otherwise noted, myogenic differentiation is defined as the ability of myoblasts to accumulate muscle-specific proteins in response to serum deprivation.

List of Cell Lines:

C2M	non-clonal mouse muscle cell line, originally isolated by Yaffe (1977)
C25	biochemically differentiating, non-fusing muscle cell line, derived from C2M
C2C12	Biochemically differentiating, fusogenic muscle cell line, derived from C2M by Blau (1983)
FD-2, FD-6	fusion deficient, biochemically non-differentiating, "muscle" cell line derived from C2M by selection
MN	C2M derived clones, transfected with pSVmyc1 and pSV2neo, and G418 selected
C5M	C25 derived clones,

	transfected with pSVmyc1 and pSV2neo, and G418 selected
C5M15	pSVmyc transfected, C25 clone, expressing the highest levels of c-myc, and differentiation inhibited
pSV	C25 derived clones, transfected with pSV2neo, and G418 selected



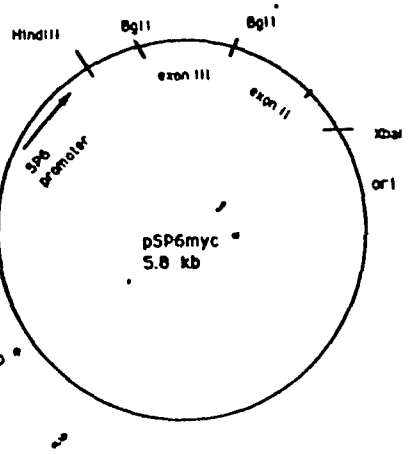


C11= 1.85 kb Fragment of myod cDNA

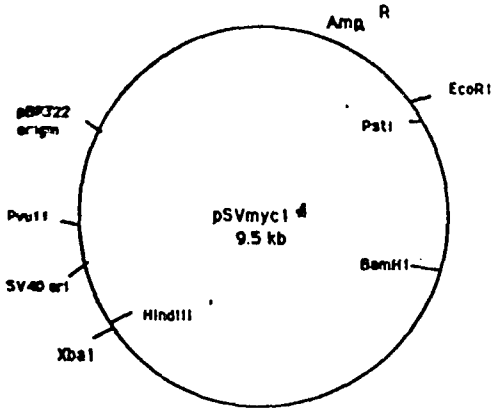
MSV-LTR is 439 bp HindIII-EcoR1 fragment
SV40 polyA (+) is 253 bp HindIII-EcoR1 fragment | from EMSV-33

The rest of the vector is Bluescribe M13(+) now EcoR1(-) (Stratagene)

*obtained from A. Lassar



* Obtained from R. Gubits
2.8 kb HindIII-XbaI fragment of pSVmyc1 in pSP64, sense orientation



C-MYC: 4.5 kb Xba-BamHI fragment

*Obtained from ATCC

Table 6.
Densitometric Scanning of Western Blots
of Differentiating C2C12 and C25 Myoblasts

	days	Embryonic MHC	Fast MHC	Desmin	Smooth Muscle Actin	TnT
C2C12:	0	0.58	0.00	3.11	0.65	0.45
	1 day	1.97	0.82	3.44	1.76	2.19
	3 day	2.90	0.92	3.25	2.00	1.93
C25:	0	2.46	0.53	3.72	2.45	1.78
	1 day	3.53	0.99	3.96	3.53	1.80
	3 day	2.65	0.58	2.20	2.83	1.50

Table 7.

Densitometric Scanning of Northern Blots of

Differentiating C2C12 and C25 Myoblasts.

		PROBE				
cell line/days		MYC	MYF5	MYOD1	MYOGENIN	28S
C2C12:	0	0.53	0.34	8.90	0.45	3.33
	1 d	0.28	0.27	8.20	9.66	0.32
	3 d	1.45	0.27	11.00	8.40	3.56
C25:	0	0.47	0.70	0.57	2.25	2.15
	1 d	0.52	0.60	0.98	6.00	2.33
	3 d	1.00	0.39	0.52	6.42	2.86

Table 8.

Densitometric Scanning of Western Blots of

pSVmyc Transfected C25 Myoblasts

C25 Transfected lines	Whole Cell Extracts					Nuclei
	MHC 0 d	TNT 0 d	MHC 4 d	TNT 4 d	DESMIN 4 d	MYC 0 d
pSVmyc + pSV2neo:						
22	0.35	0.20	1.30	5.50	2.40	
15	0.40	0.20	0.65	2.41	2.21	1.44
13	0.32	0.39	1.05	5.13	2.06	
11	0.33	0.30	1.78	4.91	1.78	
10	0.29	0.41	1.98	4.91	1.65	
9	0.22	0.37	2.13	4.34	1.50	0.96
8	1.60	0.33	1.83	4.40	1.50	
7	1.71	1.21	2.20	5.13	1.50	0.76
6	0.35	0.32	1.18	3.92	1.92	1.04
5	3.50	3.46	2.73	5.26	2.10	1.10
4	0.87	1.92	1.94	5.72	2.33	
3	0.50	0.55	2.67	4.71	2.14	1.0
2	1.15	1.14	3.35	5.00	2.13	0.96
1	0.73	0.70	2.57	5.35	2.11	1.01
19	0.76	0.20				
18	0.83	0.27				
17	1.07	0.29				
14	1.90	0.20				
pSV2neo	0.80	0.70	2.18	4.71	2.00	0.86
C25	0.90	1.07				0.75

Table 9.

Densitometric Scanning of Northern Blots of pSVmyc

Transfected C25 Myoblasts

Transfected			PROBE			
line	C-MYC	28S	MYOG	MYOG	MYOD1	MYOD1
			0 d	4 d	0 d	4 d
pSVmyc+pSV2neo						
15	10.9	1.3				
9			0.3	5.8	0.3	1.7
8			0.8	4.4	0.3	1.7
7	1.7	6.8	2.6	5.1	2.0	7.2
6	7.5	6.9	1.2		0.3	
5	5.2	6.1	3.8	5.0	0.3	1.0
4	8.3	1.1				
3	4.7	7.3	1.3		0.4	
2	2.5	8.7	3.4	4.9	1.0	2.7
1	6.3	10.2	2.8		0.5	
C25	1.3	6.5				
pSV2neo			3.3	2.7	0.7	1.0

Table 10.

Densitometric Scanning of pSVmyc Transfected C2M Myoblast Northern and Western Blots.

C2M Transfected line	MHC (Protein) 4 d	MYC (mRNA) 0 d	4 d
27	0.61	4.61	1.84
19	0.17	7.92	7.47
16	0.99	4.82	4.45
8	1.61		
6	0.92	1.85	4.47
4	0.29	6.54	7.94
3	0.55	6.20	6.85
1	0.55		5.15
C2	0.42	0.63	0.10

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