DIFFERENTIATION OF RECOMBINANT MYOBLASTS IN ALGINATE MICROCAPSULES

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ABSTRACT

A cost effective approach to the delivery of therapeutic gene products *in vivo* is to immunoprotect genetically-engineered, universal, non-autologous cells in biocompatible microcapsules before implantation. Myoblasts may be an ideal cell type for encapsulation due to their inherent ability to differentiate into myotubes, thereby eliminating the problem of cell overgrowth within the capsular space. To evaluate the interaction between the differentiation program and the secretory activity of the myoblasts within the microcapsule environment, we transfected C2C12 myoblasts to express human growth hormone and followed their expression of muscle differentiation markers, such as creatine phosphate kinase (CPK) protein and up-regulation of muscle-specific genes (ie. myosin light chains 2 & 1/3, Troponin I slow, Troponin T, myogenin and MyoD1).

As the transfected myoblasts were induced to differentiate for up to two weeks, their myogenic index (i.e. the percentage of multinucleate myoblasts) increased from 0 to ~50%. Concomitantly, up-regulation of differentiation marker RNA levels, and as much as a 23-fold increase in CPK activity, were observed. After encapsulation and the induction of differentiation, the myoblasts showed a lag phase of ~3 days before an increase in CPK was observed, although the level of CPK activity increased by as much as 63-fold. The myogenic index of the encapsulated cells remained at zero. The rate of human growth hormone secretion was relatively constant throughout the two-week differentiation period, at an average of 7.78×10^{-2} ng hGH per hour per μg protein,

however, human growth hormone secretion was slightly decreased by about twofold during the differentiation of encapsulated myoblasts.

In conclusion, the differentiation of myoblasts into myotubes is retarded after encapsulation while the secretion of a recombinant product is slightly reduced. Further studies are necessary to elucidate the cause of this atypical differentiation pattern such that the proliferation and differentiation of the encapsulated myoblasts may be optimized to provide a stable vehicle for gene delivery.

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ABBREVIATIONS

α	alpha
β	beta
ADA	adenosine deaminase
APA	Alginate-Poly-L-lysine-Alginate
bp	base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cc	cubic centimeter
CFTR	cystic fibrosis transmembrane conductance regulator
CHES	2-(N-Cyclohexylamino)ethanesulfonic acid
Ci	Curie
cm	centimeter
CO_2	carbon dioxide
CPK	creatine phosphate kinase
DAB	diaminobenzidine
dCTP	deoxycytosine 5'-triphosphate
DEPC	diethyl pyrocarbonate
D-MEM	Dulbecco's Modified Minimal Essential media
ddH_2O	distilled deionized water
HEBS	HEPES-buffered saline
HEPES	4-(2-Hydroxyathyl)-1-piperazin-athansulfonsaure
H_2O	water
EDTA	ethylenediaminetetraacetic acid disodium salt
ELISA	enzyme linked immunosorbent assay
EtBr	ethidium bromide
EtOH	ethanol
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
hGH	human growth hormone
IgG	immunoglobulin G
KAlg	potassium alginate
L	litre
LB	Luria Broth
mg	milligram
ml	millilitre
mm	millimetre
mMT	mouse metallothionein
M	molar
MHC	myosin heavy chain
MOPS	morpholinopropanesulfonic acid
NaCl	sodium chloride

NaOAc	sodium acetate
NaOH	sodium hydroxide
nm	nanometre
OD	optical density
PBS	phosphate buffered saline
PLL	poly-L-lysine
PPD	paraphenylenediamine
RNase	ribonuclease
rpm	rotations per minute
R.T.	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine hydrochloride
Tris	Tris(hydroxymethyl)aminomethane
µg	microgram
µl	microlitre

1.0 INTRODUCTION

The advent of genetic engineering and recombinant DNA technology has not only provided a means whereby the roles of various gene products may be characterized, but it has sparked the development of a promising practice in the area of clinical medicine, that being "gene therapy". Gene therapy is the treatment of genetic disease via the introduction, or transfer of, genetic material into the body (primarily directed towards the somatic cells) of the patient (germline gene therapy is currently not practised) . The majority of gene therapy studies are directed towards gene augmentation (as opposed to gene correction), which refers to the concept of introducing a gene into a patient's cells that can manufacture the required gene product, thus compensating for the deficiency associated with the disease. There are three basic requirements necessary for any gene therapy trial to be successful: (i) the gene in question must be isolated; (ii) there must be an efficient and effective method of inserting the gene of choice into the target cells; (iii) the inserted gene must not cause any deleterious side effects while providing sufficient amounts of gene product for a sustained period of time (Weatherall, 1995).

To date, there are currently more than 100 human gene therapy studies which have been approved by various governing regulatory bodies in the United States and other countries. Gene therapy protocols may involve either *ex vivo* or *in vivo* methods of delivering a specific gene product. Cells are genetically engineered *in vitro* and subsequently placed back into the patient in *ex vivo* gene therapy, while *in vivo* gene therapy involves a direct transfer of genetic material into the patient's tissues or organs. Various strategies for gene delivery that have been developed include: physical gene

transfer (i.e. direct DNA injection, transfection, electroporation/particle bombardment, and liposome-mediated), viral vectors, receptor-mediated gene transfer, site-directed recombination, artificial chromosomes and activation of genes with related functions (Weatherall, 1995). Representative *in vivo* and *ex vivo* methods of gene therapy will be addressed in this section, focusing on the use of biocompatible encapsulation devices. In addition, an overview of myoblast proliferation and differentiation is provided as the work in this thesis is directed towards the use of encapsulated, genetically engineered myoblast cell lines as a vehicle for gene delivery.

1.1 Autologous Cell-Mediated Gene Therapy

1.1.1 *In Vivo* Gene Therapy

1.1.1.1 Retroviral Vectors

Retroviruses have been employed to serve as gene delivery vehicles and have been widely used to produce various mammalian cell types engineered to secrete stable levels of expression of the foreign gene. Most gene therapy protocols are based on Murine Leukaemia Viruses (MLVs) which are designed to be "replication defective" in nature, such that they sustain the ability to effectively infect an individual target cell without the capability of further replication and infection. Such retroviral vectors can accommodate insertion of up to 8 kb of foreign genetic information, which translates to the delivery of 1 to 3 genes per vector (Vile and Russell, 1995).

Retrovirally mediated gene transfer is not, in general, ideally suited to *in vivo* applications of gene therapy, as its applicability is limited due to several features of the retroviral life cycle which impede the safe and/or effective delivery of genetic material. For example, efficient gene delivery requires that the target cell possess the appropriate receptor to allow binding of the retroviral vector and subsequent entry into the cell. Thus, certain cell types, such as hematopoietic stem cells, may not be amenable to retroviral transduction (Mulligan, 1993). Secondly, integration of the proviral DNA is dependent upon proliferation (i.e. replication) of the cell to which it is targeted, which

excludes many of the organs/tissues of the body. Lastly, the use of retroviral vectors are associated with certain safety issues, such as the potential for toxic effects due to chronic overexpression or insertional mutagenesis, the latter of which may result in neoplastic transformation of the targeted cell(s) (Crystal, 1995). The possibility of sustained infectivity remains problematic, as several "outbreaks" of replication-competent retroviruses have been reported from the use of recombinant replication-defective retroviruses (Mulligan, 1993). Other issues include low retroviral titres (i.e. the number of infectious vector particles produced per ml of tissue culture supernatant), the instability of retroviral particles and the inactivation of retroviruses by human complement *in vivo* (Vile and Russell, 1995).

Some groups have attempted to employ the recombinant retrovirus as a mode of *in vivo* gene delivery despite the aforementioned associated difficulties. Pig vascular smooth muscle cells were effectively targeted *in vivo* by a recombinant retroviral vector expressing the β-galactosidase gene, which demonstrated possible applicability in treating cases such as cardiovascular disease and vasculature malignancies (Nabel et al., 1990). Another study employed the use of retroviral vectors expressing the herpes simplex thymidine kinase gene (which confers sensitivity to the antiviral agent "ganciclovir") to transduce proliferating, tumorigenic glioma cells in rats. Gliomas in most of the rats treated with the retroviral vector/ganciclovir combination demonstrated complete regression supporting the potential clinical application of this direct retroviral gene transfer in the treatment of human brain tumors (Culver et al., 1992). A hemophilia B dog model was used to determine the efficacy of *in vivo* hepatic transduction by retroviral

vectors encoding the canine factor IX gene (Kay et al., 1993). Persistent expression of the canine factor IX was observed for more than 5 months (although the levels were only 0.1% of the normal endogenous concentration in dogs) and phenotypic improvement in the treated dogs was noted. A similar, more recent, trial involved infusion of a recombinant retrovirus encoding the rat insulin 1 gene into rats suffering from Type 1 diabetes mellitus that resulted in sustained hepatic expression of the insulin gene, thereby alleviating the acute clinical symptoms associated with diabetes (Kolodka et al., 1995). Although efficacy and safety issues exist, these studies are decidedly indicative of a role for recombinant retroviral vectors in a select group of *in vivo* gene delivery therapies.

1.1.1.2 Adenoviral Vectors

Human wild-type adenoviruses (Ads) are well suited to *in vivo* gene therapy primarily due to the fact that they efficiently infect almost all cell types, be they dividing or quiescent. Ads are also relatively safe to use: they rarely cause any adverse side effects upon infection and seldom integrate into the host genome. Unlike retroviruses, Ads are relatively stable and are quite amenable to purification and concentration (Kremer and Perricaudet, 1995). Replication-defective Ads are created by deletion of the E1 and E3 regions of the viral genome and can be propagated by providing host cells engineered to express the deleted genes (Mulligan, 1993). Current Ad vectors (i.e. Ad2 and Ad5) can theoretically accept up to 7 or 8 kb of foreign DNA sequence, which is generally placed downstream of the E1A promoter, the major late promoter, the E3 promoter, or other introduced promoters. Recombinant Ad vectors have also been constructed such that the

E1 or E3 region, or both, are replaced with a polycloning site to allow for gene insertion (Clemens et al., 1995; Bramson et al., 1996).

Certain drawbacks are inherent in the use of adenoviral vectors as gene delivery systems. It is understood that there exists the potential for a low level of adenoviral replication *in vivo*, which may not only cause deleterious suppression of host protein synthesis, but may also result in the expression of genes producing toxic effects upon host cells or malignant transformation of host cells (Mulligan, 1993). Another major problem is the nonspecific inflammatory response and antivector cellular immunity observed upon adenoviral vector administration, which may limit the duration of gene expression and render repeated administrations ineffective (Crystal, 1995). Recent studies have shown promise, however, in blocking the humoral immune response against Ad vectors, thereby allowing for repeated administrations. Transient ablation of CD4 function or specific T helper cells at the time of vector administration appears to partially combat the problem of humoral immunity (Yang et al., 1996).

Various trials exploiting the use of adenoviral vectors have been conducted over the past few years. Recombinant Ad vectors encoding the cystic fibrosis transmembrane conductance regulator (CFTR) gene were administered to three patients with cystic fibrosis (CF) in a human clinical trial (Zabner et al., 1993). Correction in the Cl^- channel defect in the nasal epithelium was detected for at least 3 weeks, although the absolute levels of CFTR expression or the percentage of cells transduced by the vector were unknown. Patients receiving the same mode of therapy were followed in an additional trial conducted by Crystal et al. (1994). Expression of CFTR in the nasal epithelium,

however, was not detected in any of the patients beyond 10 days. Although the Ad vectors did not produce any permanent adverse effects (only transient inflammation of the lower respiratory tract was observed), were not replication-competent, and did not induce any neutralizing immunity, a concern arose due to the uncontrolled "spread" of the vector beyond the area of administration.

In vivo infusion of recombinant Ad vectors expressing the low density lipoprotein (LDL) receptor gene resulted in correction of hypercholesterolemia in mutant mice (Ishibashi et al., 1993) and in LDL-receptor deficient rabbits (Kozarsky et al., 1994). Both studies cite only transient levels of LDL receptor expression (i.e. approximately 2 weeks) from the transduced hepatocytes. In addition, the problem of an immune response against the viral proteins, thus neutralizing the effectiveness of additional infusions, was observed (Kozarsky et al., 1994).

A final example of *in vivo* adenoviral gene therapy is a trial involving the subretinal injection of a recombinant replication-defective adenovirus encoding the murine cDNA for the β subunit of the cGMP phosphodiesterase (βPDE) gene into mutant mice (Bennett et al., 1996). These mice are recessive for the βPDE gene mutation and thereby demonstrate retinal degeneration. Significant delay in the degeneration of photoreceptor cells was observed and was maximal at approximately 3 weeks post injection, after which time the number of "rescued" cells decreased. Once again, the issue at hand remains the lack of sustained expression of the transgene, despite the safe and effective initial administration of the recombinant adenoviral vector.

1.1.1.3 Direct DNA Injection

The concept of directly injecting pure closed circular DNA or RNA into targeted tissues *in vivo* has been employed in many instances as a method of gene delivery. The ability to transfer foreign genes into tissues or organs without the use of viruses is generally thought of as a relatively simple, yet non-toxic approach to gene therapy. RNA and DNA expression vectors or plasmids containing various reporter genes (i.e. chloramphenicol acetyltransferase, luciferase and β-galactosidase) were directly injected into mouse skeletal muscles *in vivo* (Wolff et al., 1990). Expression of the luciferase gene in the muscle cells was observed for at least 2 months in substantial amounts. Similar reporter gene constructs were injected into the tibialis anterior skeletal muscle of mice in which the injection site had previously been induced to undergo cell regeneration via the administration of a snake cardiotoxic venom (Davis et al., 1993). This study demonstrated increased cellular uptake of the plasmid constructs as a result of the regenerating muscle tissue. In fact, it was further shown that the administration of adenoviral and retroviral vectors produced a lower degree of expression of the reporter gene than did the pure plasmid DNA.

Another study involved the injection of the luciferase reporter gene into monkey myofibres in which expression was detected for at least 4 months post-injection (Jiao et al., 1992). Although the expression level was lower than what had been observed in rodents, it was noted that no adverse side effects were seen, including the production of antibodies against the foreign DNA.

Direct injection of DNA is therefore a simple, inexpensive and non-toxic method of gene delivery which can potentially allow for the transfer of very large DNA constructs (unlike viral vectors). Repeated administrations of the purified DNA may be necessary, however, as the expression levels are relatively low and are not persistent over time.

1.1.1.4 Liposome Mediated DNA Transfer

The technique of liposome mediated gene transfer relies upon the binding of negatively-charged DNA molecules to positively-charged cationic lipids to form a soluble complex, which is then attracted to the negatively-charged cell surfaces *in vivo* (Schofield and Caskey, 1995). DNA molecules are encapsulated in various types of liposomes, many of which are composed of lipids such as phosphatidylcholine, phosphatidylserine and cholesterol (Nicolau et al., 1980). A mixture of these components was complexed to decorin cDNA and injected into rat skeletal muscles, the results demonstrating a marked therapeutic effect on progressive tissue fibrosis in a rat model of glomerulonephritis (Isaka et al., 1996).

Many other investigators have used different compounds to form liposomes, such as poly-L-lysine (Wu et al., 1989), short synthetic peptides which emulate viral proteins (Gottschalk et al., 1996), dioctadecylamidoglycylspermine (DOGS) (Tsukamoto et al., 1995), or polycationic liposomes formed from mixtures of cholesterol and dioleoyl phosphatidylelthanolamine (DOPE). The latter type of liposome was employed to deliver reporter genes (i.e. *E. coli* lacZ, β-galactosidase) intravenously and intratumourally into mice (Stewart et al., 1992), in which case no acute adverse reactions were observed. The

DOPE/cholesterol liposomes were also used to deliver L-dopa to brain cells in Parkinson disease model rats (Cao et al., 1995) and in human clinical trials, including treatment of a patient with metastatic melanoma with HLA-B7 (Nabel et al., 1994) and therapeutic transfer of the CFTR gene to nine cystic fibrosis patients (Caplen et al., 1995). Once again, no treatment-related toxicity was evident, and in the Caplen study, partial correction (i.e. an increase of approximately 20%) of the ion-transport abnormality was detected in treated patients. A modified version of the DOPE/cholesterol liposome method by San et al. (1993), which utilized the more effective dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium (DMRIE) instead of cholesterol to deliver the HLA-B7 gene to mice and pigs, found that 1,000-fold higher concentrations of DNA-lipid complexes could be tolerated *in vivo* than what was seen with the DOPE/cholesterol method.

In an effort to further improve the efficiency of gene transfer, some groups are combining the method of liposome-mediated DNA transfer with the use of viruses, such as adenovirus (Curiel et al., 1991; Cotten et al., 1992; Cristiano et al., 1993) or the HJV/Sendai virus (Kaneda et al., 1989). Specific tissues or cells may be targeted *in vivo* by coupling the liposome / DNA complexes with carrier ligand molecules which bind to cell surface receptors. Examples include transferrin-polylysine/DNA conjugates (Wagner et al., 1992) and hepatocyte-specific asialoglycoprotein polycation/DNA complexes (Wu et al., 1989), the latter of which was applied successfully in a study that targeted the low density lipoprotein receptor gene to the liver of hyperlipidaemic Watanabe rabbits (Wilson et al., 1992). Alternatively, plasmid DNA may be complexed to certain

antibodies, a method termed "antifection", which can target specific cells (i.e. lymphoid-related cell lines were targeted by CD34 and CD3 antibodies complexed to DNA) (Poncet et al., 1996).

Liposome-mediated gene transfer may, therefore, be an attractive alternative to other methods of gene delivery due to the relatively low immunogenicity, ease of preparation and the lack of DNA-insert constraints, however, the challenge of long-term gene expression remains to be met.

1.1.2 *Ex Vivo* Gene Therapy

Most of the above methods of *in vivo* gene therapy described previously can also be applied to *ex vivo* gene delivery. It should be noted, however, that direct injection of DNA and ligand-DNA conjugates (with or without the aid of an adenoviral vector) are not generally utilized for transferring genes into cells *in vitro* (Mulligan, 1993).

A great deal of the research efforts in the area of *ex vivo* gene therapy have focused on treating diseases which are based upon hematopoietic stem cell disorders, primarily because the transplantation of genetically modified stem cells would provide a theoretically infinite supply of the required deficient protein (Mulligan, 1993). A wide range of hematologically-related afflictions exist, such as various types of hematological malignancies (i.e. acute and chronic leukemias, lymphomas, multiple myeloma etc.) and genetic diseases (i.e. severe combined immunodeficiency or SCID, Wiskott-Aldrich syndrome, thalassemia, Gaucher's disease, various lysosomal storage diseases etc.). Traditional therapies for treating such disorders have been based upon the process of

autologous bone marrow transplantation, whereby pluripotential stem cells are prepared *ex vivo* and infused into the patient to restore normal hematopoiesis and/or lymphopoiesis following ablation of the host's immune system (Rappeport, 1991). Later studies demonstrated the possibility of using self-renewing, long-term bone marrow cultures (LTMCs) as gene target cells, whereby marrow-ablative preconditioning of the host was not required (Beinzle et al., 1994). The first successful allogeneic bone marrow transplant was performed in 1968 on a patient with SCID (Gatti et al., 1968). Allogeneic transplants require, however, that the donor and recipient carry identical class I and class II HLA antigens. The scarcity of bone marrow donors is a harsh reality which impedes the treatment of many patients in need of a transplant.

There are many technical problems surrounding the *ex vivo* transduction of hematopoietic stem cells, mainly relating to the difficulty in obtaining sufficient numbers of stem cells, which are only found in small numbers in bone marrow and tend to be quiescent in nature (Mulligan, 1993). Many trials have, therefore, focused on the transduction of stem cell-derived lineages, such as T lymphocyte cells, as was the case in a clinical trial which involved the transduction of two SCID patient's T cells with the adenosine deaminase (ADA) gene (Blaese et al., 1995). Gaucher's disease patients have been treated in trials which followed a similar protocol, with bone marrow transplantation resulting in amelioration of the disease phenotype (Ringdon et al., 1988). Many current trials involve the transplantation of genetically modified hematopoietic stem cells or their derivatives. In most cases, viral vectors (i.e. retroviruses) are utilized to transfect these cells *in vitro* for subsequent delivery back into the patient.

1.1.2.1 Viral Vectors

Viral vectors are utilized to deliver genes into various cell types *ex vivo* in much the same way as was described for *in vivo* gene therapy. Retroviruses are primarily used in *ex vivo* gene therapy, while the use of adenoviruses is somewhat limited, as they do not produce stably-transduced cell lines *in vitro*.

Various cell types have been successfully transduced by retroviral vectors *in vitro*, including the often targeted hematopoietic stem cells. Murine bone marrow cells, including peripheral blood lymphocytes, are efficiently transduced, as evidenced in studies which involved the *ex vivo*, retrovirally-mediated transfer of the ADA gene into murine stem cell populations, followed by transplantation of the recombinant cells into mice (Williams et al., 1986; Ferrari et al., 1991). The latter study found some degree of phenotypic correction in the treated ADA-deficient mice, accompanied by a long-term survival of the transduced cells (i.e. up to 10 weeks). Stem cells from primates have also been successfully transduced by retroviral vectors, including those of the rhesus monkey (Donahue et al., 1992) and human stem cells (Nolta et al., 1996; Kohn et al., 1995). Both studies demonstrated the continued presence of recombinant progenitor cells, and in the latter trial, conducted by Kohn et al. (1995), the stable presence of ADA-expressing leukocytes from bone marrow and peripheral blood of the three neonate patients (i.e. 18 months) was achieved without cytoablation prior to the transplant. The neonates did, however, require enzyme replacement therapy, as the levels of ADA produced from the transduced cells alone was insufficient. Due to the low level of expression obtained with transduced human stem cells, some groups have directly targeted human T cell

lymphocytes, as in the case of a clinical trial directed towards the treatment of SCID in two children via transfer of T cells that were transduced with a retroviral vector containing the ADA gene (Blaese et al., 1995). Expression of the ADA transgene has persisted *in vivo* in one patient for at least 5 years, and no adverse effects of the retroviral-mediated gene transfer have been detected in either patient.

Other human clinical trials involving retroviral-mediated gene transfer include the treatment of familial hypercholesterolemia with hepatocytes transduced to express low density lipoprotein receptor (LDLR) (Grossman et al., 1994) and the administration of recombinant fibroblasts expressing interleukin-4 (IL-4) (Elder et al., 1996) or interleukin-12 (IL-12) (Tahara et al., 1995) for the treatment of solid tumors. Although concerns regarding the use of retroviral vectors exist, such as the possibility of insertional mutagenesis *in vivo*, no adverse effects have been seen to date (Crystal, 1995). The challenge is clearly to create a more effective method of gene delivery, providing higher, more consistent levels of transgene expression than what is presently obtained with retroviral-mediated methods.

1.1.2.2 Recombinant Cell Grafts

A large portion of *ex vivo* gene therapy protocols involve the transfection of allogeneic or syngeneic cell lines with a vector designed to engineer the gene product of choice, either by viral or non-viral methods, for subsequent injection back into the

patient. This method of gene delivery has been widely adapted, targeting various explantable tissues, such as bone marrow cells, fibroblasts, hepatocytes, and myoblasts.

The transkaryotic implantation of murine fibroblasts, transfected with the human growth hormone (hGH) gene, into various intraperitoneal sites in mice resulted in varying degrees of hGH expression which, at best, persisted for at least 3 months when accompanied by the administration of one, or more, immunosuppressive agent(s) (Seldon et al., 1987). Although high levels of circulating plasma hGH levels were detected, such prolonged hGH expression, in addition to the formation of peritoneal plaques and ascites, resulted ultimately in death. The use of transformed cell lines is also problematic due to their inherent tumorigenic nature. Tumor formation was also observed in a study in which recombinant fibroblasts expressing bilirubin UDP-glucuronosyltransferase (B-UGT) were transplanted intraperitoneally into Gunn rats deficient in B-UGT (Seppen et al., 1997). Other groups have therefore harvested primary fibroblasts for DNA-mediated transfection and subsequent implantation back into the host from which they were harvested. Primary rat fibroblasts transfected with hGH were implanted into rats intraperitoneally, intramuscularly, subcutaneously (Chang et al., 1990) and into the cerebral cortex (Doering and Chang, 1991). Immuno-rejection of the foreign gene product was, once again, problematic, and fibrosarcomas were evident in the implanted mice (with the exception of those receiving brain implants, in which case optimal expression persisted for merely 1 month). In another study, a sustained, lifetime expression of hGH in nude mice receiving recombinant primary rabbit fibroblasts was achieved without the presence of any adverse side effects, such as the formation of

fibrosarcomas (Heartlein et al., 1994). Clinical trials using an approach based on the transplantation of recombinant autologous fibroblasts for the treatment of solid tumors have shown promise recently. Such studies have involved the retroviral transduction of human fibroblasts with the cytokine IL-4 (Elder et al., 1996) and IL-12 (Tahara et al., 1995). Autologous human lung adenocarcinoma cells were transduced *ex vivo* with a vector expressing human IL-2 for the treatment of mice harboring lung tumors (Heike et al., 1997). The data suggested possible clinical benefit for lung cancer patients (i.e. regression of lung tumors was shown), however, no therapeutic effects were derived from the treatment with respect to pre-existing tumors in the subcutaneous region distal from the region of the transplant. The concept of using autologous cells is undoubtedly very labour intensive and is also dependent upon successful proliferation of the patient's cells accompanied by substantial levels of transgene expression.

An *ex vivo* gene therapy clinical trial which employed the use of autologous hepatocytes expressing the LDL receptor to treat a patient with familial hypercholesterolaemia demonstrated successful engraftment of the recombinant cells concomitant with a stable correction of the deficiency for 18 months (Grossman et al., 1994). Although seemingly effective, this method of therapy is, yet again, time consuming and impractical for wide-scale use.

Myoblast cell lines have also been utilized to deliver various gene products *in vivo* upon transplantation via intramuscular injection, including hGH (Barr and Leiden, 1991; Dhawan et al., 1991), human factor IX (Roman et al., 1992; Dai et al., 1992; Yao and Kurachi, 1992; Yao et al., 1994), human β-glucuronidase (Naffakh et al., 1996) and

canine α-L-iduronidase (Shull et al., 1996). A small proportion of recombinant myoblasts, upon implantation, survive and ultimately fuse with the endogenous muscle fibres of the recipient (Roman et al., 1992). The surviving donor myoblasts, which are generally confined to the area surrounding the injection site, act as a relatively stable and persistent gene delivery system in mice, however, in human transplant experiments involving Duchenne Muscular Dystrophy (DMD) patients, little or no trace of the donor myoblasts is evident subsequent to the injection (Partridge and Davies, 1995). It is suggested that an immune response against the implanted myoblasts is responsible, even in cases where donor and recipient are fully matched at the major histocompatibility locus.

1.2 Non-Autologous Cell-Mediated Gene Therapy

1.2.1 Biocompatible Encapsulation Devices

Many different techniques for immobilizing, entrapping, or encapsulating a variety of microbial, plant and animal cells, proteins, enzymes and drugs have been proposed. The concept was initially suggested as a method of encapsulating aqueous solutions of protein within polymer membranes in 1964 by T.M.S. Chang. The encapsulation device may be formed from several different natural or synthetic materials, and takes the form of a sphere, a rod-shape, a hollow longitudinal fibre, or a disc-shape. Encapsulation devices are designed to act as biocompatible, perm-selective membranes which allow passage of nutrients and the desired product for *in vivo* delivery to the

recipient animal / human, while acting as an immune-barrier to exogenous cells, complement, antibodies and viruses (Aebischer et al., 1988). The major factors involved in the selection of the most effective immuno-isolation device are the pore size in the membrane (defined by the molecular size and structure of the compound utilized to form the capsule) and the size of the substrate or product expected to diffuse across the membrane / matrix (Tanaka et al., 1984).

Encapsulation devices can be divided into two main categories: macrocapsules and microcapsules. Macroencapsulation is generally thought to involve filling a hollow, cylindrical tube or membrane with cells / tissue (Emerich et al., 1992), although some are spherical in nature. The compounds used to form these hollow fibres include polymers such as polyvinyl chloride acrylate (Aebishcer et al., 1988; Lacy et al., 1991; Sagen et al., 1993), acrylonitrile / sodium methallyl sulfonate (Kessler et al., 1991), poly-ether-sulfone (Aebischer et al., 1996), poly-vinyl-alcohol (Burczak et al., 1994), poly(hydroxyethyl methacrylate) (pHEMA) (Uludag and Sefton, 1993; Rao et al., 1994) and polypropylene (Takebe et al., 1996). Macrocapsules tend to be, mechanically, very stable due to the thickness of the membrane wall and also carry the advantage of being retrieved with relative ease from the patient following implantation (Emerich et al., 1992). The thickness of the tubular membrane, in conjuction with the large inner diameter of the capsule may, however, compromise cell viability and somewhat impair the diffusion of various factors. Microencapsulation, on the other hand, consists of enclosing cells in a thin, spherical, porous membrane, which is generally quite optimal for diffusion and cell

viability. These capsules are more fragile on a mechanical or chemical level, however, and once implanted, are not readily removable (Emerich et al., 1992).

Hollow fibre encapsulation devices have been employed in many instances to isolate a variety of cell types or tissues, such as embryonic mouse mesencephalon (Aebischer et al., 1988), pancreatic islets (Kessler et al., 1991; Lacy et al., 1991; Lanza et al., 1992), PC12 (rat pheochromocytoma) cells (Aebishcer et al., 1991), adrenal medullary chromaffin cells (Sagen et al., 1993), rat hepatocytes (Honiger et al., 1995), and hamster kidney cells (Aebischer et al., 1996). Many of these studies demonstrated effective, relatively long-term, expression of gene products from encapsulated tissues or cells, with little or no adverse side effects, including signs of an immune response against the implanted devices, such as fibrosis and immune cell adherance surrounding the capsules.

Microcapsules, like macrocapsules, are also formed from a variety of compounds including albumin-heparin (Cremers et al., 1994), agarose hydrogel (Yang et al., 1994), hydroxyethyl methacrylate - methyl methacrylate (HEMA-MMA) (Uludag and Sefton, 1993) and alginate (O'Shea et al., 1984; Darquy and Reach, 1985; Lum et al., 1991; Chang et al., 1994). The latter of these types, composed of the polysaccharide alginate, is the most widely used to date.

Overall, the concept of employing a biocompatible device to immuno-isolate tissue or cells for therapeutic purposes alleviates some of the risk involved, such as that inherent in protocols utilizing viral vectors, although the possibility of the entrapped cells escaping the capsule (i.e. due to capsule instability) remains an issue. The encapsulation

method does, however, show promise in providing an effective and practical approach to the long-term systemic delivery of gene products.

1.2.1.1 Alginate Polylysine Microcapsules

1.2.1.1.1 Chemical Composition

Alginates are composed of unbranched copolymers, namely, 1-4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). The M and G monomers are arranged in various random patterns along the polymer chain, which explains the numerous types of alginates that exist in nature. This chemical composition (i.e. M/G structure) correlates directly with the functional properties of the alginate itself (Smidsrod and Skjak-Braek, 1990). Various properties intrinsic to the particular alginate which is to be utilized as a component of a cell-immobilization device must be considered, such as the chemical and mechanical strength of the alginate and the percentage of toxic, pyrogenic and immunogenic contaminants in the alginate isolate (Smidsrod and Skjak-Braek, 1990). Alginate possesses the ability to form a gel upon exposure to divalent ions, such as calcium (Ca^{2+}) due to the interchain chelation of the ions between homopolymeric blocks of G residues (Martinsen et al., 1989).

The permeability of the alginate gel is controlled, such that a desired pore size may be created, via the use of an additional polymer, such as poly-L-lysine (PLL). The ionic bonds formed between the positively-charged PLL molecules and the negatively-charged carboxyl ($-COO^-$) groups of the alginate produce a relatively stable membrane (Ma et al., 1994). The molecular weight of the PLL used as a copolymer plays a large

role in determining the porosity of the resulting microcapsule, as well as the strength of the capsule. In general, as the length of the PLL chain increases (i.e. increased molecular weight), the number of PLL/alginate ionic interactions decreases (Goosen et al., 1985). It has been demonstrated that PLL with an average molecular weight of 1.7×10^4 produces microcapsules with an optimal pore size, such that the capsule membrane is impermeable to extraneous antibodies, including the lower molecular weight immunoglobulins (Goosen et al., 1985). Recent work, however, has shown that alginate-polylysine microcapsules (which were made with PLL possessing a molecular weight of 2.1×10^4) were permeable to human IgG (~ 150,000 kd) and, to a lesser extent, mouse β-glucuronidase (~ 300,000 kd) (Awrey et al., 1996). In this study, a PLL preparation with a molecular weight range of 1.5×10^4 to 3.0×10^4 was employed for the formation of APA microcapsules. The PLL copolymer further stabilizes the alginate gel upon exposure to chelating compounds which possess an affinity towards Ca^{2+} ions, such as phosphates or citrates (Smidsrod and Skjak-Braek, 1990), the latter of which (i.e. sodium citrate) is used in this study to liquefy the core of the Alginate-Poly-L-lysine-Alginate (APA) microcapsule. Extended exposure to a 55mM sodium citrate solution ultimately results in the dissolution of the APA capsules due to the sequestering of the Ca^{2+} ions by the citrate molecules (Smidsrod and Skjak-Braek, 1990), which proves to be useful for the recovery of entrapped cells or tissues.

Overall, there exist many parameters which must be taken into consideration when designing an effective biocompatible immobilization device, such as the chemical composition of the polymers used, the duration of the reaction times between the

polymers and chelating or cross-linking agents, the size/shape of the capsule itself and the nature of the material to be encapsulated.

1.2.1.1.2 APA Encapsulation Studies

A variety of studies have employed the use of APA microcapsules to encapsulate different cell types. Many groups have implanted alginate-polylysine microcapsules containing pancreatic islet cells isolated from rats (Lim and Sun, 1980; O'Shea et al., 1984; Darquy and Reach, 1985; Fan et al., 1990; Weber et al., 1990; Mazaheri et al., 1991; Lum et al., 1992), mice (Soon-Shiong et al., 1993) and humans (Soon-Shiong et al., 1994; Lanza et al., 1995) for the treatment of diabetes. Other cell types which have been encapsulated in alginate-polylysine membranes include: rat parathyroid cells for the treatment of hypoparathyroidism (Fu and Sun, 1989), rat hepatocytes (Wong and Chang, 1991), bovine chromaffin cells for the treatment of Parkinson's disease (Aebischer et al., 1991), mouse fibroblasts expressing hGH (Tai and Sun, 1993; Chang et al., 1993; Chang et al., 1994), human factor IX (Liu et al., 1993), or adenosine deaminase (Hughes et al., 1994) in *in vitro* studies and mouse myoblasts producing human factor IX *in vivo* as a method of correcting the Hemophilia B phenotype (Hortelano et al., 1996).

Mouse Ltk^- fibroblasts expressing human growth hormone (hGH) were encapsulated in an APA membrane and implanted intraperitoneally into allogeneic mice for the purpose of analyzing the capsule biocompatibility, cell growth and recombinant gene expression *in vivo* (Tai and Sun, 1993; Chang et al., 1993). These studies demonstrated persistent systemic delivery of hGH in the transplanted mice, in addition to

the sustained proliferative capacity and viability of the recombinant fibroblasts, as measured *in vitro* following the removal of the capsules. In both cases, no adverse side effects such as intraperitoneal adhesions or tumorigenic growth, was evident as a result of the implantation. In order to test the efficacy of using alginate microcapsules as an *in vivo* method of gene therapy, mouse myoblasts engineered to secrete mouse growth hormone (mGH) were implanted intraperitoneally into allogeneic Snell dwarf mice (Al-Hendy et al., 1995). Several biological effects were observed in the treated mice, when compared to control mice, as a direct result of the mGH systemic delivery which included increases in linear growth, overall body weight, peripheral organ weights and tibial growth plate thickness. The encapsulated myoblasts remained viable *in vivo* and demonstrated persistent expression of the mGH transgene for up to 6 months post-implantation, as measured by analysis of the retrieved capsules *in vitro*. In addition, no inflammatory cell adhesions or other adverse effects were identified in the implanted mice. The use of encapsulated myoblasts as a gene delivery system is seemingly advantageous due to the fact that they possess the ability to differentiate into stable structures called myotubes upon reaching cell confluency within the capsular space. This capacity to terminally differentiate appears to aid in the sustained viability of the encapsulated cells *in vivo*, unlike encapsulated fibroblasts, which demonstrate a reduction in viability over time due to continued proliferation and over-crowding within the capsular space, thereby resulting in limited exchange of nutrients and metabolic waste throughout the capsule (Chang et al., 1994).

A more recent study employed the use of encapsulated murine myoblasts secreting human factor IX (hFIX) as a potential method of gene therapy to treat hemophilia B

(Hortelano et al., 1996). Sustained expression of hFIX was observed throughout a time span of 213 days (i.e. hFIX was detected in mouse plasma for up to 14 days at a maximum level of ~ 4 ng/ml, and thereafter, continued circulating hFIX antibodies suggested hFIX secretion *in vivo*). Despite the remarkable persistence of hFIX production over time, accompanied by the long-term viability of the encapsulated myoblasts, only low levels of circulating hFIX were detected (i.e. approximately 0.1% of the normal physiologic levels in humans). It should be noted, however, that the detection of circulating hFIX antibodies most likely resulted in an underestimation of the true level of secreted hFIX. It has also been observed that the durability of the alginate-polylysine capsules is somewhat limited *in vivo* and requires some improvement (Hortelano et al., 1996; M. Peirone, unpublished observations). The APA capsules may, in fact, be increased in strength and durability by altering the type of alginate used (i.e. alginates with an increased percentage of G monomers form capsules of increased mechanical strength) and/or the divalent cation used to gel the alginate (i.e. some cations have a greater affinity for alginate, such as barium (Ba^{2+}) and strontium (Sr^{2+})) (Smidsrod and Skjak-Braek, 1990).

Despite certain problematic issues, the microencapsulation method shows promise in terms of providing an effective method of gene delivery that does not pose a lot of the previously mentioned risks involved in using other systems, such as viral vectors. The cell immobilization concept not only allows for the use of allogeneic or xenogeneic transplants without immune-suppression, but provides a practical and cost-effective approach to somatic gene therapy that can be ultimately universally applicable.

1.3 Myoblast Cell Lines

1.3.1 Myogenesis

During the course of vertebrate myogenesis, myogenic progenitor cells, or "myoblasts", arise within the somites which, in turn, arise from the segmental plate mesoderm of the vertebrate embryo (Sassoon, 1993). The myoblasts migrate to destinations in the thorax and limb whereby proliferation of the committed myoblasts, and their subsequent fusion into multinucleate "myotubes", ultimately result in the generation of skeletal muscle mass in the adult. Adult muscle is, in fact, a very specialized tissue composed of various types of differentiated muscle fibres, each type expressing unique muscle protein isoforms. Various extracellular signals, such as neuronal and humoral factors, play a role in the fibre type determination (Buckingham, 1992). Not only do muscle fibres differ, but the myoblasts from which they originate also demonstrate a large degree of diversity at a molecular level. Furthermore, there exist defined sub-types of myoblasts at specific developmental ages, which include the somitic, embryonic, fetal, neonatal and adult stages (Stockdale, 1992). A striking complexity is inherent in the process by which different types of muscle fibres are formed during the embryonic and fetal stages of development, as new fibres are rapidly added in a seemingly well-choreographed manner. Fibre-type specialization is evident upon morphological analysis, accompanied by distinct patterns of muscle specific gene expression, particularly that of the myosin gene family. Primary fibres, which are embryonically-derived, are therefore quite distinguishable from the secondary fibres that

are formed in the fetal stage. It is suggested that myoblasts carry a kind of "positional identity" whereby distinct patterns of homeobox gene expression may be partly responsible for dictating the migration path of the muscle precursor cells (Miller, 1992).

It has been shown via analysis of muscle biopsies taken from chickens, mice and humans that the types of fibres formed *in vitro* are dependent upon the stage of development of the organism from which the biopsy was taken, as well as the cell culture conditions used (Hauschka, 1974). Embryonic myoblasts tend to form short myotubes in culture which contain very few nuclei and express specific types of myosin heavy chain (MHC) gene. Fetal and adult myoblasts, however, form long, multinucleated fibres which express many different MHC isoforms depending on the developmental stage at the time of isolation (Stockdale, 1992). MHC isoforms differ with respect to their intrinsic ATPase activities, which ultimately plays a role in the contraction rate of a mature muscle fibre (Miller et al., 1993). This diversity is evident in cloned embryonic myoblast cultures in which 70-80% of differentiated myotubes express "fast" MHC, 20-30% coexpress "fast" and "slow" MHCs and approximately 1% express exclusively "slow" MHC (Miller and Stockdale, 1986).

All of the exact signals which play a role in the induction of myogenesis *in vivo* have yet to be fully elucidated, including the plethora of cellular signals involved in the activation / inhibition of myoblast proliferation and differentiation. When cultured *in vitro*, however, myoblasts are subject to a tightly controlled mechanism that prevents differentiation. This differentiation-inhibition is regulated by exposure to serum and various exogenous peptide growth factors, which, when maintained above a critical threshold, ensure the continued proliferative growth of the myoblasts (Olson, 1992). It is

known that maintenance of myoblasts in media containing 10-20% fetal bovine serum (growth medium) suppresses differentiation, while medium containing 2-10% horse serum (differentiation medium) promotes the formation of myotubes (Wells and Strickland, 1994). More specifically, there exist countless extracellular molecules, such as peptide growth factors, which play a role in myogenic determination and differentiation, either in a positive or a negative regulatory fashion. Withdrawal of mitogen-rich growth medium not only induces differentiation in myoblast cell cultures, but it also results in programmed cell death, termed "apoptosis", in a portion of the cells. An *in vitro* study examining the degree of cell death among a differentiating murine myoblast cell line (C2C12) found that a maximum of 20 to 30% of the cells appeared to be undergoing apoptosis 48 hours following exposure to differentiation medium (Wang and Walsh, 1996). Differentiated myoblasts are, however, resistant to programmed cell death due to the induction of cyclin-dependent kinase (Cdk) inhibitors such as $p21^{CIP1}$ and $p16^{INK4A}$.

1.3.2 Myoblast Differentiation

Skeletal muscle differentiation involves the conversion of proliferating myoblasts into terminally differentiated, non-cycling myotubes. The events surrounding this transformation include: (i) irreversible withdrawal from the cell cycle (also referred to as "commitment"); (ii) the activation of numerous un-linked muscle-specific genes ("biochemical differentiation"); (iii) cell fusion (Crescenzi et al., 1994). Due to the close temporal correlation of these three events, the possibility of some type of cause-effect

relationship between two or all of these differentiation-related modifications has been considered. A study utilizing a myogenic cell line that is temperature-sensitive for fusion and irreversible cell cycle withdrawal attempted to determine if any causal relationship existed (Nguyen et al., 1983). It was shown that cell fusion and commitment were not required for the induction of muscle-specific genes, the latter of which is merely transient in nature, as the gene expression is reversible upon growth stimulation. Furthermore, cell cycle withdrawal and muscle-specific gene activation are independently induced, although both are absolutely required for the stable and irreversible differentiation of the muscle cell. Analysis of myoblast differentiation has provided evidence that the "commitment" step towards differentiation occurs in the G1 stage of the cell cycle, and that DNA synthesis or cell division is not necessary for myoblast fusion to occur (Buckley and Konisberg, 1974; Nadal-Ginard, 1978; Turo and Florini, 1982). The stability of the myogenic phenotype, or the maintenance of the irreversible differentiated state of the myoblasts, is suggested to be a result of gene expression patterns and/or changes in chromatin structure (Ludolph and Konieczny, 1995).

Upon fusion of the mononucleate myoblasts into post-mitotic, multinucleate muscle fibres, skeletal muscle-specific proteins are synthesized in a coordinate manner, including the accumulation of contractile / myofibrillar proteins which comprise the contractile apparatus (Devlin and Konisberg, 1983), as well as metabolic enzymes and membrane proteins (Chamberlain et al., 1985). The synthesis of contractile proteins appears, in turn, to be accompanied by an overall restriction of most DNA and RNA synthesis.

The marked increase in the production of various enzyme systems upon differentiation of muscle tissue has been studied *in vitro*, and more specifically, the interrelationship between morphogenetic events and macromolecule synthesis has been investigated. Examples of enzyme systems which change dramatically during the course of differentiation include that of phosphoglycerokinase, glycogen synthetase, glycogen phosphorylase and creatine phosphokinase, albeit none of which are produced exclusively in muscle tissue (Shainberg et al., 1971). Creatine phosphokinase (CPK) is a good indicator of myoblast differentiation and fusion due to the fact that muscle fibres demonstrate extremely elevated levels of CPK, unlike their proliferating, non-committed myoblast predecessors which possess a very low basal level of CPK activity similar to that seen in non-muscle tissues.

Thus, the differentiation of myogenic cell lines involves the coordinate expression of various genes, accompanied by specific morphological changes that transform a culture of myoblasts into multinucleate myofibres. This differentiation program is, indeed, highly sensitive to a variety of external cues such that the conditions under which myogenic cultures are maintained plays a large role in their rates of growth and differentiation.

1.3.2.1 Muscle-specific Gene Expression

Myogenic differentiation is accompanied, as mentioned previously, by the transcriptional activation of several muscle-specific genes. Genes encoding structural and contractile proteins, such as desmin, actin, myosin, troponin and tropomyosin are turned

on during muscle differentiation, as are various regulatory genes involved in muscle development. To date, there are four major transcription factor families which play a regulatory, likely non-overlapping, role in skeletal development: the muscle regulatory factors (MRFs), the myocyte enhancer factor-2 (MEF2) family, the *paired box* (*Pax*) gene family, and the LIM gene family (Ludolph and Konieczny, 1995), of which only the first of these (i.e. MRFs) will be discussed in detail for the purpose of this study. The regulatory protein products are involved in DNA binding or protein:protein interactions, and it has been shown that many regulatory proteins form complexes with DNA, such as heterodimers via leucine zipper or helix-loop-helix motifs, which is suggestive of "active" regulation of muscle-specific genes (Blau and Baltimore, 1991). Much remains to be discovered with respect to the complete role each regulatory factor plays in controlling skeletal myogenesis, in addition to understanding the complex molecular pathways and control points necessary for muscle development. Investigations of skeletal myogenesis during vertebrate embryogenesis is necessary to elucidate the highly orchestrated process of cell proliferation and differentiation that occurs *in vivo*, as opposed to merely analyzing myoblast differentiation in cell culture.

Muscle-specific gene expression is largely controlled by the aforementioned MRF family which activates gene transcription by, firstly, dimerizing with helix-loop-helix (HLH) proteins of the E2A family, followed by subsequent binding to a promoter DNA sequence termed an "E-box" (i.e. a consensus DNA sequence: "CANNTG"). Four mammalian MRFs have been identified: MyoD1, myogenin, Myf-5 and MRF4/herculin/Myf-6 (Miller et al., 1993). Studies have shown that the MRFs are, at least in part, functionally equivalent, in that there exists some overlap in the activation of

gene expression between some factors, as well as the fact that each MRF is able to cross-activate expression of one or more of the other MRFs (an "autoregulatory" feature which may function in the maintenance of the stably differentiated state (Blau and Baltimore, 1991)). This functional redundancy has been nicely demonstrated in studies of MyoD1 or Myf-5 knock-out mice whereby the expression of one gene compensates for the gene which is lacking, at least with respect to muscle development (Rudnicki et al., 1992; Braun et al., 1992). In myogenic cultures, proliferating, undifferentiated cells express MyoD and/or Myf-5, while myogenin expression is significantly upregulated once the myoblasts become "committed" to terminally differentiate (Ludolph and Konieczny, 1995).

Various growth factors and proteins have been shown to inhibit myogenesis by repressing muscle-specific gene expression. One HLH protein, termed "Id", is expressed at high levels in proliferating cells and forms hetero-oligomers primarily with E2A products, which, in turn, prevents their association with MyoD thereby inhibiting activation of the myogenic differentiation program (Olson, 1992). Other examples of myogenic protein inhibitors which are involved in the repression of muscle-specific gene expression include "c-Fos" and "c-Jun", both of whose expression is induced by serum and certain growth factors (Olson, 1992).

Other genes which are primarily expressed in muscle include those which encode proteins involved in the myofibril contractile apparatus, such as actin, myosin, tropomyosin and troponin. These proteins are synthesized in a highly coordinate manner in myoblasts which have initiated the differentiation program and have begun to form multinucleate myofibres. For the purposes of this thesis, only troponin and myosin will

be discussed in detail. Troponin I (TnI) prevents the contractile interaction of actin and myosin in striated muscle cells in the relaxed state and exists as two distinct isoforms, TnI_{slow} and TnI_{fast} (Koppe et al., 1989). Individual muscle fibres express predominantly either TnI_{slow} or TnI_{fast}, which may be partly due to neuronal regulation/innervation *in vivo*, as opposed to *in vitro*, whereby myogenic cell lines, such as C2 and L6 myoblasts, express both TnI isoforms (Koppe et al., 1989). The other subunits of the troponin complex include Troponin C (TnC) and Troponin T (TnT), which also possess fast and slow isoforms. Troponin C is involved in the binding of calcium, while Troponin T binds tropomyosin (Pearlstone et al., 1976).

There exist three myosin light chains which compose part of the muscle contractile apparatus: myosin light chain 2 (MLC2), myosin light chain 1 (MLC1) and myosin light chain 3 (MLC3), of which the latter two are transcribed from the same gene (Garfinkel et al., 1982). The gene coding for myosin light chain 1/3 has been demonstrated to contain muscle-specific DNA regulatory sequences, including an "E box" sequence that is subject to regulation by the MRF regulatory protein family (Lin et al., 1991), as well as a $CC(A/T)_6CG$ (CArG) sequence which binds a 67-kD serum-responsive nuclear factor ($p67^{SRF}$) (Vandromme et al., 1992). Such regulatory binding sites/sequences have also been identified in members of the troponin family of proteins (Lin et al., 1991; Vandromme et al., 1992).

The various muscle-specific genes involved in myogenesis have, in large part, been elucidated. However, the highly controlled manner with which these genes are regulated is only just recently becoming evident. Although many studies have been

carried out along these lines utilizing specific myogenic cultures under controlled environments, the picture gained is a simplistic one in comparison to the events which unfold *in vivo* in the developing embryo.

1.3.3 Myoblast Fusion

The differentiation of mammalian myoblasts into mature myotubes not only depends upon the up-regulation of various muscle-specific genes, but also requires that extensive changes in cell morphology take place (Mangan and Olmsted, 1996). In order to facilitate myoblast fusion, the myoblasts must exist in close proximity to one another. The process of myoblast fusion is calcium regulated whereby external concentrations of approximately 0.1 μM Ca^{2+} are required for the alignment and aggregation of the myoblasts, while higher concentrations of calcium (1.4 μM) are necessary for membrane fusion and are generated from the internal stores of Ca^{2+} present in the myoblasts (Seigneurin-Venin et al., 1996). Myogenic cells, once aligned, communicate via gap junctions, which allows for their synchronization during myogenesis. The excitation-Ca^{2+} release machinery may serve as a detector of electrical signals (via the dihydropyridine receptor which is localized in the transverse tubular system) and as a trigger for myoblast differentiation and fusion (via the mobilization of Ca^{2+} ions through ryanodine receptors which act as release channels in the sarcoplasmic reticulum) (Seigneurin-Venin et al., 1996). The mobilized Ca^{2+} may, in fact, activate a plethora of muscle-specific genes and/or enzyme systems involved in the myoblast to myotube conversion. This calcium-dependent adhesion process in myoblasts has been associated

with the expression of a particular set of cell adhesion molecules (CAMs) called cadherins (Peck and Walsh, 1993) which are thought to act in the establishment and maintenance of tissue organization (Zeschnigk et al., 1995). Three cadherins have been identified in skeletal muscle, namely N-cadherin (Knudsen et al., 1990), M-cadherin (Donalies et al., 1991) and T-cadherin (Ranscht, 1991). Studies employing the use of antagonistic peptides directed towards N-cadherin and M-cadherin have demonstrated the inhibition of myoblast fusion (Zeschnigk et al., 1995) thereby suggesting that both proteins are required for the fusion process to occur. It is the current thinking that M- and N-cadherins may influence the formation or organization of cytoskeletal or myofilament structures (Zeschnigk et al., 1995).

It has been shown that myoblasts cultured *in vitro* also display a calcium-independent cell adhesion system as well (Peck and Walsh, 1993). Examples of such calcium-independent cell adhesion systems include members of the Ig superfamily namely CAMs (cell adhesion molecules) including NCAM (neural CAM) and VCAM-1, as well as a novel form of β1 integrin called β1D which is a transmembrane heterodimeric receptor present only in skeletal and cardiac muscles that mediates the association between the extracellular matrix (ECM) and cytoskeletal elements (Belkin et al., 1996). It has been suggested that the level of CAM expression is a critical factor involved in the alignment and fusion of myoblasts (Fazeli et al., 1996) Studies have demonstrated that overexpression of a 125-Kd NCAM isoform in C2 myoblasts resulted in enhanced cell fusion (Peck and Walsh, 1993) although the exact mechanism by which NCAMs mediate myoblast differentiation and/or cell fusion is still not clear.

Clustering of integrins in the plasma membrane with extracellular matrix ligands [i.e. laminin, fibronectin and lectin (Gu et al., 1994)] triggers the association of various cytoskeletal proteins such as talin, vinculin, α-actinin, tensin, paxillin and zyxin as well as activating a number of signalling pathways within the cell which likely play a role in muscle-specific gene expression (Belkin et al., 1996). Studies employing the use of an antibody directed against β1-integrin demonstrated the inhibition of myogenic differentiation and fusion concomitant with the rounding-up of the myoblasts and detachment from the extracellular matrices (Hyodo and Kim, 1994).

Another family of cell surface proteins which has been implicated in modulating cell-cell and cell-extracellular matrix interactions are the ADAM (*a* *d*isintegrin *a*nd *m*etalloprotease) proteins (Yagami-Hiromasa et al., 1995; Alfandari et al., 1997). ADAMs contain four putative domains: (i) a metalloprotease domain, (ii) a cell-cell adhesive domain, (iii) a cell fusion domain, and (iv) a C-terminal cell-signalling cytoplasmic domain (Alfandari et al., 1997). ADAMs interact with specific integrins thereby functioning in cell-cell adhesion. One member of the ADAM family, namely ADAM 12 or meltrin α, has been directly linked to the process of myoblast fusion, as studies which involved the repression of ADAM 12 resulted in the inhibition of myoblast fusion (Alfandari et al., 1997).

Other extracellular matrix proteins have been implicated in the differentiation and fusion of myoblasts including merosin, laminin, fibronectin (Vachon et al., 1996) and thrombospondin-1 (Adams and Lawler, 1994). There appears to exist a highly-controlled cascade of events which regulates the differentiation and fusion of myogenic cells, with

many of the regulatory systems acting independently of one another. The mechanism by which each of the muscle proteins discussed control the process of cell fusion is still largely undetermined, and it is likely that many of the key players involved in muscle differentiation and fusion have yet to be discovered.

1.3.4 Regulation of Myoblast Proliferation/Differentiation by bFGF

As mentioned previously, a variety of growth factors and hormones have been shown to affect the proliferation and the differentiation of myogenic cells. These polypeptide growth factors fall into three separate categories: (i) the fibroblast growth factor (FGF) family; (ii) the insulin-like growth factor (IGF) family; (iii) the transforming growth factor-beta (TGF-beta) family (Allen and Boxhorn, 1989). For the purposes of this study, the effects of FGF alone on myoblast proliferation and differentiation will be discussed.

FGF exists in one of two isoforms, those being the acidic (aFGF) and the basic (bFGF) forms, of which the basic is more potent. bFGF is a very effective mitogen for mesoderm-derived cells, and when added to cultures of these cells, there is a marked reduction in the average doubling time (Gospodarowicz et al., 1987). The mitogenic effects of bFGF are synchronous with a block in the terminal myogenic differentiation pathway, and only 0.05 ng/ml of bFGF are required for half-maximal inhibition of differentiation (Florini et al., 1991). The differentiation-inhibitory effect of bFGF, however, is only transient. Once it is removed from the media, myoblasts resume their

natural course of differentiation, which includes the subsequent expression of muscle-specific genes (Florini et al., 1991).

Myoblast differentiation is inhibited by bFGF, in part, via a post-translational mechanism that involves protein kinase C phosphorylation of the basic region in myogenic basic helix-loop-helix (bHLH) proteins. This phosphorylation mechanism impedes the DNA-binding activity of the bHLH protein regulators, thereby suppressing the transcriptional activation of various muscle-specific genes (Rao and Kohtz, 1995). bFGF also appears to also sustain the upregulation of cyclin D1, a cyclin-dependent kinase (cdk) regulatory subunit, which has been associated with repression of muscle gene transcription (Rao and Kohtz, 1995). The inhibitory role of bFGF is supported by studies which have demonstrated the significant reduction in the production of MyoD1 and myogenin mRNA and protein in C2C12 cells exposed to bFGF (Yoshida et al., 1996). This indirect method of gene inactivation, in turn, maintains the myoblasts in a proliferative state. It has been observed, as well, that bFGF is able to override intracellular signals activated by cell-cell contact in myogenic cultures which promote differentiation (Yoshida et al., 1996). FGF has also been suggested to play a role in muscle regeneration and embryonic myogenesis, where, in the latter case, it may be important in allowing myoblast proliferation to occur despite a differentiation promoting environment (Yoshida et al., 1996).

bFGF, thereby, effectively prevents the differentiation of C2C12 myoblasts, among other myogenic cell types, and was employed in this study to aid in the

proliferation and the prevention of differentiation of microencapsulated C2C12 cells during their maintenance in regular growth media.

1.4 Rationale and Goals for Thesis

Biocompatible microcapsules have been employed as therapeutic gene delivery vehicles in a wide variety of studies. Alginate-polylysine microcapsules have been shown to be effective in delivering recombinant gene products to mice (Tai and Sun, 1993; Chang et al., 1993; Al-Hendy et al., 1995; Hortelano et al., 1996) utilizing fibroblast and myoblast cell lines. Myogenic cell lines possess an advantage over other cell types, such as fibroblasts, with respect to encapsulation due to their ability to terminally differentiate. This differentiation capability allows for long term, *in vivo* maintenance and/or viability of the encapsulated myoblasts (Hortelano et al., 1996), without the problem of continued proliferation and overcrowding within the capsular space, such as is evident with microencapsulated fibroblasts.

A great deal of knowledge has been gained in the area of myoblast growth and differentiation, in both culture conditions as well as in the developing embryo, including the elucidation of many of the factors which exert either stimulatory or inhibitory effects upon the process of myogenesis. It is relatively unknown, however, how myoblasts respond, particularly on a molecular level, when grown in a microencapsulated environment.

The work carried out in this thesis has attempted to analyze the differentiation of murine C2C12 myoblasts *in vitro* under two separate conditions: (i) unencapsulated; (ii) encapsulated in alginate-polylysine microcapsules. C2C12 cells were transfected with human growth hormone (which was used as a marker protein in the analysis of protein

secretion rates) and allowed to differentiate under both conditions via exposure to media containing 2% horse serum. The C2C12 differentiation program was analyzed on a morphological level by assessing the amount of myoblast fusion which had occurred. In addition, various biochemical parameters were studied in order to compare differentiating unencapsulated with encapsulated C2C12 cells: (i) the amount of creatine phosphate kinase (CPK) and myosin heavy chain (MHC) protein produced (quantitative and qualitative measurements, respectively); (ii) muscle-specific gene expression (i.e. MyoD1, myogenin, myosin light chains 1/3 and 2, Troponin I and Troponin T); (iii) hGH secretion rates (qualitatively by immunofluorescent staining and quantitatively by ELISA).

The overall goal of this work was to determine whether or not the secretion of a recombinant protein, namely hGH, was affected upon myoblast differentiation, and how the myoblast differentiation program would progress under the atypical conditions associated with encapsulation. These queries are necessary in order to understand, at least in part, the fate of the encapsulated myoblast, such that this gene therapy tool may be exploited in order to optimize the efficiency with which recombinant gene products are delivered systemically.

2.0 METHODS

2.1 Cell Lines

All cell lines employed consisted of, or were derived from, the murine C2C12 cell line (ATCC, Rockville, Md; catalogue #CRL-1772) which is derived from normal adult C3H mouse leg muscle. The cells were maintained on 100 mm tissue culture dishes (Falcon, Product #3003) and incubated at 37°C in a 5% CO_2 water-jacketed incubator. Standard growth media consisted of Dulbecco's Modified Eagle's medium (D-MEM) (Gibco) supplemented with 10% fetal bovine serum (Gibco, Catologue #26140-079) and 1% penicillin/streptomycin (Gibco). Differentiation media consisted of Dulbecco's Modified Eagle's medium supplemented with heat-inactivated, 2% horse serum (Gibco, Catologue #26050-070) along with 1% penicillin/streptomycin.

2.2 Preparation of Competent DH5α Cells

The method used for the preparation of competent DH5α cells was based upon the protocol described by D. Hanahan (1983). A scraping of *E. Coli* DH5α cells was streaked onto an LB plate and left to grow overnight. A single colony was chosen to inoculate 20 mls of TYM broth (see Appendix A) in a 250 ml flask. Cells were grown to mid-log phase (OD_{600} of 0.2 to 0.8). The culture was then added to 100 mls of TYM broth contained in a 2 L flask and vigorously agitated until the OD_{600} was between 0.5 to 0.9. 500 mls of TYM broth was then used to dilute the culture further, which was, in

turn, cooled rapidly by shaking in ice water once an OD_{600} of 0.6 was reached. The cooled broth was then placed in 250 ml Sorvall bottles and spun at 5,000 rpm for 15 minutes at 4°C. The supernatant was discarded and the remaining pellet resuspended by gently shaking on ice in 100 mls of ice cold TfBI (see Appendix A). A second spin (8 minutes in duration) produced a pellet which was subsequently suspended in 20 mls of cold TfBI by gentle shaking on ice. Pre-chilled 1.5 ml eppendorf tubes received aliquots of the culture (250 µl each) which were subjected to a -20°C isopropanol bath resulting in immediate freezing of the culture. Frozen competent DH5α cells were then stored at -70°C.

2.3 Heat Shock Transformation of DH5α Cells

The following protocol for the transformation of DH5α cells was based upon methods described by Mandel and Higa (1970). Plasmid DNA (approximately 5 ng) was aliquoted into 1.5 ml eppendorf tubes (2.5 µl) and placed on ice. 2.5 µl of ddH_2O was used as a negative control. Each eppendorf tube received 50 ul of competent DH5α cells (thawed on ice) followed by a time period of 30 minutes on ice. The tubes were then placed in a 37°C waterbath for 20 seconds, at which time 0.5 mls of 37°C-LB medium was added and allowed to incubate for 40 minutes. Aliquots of this transformation mixture (10 µl and 90 µl aliquots) were streaked onto LB-ampicillin agar plates and allowed to grow at 37°C overnight in a dry incubator.

2.4 Small Scale Preparation of Plasmid DNA

The protocol used for the small scale preparation of plasmid DNA was based upon the method described by Birnboim and Doly (1979). LB-ampicillin media (5 mls) was inoculated with a single colony of transformed DH5α cells and agitated overnight at 37°C in a dry shaker. 1.5 mls of the cell suspension was subsequently spun in a microcentrifuge for approximately 10 seconds, following which the cell pellet was resuspended in 50-100 μl of supernatant. The suspension was vortexed briefly (5-10 seconds) following the addition of both 300 μl of TENS and 150 μl of 3.0M NaOAc, pH 5.2. The mixture was microfuged for approximately 2 minutes, after which the supernatant was transferred to a clean eppendorf tube and the DNA precipitated with 900 μl of cold EtOH. The DNA was pelleted (2-minute spin down) the supernatant discarded and the pellet washed with 70% EtOH. The DNA pellet was then dried under vacuum and resuspended in approximately 50 μl of TE.

2.5 Large Scale Preparation of Plasmid DNA

The large scale purification of plasmid DNA from cultures of E.coli was carried out via the Qiagen Maxi plasmid purification kit (Qiagen, Chatsworth, CA; catologue #12163) according to kit instructions. Briefly, a single colony from the bacterial culture of choice was used to inoculate 5 mls of LB media (containing ampicillin) which was grown to saturation. This saturated culture was further diluted 1:100 in LB media,

allowed to grow overnight, and centrifuged for 15 minutes at 6,000 rpm the following day in order to harvest the bacterial cells. The bacterial pellet was resuspended in 10 mls of buffer P1, and the cells lysed upon the addition of 10 mls of buffer P2. The cell lysate solution was then neutralized with 10 mls of buffer P3 (see Appendix A for details on solutions). Following a 20-minute incubation on ice, the sample was centrifuged for 30 minutes at 15,000 rpm. The supernatant was removed, filtered through gauze and loaded directly onto the Qiagen-tip column. The sample was washed twice with buffer QC, following which the plasmid DNA was eluted from the column with buffer QF and then precipitated with 0.7 volumes of isopropanol. The plasmid DNA was subsequently pelleted via centrifugation at 10,000 rpm for 30 minutes, washed with 15 mls of 70% ethanol, air dried for 5 minutes, redissolved in 100 µl of TE buffer, and transferred to an eppendorf tube.

2.6 Transfection of C2C12 Cells

Murine C2C12 myoblasts were transfected with the pNMG3 plasmid created by Chang et al., 1990 (encoding human growth hormone (hGH) under the control of the mouse metallothionein (mMT) promoter, in addition to an SV40 sequence upstream of the hGH gene and a polyadenylation signal downstream). A calcium phosphate-mediated DNA precipitation method (Graham and Van der Eb, 1973) was utilized for transfection of the C2C12 cells, which were seeded onto 100mm tissue culture dishes one day prior to transfection such that they were approximately 30 to 40% confluent at the time of

transfection. Fresh growth media was added to the cells 4 hours prior to transfection.

A 0.5 ml DNA/$CaCl_2$ mixture (containing 11.1 µl (10 µg) of pNMG3 plasmid DNA, 50 µl of 2.5 M $CaCl_2$ and 439 µl ddH_2O) was added dropwise, at a rate of 1 drop per second, to 0.5 ml of sterile 2X HEBS solution (see Appendix A). A control sample contained 10 µg of sonicated salmon sperm DNA (Sigma) as opposed to the pNMG3 plasmid DNA. The 2X HEBS solution was gently "bubbled" with a 1ml sterile pipette attached to an electric pipetman as the DNA/$CaCl_2$ mixture was added. The resulting solution was incubated at room temperature for approximately 30 minutes to allow for the formation of a precipitate, and then added directly to the media of the cells to be transfected. Subsequent to a 4 hour incubation period at 37°C, the growth media was removed and the cells were washed as follows: 2X in serum free Dulbecco's media, once in sterile 15% glycerol in serum free media (for exactly 30 seconds) and 2X in serum free media. The cells were then grown in regular growth media for 24 hours, after which time they were split via trypsinization (see Appendix B) at a ratio of 1:5 and 1:10 onto 150 mm tissue culture dishes and grown in growth media containing the antibiotic G418 (Boehringer Mannheim, Laval, Quebec; catologue #1464-981) at an effective concentration of 40 µg/ml to select for positive transfectants. Following growth in selection (G418) media for approximately one week, the recombinant cells were pooled together and designated "pNMG3-pool".

2.7 Determination of hGH Secretion Rate

The rate of hGH secretion from microencapsulated or unencapsulated pNMG3-pooled cells contained in 35 mm tissue culture dishes was determined by taking 50 µl samples of media at 0, 1, 2 and 4 hours following the addition of fresh, equilibrated differentiation media (**note**: microencapsulated cell samples were washed 3-4 times with equilibrated media to remove excess hGH protein). At each time interval, 50 µl of fresh, equilibrated media was added to each dish to maintain the original volume of media used. Samples were subsequently run on an hGH enzyme linked immunosorbent assay (ELISA) (UBI-Magiwell hGH Quantitative kit, United Biotech Inc., CA) according to kit instructions. Based upon standards of known hGH concentration, the amount of hGH protein present in the sampled media was determined via the use of a microwell reader which provided absorbance values at an optical density of 450 nm. The absorbance values were plotted against the sampling time and the slope of the best-fit line was recorded to be the hGH secretion rate in units/hour. The amount of total protein present in each sample was determined by the Lowry assay (see Methods: "Protein Determination") and the rate of hGH secretion was further expressed in ng hGH/h/µg protein.

2.8 RNA Isolation

RNA was isolated from C2C12 myoblasts via the use of TRIzol Reagent (Gibco; catalogue #15596-026) which is a mono-phasic solution of phenol and guanidine isothiocyanate. RNA was isolated according to the instructions provided by Gibco. The growth media was removed and 3 mls of TRIzol reagent was added directly to the 100 mm tissue culture dish of cultured myoblasts. The lysed cells were pipetted up and down several times and placed into a capped 16 ml polypropylene, round-bottom tube (Dupont; catalogue #03244). Following a 5 minute incubation period, 600 µl of chloroform (Sigma) was added to the cell lysate accompanied by vigorous mixing for approximately 15 seconds. The sample was incubated at room temperature for 2-3 minutes and then centrifuged at 10,000 rpm for 15 minutes at 5°C. The aqueous phase of the centrifuged sample was removed, placed into a fresh tube and the RNA precipitated at room temperature for 10 minutes via the addition of 1.5 mls of isopropyl alcohol (Sigma). The precipitated RNA was pelleted by centrifugation at 10,000 rpm for 10 minutes at 5°C, the supernatant removed, the pellet washed with 3 mls of 75% ethanol by vortexing and then centrifuged at 8,000 rpm for 5 minutes at 5°C. The washed RNA pellet was subsequently air-dried for 5-10 minutes, dissolved in RNase-free water and incubated at 55-60°C prior to storing at -70°C.

2.9 Northern Blot

The method used for Northern blotting was adapted from a protocol supplied by Dupont ("Company Research Tips" guide). A 1% agarose gel was prepared by the addition of 1.0 g of agarose (Gibco; catalogue #15510-027) to 62 mls of DEPC-treated ddH_2O and 20 mls of 5X MOPS buffer. This solution was brought to a boil in a sterile beaker and subsequently cooled to approximately 70°C. In a fume hood, 18 mls of 37% formaldehyde (Sigma) was added to the mixture, mixed immediately, and poured into a gel form (previously treated with SigmaClean detergent (Sigma) and rinsed with DEPC-ddH_2O). The gel was allowed to harden for at least 30 minutes and then placed into the gel tank with an appropriate amount of 1X MOPS running buffer. RNA samples (10 μg in 4.5 μl DEPC-ddH_2O) were prepared by the addition of 5X MOPS, pH 7.0 (2 μl), 37% formaldehyde (3.5 μl) and deionized formamide (10 μl). Samples were heated at 60°C for 15 minutes and then loaded onto the gel following the addition of 3 mg/ml EtBr solution (1 μl) and sterile gel loading buffer (5 μl). The gel was run at 60 volts for approximately 2 hours.

The agarose / formaldehyde gel was immersed in the following solutions prior to the overnight transfer of RNA: (i) DEPC-ddH_2O (5X for a duration of 1 minute each); (ii) 50 mM NaOH (for 15 minutes); (iii) 100 mM Tris-HCl, pH 7.0 (for 30 minutes). The transfer was set up (using 10X SSC as the transfer buffer) in a layered fashion as follows (in order from bottom to top): 2 sheets of 3MM Whatman paper (Whatman; catalogue #3030-917) for the bridge, agarose gel, Gene Screen Plus membrane (Dupont, Canada;

product #NEF-976) (previously soaked in 10X SSC for 15 minutes), 2 sheets of 3MM Whatman paper (wet in 10X SSC), 4 sheets of dry 3MM Whatman paper and a stack of dry paper towels. The Gene Screen Plus membrane was rinsed twice in 2X SSC (15 minutes each) and dried at room temperature. The RNA was then fixed onto the membrane by baking in a hybridization oven (Robbins Scientific Corporation, Sunnyvale, CA; model #310) for 2 hours at 80°C.

The membrane was pre-hybridized (after wetting in 2X SSC) in 5 mls of pre-hybridization buffer containing 50 µl of denatured, sonicated herring sperm DNA (Promega; catalogue #D1811) (10 mg/ml), at 42°C (in a rotating hybridization oven) for a minimum of 4 hours in a 50 ml conical tube (Falcon; product #2070) which was, in turn, placed inside a capped glass hybridization cylinder (Robbins Scientific Corporation). The pre-hybridization buffer was then removed and replaced with 2-5 mls of hybridization buffer containing 50 µl of [^{32}P-alpha]dCTP-labelled probe (see Appendix C for detailed protocol on labelling of probes) in addition to 50 µl of 10 mg/ml of sonicated, denatured herring sperm DNA. The membrane was hybridized overnight (approximately 16 hours) at 42°C. The membrane was then washed as follows: (i) (2X) 15 minute washes at R.T. in 2X SSC / 1% SDS; (ii) (2X) 30 minute washes at 65°C in 0.1X SSC / 0.5% SDS. The washed membrane was blotted with 3MM Whatman paper, sealed in plastic wrap (Roll-O-Sheets, Canada) and exposed to a phosphor screen (Molecular Dynamics, CA) for approximately 2 hours, after which the image captured on the screen was read by a phosphorimager (Molecular Dynamics).

2.10 Dot Blot

The dot blot protocol employed for the analysis of total mRNA was adapted from C. Hartley (1987), with the exception of the RNA preparation and membrane treatment, which was based upon methods outlined in the Hybond-N guide booklet (Amersham, UK). The RNA samples (4-8 μg each) were brought up to a volume of 25 μl to which 3 volumes of the following solution was added: 500 μl of formamide (Sigma); 162 μl of 37% formaldehyde (Sigma); and 100 μl of 10X MOPS buffer. The RNA samples were incubated at 65°C for 5 minutes, chilled on ice, following which 1 volume of cold 20X SSC was added. The Bio-dot apparatus (Bio-Rad, cat. #170-6545) was set-up according to the instructions provided and the RNA samples were spotted onto Hybond-N nylon membrane (Amersham) which had been pre-wetted in 10X SSC. A buffer solution (6 mls of 20X SSC; 4 mls of 37% formaldehyde; 10 mls of RNase-free ddH_2O) was rinsed through all of the sample wells once prior to the sample loading (200 μl per well) and twice following the application of the RNA to the membrane (**note**: the RNA was allowed to sit on the membrane for approximately 30 minutes before any vacuum pressure was applied). The blotted membrane was subsequently removed from the apparatus, placed between two pieces of 3MM Whatman paper and baked for 10 minutes at 80°C. The membrane was then placed in plastic wrap and the RNA was fixed to the membrane by exposure to ultraviolet light for 3 minutes. The membrane was ultimately put through pre-hybridization, hybridization and wash steps followed by exposure via the

use of a phospor screen (Molecular Dynamics) as outlined previously in the "Northern Blot" protocol.

2.11 Probe Preparation

The preparation of double-stranded plasmid DNA fragments, which would serve as RNA-hybridization probes, was based upon methods outlined in Sambrook et al. (1989). Initially, 5 μg of plasmid DNA was digested with the appropriate restriction enzyme in order to release the insert DNA to be used as a probe (i.e. plasmid vectors contained the following genes: human growth hormone (hGH), human phosphoglycerate kinase (PGK1), mouse MyoD1, mouse myogenin, rat fast muscle myosin light chain 2 (LC2), rat fast muscle myosin light chain 3 (LHC3), rat fast muscle troponin T (TnT), and rat troponin I slow skeletal muscle isoform (TnIslow) (see Appendix D for details on plasmid vectors). Plasmid vectors were digested according to the manufactuer's specifications (Boehringer Mannheim) for 1-2 hours at 37°C with the reaction solution consisting of 5 μg of plasmid DNA, 1 μl of enzyme (EcoR1 or Pst1, Boehringer Mannheim) and 2 μl of the appropriate 10X reaction buffer, to a total volume of 20 μl. Gel-loading buffer (5 μl) was added to the digested samples and the resulting fragments were separated out on a 0.7% agarose (Gibco) gel, which was run at approximately 80 volts for 1.5 hours, along with a standard marker lane (λ HindIII fragments). The band representing the insert DNA of choice was isolated from the gel by, firstly, cutting out the fragment with a sterile razor blade and then purifying the DNA from the gel with "Gene-

Clean" (Gibco) according to kit instructions. The insert DNA was ultimately resuspended in approximately 30 µl of TE buffer and the concentration of double-stranded DNA in the sample was determined via the use of a spectrophotometer.

2.12 Myoblast Differentiation Determination - CPK Assay

The level of myoblast differentiation was determined by measuring the amount of creatine phosphate kinase (CPK) present in the cells. Cells were harvested by trypsinization (see Appendix B), pelleted, resuspended in 500 µl of 1X PBS and sonicated three times (10 seconds each) on HIGH power via the use of a "micro-ultrasonic cell disrupter" (Kontes). The resulting suspension was analyzed via the use of the CK-MPR 1 diagnostic kit (Boehringer Mannheim, Montreal; catalogue #1087533) according to kit instructions. The cell lysate was diluted 1:25 (cell lysate: MPR 1 reagent solution), mixed well and incubated at R.T. for 3 minutes. The absorbance at 340 nm was then read at 1 minute intervals (for a total of 4 readings) with an Ultrospec 2000 (Pharmacia, Uppsala, Sweden). The CK activity was then calculated by determining the mean absorbance change per minute (ΔA/min) and multiplying this value by 4127 to get a CK activity value, measured in International Units (U/I).

2.13 Protein Determination

The total protein present in naked and encapsulated myoblast samples (which had been resuspended in 1X PBS to a total volume of 500 µl and sonicated for 20 seconds (3X)) was performed according to the method of Lowry et al. (1951). Briefly, 1 ml of reagent C was added to 200µl of sample placed into a disposable, borosilicate, round-bottom, 10 x 75 mm culture tube (Maple Leaf Brand, Canada; catalogue #60825-400). Following a 10 minute incubation period, 100 µl of Folin-Phenol reagent (Sigma) was added while simultaneously vortexing the sample. Following a 30 minute incubation period, the samples were transferred into disposable, 1.5 ml plastic cuvettes (Mandel Scientific; catalogue #DN-302205-0002), the absorbance was read at an optical density of 750 nm and the protein determined based on a set of bovine serum albumin (BSA) standards.

2.14 hGH Immunofluorescence

Myoblasts were stained for hGH based on a protocol by Doering and Chang (1991) (modified by J. VanStralen, 1995; unpublished observations). Briefly, cells were grown on gelatin-coated coverslips (or, in the case of encapsulated cells, spun onto slides via cytocentrifugation - see Appendix E), fixed in 4% paraformaldehyde for 30 minutes, exposed to acetone for 3 seconds, and rinsed in 1X PBS for 5 minutes (3X). Following fixation, an overnight exposure (in a humidified container at 4°C) to the polyclonal

primary rabbit anti-hGH antibody (Monosan, Netherlands; catalogue #PS-036) was performed. The primary antibody had been previously absorbed for one hour with an untransfected C2C12 homogenate in which it was diluted 1:100. The following morning, the coverslips/slides were rinsed with 1X PBS for 20 minutes (3X) and exposed to the secondary FITC-Goat anti-rabbit IgG antibody (Zymed, CA; catalogue #65-6111), diluted 1:50 in Antibody Diluting Buffer (Dimension Laboratories Inc., Mississauga, Ontario; catalogue #D2000S), for 30 minutes at room temperature. The coverslips/slides were then washed three times in 1X PBS for 10 minutes, followed by three rinses in ddH_2O for 1 minute. Air-dried slides/coverslips were mounted in a solution of 90% glycerol, 0.1% para-phenylenediamine (PPD) and 10% 0.01 M phosphate buffer (v/v) in 0.15 M NaCl. Clear nailpolish was used to seal the edges of the mounted slides/coverslips. Photographs were taken on a Zeiss fluorescent microscope.

2.15 Myogenic Index Determination

The determination of the myogenic index (i.e. the number of nuclei in multinucleate cells expressed as a percentage of the total number of nuclei observed) was aided by the detection of myosin heavy chain (MHC) protein in order to delineate which myoblasts had begun the process of biochemical differentiation (note: MHC is largely expressed by multinucleate cells in a differentiating culture of myoblasts). The MHC-staining protocol was obtained from R. Perry (McMaster University) and was based upon methods outlined in Bader et al. (1982). The myogenic index was determined, firstly, by fixing cells grown on gelatin-coated, 35 mm tissue culture dishes (or spun onto slides, as

were the encapsulated cells - see Appendix E) in 90% methanol for 6 minutes at -20°C. The fixed cells were washed (3-4 X) in 1X PBS and immersed in a blocking agent (5% NFDM (non-fat dry milk; Carnation) in 1X PBS) for 30 minutes at 37°C. The blocking agent was then removed and the fixed cells were exposed to the primary monoclonal antibody, MF20 (ATTC, Rockville, MD), diluted 1:10 in 5% NFDM, for the detection of myosin heavy chain (MHC) protein for 1 hour at room temperature. A secondary HRP-conjugated goat anti-mouse IgG antiserum (Biorad; catalogue #170-6516), diluted 1:1000 in 5% NFDM, was applied to the samples for 1 hour at room temperature on a rocking table following washes in 1X PBS (3X for 5 minutes). Samples were again washed (as previously) and exposed to a 0.06% solution of diaminobenzidine (DAB) (Sigma) for approximately 5 minutes, washed in ddH_2O, and stained with diluted filtered Giemsa stain for approximately 20 minutes. The myogenic index was then determined by scoring the number of nuclei residing in multinucleate cells via a Zeiss light microscope under 400X power and the average was calculated from 10 randomly counted fields.

2.16 Microencapsulation of Cells

The microencapsulation procedure was carried out according to the protocol refined by Chang et al., 1993. Cells were prepared for microencapsulation by harvesting via trypsinization (see Appendix B), resuspended in growth media, counted with a Coulter counter (Coulter Electronics, Hialeah, Florida) and spun at 1000 rpm for 10 minutes at 4°C. The cell pellet was then washed in 35 mls of ice cold 1X PBS and spun again as above. The pelleted cells were subsequently resuspended in 500 µl of 0.85%

NaCl and then mixed with 10 to 15 mls of cold 1.5% Kelmar potassium alginate, which is a seaweed extract composed of the polysaccaride polymannuronic and polyguluronic acid (Kelco, Merck, Chicago, Il). The alginate solution had been previously heated to 50°C and sterilized with a 0.45 um syringe filter (Gelman Sciences, product #4184). A standard mixture of 3×10^6 to 5×10^6 cells per ml of alginate was transferred to a 20 ml syringe (Becton Dickinson, Mississauga, Canada). The syringe was placed into a delrin holder attached to the microcapsule machine, the latter of which consisted of an Orion Sage pump (model M362) supported by a plexiglass housing unit (Health Sciences machine shop, McMaster University). The alginate-cell mixture was extruded through a 27 gauge needle (Cat. 7400, Popper & Sons, New York) at a rate of $99.9 \times {}^{1}/_{100}$ cm^3 per hour. The delrin holder was supplied with approximately 4,000 cm^3 of air per minute which flowed in a concentric pattern around the needle, thereby causing the extruded alginate to form small spheres (approximately 500-700 μm in diameter) that were allowed to drop into a cold 1.1% $CaCl_2$ bath placed approximately 7 to 8 centimeters below the needle tip. The calcium ions in the $CaCl_2$ solution crosslinked with the alginate, forming a calcium-alginate matrix throughout the "capsule", in which the cells were then embedded. These capsules were transferred to conical 50 ml centrifuge tubes (Sarstedt; catalogue #62-547-205) for further treatment with various solutions (see Appendix F for specific procedure), among which included the addition of a 0.05% (w/v) solution of poly-L-lysine (a polycation with a Mol Wt of 15,000-30,000; Sigma, MO). These negatively-charged droplets were subsequently neutralized by a coat of polyanionic alginate. The intracapsular space was ultimately liquified by the addition of sodium

citrate for 6 minutes. The microcapsules were kept in regular growth media supplemented with basic fibroblast growth factor (bFGF) (Sigma; catalogue #F-0291) at a concentration of 10 ng/ml and incubated under regular tissue culture conditions.

2.17 Characterization of Encapsulated Cells

2.17.1 Cell Viability

Encapsulated cell viability was determined by placing approximately 50-100 µl of capsules onto a plain microscope slide (Corning Glass Works, N.Y.; product #2947), removing the excess media with a Kimwipe and adding a drop of trypsin for 2-3 minutes. A drop of 0.4% Trypan blue stain (Gibco, catalogue #15250-061) was then added to the capsules, after which a glass coverslip was lowered onto the capsule sample accompanied by the application of gentle pressure to ensure the release of the cells from the capsules. Cell viability was determined by scoring for the percentage of cells which demonstrated exclusion of the trypan blue stain via a Zeiss light microscope at a magnification of 160X.

2.17.2 Cell Release

Myoblasts were released from the microcapsules by, initially, rinsing the capsules in 1X PBS approximately 3-4 times followed by a single rinse in 0.055 M sodium citrate for 6-8 minutes on ice on a rocking table. The washed capsules were allowed to settle and then drawn into a 10cc syringe (Becton Dickinson; product #309604) and extruded though a 26 gauge needle (Becton Dickinson; product #305111). The viscous suspension

was then transferred to a 15 mL conical tube (Sarstedt; catalogue #62-554-205), mixed with cold 1X PBS (to a total of 14 mls) and spun at 1500 rpm for 10 minutes to pellet the cells. The majority of the crushed capsule material was aspirated with a Pasteur pipette and the cell pellet was again rinsed in 14 mls of 1X PBS and spun down as before.

2.17.3 Determination of Cell Number per Capsule

A volume of 50-100 µl of microcapsules was transferred to a 1.5 ml eppendorf tube and the number of capsules in the sample was determined by adding a drop of trypan blue stain to the sample, placing the sample into approximately 3 mls of 1X PBS in a 60 mm tissue culture dish (Falcon) which was situated over a grid, and counting the total number of capsules present. The count was repeated three times and the average of the three values was calculated.

To determine the number of cells per capsule, the cells were released from a known number of capsules by crushing the capsules with a small plastic pestle (Baxter; catalogue #749520-0000). The resulting suspension was brought up to a known volume (50-100 µl) and approximately 15 µl was transferred to a haemocytometer for counting. The count was repeated ten times, from which the average count was calculated. The total number of cells in the sample was then divided by the total number of capsules present in the same sample.

2.18 Data Analysis

All statistical analyses were performed with the use of Microsoft Excel™. A two-tailed student's T-test was carried out in each case ($p=0.05$).

3.0 RESULTS

3.1 Expression of Human Growth Hormone

In order to compare the secretion of a marker protein from encapsulated versus unencapsulated C2C12 myoblasts, a protein which was known to be readily secreted from alginate-polylysine capsules was chosen, namely human growth hormone (hGH) (Tai and Sun, 1993; Chang et al, 1993; Chang et al, 1994). C2C12 myoblasts were transfected via a calcium phosphate-mediated method (as described in the Methods section). The transfected cells were grown in selection media, consisting of D-MEM media supplemented with 10% FBS and G418 (40 μg/ml) for approximately one week. The recombinant cells were then pooled together and designated "pNMG3-pool". The rate of hGH secretion was determined by ELISA (UBI Magiwell, CA), whereby 50 μl samples of media were taken at 0, 1, 2 and 4 hours following replacement with fresh, equilibrated growth media. These media samples were run on an hGH ELISA, along with the provided hGH standards (0 ng/ml, 1 ng/ml, 2.5 ng/ml, 5 ng/ml, 10 ng/ml and 20 ng/ml), and the absorbance was read at an O.D. of 450 nm. The amount of hGH protein present in each of the media samples was calculated, after which the rates of hGH secretion were determined by plotting the amounts of hGH protein (ng/ml) against time (hours). The average rate of hGH secretion from the pNMG3-pool myoblasts was calculated to be 50.5 ± 6.8 ng hGH/hour/10^6 cells (see Appendix G).

3.1.1 hGH Secretion Rates: Comparison of Encapsulated and Unencapsulated Myoblasts

The recombinant myoblasts engineered to secrete hGH, namely pNMG3-pool cells, were analyzed with respect to rates of hGH secretion over a period of two weeks of growth in differentiation media (D-MEM media supplemented with 2% horse serum). The rate of hGH secretion, expressed in ng hGH/hour/μg protein, was determined on days 0, 3, 5, 9, 11 and 14 of differentiation for both naked (unencapsulated) and encapsulated pNMG3-pool myoblasts (Experiment #2). This experiment attempted to ascertain whether or not the process of myoblast differentiation would have an effect on the rate of hGH secretion. In addition, it was of interest to determine whether or not the process of microencapsulation played a role in altering the secretion of hGH.

Figure 1 compares the rates of hGH secretion from differentiating naked (unencapsulated) myoblasts and differentiating encapsulated myoblasts. The secretion of hGH does not appear to be affected by the process of myogenic differentiation, as demonstrated by the sustained expression of hGH in naked pNMG3-pool cells over a period of two weeks, which ranged from 6.04×10^{-2} to 9.56×10^{-2} ng hGH/hour/μg protein. The encapsulated pNMG3-pool cells, however, demonstrated a decrease in hGH secretion of approximately 44%, from 8.38×10^{-2} ng/hour/μg on day 0 of differentiation, to 3.72×10^{-2} ng/hour/μg on day 14 of differentiation.

Upon comparison of the naked myoblasts with the encapsulated myoblasts (Figure 1), the rates of hGH secretion between the two are not significantly different ($p>0.05$) on days 0, 3, 5 and 9 of differentiation. On days 11 and 14, however, the rates of hGH

secretion between the naked and encapsulated cells are significantly different ($p<0.05$).

Figure 2 compares the rates of hGH secretion between differentiated naked myoblasts and differentiated encapsulated myoblasts, as in Figure 1, however, in this case, the secretion rate was calculated using total protein values which had been adjusted according to the percentage of viable cells present at the time of sampling. This adjustment is relevant for the encapsulated cells, whose viability decreased approximately 41% throughout the two weeks in differentiation media, from ~90% on day 0 of differentiation to ~53% on day 14 of differentiation. It was assumed that the dead cells remaining in the capsular space contributed to the total protein measurement, and therefore, this percentage of non-viable cells had to be taken into account. The rates of hGH secretion between naked and encapsulated pNMG3-pool cells are not significantly different ($p>0.05$) on days 0, 3, 5, 9 and 14 of differentiation, however, the rates of hGH secretion are significantly different ($p<0.05$) on day 11 of differentiation. Overall, correcting the total protein values to reflect the cell viability only demonstrates a difference in hGH secretion rate for encapsulated pNMG3-pool cell on day 14 of differentiation when compared to the hGH secretion rates derived from uncorrected protein values, as in Figure 1.

Figure 1.

Secretion rates of human growth hormone (hGH) expressed in ng hGH per hour per μg protein of: (a) encapsulated and, (b) unencapsulated differentiating pNMG3-pool myoblasts. 50 μl samples were taken at time points of 0, 1, 2 and 4 hours on each of 0, 3, 5, 9, 11 and 14 days following the addition of differentiation media. Microencapsulated samples were exposed to bFGF for approximately 11 days prior to the addition of differentiation media. hGH secretion was detected by ELISA, and the resulting data presented represents the average of triplicate measurements ± SD.

Figure 1

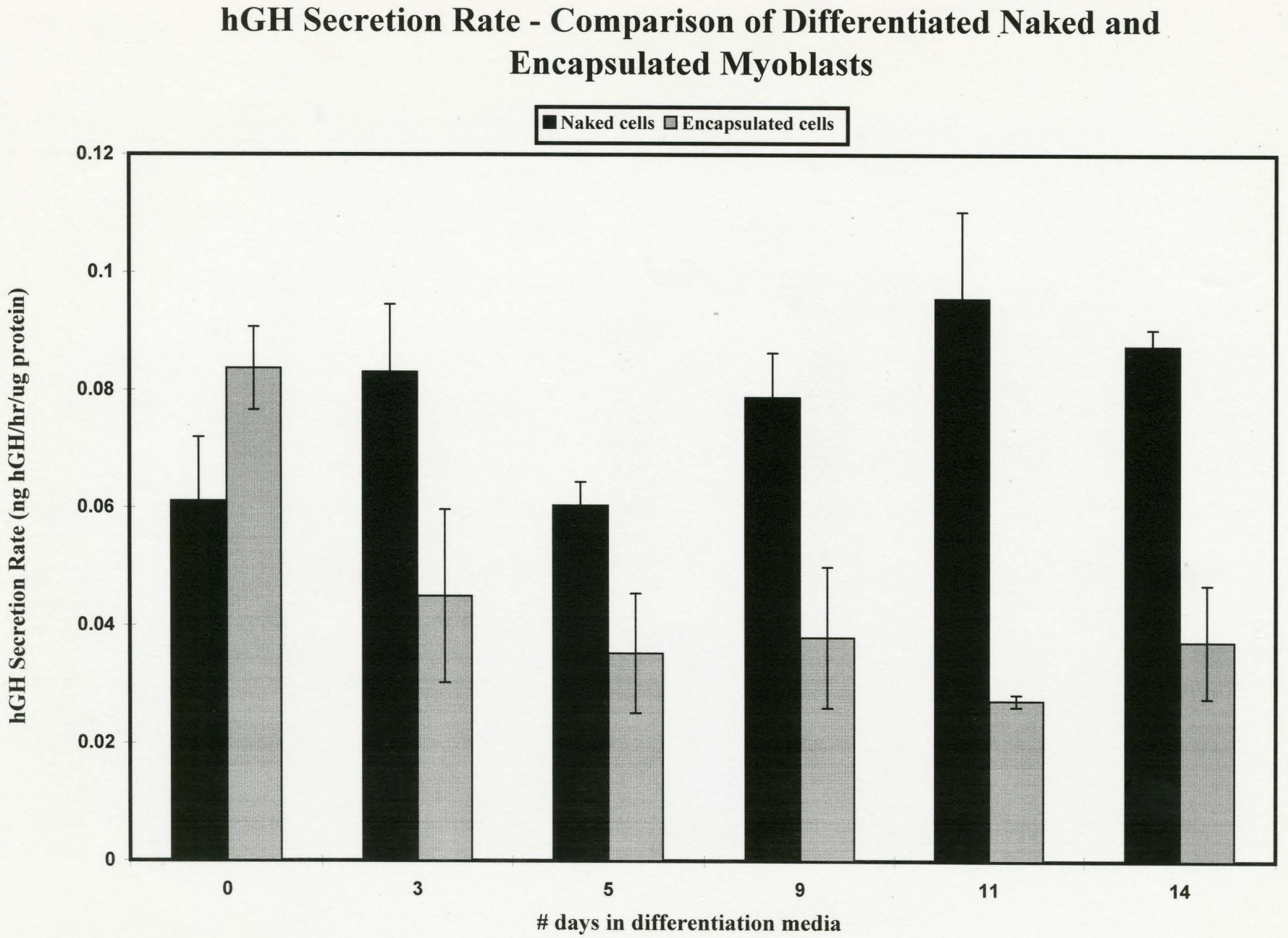

hGH Secretion Rate - Comparison of Differentiated Naked and Encapsulated Myoblasts
Naked cells
Encapsulated cells
hGH Secretion Rate (ng hGH/hr/ug protein)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
3
5
9
11
14
days in differentiation media

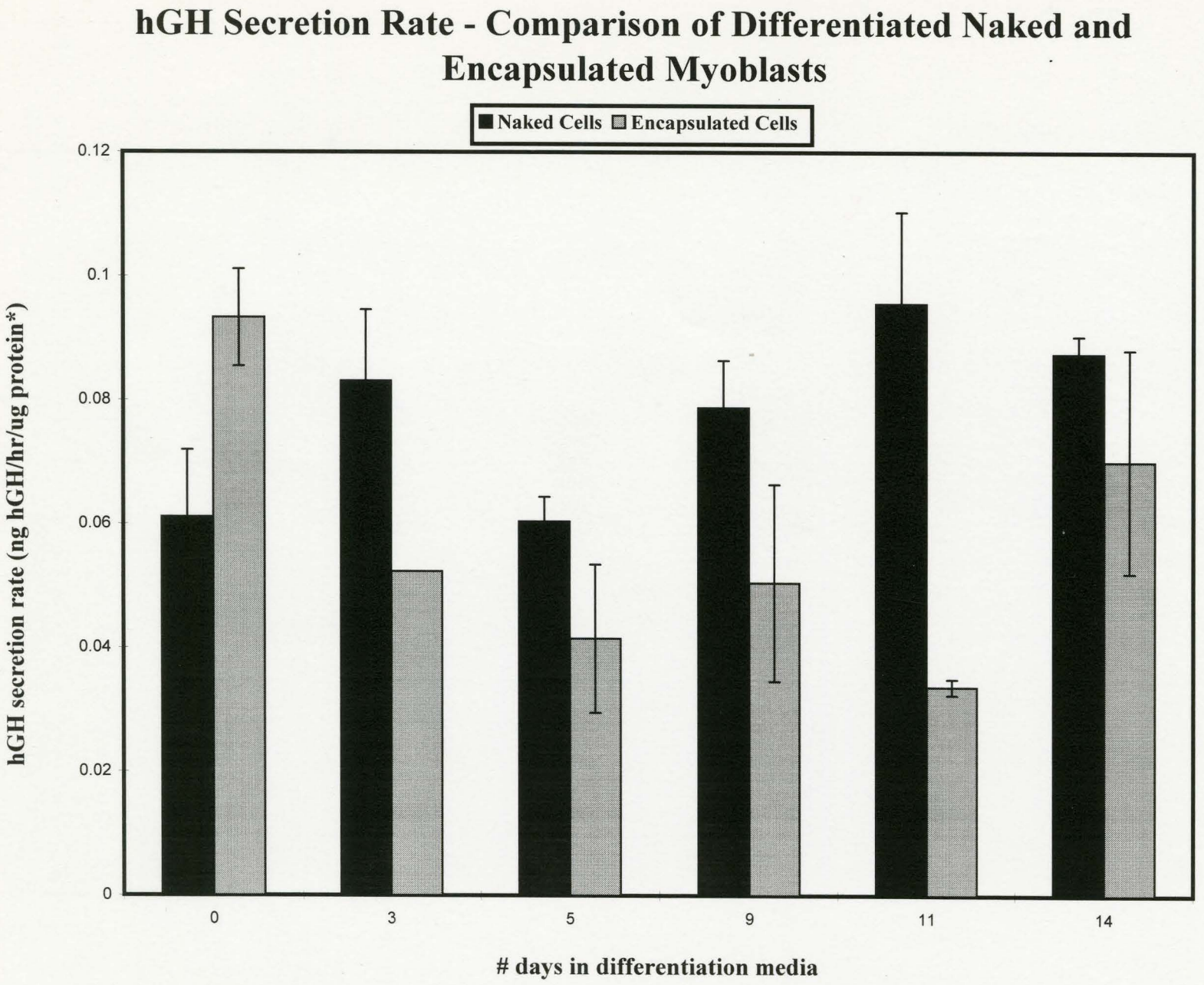
Figure 2.

Secretion rates of human growth hormone (hGH) expressed in ng hGH per hour per μg protein of (a) naked (unencapsulated) pNMG3-pool myoblasts and (b) encapsulated pNMG3-pool myoblasts, both of which were grown in differentiation media for a period of two weeks. 50 μl samples were taken at time points of 0, 1, 2 and 4 hours on each of 0, 3, 5, 9, 11 and 14 days following the addition of differentiation media. Microencapsulated samples were exposed to bFGF for approximately 11 days prior to the addition of differentiation media. hGH secretion was detected by ELISA and the final values, expressed in ng hGH/hr/μg protein, were calculated using protein values that had been corrected according to the percentage of cell viability (see Table 1 below) determined at the time of sampling. The data presented represents the average of triplicate measurements ± SD (with the exception of the encapsulated "day 3" sample, which represents the average of two measurements).

Table 1. Encapsulated Cell Viability During Differentiation.

# Days in Differentiation Media:	% of Viable Cells:
0	90 ± 7
3	86 ± 3
5	85 ± 5
9	75 ± 5
11	80 ± 5
14	53 ± 7

Figure 2



(* Protein values have been corrected according to encapsulated cell viability)

3.1.2 Immunofluorescent Staining of hGH

The amount of hGH produced by pNMG3-pool myoblasts, either unencapsulated or encapsulated, was studied qualitatively by immunofluorescent staining. Encapsulated and unencapsulated pNMG3-pool cells were grown in differentiation media for a period of 2 weeks and the cells were fixed and stained with an anti-human growth hormone antibody for the detection of hGH. The addition of a secondary goat-anti-rabbit IgG antibody conjugated to FITC allowed for the visualization of the hGH signal via the use of a fluorescent microscope. Cells were fixed and stained on days 0, 3, 5, 9, 11 and 14 of differentiation, and then photographed with a camera attached to a fluorescent microscope (Figures 3a, 3b, 3c and 3d). Naked myoblast samples were exposed for 15 seconds while the encapsulated myoblast samples were subjected to a 90-second exposure time (longer exposure times were required with the encapsulated samples as the fluorescent signal was not as intense due to the decrease in the number of viable cells present). The exposure time would have been kept constant among all naked and encapsulated samples had a direct comparison been made between the levels of hGH observed in differentiating naked myoblasts as opposed to encapsulated myoblasts, which in this case, it was not. The results merely demonstrated that hGH was detectable throughout the two week period in both naked (Figures 3a and 3b) and encapsulated (Figures 3c and 3d) cells. The hGH signal among the pNMG3-pool population was quite variable, given the fact that the cells stained are representative of a pooled population and are not clonal in origin. The hGH signal did, however, appear to become more diffuse throughout the myotube as myoblast fusion occurred (Figures 3b-E and 3b-F), as

evidenced in the naked myoblasts, such that the "perinuclear" staining was not as distinct as what was seen in the mononucleate cells (Figure 3a-B: arrow indicates hGH signal, yellow in colour, around nucleus). This perinuclear staining is not evident in Figure 3a-A, as it is an untransfected C2C12 cell sample (negative control). There appeared a greater portion of encapsulated cells which lacked a positive signal for hGH, however, it must be noted that this is due to the fact that the encapsulated cell preparations used for hGH staining contained a significant percentage of dead cells (note: the viability of the encapsulated pNMG3-pool cells decreased from ~90% on day 0 of differentiation, to ~66% on day 14 of differentiation).

Figures 3a-D and 3b-E both represent samples of naked myoblasts at day 5 of differentiation. Figure 3a-D shows that there are still a portion of mononucleate cells present at day 5 that have not yet fused into multinucleated myotubes, unlike Figure3b-E, in which multinucleate myotubes are evident. It is difficult to detect the typical hGH staining patterns around the cell nuclei in Figures 3b-G and 3b-H (days 11 and 14 of differentiation, respectively) as the myoblasts/myotubes in these samples have begun to "clump" together as they differentiate. Overall, conclusions regarding the levels of hGH staining observed throughout the two week differentiation period cannot be ascertained, at least with respect to comparing the different days of differentiation, due to the fact that the staining is variable among each myoblast. It is evident, however, that hGH is still being produced by 14 days of differentiation, in both naked and encapsulated myoblasts.

Figure 3 (a,b,c,d).

Immunofluorescence staining for the detection of human growth hormone (hGH) in: (i) encapsulated pNMG3-pool myoblasts, and (ii) unencapsulated pNMG3-pool myoblasts, both of which were induced to differentiate over a two week period. Cells were fixed and stained on days 0, 3, 5, 8, 11 and 14 of differentiation. Unencapsulated (naked) samples were exposed for 15 seconds, while encapsulated cells were exposed for 90 seconds.

Figure 3a: (A) Untransfected C2C12 cells (negative control) (Mag.: 1000 X)

(B) Naked pNMG3-pool cells - day 0 (Mag.: 630 X)

(C) Naked pNMG3-pool cells - day 3 (Mag.: 630 X)

(D) Naked pNMG3-pool cells - day 5 (Mag.: 1000 X)

Figure 3b: (E) Naked pNMG3-pool cells - day 5 (Mag.: 630 X)

(F) Naked pNMG3-pool cells - day 9 (Mag.: 630 X)

(G) Naked pNMG3-pool cells - day 11 (Mag.: 630 X)

(H) Naked pNMG3-pool cells - day 14 (Mag.: 630 X)

Figure 3c: (I) Encapsulated pNMG3-pool cells - day 0 (Mag.: 630 X)

(J) Encapsulated pNMG3-pool cells - day 3 (Mag.: 630 X)

(K) Encapsulated pNMG3-pool cells - day 5 (Mag.: 630 X)

(L) Encapsulated pNMG3-pool cells - day 9 (Mag.: 630 X)

Figure 3d: (M) Encapsulated pNMG3-pool cells - day 11 (Mag.: 630 X)

(N) Encapsulated pNMG3-pool cells - day 14 (Mag.: 630 X)

Figure 3a

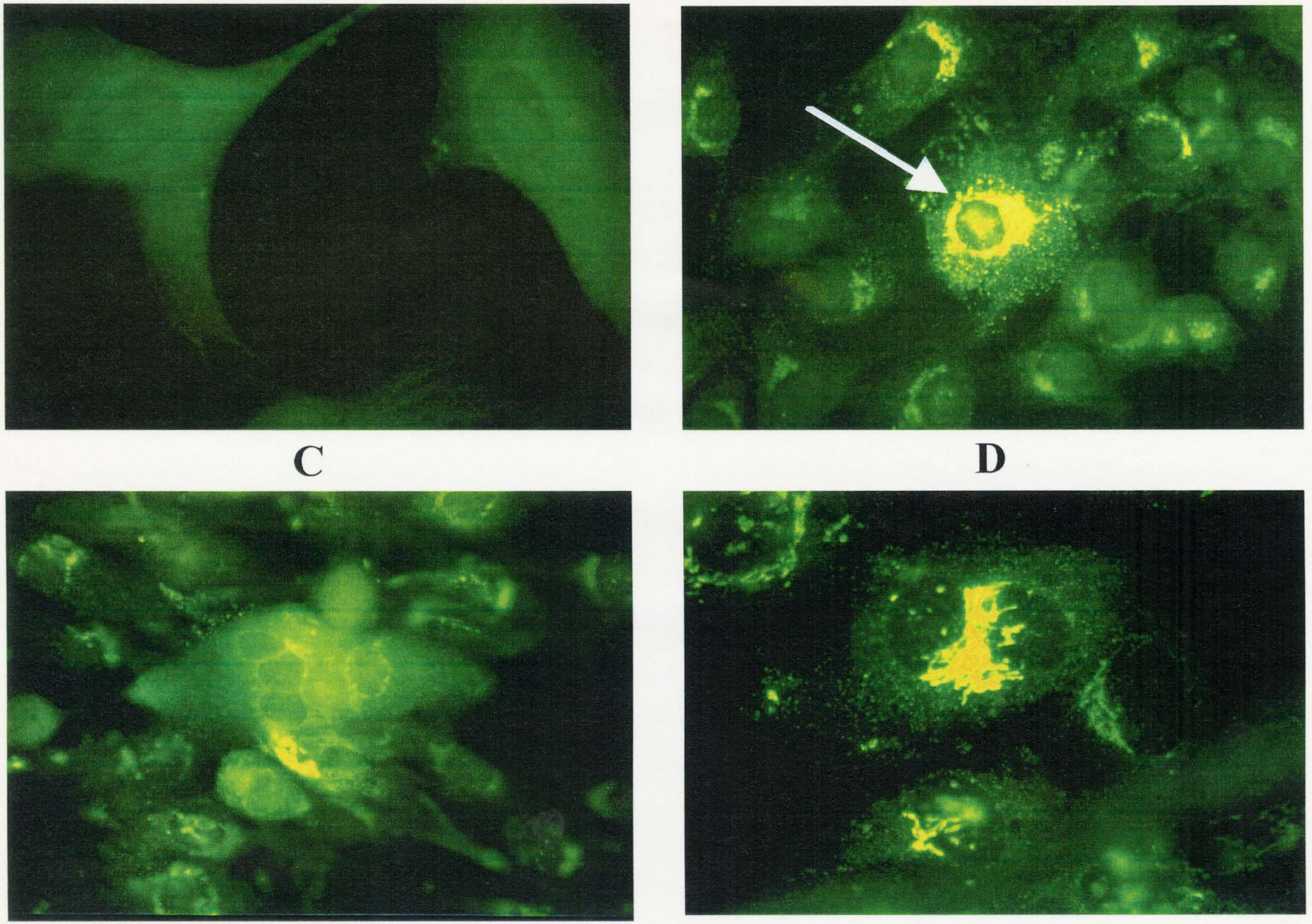
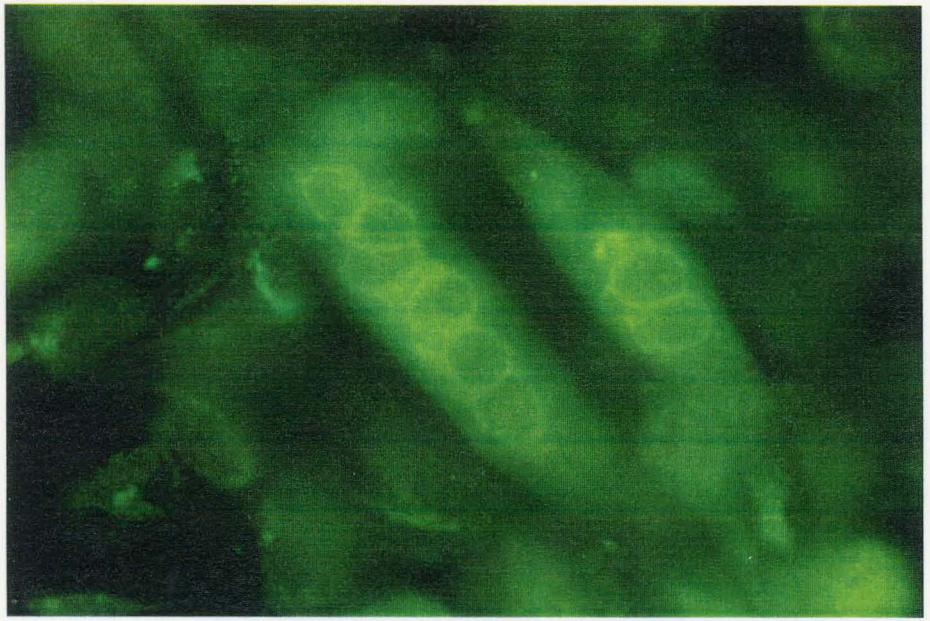
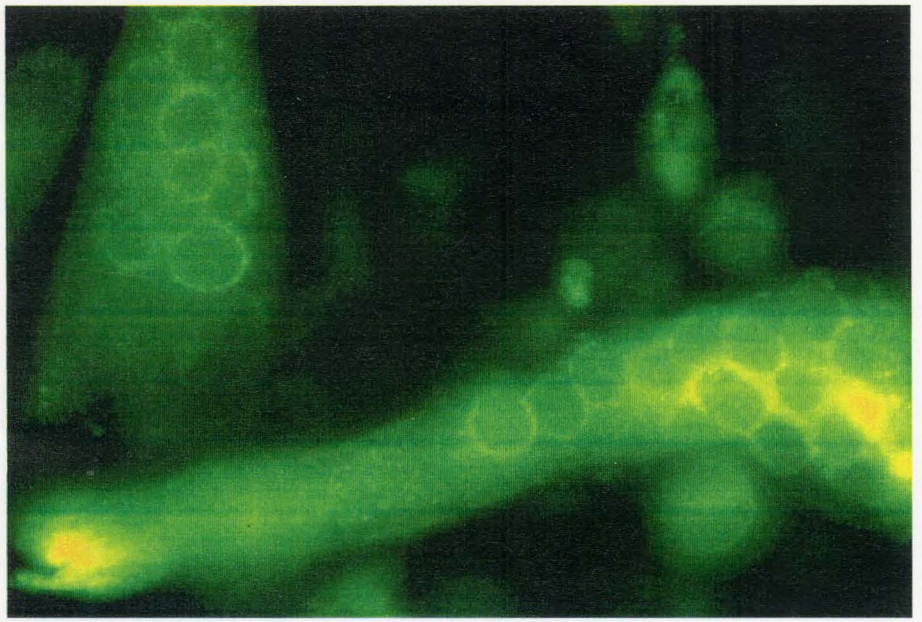


Figure 3b

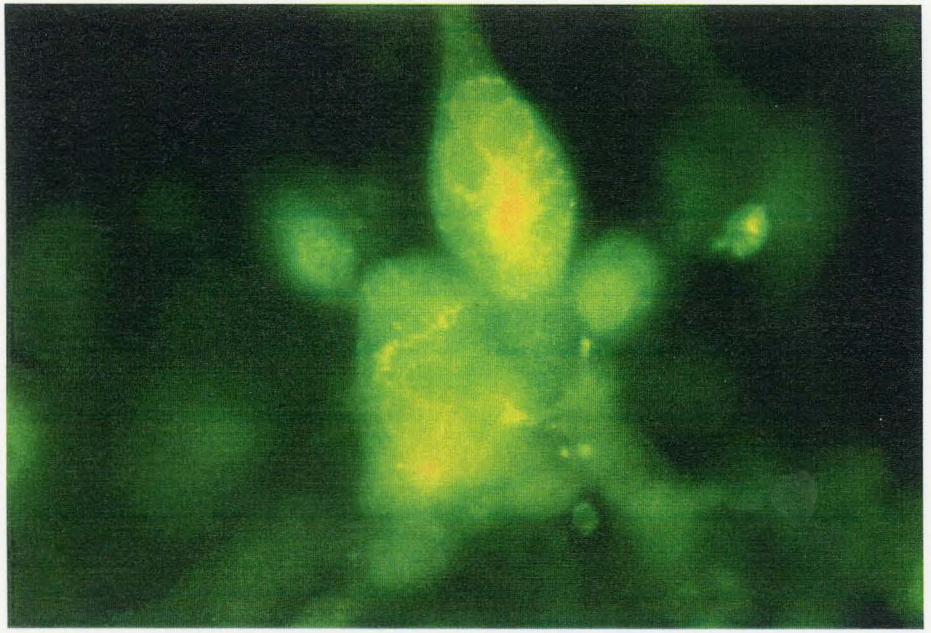
E



F



G



H

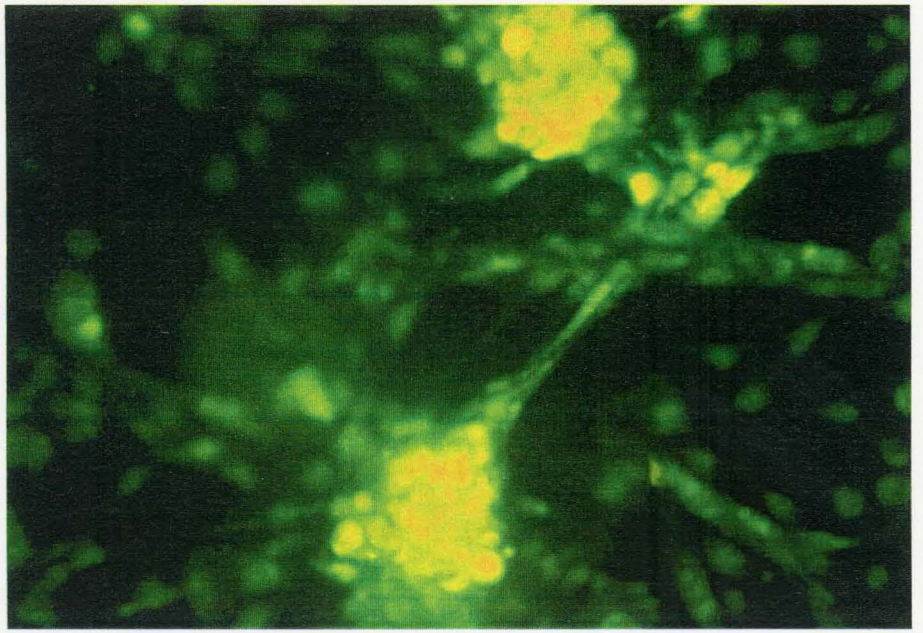
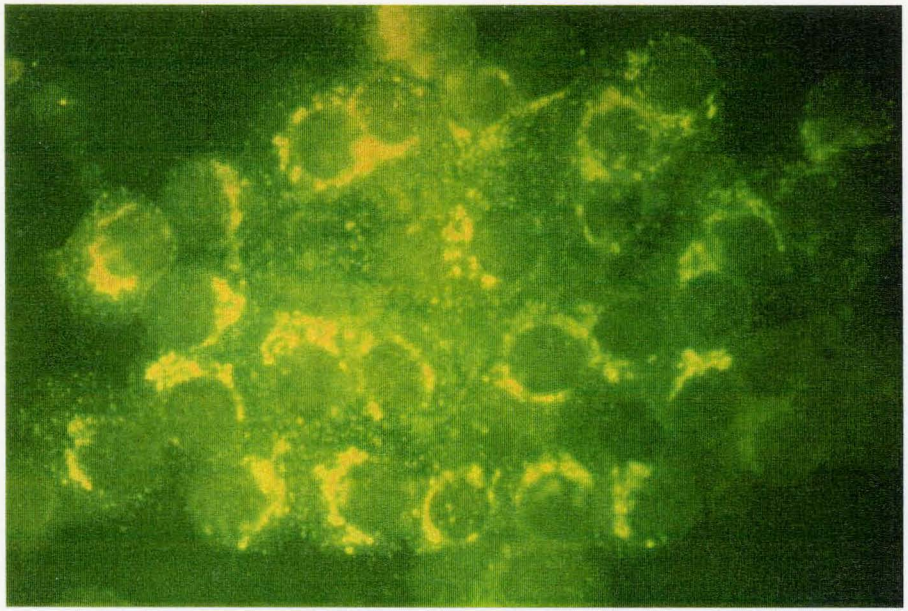
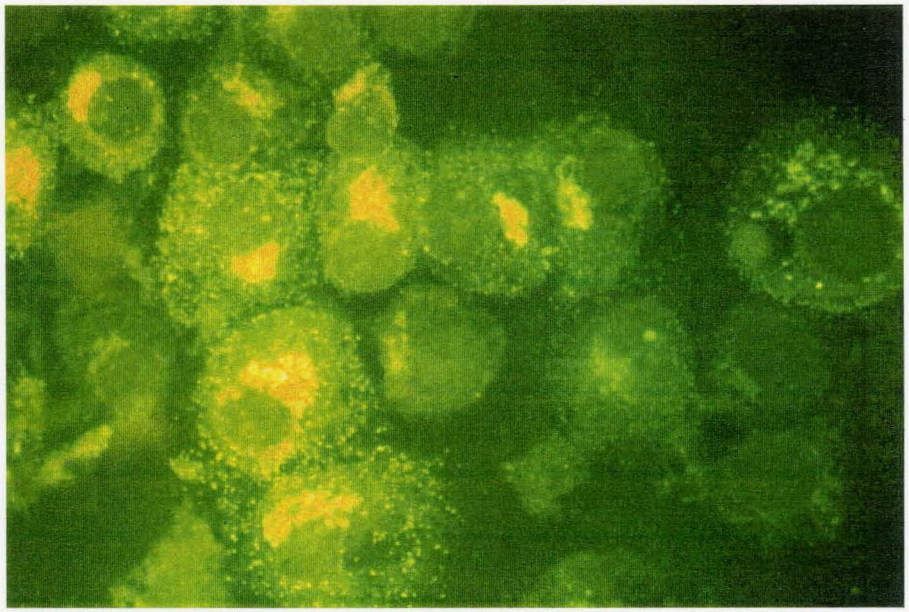


Figure 3c

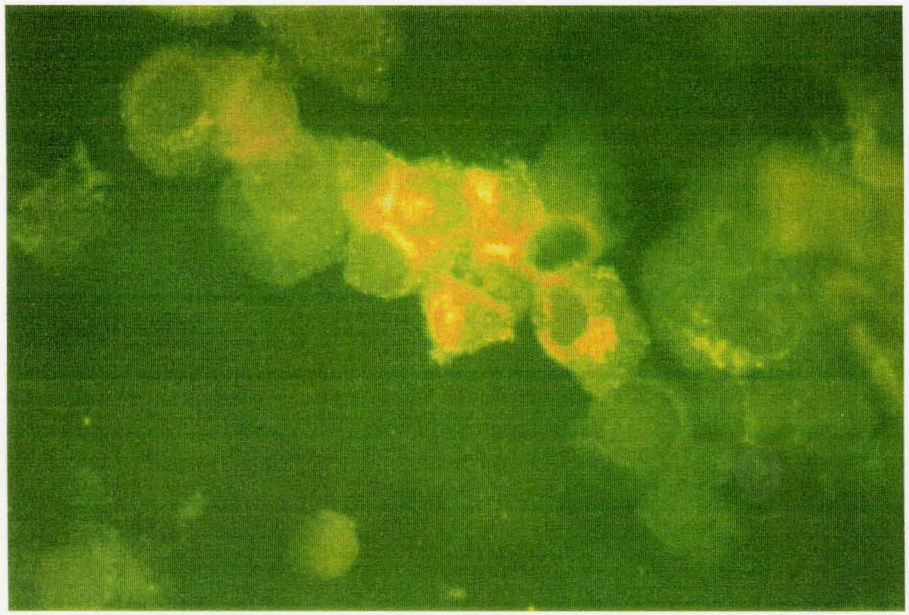
I



J



K



L

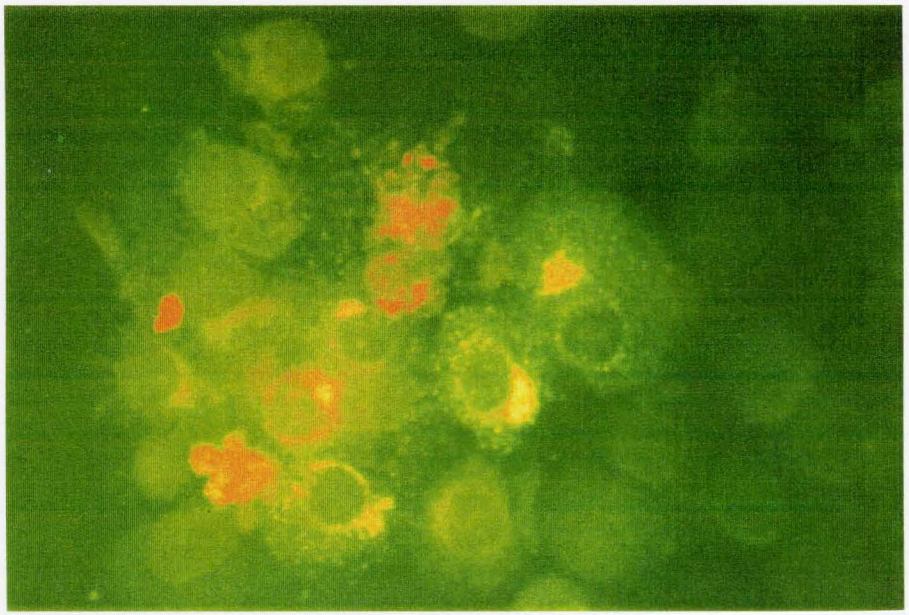
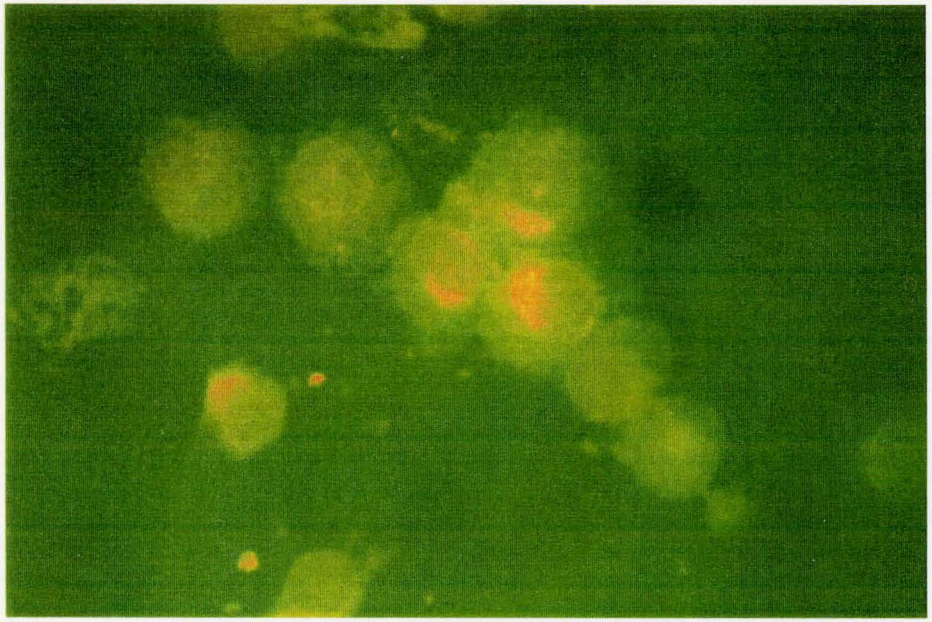
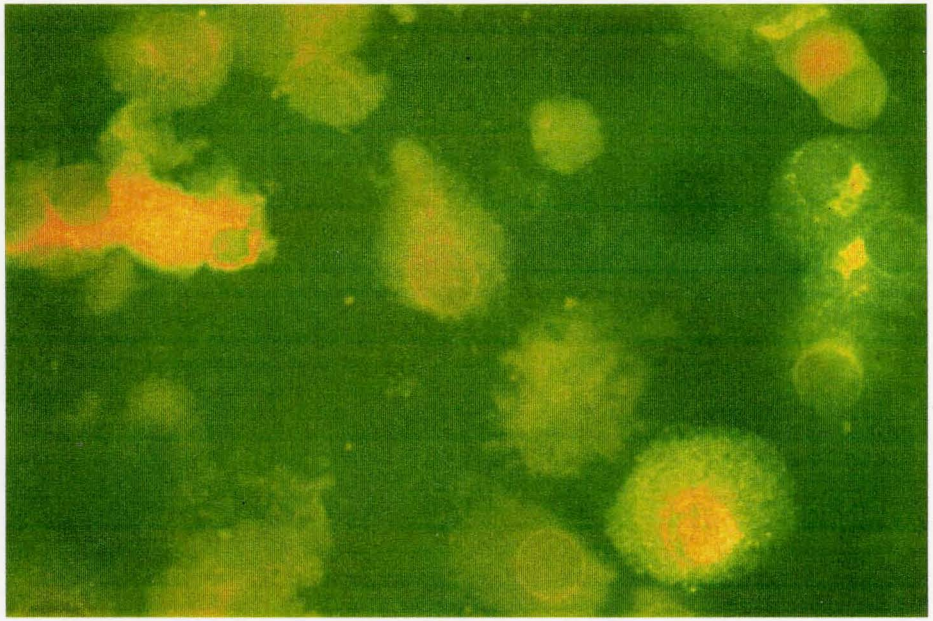


Figure 3d

M



N



3.2 Qualitative Analysis of Myoblast Differentiation

3.2.1 Myogenic Index

In order to study the differentiation of pNMG3-pool myoblasts on a morphological level, both in encapsulated and unencapsulated conditions, the cells were fixed and stained with a Myosin Heavy Chain (MHC) antibody conjugated to horseradish peroxidase, in addition to Giemsa stain. The stained cells were scored for the myogenic index (MI) (i.e. the percentage of cells with 3 or more nuclei in a given population of myoblasts), which provided a measure of the degree of myoblast fusion. Figure 4 demonstrates the MI for naked and encapsulated pNMG3-pool cells throughout a two week period, specifically on days 0, 3, 5, 9, 11 and 14 of differentiation. The MI remained at 0% for the encapsulated myoblasts, as no cell fusion was evident throughout the differentiation time course. The MI of the naked myoblasts, however, increased dramatically from 0% on day 0 of differentiation to ~ 47% by day 9 of differentiation. In fact, the MI of the naked myoblasts appeared to be leveling off after day 9 of differentiation, as the MI on day 14 of differentiation (~ 49%) was similar to that of day 9 (~ 47%). Table 2 lists the MI values calculated for both naked and encapsulated myoblasts for days 0, 3, 5, 9, 11 and 14 of differentiation.

Figure 4.

Myogenic index values (i.e. percentage of multinucleate cells in a given population of myoblast nuclei) for (a) unencapsulated (naked) pNMG3-pool cells and (b) encapsulated pNMG3-pool cells. The myogenic index was scored for on days 0, 3, 5, 9, 11 and 14 of differentiation for both encapsulated and unencapsulated myoblasts. The cells were fixed, stained with a Myosin Heavy Chain (MHC) antibody and analyzed via a light microscope under 400 X power. The data shown represents an average of ten scored fields of view ± SD. Table 2 lists the data presented in Figure 4 as an average of these ten fields ± SD.

Figure 4

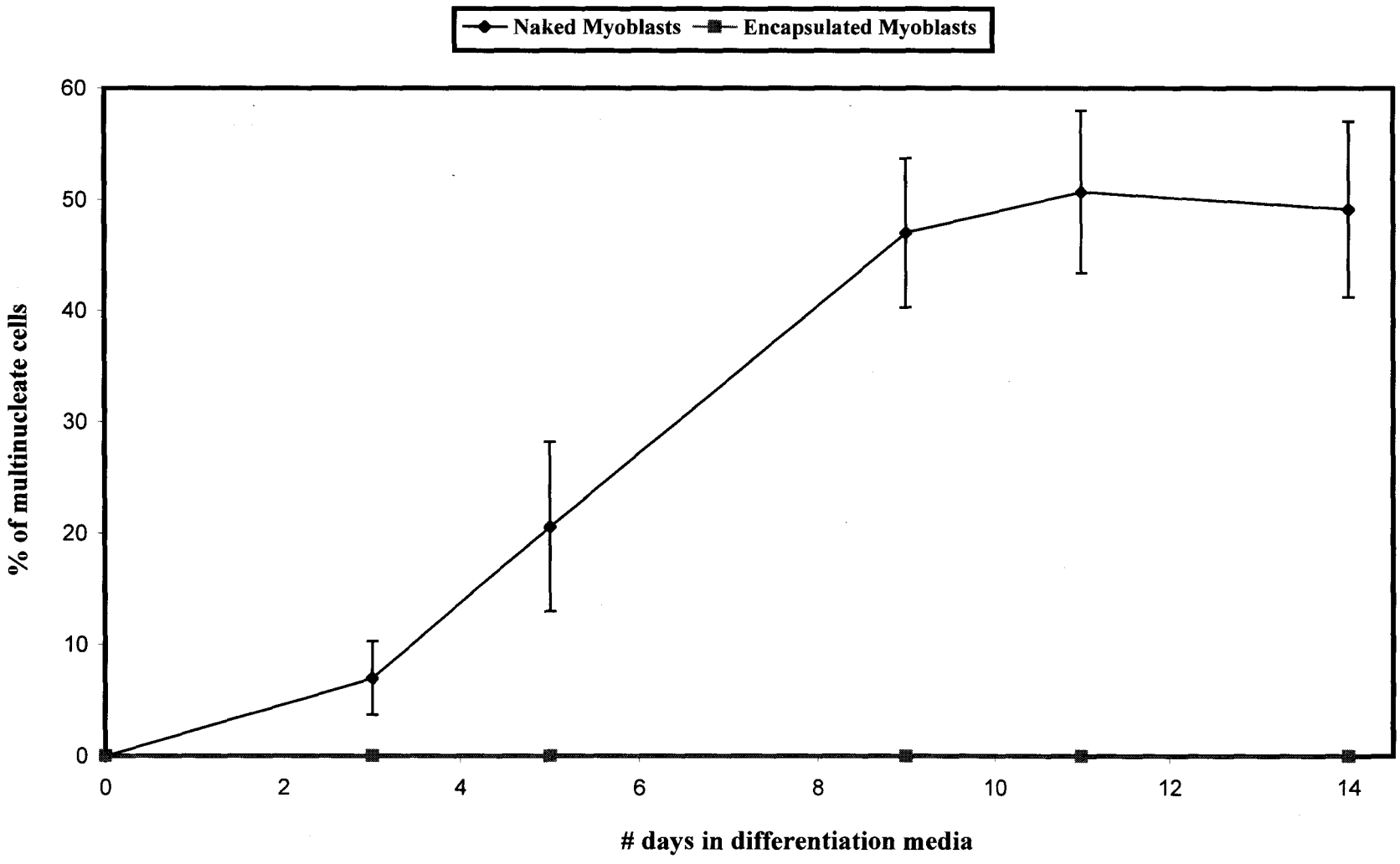

Myogenic Index: Comparison of Naked and Encapsulated pNMG3-pool Myoblasts
Naked Myoblasts
Encapsulated Myoblasts
% of multinucleate cells
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
days in differentiation media

Table 2.

# Days of Differentiation:	% Multinucleate Cells:	
	Naked Cells:	Encapsulated Cells:
0	0	0
3	7±3.3	0
5	20.6±7.6	0
9	47±6.7	0
11	50.7±7.3	0
14	49.1±7.9	0

# Days of Differentiation:	% of Cells Staining +(ve) for MHC:	
	Naked Cells:	Encapsulated Cells:
0	0	0
3	22.7± 6.8	0
5	21.4±2.1	0
9	61.9±7.8	3.3±1.8
11	50.7±7.7	6.2±3.5
14	60.9±9.3	12.9±4.8

3.2.2 Myosin Heavy Chain Staining

Although the myogenic index was useful in approximating the percentage of cells which had differentiated based upon the degree of myoblast fusion that had occurred, the true percentage of cells which had begun differentiating is more accurately determined by analyzing the number of cells expressing a differentiation-dependent muscle-specific protein, such as myosin heavy chain (MHC). It is known that during myoblast differentiation, the up-regulation of MHC, among other genes, occurs prior to cell fusion (Andres and Walsh, 1996). Thus, the percentage of cells expressing MHC is a more accurate reflection of the number of cells which have begun the differentiation process, albeit only on a molecular level.

Figure 5 is a graphical representation of the MHC expression in differentiating naked and encapsulated pNMG3-pool myoblasts. The cells were fixed and stained with a MHC antibody conjugated to horseradish peroxidase and Giemsa stain on days 0, 3, 5, 9, 11 and 14 of differentiation and the percentage of cells which demonstrated a positive signal for MHC were counted. It should be noted that the counts were representative of mononucleate cells expressing MHC (i.e. the number of nuclei in MHC-expressing myotubes were counted and each nucleus counted was considered to be representative of a single myoblast). The MHC protein was visualized by the addition of a horseradish peroxidase-conjugated secondary antibody, which resulted in the appearance of a brownish coloration upon exposure to diaminobenzidine (DAB). The myoblasts expressing MHC appear brown in colour throughout the cytoplasm of the cell (Figures 6

and 6a, demonstrating stained naked and encapsulated pNMG3-pool myoblasts, respectively).

No MHC staining was evident on day 0 of differentiation, which is to be expected, given the fact that the cells have not yet been exposed to differentiation media. The naked pNMG3-pool cells demonstrated a significant increase in MHC staining throughout the two week period, reaching a level of ~ 61% of cells expressing MHC on day 14 of differentiation. It should be noted that this value exceeds the MI value (Figure 4) by approximately 20%, which is representative of the fact that by day 14, 20% of the cells have begun to differentiate on a molecular level, as evidenced by the MHC expression, but have not yet initiated the final step towards terminal myogenic differentiation, that being cell fusion.

The encapsulated pNMG3-pool cells did not show any appreciable increase in the percentage of cells expressing MHC over the two week period of differentiation. MHC staining is not evident until day 9 of differentiation (although it may have begun as early as day 6), with only ~ 3% of myoblasts staining positively for MHC. By day 14 of differentiation, only ~ 13% of encapsulated myoblasts are expressing MHC. The pNMG3-pool myoblasts, therefore, appear to express MHC to a much lesser degree upon encapsulation.

Figures 6 and 6a provide some examples of MHC-stained myoblasts. It was observed that the number of naked cells expressing MHC (as evidence by the "reddish-brown" staining throughout the cytoplasm of the myoblast/myotube) increased significantly throughout the course of exposure to differentiation media (although the

number of encapsulated cells expressing MHC by day 14 of differentiation was extremely low). The photographs do not show the Giemsa-stained (undifferentiated) myoblasts.

Figure 5.

Comparison of Myosin Heavy Chain (MHC) staining in differentiating (a) encapsulated and (b) unencapsulated myoblasts, (pNMG3-pool cells). Encapsulated and unencapsulated (naked) pNMG3-pool cells were fixed and stained with a MHC antibody following exposure to differentiation media for up to 2 weeks. The percentage of cells which demonstrated positive staining for MHC were scored for under 400 X power via a light microscope on days 0, 3, 5, 9, 11 and 14 of differentiation. The data shown represents an average of 10 measurements (fields of view under the microscope) ± SD and Table 2 lists the data presented in Figure 5 ± SD.

Figure 5

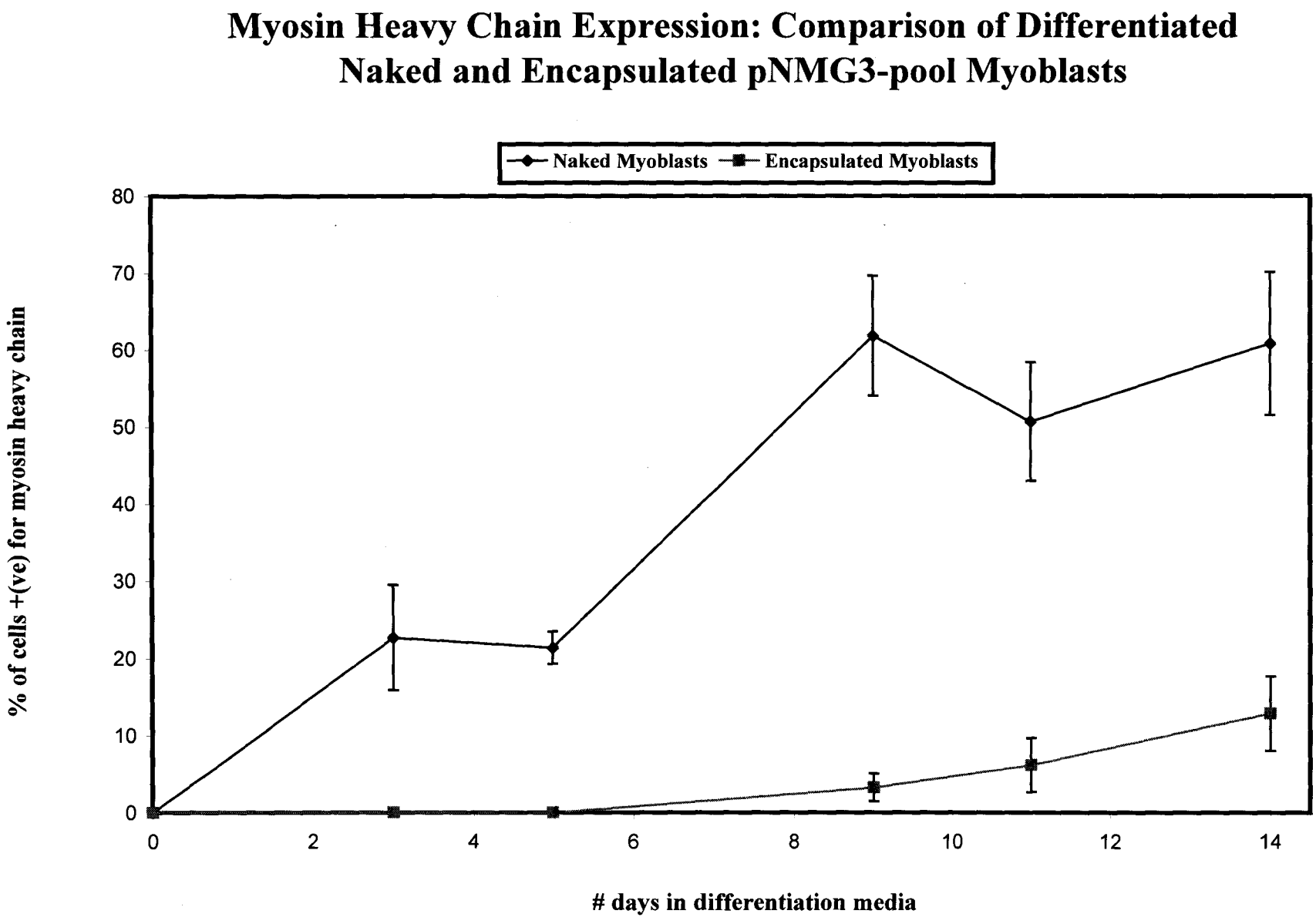

Myosin Heavy Chain Expression: Comparison of Differentiated Naked and Encapsulated pNMG3-pool Myoblasts
Naked Myoblasts
Encapsulated Myoblasts
% of cells +(ve) for myosin heavy chain
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
days in differentiation media

Figure 6.

Myosin Heavy Chain (MHC) staining of unencapsulated (naked) pNMG3-pool myoblasts. Cells were grown on gelatin-coated coverslips, maintained in differentiation media, fixed and stained with MF20 (an anti-MHC antibody) and scored for the percentage of MHC-positive cells on days 0, 3, 5, 9, 11 and 14 of differentiation, (only samples from days 1, 3 and 5 are shown in Figure 6). The presence of MHC protein is indicated by the reddish-brown staining observed in the cytoplasm of the myoblasts/myotubes. Samples were viewed, and photographs taken, via the use of a Zeiss microscope and camera.

Figure 6-A: Day 1 of differentiation (magnification: 160 X)

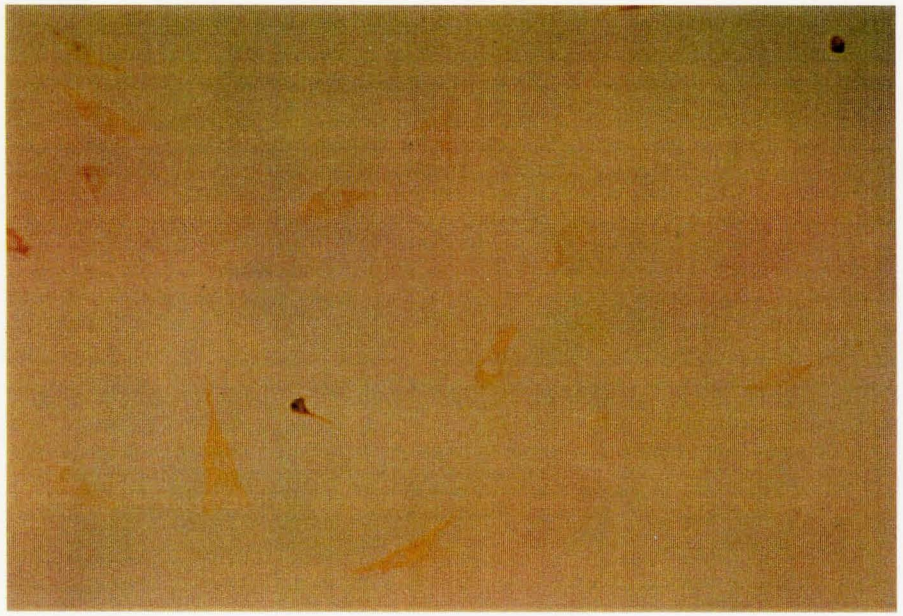
Figure 6-B: Day 3 of differentiation (magnification: 160 X)

Figure 6-C: Day 5 of differentiation (magnification: 160 X)

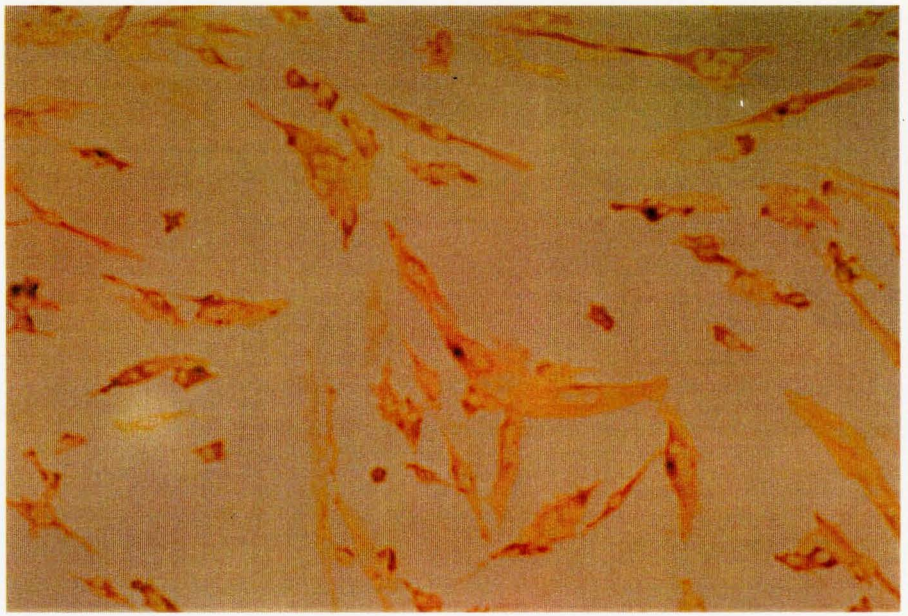
Figure 6-D: Day 5 of differentiation (magnification: 630 X)

Figure 6

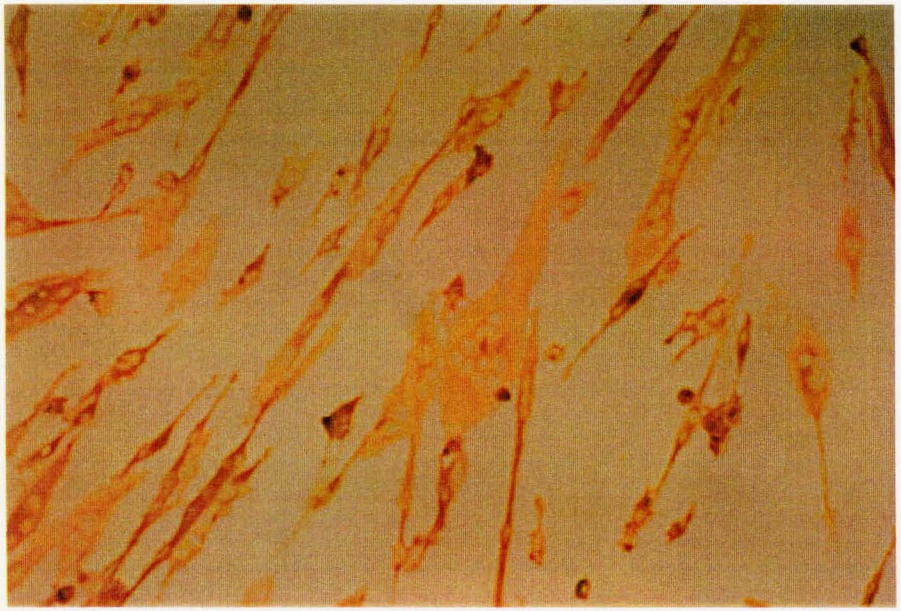
A



B



C



D

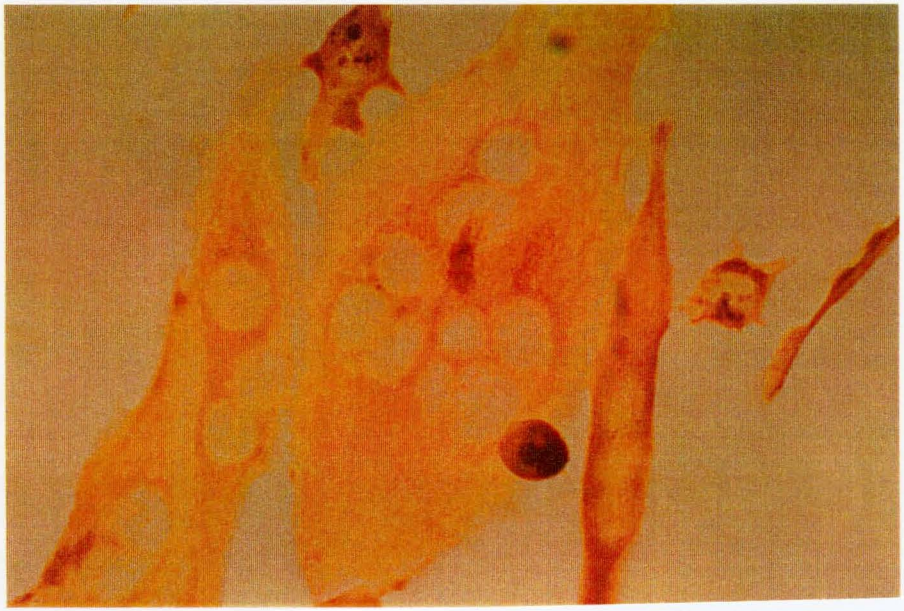


Figure 6a.

Myosin Heavy Chain (MHC) staining of encapsulated pNMG3-pool myoblasts. Cells were released from the alginate-polylysine microcapsules as previously described (see Methods) and were spun onto slides via cytocentrifugation (see Appendix E). The cells were then fixed onto the slides with 90% methanol and stained with MF20 (an anti-MHC antibody) on days 0, 3, 5, 9, 11 and 14 of differentiation (only samples from days 0, 3, 5 and 9 are shown). The presence of the MHC protein is indicated by the dark brown staining observed in the cytoplasm of the myoblast(s) (a MHC-positive cell is indicated by an arrow on Day 9). Photographs were taken via the use of a Zeiss microscope and camera.

Figure 6a-E: Day 0 of differentiation (magnification: 1000 X)

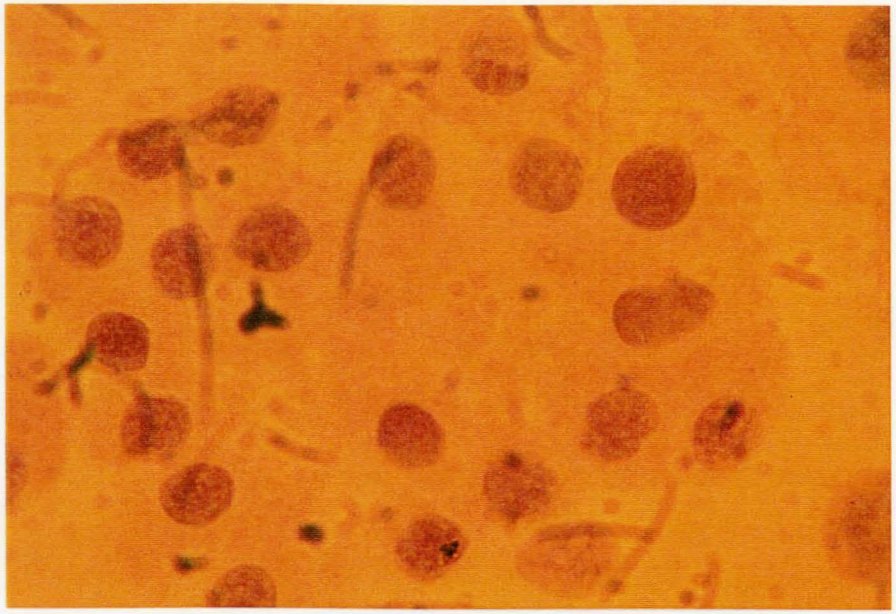
Figure 6a-F: Day 3 of differentiation (magnification: 1000 X)

Figure 6a-G: Day 5 of differentiation (magnification: 1000 X)

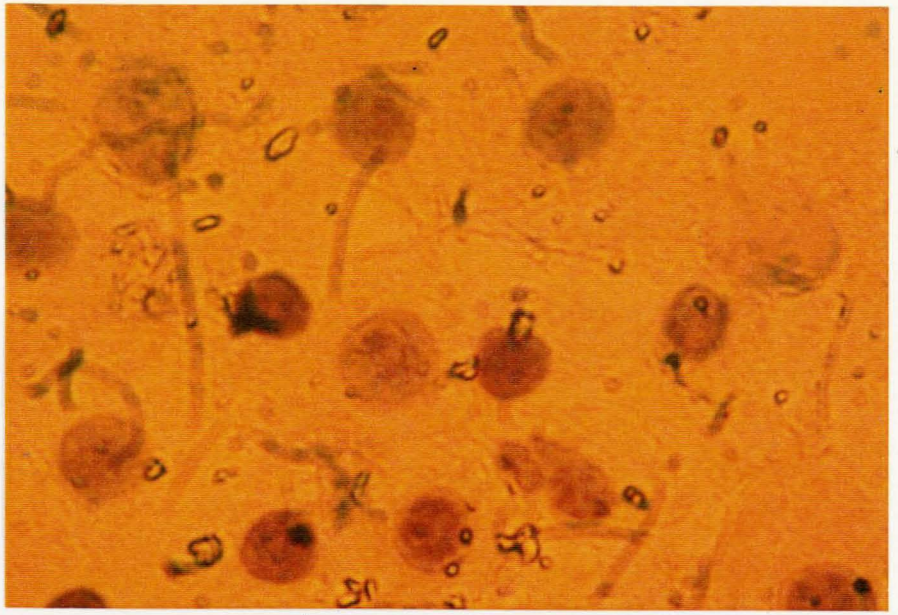
Figure 6a-H: Day 9 of differentiation (magnification: 1000 X)

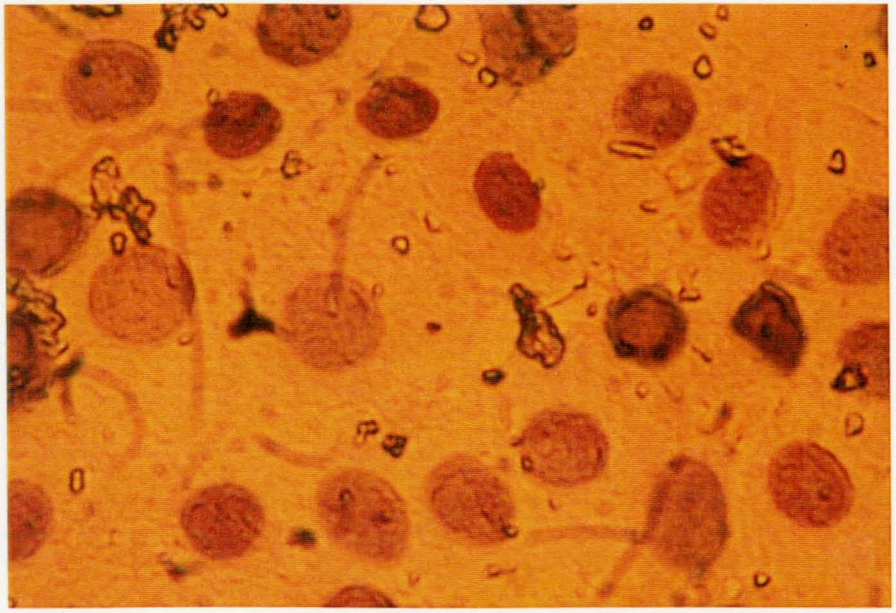
Figure 6a

E

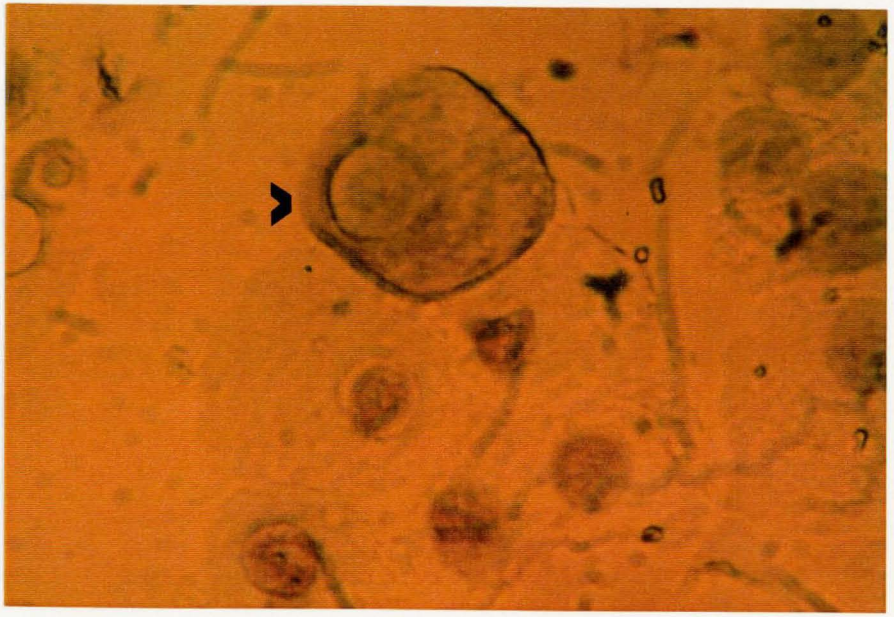


F





G



H

3.3 Analysis of Myoblast Differentiation: CPK Assays

3.3.1 Comparison of CPK in Differentiating Naked and Encapsulated Myoblasts

Differentiating myoblasts are characterized, in part, by the rapid up-regulation of various muscle-specific genes, one of which is creatine phosphate kinase (CPK). It has been shown that CPK protein and mRNA levels start increasing approximately 5 to 6 hours following exposure to mitogen-depleted media (Chamberlain et al., 1985). Therefore, levels of CPK activity in differentiating myoblasts provide a good measure of the relative number of cells which have committed to terminal myogenic differentiation.

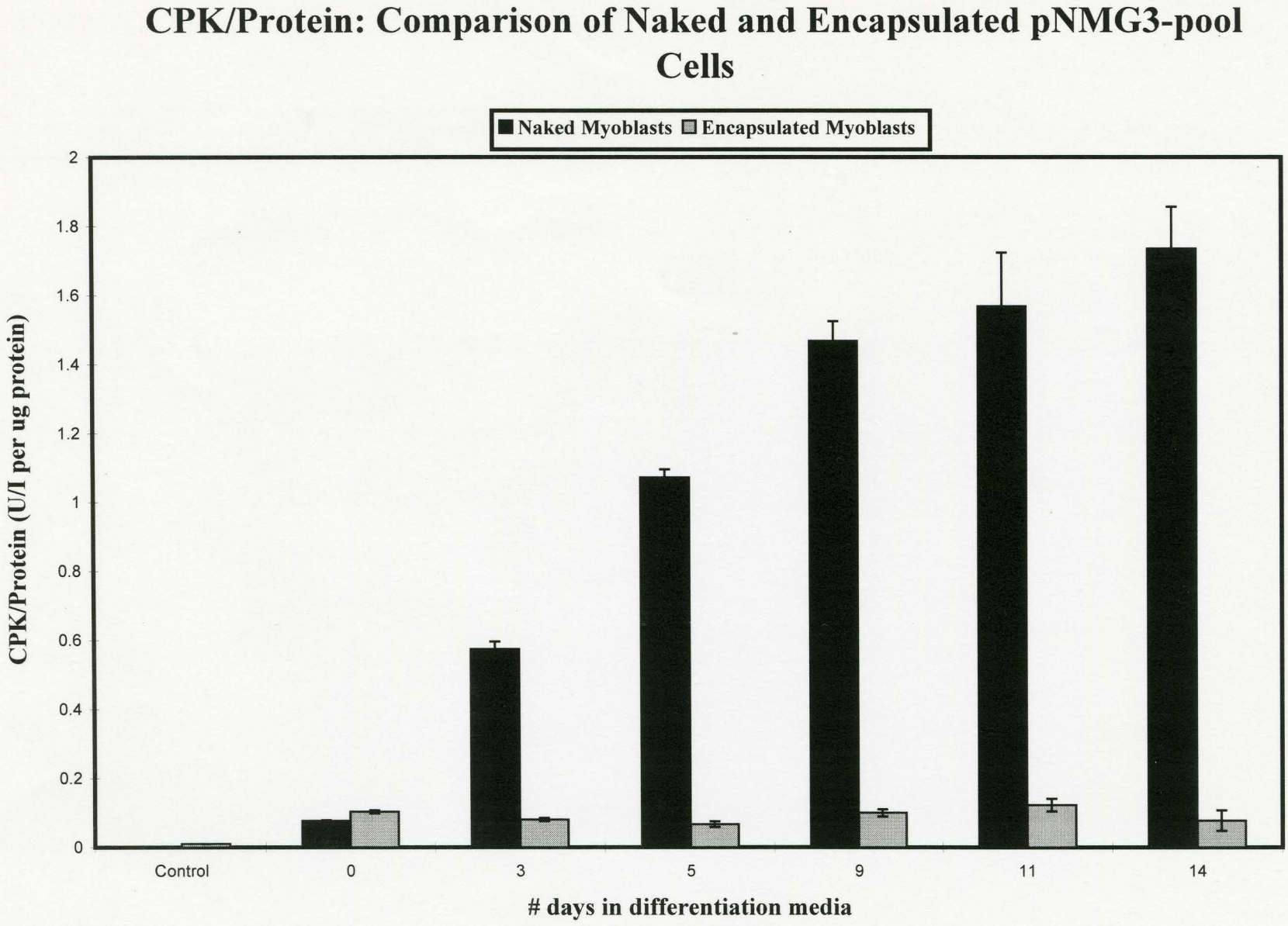
pNMG3-pool myoblasts, both encapsulated and unencapsulated, were maintained in differentiation media for a period of two weeks, and the CPK activity, expressed in U/I (International Units) per μg protein, was measured on days 0, 3, 5, 9, 11 and 14 of differentiation. Differentiating unencapsulated (naked) pNMG3-pool myoblasts demonstrated a dramatic rise in CPK levels which increased approximately 23-fold over the two week period (from 7.68×10^{-2} U/I /μg to 1.73 U/I /μg) of growth in differentiation media (Figure 7). The CPK levels in the naked myoblasts appeared to be leveling off after day 9 of differentiation, and, in fact, there is no significant difference ($p>0.05$) between the CPK levels on day 11 and day 14 of differentiation. The encapsulated pNMG3-pool myoblasts, however, did not demonstrate any appreciable increase in CPK activity throughout the two week differentiation period, with CPK levels ranging from 6.70×10^{-2} U/I /μg to 1.23×10^{-1} U/I /μg, the average CPK level being 9.19×10^{-2} U/I/μg.

The levels were above that of the control sample (~ 0 U/I /μg) which consisted of empty alginate-polylysine capsules. It should be noted that the viability of the encapsulated myoblasts was taken into account such that the calculated value representative of CPK activity, expressed against the total protein present in the sample, was accurate with respect to the percentage of viable cells which remained in the capsule at the time of sampling. The encapsulated cell viability decreased from ~ 89% on day 0 of differentiation to ~ 60% on day 14 of differentiation. Overall, the unencapsulated myoblasts appeared to be differentiating on a molecular level over the two week period of growth in the differentiation media based upon the increasing CPK levels. Once encapsulated, however, the pNMG3-pool cells did not demonstrate any increase in CPK levels, which would indicate that the myoblasts are not committing to terminally differentiate.

Figure 7.

Comparison of Creatine Phosphate Kinase (CPK) activity in (a) microencapsulated and (b) unencapsulated (naked) differentiating C2C12 myoblasts (study designated "Experiment #2"). CPK activity was measured via the CK-MPR1 kit (Boehringer Mannheim) at 0, 3, 5, 9, 11 and 14 days following the addition of differentiation media and expressed in U/I (International Units) per μg of total protein. Microencapsulated myoblasts were exposed to bFGF (10 ng/ml) for approximately 10 days prior to the addition of differentiation media. The control sample represents the CPK/protein level obtained from empty capsules (i.e. devoid of any cells). Data presented represents an average of triplicate measurements ± SD.

Figure 7


CPK/Protein: Comparison of Naked and Encapsulated pNMG3-pool Cells
Naked Myoblasts
Encapsulated Myoblasts
CPK/Protein (U/l per ug protein)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
0
3
5
9
11
14
days in differentiation media

3.3.2 CPK Activity: Comparison of Naked Myoblasts

The CPK activity present in differentiating pNMG3-pool cells was measured in two separate studies, namely "Experiment #1" (preliminary pilot study) and "Experiment #2". The two experiments utilized the same cell population, which was thawed out on two separate occasions and grown on 35-mm tissue culture dishes in triplicate samples, (~300,000 cells seeded per dish). The pNMG3-pool cells utilized in Experiment #1 were passaged approximately three (3) times prior to differentiation, while the pNMG3-pool cells employed in Experiment #2 were passaged approximately seven (7) times. These pNMG3-pool samples were exposed to differentiation media 24 hours post-seeding for five days (Experiment #1), or 14 days (Experiment #2). The myoblasts were harvested and tested for CPK activity on days 0-5 of differentiation (Experiment #1) or days 0, 3, 5, 9, 11 and 14 of differentiation (Experiment #2). Figure 8 compares the CPK levels, expressed in U/l /μg protein, for both Experiment #1 and #2, for the period of 0 through 5 days of differentiation. The CPK levels in Experiment #1 are significantly different ($p<0.05$) than the CPK levels measured in Experiment #2.

3.3.3 CPK Activity: Comparison of Encapsulated Myoblasts

The CPK activity present in encapsulated pNMG3-pool cells was determined in three separate experiments: (i) Experiment #1a, (ii) Experiment #1b and (iii) Experiment #2, of which the first two experiments (preliminary pilot studies) involved the use of the same vial of pNMG3-pool cells thawed from liquid nitrogen storage. The pNMG3-pool

myoblasts were passaged (P) as follows: P3 (Experiment #1a); P7 (Experiment #1b); and P7 (Experiment #2). Cells were assayed for CPK activity as follows: days 0, 1, 2, 3, 4, 6, 7 and 8 of differentiation (Experiment #1a); days 0, 1, 2, 3, 4, 5, 6 and 7 of differentiation (Experiment #1b); days 0, 3, 5, 9, 11 and 14 of differentiation (Experiment #2). The levels of CPK/protein, expressed in U/I /μg, are quite varied among the three experiments (Figure 9). The CPK activity increased approximately 63-fold in Experiment #1a, (from 5.89×10^{-2} U/I /μg on day 0 of differentiation to 3.73 U/I /μg on day 9 of differentiation) and approximately 8-fold in Experiment #1b (from 7.80×10^{-2} U/I /μg on day 0 of differentiation to 6.38×10^{-1} U/I /μg on day 7 of differentiation). In Experiment #2, however, the CPK activity remains at an average level of 9.19×10^{-2} U/I /μg and did not demonstrate any significant increase throughout the differentiation period of 0 to 14 days.

Figure 8.

Comparison of CPK activity in unencapsulated (naked) C2C12 myoblasts (pNMG3-pool) from (a) Experiment #1 (preliminary pilot study) and (b) Experiment #2. The two experiments represent data obtained from pNMG3-pool cells which were thawed out on separate occasions, cultured and allowed to differentiate in differentiation media for a period of five days. The CPK activity was expressed in U/I (International Units) per μg total protein and was measured at 0, 1, 2, 3, 4 and 5 days of differentiation in the case of Experiment #1, and at 0, 3 and 5 days of differentiation in the case of Experiment #2. The data shown represents an average of triplicate measurements ± SD.

Figure 8

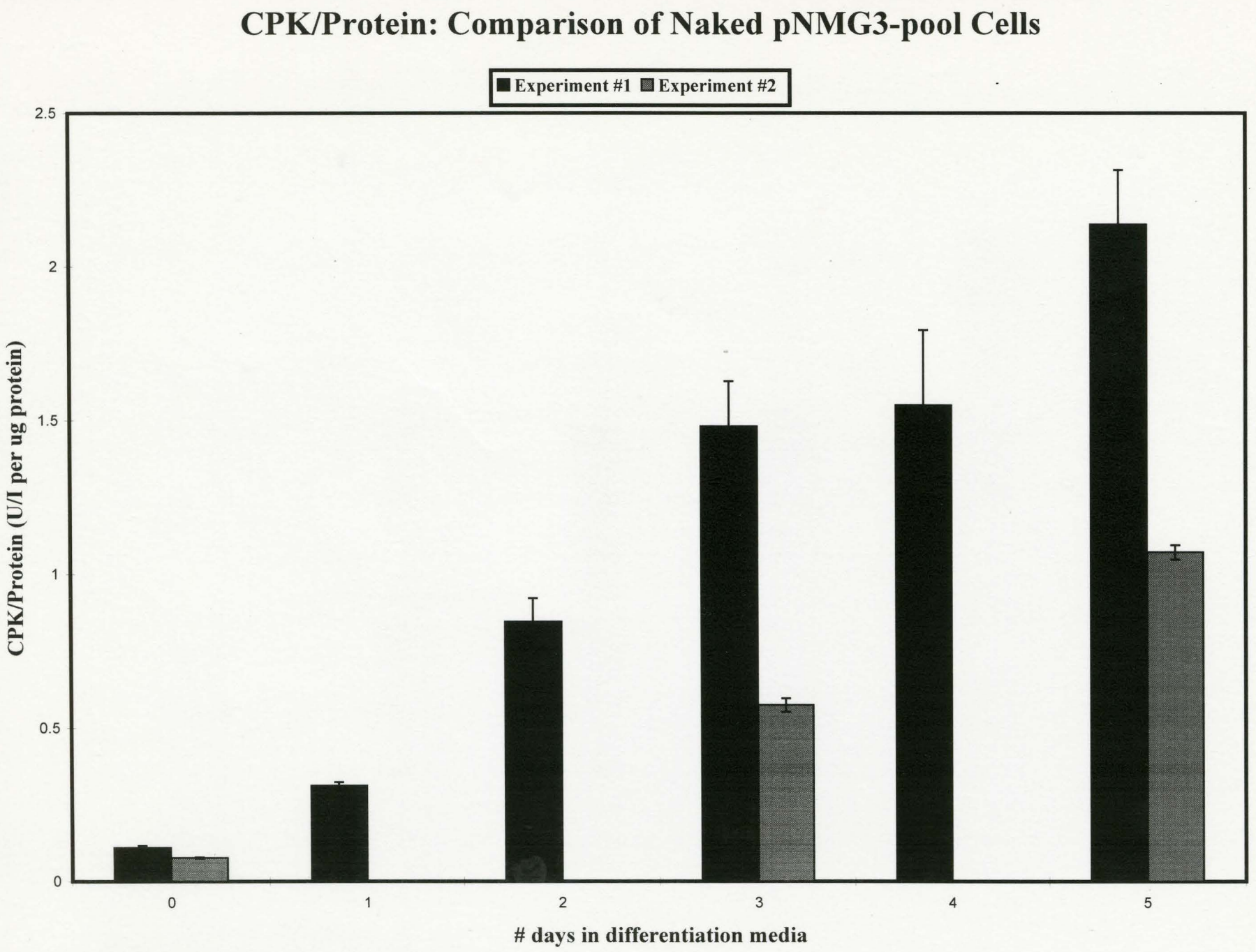
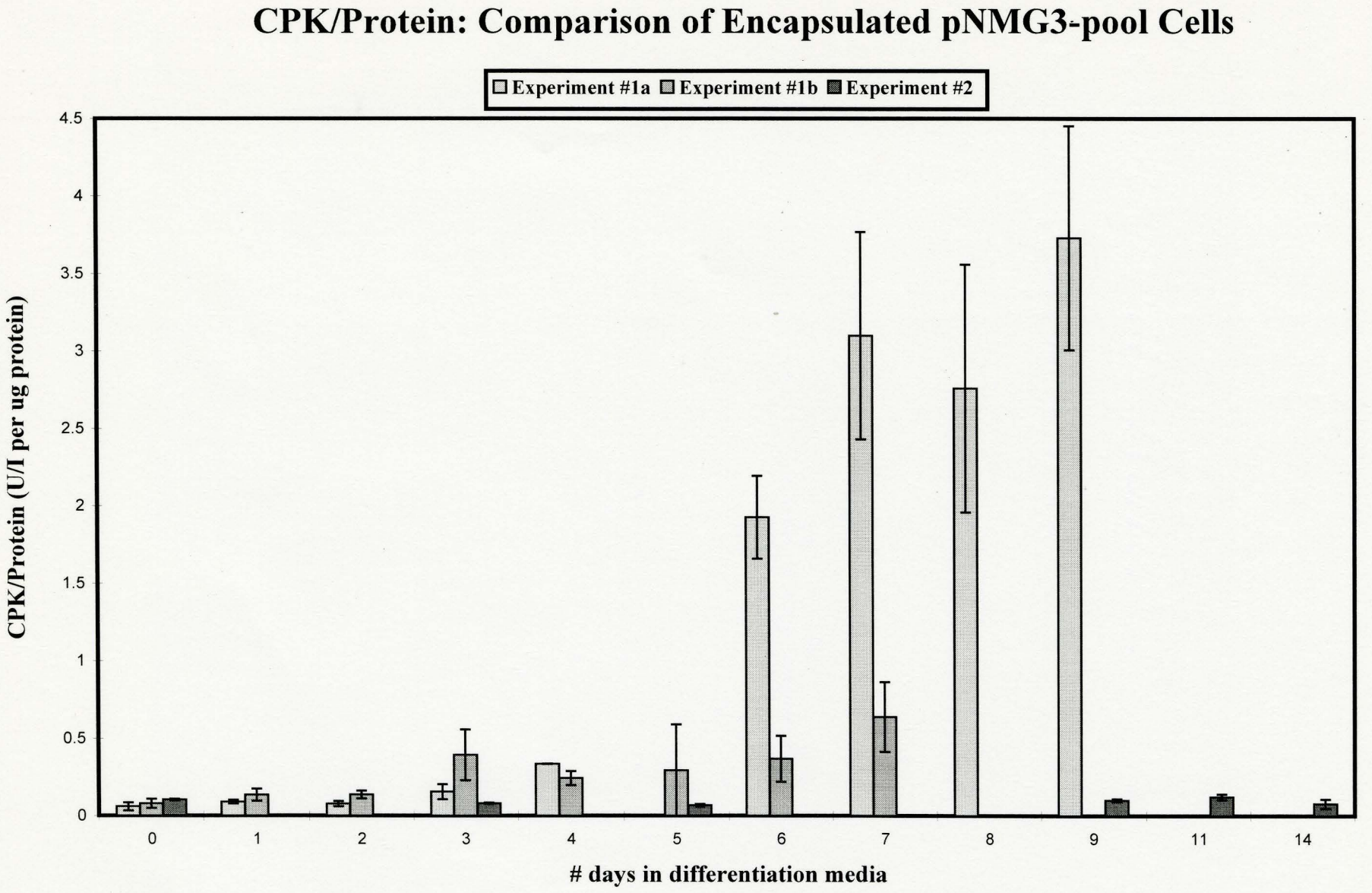

CPK/Protein: Comparison of Naked pNMG3-pool Cells
Experiment #1
Experiment #2
CPK/Protein (U/l per ug protein)
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
days in differentiation media

Figure 9.

Comparison of CPK activity in differentiating microencapsulated C2C12 (pNMG3-pool) myoblasts from (a) Experiment #1a, (b) Experiment #1b and (c) Experiment #2. Experiments #1a and #1b (preliminary pilot studies) represent data which was derived from differentiating pNMG3-pool cells which were thawed out simultaneously, but passaged differentially prior to growth in differentiation media (i.e. the cells employed in Experiment #1b were passaged to a greater extent than those used in Experiment #1a). Experiment #2 utilized C2C12 cells (pNMG3-pool) which were thawed out and passaged at a later date than those of Experiments #1a and #1b. All encapsulated myoblasts employed in Experiments #1a, #1b and #2 were exposed to 10 ng/ml of bFGF for approximately 10 days prior to the addition of differentiation media. CPK analysis was performed on days 0, 1, 2, 3, 4, 6, 7, 8, and 9 for Experiment #1a, days 0, 1, 2, 3, 4, 5, 6, and 7 for Experiment #1b, and days 0, 3, 5, 9, 11, and 14 for Experiment #2. The data shown represents the average of triplicate measurements ± SD.

Figure 9


CPK/Protein: Comparison of Encapsulated pNMG3-pool Cells
Experiment #1a
Experiment #1b
Experiment #2
CPK/Protein (U/I per ug protein)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
8
9
11
14
days in differentiation media

3.3.4 Effects of bFGF on Differentiating Encapsulated Myoblasts

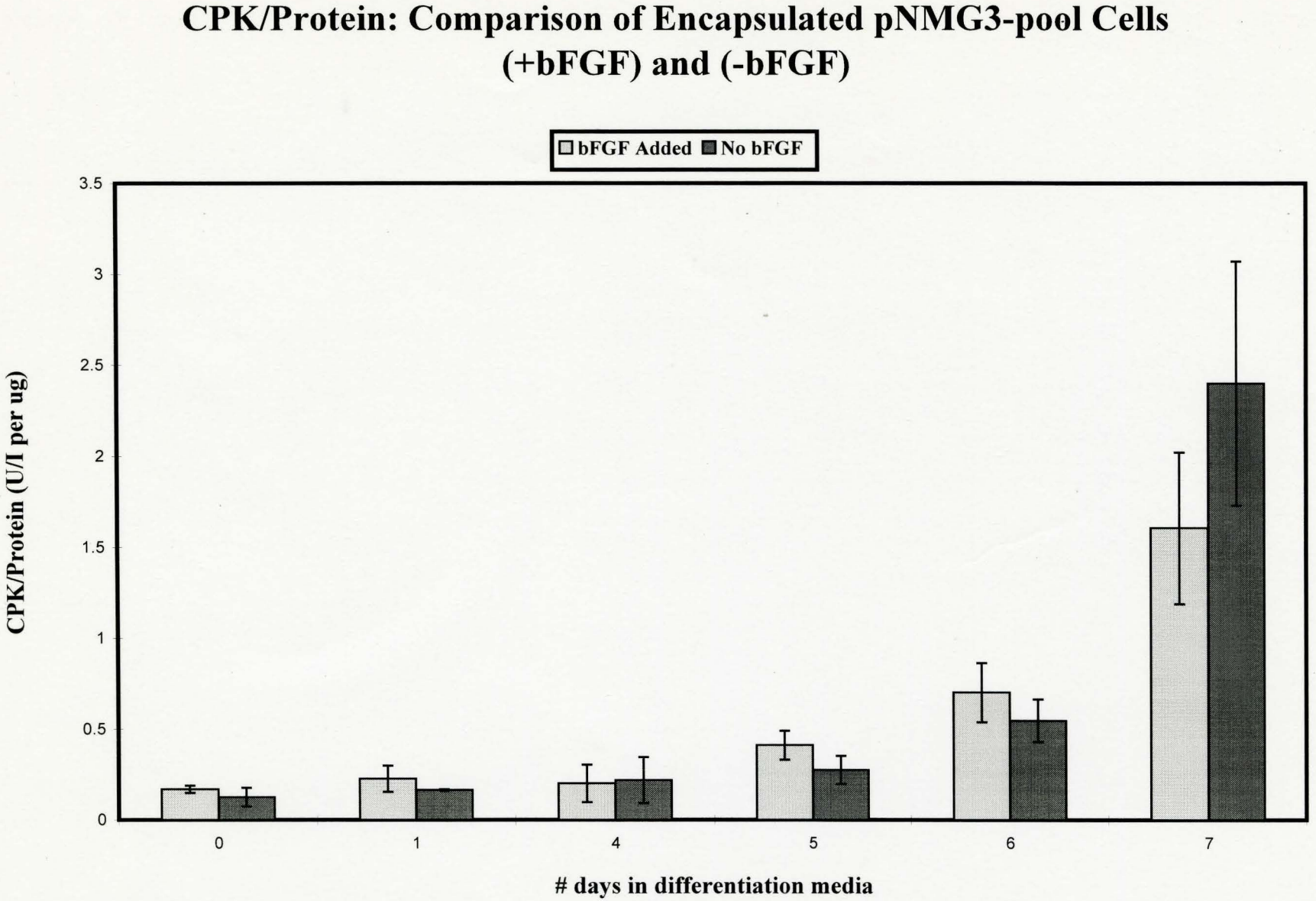
The encapsulated pNMG3-pool myoblasts were grown for approximately 10 days in growth media supplemented with 10 ng/ml of basic fibroblast growth factor (bFGF) (Experiments #1a, #1b and #2) prior to CPK analysis, hGH secretion rate analysis, myogenic index analysis or RNA analysis. The bFGF was added prior to the addition of differentiation media to enhance the growth of the myoblasts within the capsular space. In addition to its mitogenic effects, bFGF also suppresses the expression of various muscle-specific genes (Rao and Kohtz, 1995), thereby inhibiting myoblast differentiation. Thus, bFGF was also useful in aiding in the prevention of myoblast differentiation prior to the start of the differentiation experiments. In order to determine whether the bFGF had any lasting or persistent effects on the differentiation of the myoblasts following its removal from the media (such as suppression of CPK expression), encapsulated pNMG3-pool cells (which used pNMG3-pool cells thawed out prior to Experiments #1a and #1b) were split into two treatment groups: (a) those grown in growth media plus 10 ng/ml bFGF; (b) those grown in growth media only. The encapsulated cells were allowed to grow for a period of 24 hours under the differing media conditions, and then they were placed into differentiation media for one week. The level of CPK activity, expressed in U/l /μg protein, was determined for each of the two groups of encapsulated pNMG3-pool cells [(+bFGF) and (-bFGF)] on days 0, 1, 4, 5, 6 and 7 days of differentiation (Figure 10). The levels of CPK activity are not significantly different ($p>0.05$) between the two treatment groups, which suggests that the bFGF does not have any persistent effects on

myoblast differentiation once it is no longer present in the extracellular environment, at least with respect to the induction of CPK.

Figure 10.

Comparison of CPK activity in differentiating microencapsulated C2C12 myoblasts (pNMG3-pool cells) that were: (a) exposed to bFGF (10 ng/ml) for 24 hours prior to differentiation; (b) grown in regular growth media in the absence of purified bFGF for 24 hours prior to differentiation. The CPK activity was measured in U/I (international units) per μg total protein and was assayed for on days 0, 1, 4, 5, 6, and 7 following exposure to differentiation media. The data is representative of triplicate measurements ± SD.

Figure 10


CPK/Protein: Comparison of Encapsulated pNMG3-pool Cells (+bFGF) and (-bFGF)
bFGF Added
No bFGF
CPK/Protein (U/I per ug)
3.5
3
2.5
2
1.5
1
0.5
0
0
1
4
5
6
7
days in differentiation media

3.4 Muscle-Specific RNA Expression During Myoblast Differentiation

3.4.1 Confirmation of Probe Specificity

In order to ensure that the plasmid insert DNA sequences used as probes (see Appendix D) were specific for the individual mRNA sequences to be detected by dot blotting, a Northern blot was run and probed (see Appendix C for labelling protocol) with 25 ng of each of the following plasmid vector inserts: myosin light chain 1/3 (LHC3), Troponin T (TnT), Troponin I slow (TnIslow), myogenin, myosin light chain 2 (LC2), MyoD1, phosphoglycerate kinase (PGK1) and human growth hormone (hGH) (Figure 11). Total RNA was isolated from undifferentiated (day 0) pNMG3-pool cells and differentiated (day 3) pNMG3-pool cells. Each lane of a 1% formaldehyde/agarose gel was loaded with 10 μg of total pNMG3-pool RNA and the migration of the 28S and 18S rRNA bands was recorded (i.e. the migration distance was measured in cm from the bottom of the loading well to the middle of the RNA band). The migration distances of the 28S and 18S rRNA bands were then used to calculate the approximate band sizes of the RNA signals which were observed on the phosphor image of each of the probed blots. The band sizes shown in Figure 11 confirmed the specificity of each of the probes as they approximate the actual mRNA sizes, which are as follows: LHC3 (972 bp); TnT (1200 bp); TnIslow (1200 bp); myogenin (1500 bp); LC2 (1000 bp); MyoD (1700 bp); PGK1 (1650 bp); hGH (1300 bp).

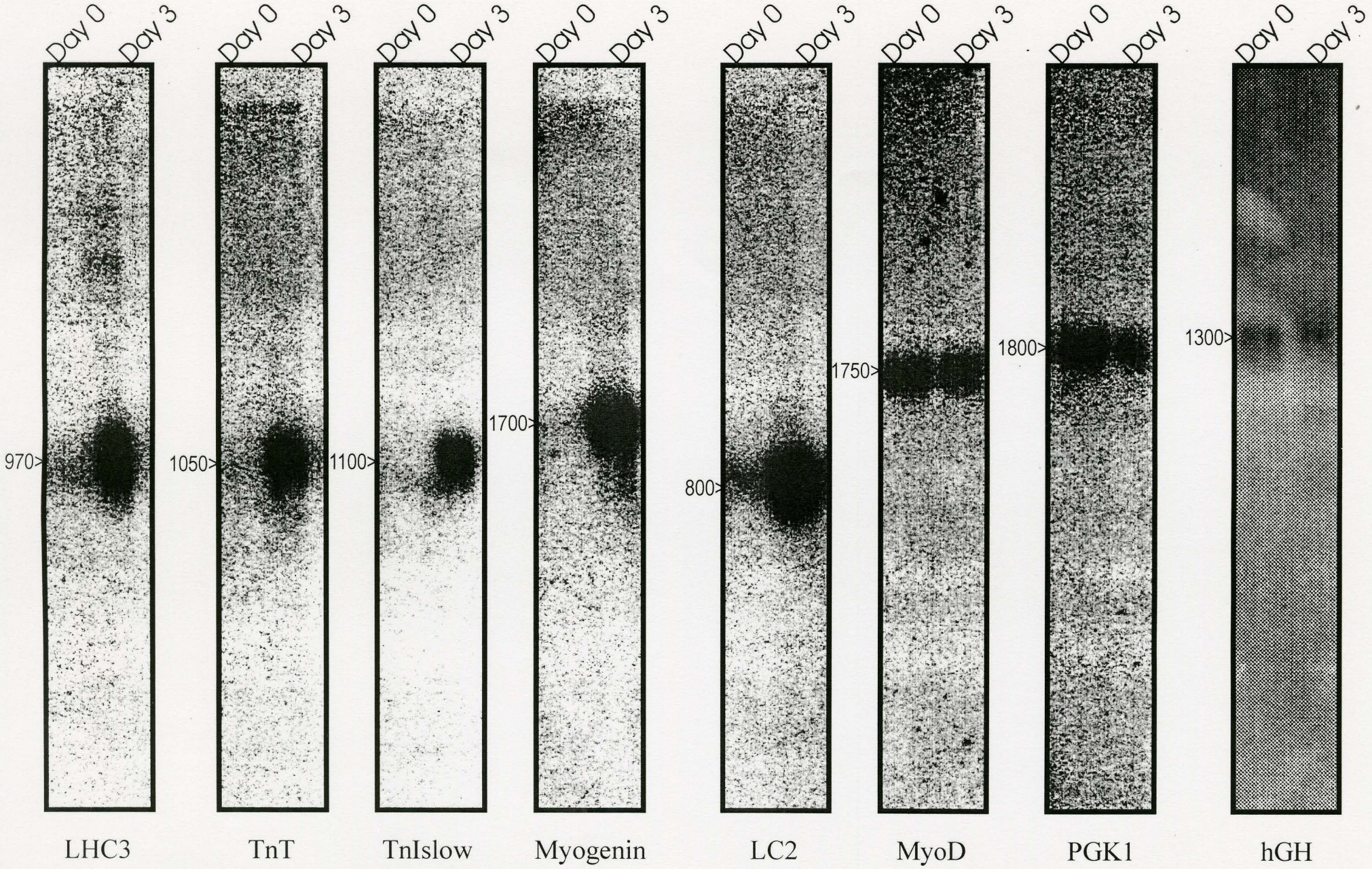
Total RNA was extracted on day 0 of differentiation, in addition to day 3, in order to provide an internal negative control. It would be expected that little or no signal would

be present in the day 0 lanes for those blots probed with the muscle-specific gene inserts that are up-regulated primarily upon myogenic differentiation, such as LHC3, TnT, TnIslow, myogenin and LC2. Indeed, the day 0 samples probed with these muscle-specific gene inserts did not demonstrate any appreciable signal (i.e. hybridization). MyoD1 is muscle-specific and is expressed in proliferating as well as differentiating myoblasts, which is supported by the bands present in day 0 and day 3 samples. PGK1 and hGH also demonstrated bands in the day 0 and day 3 lanes as well, which is to be expected due to the fact that they are both constitutively expressed.

Figure 11.

Northern blots of total RNA extracted from pNMG3-pool myoblasts on day 0 and day 3 of differentiation. Each lane was loaded with 10 μg of total RNA and each day 0 and day 3 sample was probed with one of the following cDNA sequences: LHC3, TnT, TnIslow, myogenin, LC2, MyoD1, PGK1 and hGH. Each probe (25 ng) was labelled with 50 μCi of [α-32P]dCTP at ~3,000 Ci/mmol, and each blot was hybridized for approximately 16 hours. All blots were exposed to a phosphor screen for 4 hours, with the exception of PGK1 and MyoD1, which were exposed for 14 hours. The approximate sizes of the mRNA bands detected by phosphorimaging technology are indicated at the side of each band with an arrow (note: band sizes were calculated based on the relative migration distance of the corresponding 28S and 18S rRNA bands on the gel).

Figure 11


Day 0
Day 3
970
1050
1100
1700
800
1750
1800
1300
LHC3
TnT
TnIslow
Myogenin
LC2
MyoD
PGK1
hGH

3.4.2 Muscle-specific RNA Levels in Differentiating Myoblasts

In order to delineate the pattern of muscle-specific gene expression in differentiating C2C12 myoblasts, as well as the levels of hGH mRNA, total RNA was isolated from pNMG3-pool cells on days 0, 1, 2, 3, 4 and 5 of differentiation. These RNA samples were blotted onto nylon membrane and were subsequently probed with muscle-specific cDNAs (i.e. LC2, TnIslow, LHC3, MyoD1, myogenin and TnT), an hGH cDNA and a PGK cDNA (control). Several studies have demonstrated key data with respect to muscle-specific gene expression in C2C12 cells (Pinset et al., 1988; Miller, 1990; Crescenzi et al., 1994) which provide a basic knowledge with respect to the general trends of muscle-specific gene expression that can be expected in this study.

Total RNA isolated from differentiating pNMG3-pool myoblasts was dot blotted onto nylon membrane (Amersham) (6 μg per well), and then probed with 25 ng of the above-mentioned cDNAs, each labelled with 50 μCi of [α-32P]dCTP at ~3,000 Ci/mmol (see Appendix C for probe labelling protocol; see Methods section for dot blot and hybridization protocols). Figure 12a and Figure 12b are computer-generated images of the dot blots which had been exposed to a phosphor screen for approximately 4 hours and then scanned with a phosphorimager (Molecular Dynamics). Each blot contains day 0, day 1, day 2, day 3, day 4 and day 5 total RNA samples, in triplicate, isolated from differentiating pNMG3-pool cells. The hGH blot contained two extra samples, "Dy 0 C2C12" and "Dy 3 C2C12", which served as control samples, since the hGH probe appeared to have some homology or reactivity with other sequence(s) in addition to the

hGH gene alone. The signal present in these two control samples was subtracted from the total signal intensity observed in the day 0 to day 5 samples probed with hGH.

Figure 12c is a graphical representation of the information derived from the dot blots presented in Figure 12a and Figure 12b. In brief, the intensity of each dot/sample was determined via the use of software (Molecular Dynamics) which integrated the volume of the signal (i.e. the total # pixels recorded by the "ImageQuant" software which was linked to the phosphorimager) and subtracted the intensity of the membrane background. The results were expressed relative to the corresponding PGK1 transcript level, which was set at 1 (i.e. a ratio of sample volume to PGK1 control volume was determined for each sample) in order to provide a standard for comparison. The average corrected volume was plotted against time (i.e. # days of differentiation). The results in Figure 12c demonstrate an appreciable up-regulation in TnIslow, LC2, myogenin, LHC3 and TnT transcript levels throughout the five days of differentiation. MyoD1 transcript levels are only slightly elevated in comparison, however, the amount of MyoD1 mRNA present on day 0 of differentiation is significantly different ($p<0.05$) from the levels seen on days 2, 3, 4 and 5 of differentiation. The levels of hGH mRNA are somewhat erratic in nature over the course of the five days of differentiation, ranging from 6.23 to 14.2 volume units.

Figures 12a & 12b.

Dot blots of differentiating pNMG3-pool myoblasts. Total RNA extracted from pNMG3-pool myoblasts on days 0, 1, 2, 3, 4 and 5 of differentiation was blotted (6 μg per sample) onto nylon membrane (Amersham) and probed with the following cDNA sequences: hGH, LC2, PGK1, TnIslow, LHC3, MyoD1, myogenin and TnT. Each cDNA insert (25 ng) was labelled with 50 μCi of [α-32P]dCTP at ~3,000 Ci/mmol and the resulting hybridized blots were exposed to a phosphor screen for approximately 4 hours. Each blot contains the day 0 to day 5 samples in triplicate. The hGH blot contains two C2C12 (negative control) samples, extracted at day 0 and day 3 of differentiation, in order to determine the degree to which the hGH probe bound non-specifically to sequences other than hGH.

Figure 12a

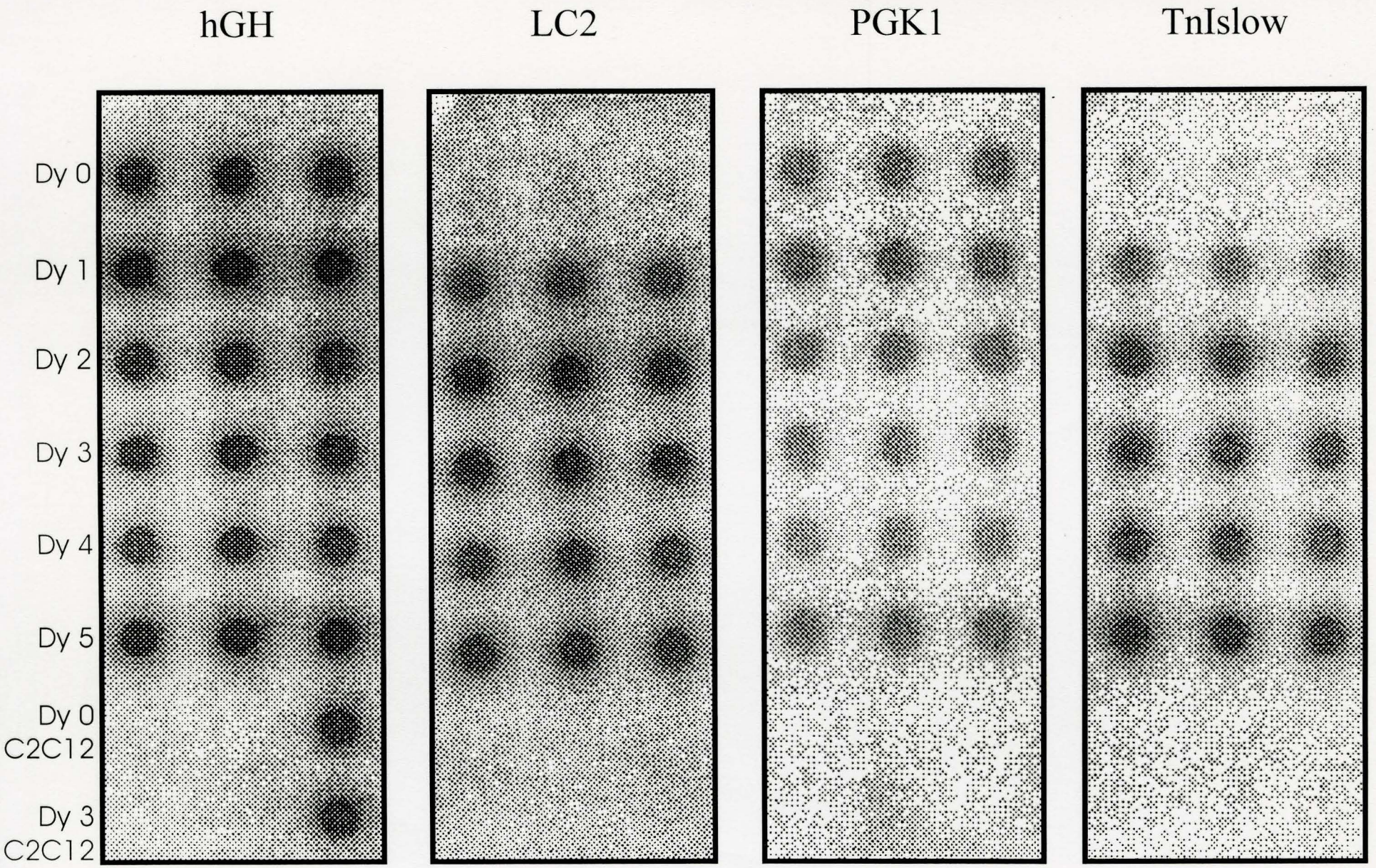

hGH
LC2
PGK1
TnIslow
Dy 0
Dy 1
Dy 2
Dy 3
Dy 4
Dy 5
Dy 0 C2C12
Dy 3 C2C12

Figure 12b

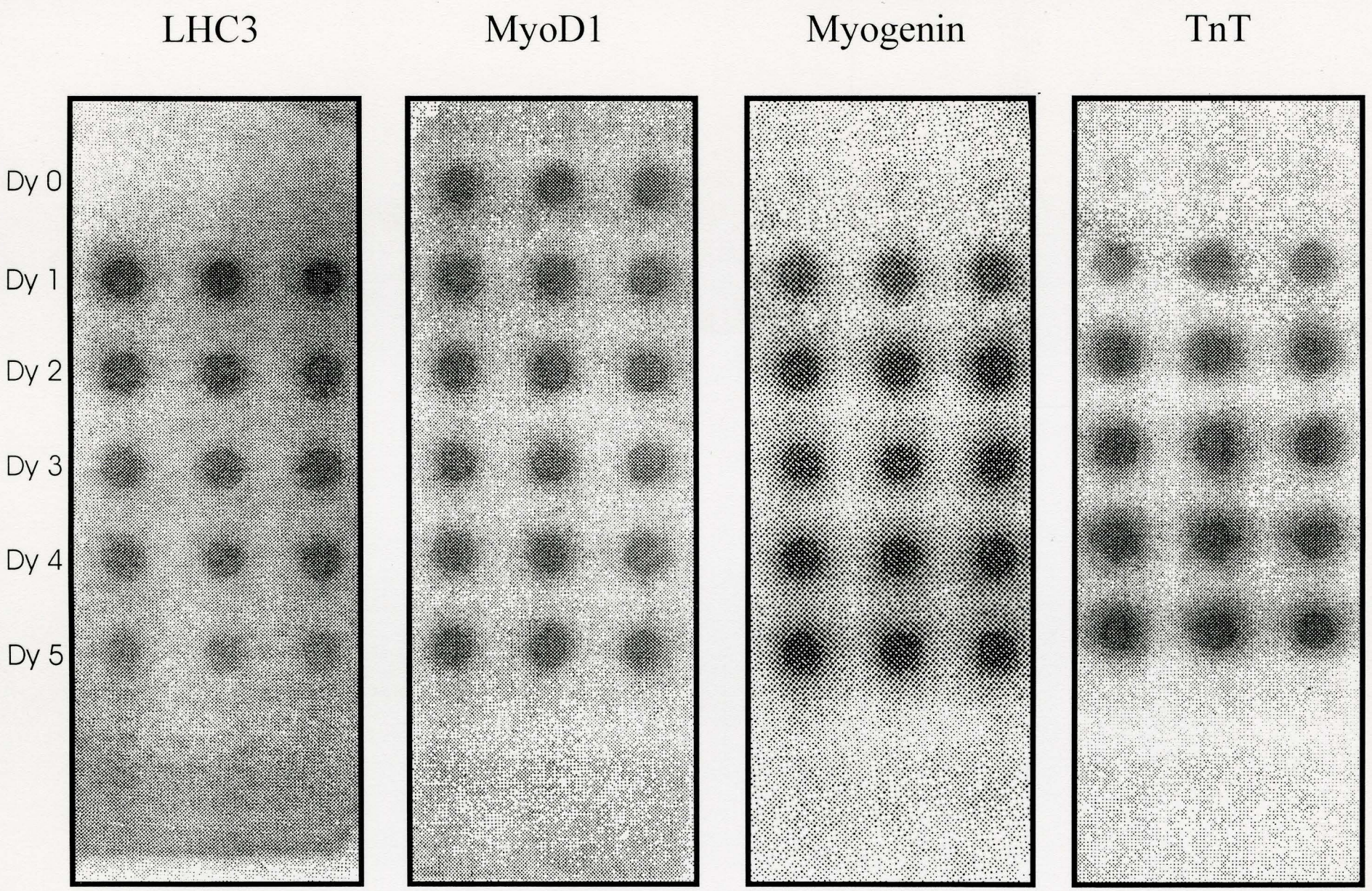
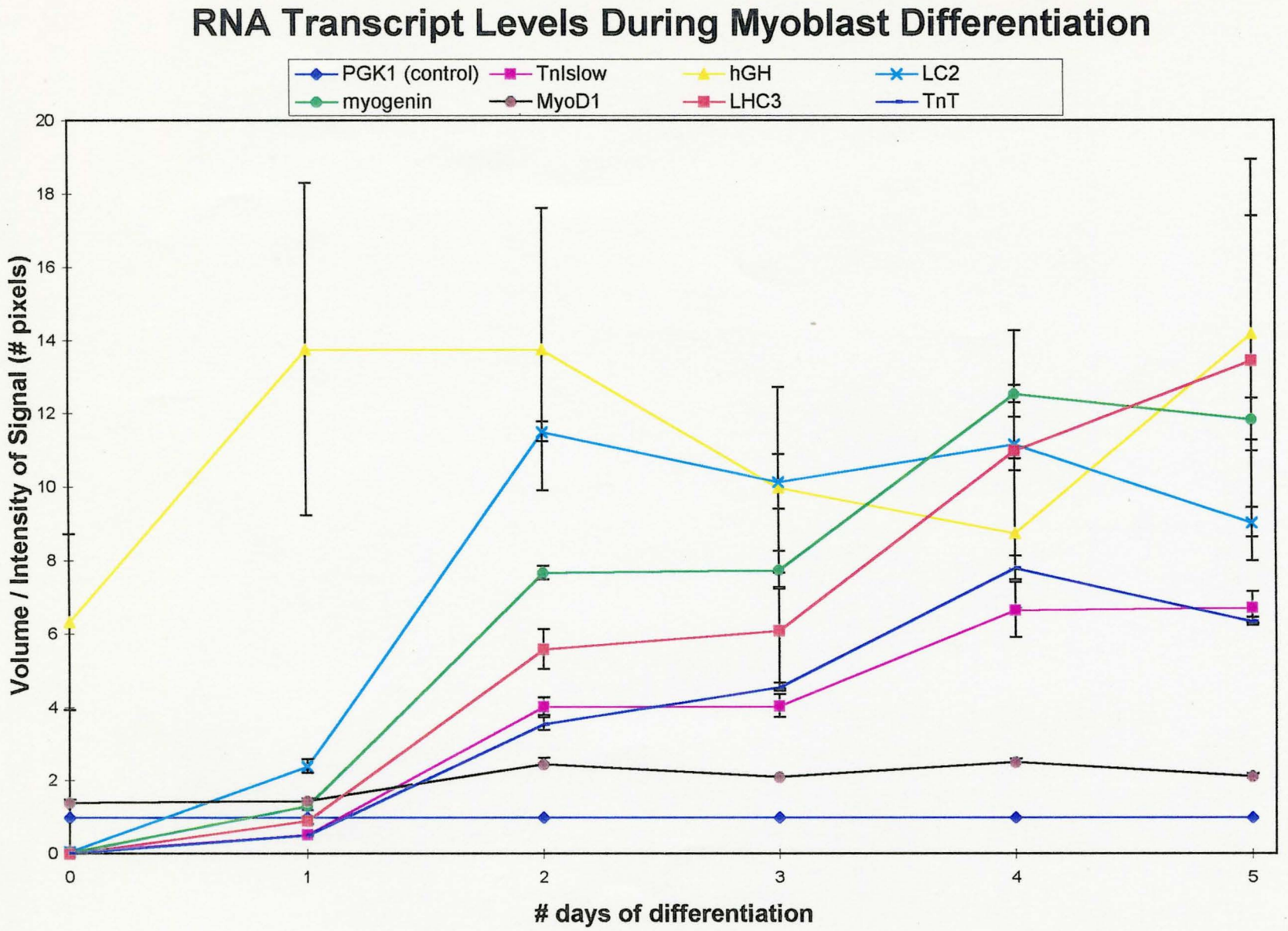


Figure 12c.

Graph of RNA transcript levels during pNMG3-pool myoblast differentiation. The intensity of each sample dot present on the blots shown in Figures 12a and 12b was determined via the use of ImageQuant software (Molecular Dynamics), which calculated the number of pixels (i.e. volume) of each sample and subtracted any background signal present. The volume / intensity of each sample, which was expressed relative to the corresponding PGK1 transcript level (set at a value of 1), was plotted against the number of days in differentiation media. Each data point represents the average of triplicate measurements ± SD.

Figure 12c


RNA Transcript Levels During Myoblast Differentiation
PGK1 (control)
TnIslow
hGH
LC2
myogenin
MyoD1
LHC3
TnT
Volume / Intensity of Signal (# pixels)
0
2
4
6
8
10
12
14
16
18
20
0
1
2
3
4
5
days of differentiation

3.4.3 RNA Expression Levels During Differentiation: Comparison of Naked and Encapsulated Myoblasts

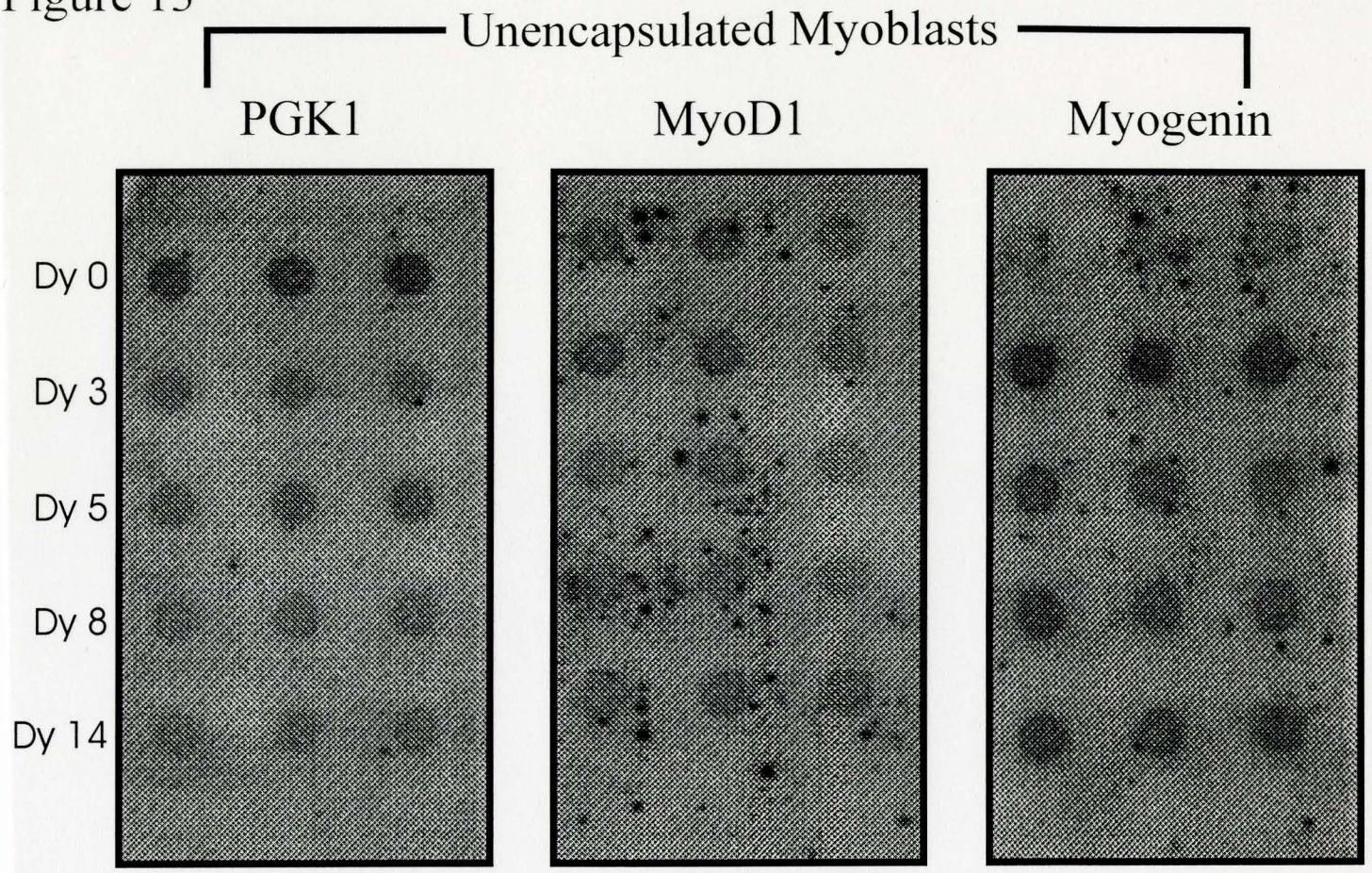
In order to compare the muscle-specific gene expression patterns observed in differentiating naked myoblasts with that of encapsulated myoblasts, total RNA was extracted from pNMG3-pool cells differentiated under both conditions (i.e. encapsulated and unencapsulated). Total RNA was dot blotted (see Methods) onto nylon membrane (Amersham) (5 μg per well) and probed with MyoD1, myogenin and PGK1 (control) cDNAs, of which 25 ng of each were labelled with 50 μCi of [α-32P]dCTP at ~3,000 Ci/mmol (see Appendix C for detailed protocol). The other muscle-specific gene sequences used as probes in previous experiments, including LC2, LHC3, TnT and TnIslow, were not used in this experiment due to the limited yields of total RNA obtained from the encapsulated pNMG3-pool myoblasts. Total RNA was extracted from naked pNMG3-pool cells on days 0, 3, 5, 8 and 14 of differentiation, and on differentiation days 0, 3, 5, 9, 11 and 14 from encapsulated pNMG3-pool cells. Figure 13 is a computer-generated image (ImageQuant, Molecular Dynamics) of the dot blots for both naked (unencapsulated) and encapsulated myoblasts. It should be noted that the encapsulated myoblast samples were not done in triplicate due to limiting amounts of available RNA. Figures 14a and 14b demonstrate the MyoD1 and myogenin transcript levels present in naked and encapsulated myoblasts, respectively. Both the MyoD1 and myogenin transcript levels in naked differentiating myoblasts (Figure 14a) demonstrated a peak at about day 3 of differentiation, and the levels appeared to plateau between day 8 and day

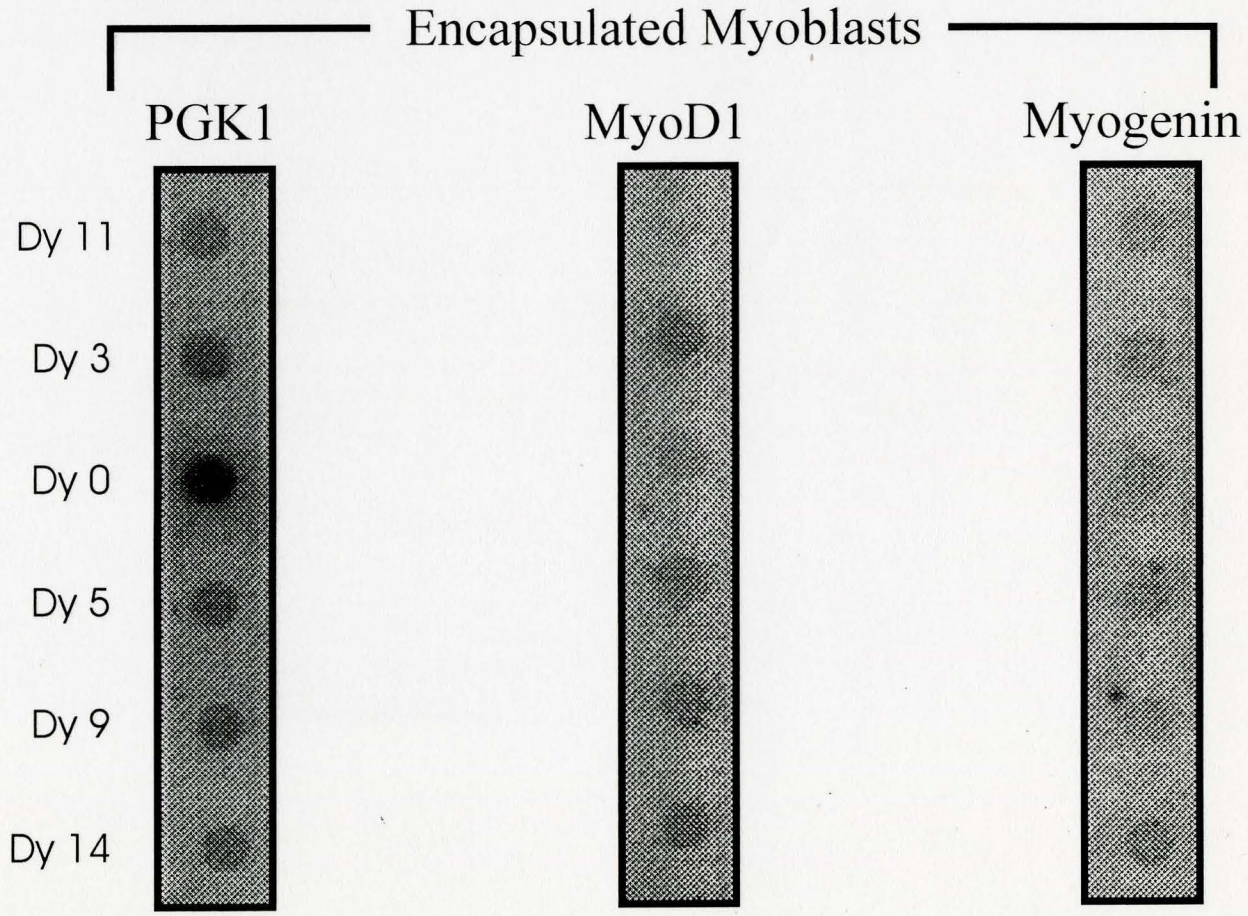
14 of differentiation. In general, the level of myogenin transcript was significantly higher than that of MyoD1 on each of the days sampled. This is in contrast to what is observed in the encapsulated myoblast samples, whereby the levels of MyoD1 transcript are generally higher than those of myogenin, and the levels of MyoD1 and myogenin mRNA appear to be significantly lower overall when compared to the standard (PGK1). It should be noted, as well, that the myogenin mRNA levels (and to a lesser degree, MyoD1) do not appear to begin rising until about day 12 to 14 of differentiation in the encapsulated myoblast samples.

Figure 13.

Dot blots of (a) unencapsulated (naked) myoblasts and (b) encapsulated myoblasts grown in differentiation media for a period of two weeks. Total RNA was isolated from naked pNMG3-pool myoblasts on days 0, 3, 5, 8 and 14 of differentiation (samples done in triplicate), while RNA was isolated from encapsulated pNMG3-pool cells on days 0, 3, 5, 9, 11 and 14 of differentiation (samples not done in triplicate). Each sample consisted of 5 μg of total RNA, from both naked and encapsulated cells, which were probed with 25 ng of one of the following: PGK1 (control), MyoD1 or myogenin (each labelled with 50 μCi of [α-32P]dCTP at ~3,000 Ci/mmol). The blots were analyzed via the use of ImageQuant software (Molecular Dynamics) to determine the intensity of each sample. The intensity (volume) of samples probed with MyoD1 and myogenin were determined by calculating the ratio of sample intensity relative to the corresponding PGK1 level of intensity (set at a value of 1).

Figure 13


Unencapsulated Myoblasts
PGK1
MyoD1
Myogenin
Dy 0
Dy 3
Dy 5
Dy 8
Dy 14


Encapsulated Myoblasts
PGK1
MyoD1
Myogenin
Dy 11
Dy 3
Dy 0
Dy 5
Dy 9
Dy 14

Figures 14a & 14b.

Graph of RNA transcript levels during pNMG3-pool myoblast differentiation: comparison of unencapsulated (naked) and encapsulated cells. The levels of MyoD1 and myogenin mRNA in naked myoblasts (Figure 14a) and encapsulated myoblasts (Figure 14b) were determined by analyzing the blots (Figure 13) via the use of ImageQuant software (Molecular Dynamics). The sample intensity (volume) was plotted against the number of days of differentiation for both naked and encapsulated cells, using the level of PGK1 transcript (set at a value of 1) as a standard reference point. Data presented in Figure 14a represents the average of triplicate measurements ± SD, while Figure 14b is illustrative of single sample measurements.

Figure 14a

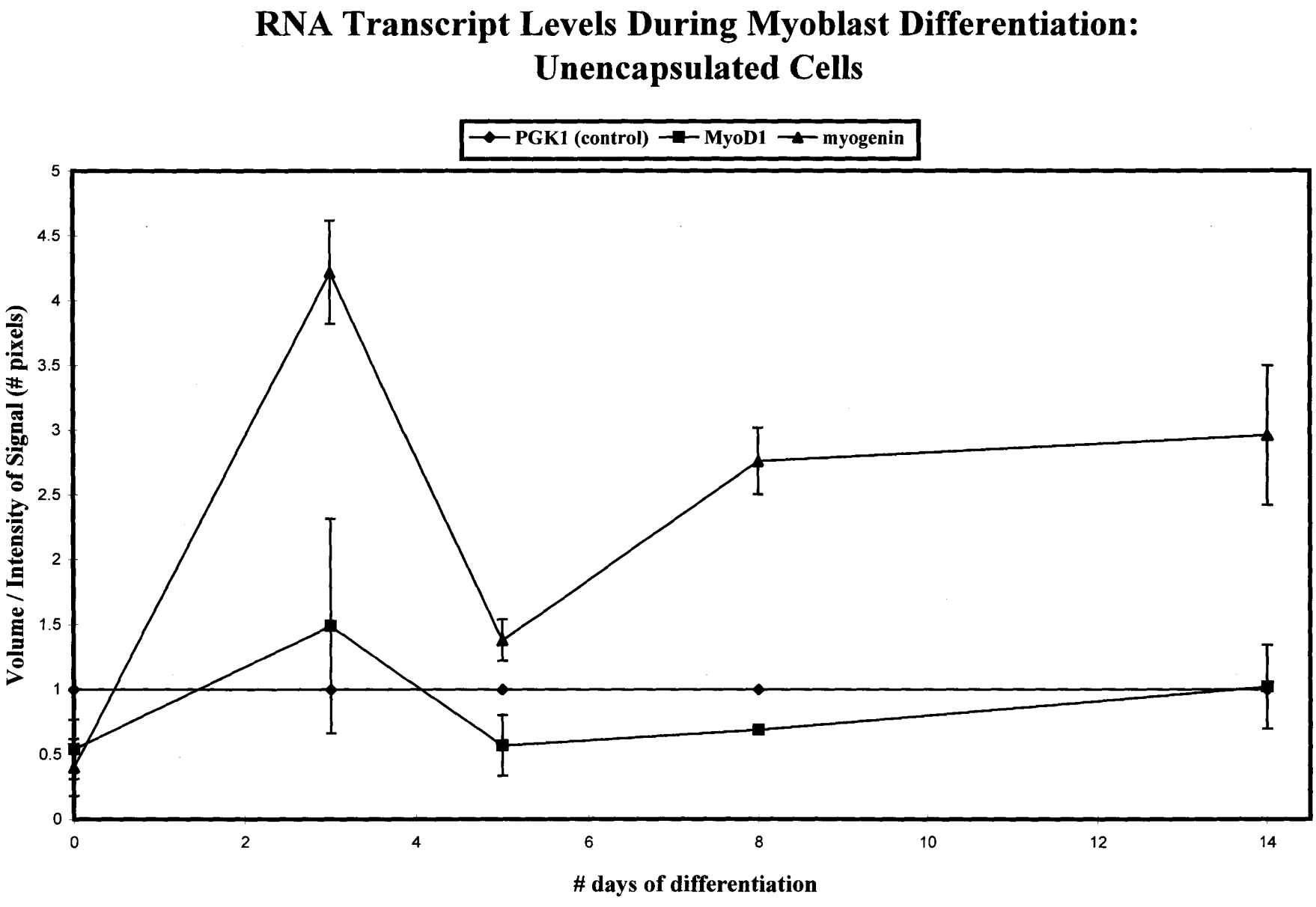
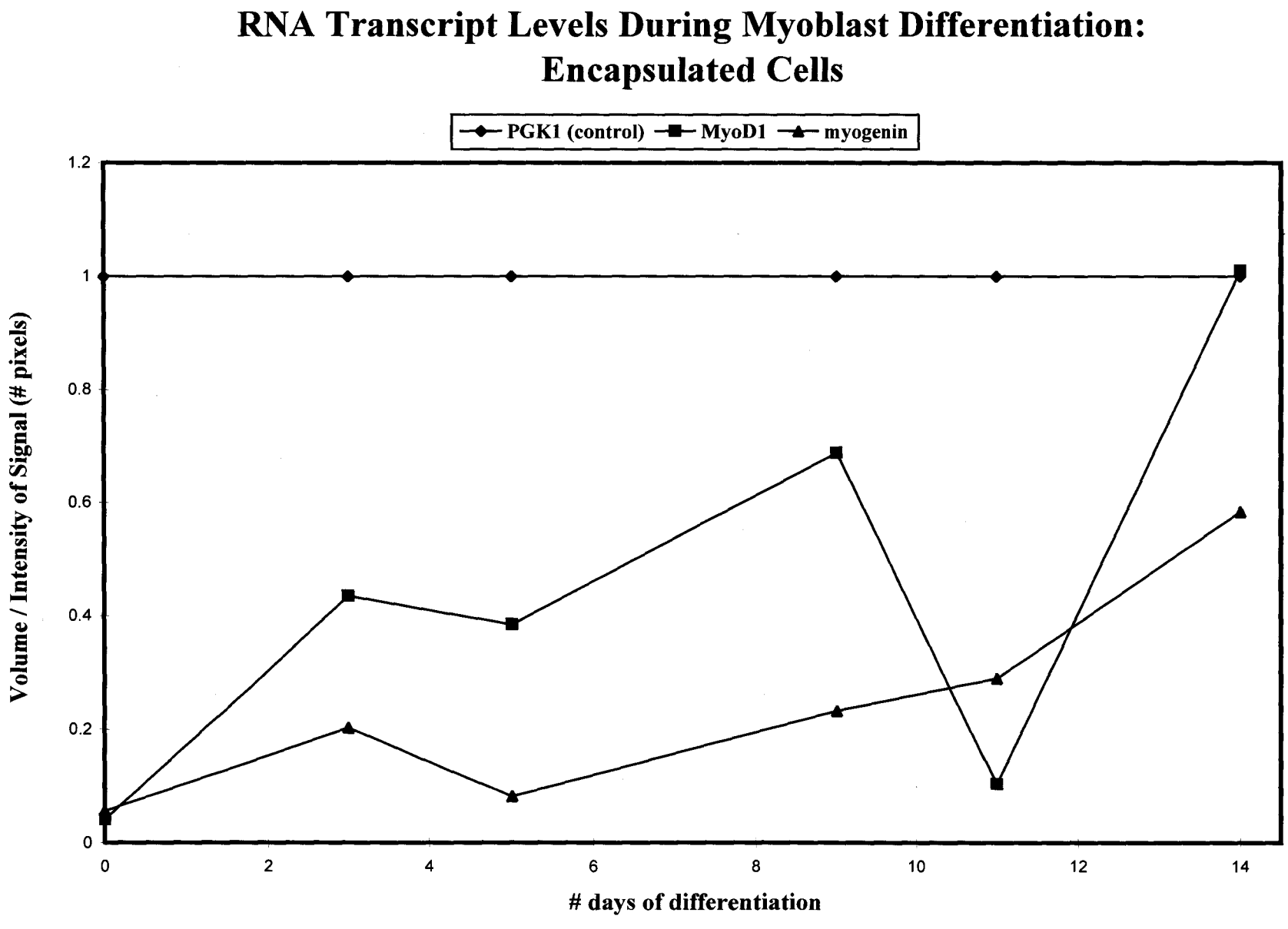

RNA Transcript Levels During Myoblast Differentiation: Unencapsulated Cells
PGK1 (control)
MyoD1
myogenin
Volume / Intensity of Signal (# pixels)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
2
4
6
8
10
12
14
days of differentiation

Figure 14b


RNA Transcript Levels During Myoblast Differentiation: Encapsulated Cells
PGK1 (control)
MyoD1
myogenin
Volume / Intensity of Signal (# pixels)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
days of differentiation

3.5 Characterization of Encapsulated Myoblasts

3.5.1 Analysis of Encapsulated Myoblasts

Alginate-polylysine microcapsules, approximately 700 um in diameter, were prepared, containing pNMG3-pool myoblasts. Approximately 5 x 10^6 cells per ml of 1.5% alginate were encapsulated. The encapsulated cells were grown in growth media supplemented with 10 ng/ml bFGF for approximately 10 days and then transferred to differentiation media for a period of two weeks. The CPK analysis, hGH secretion analysis and RNA analysis were performed using the encapsulated myoblasts shown in Figure 15. The differentiation media was changed every three days. The cell viability and the number of cells per capsule (see Methods for protocols) were calculated on days 0, 3, 5, 9, 11 and 14 of differentiation (data summarized below in Table 3). The cell viability data represents an average of seven measurements ± SD and the number of cells per capsule counts are derived from the average of ten measurements ± SD (note: N/D indicates those samples which were Not Done).

Table 3.

# Days in Differentiation Media:	Cell Viability (%):	# Cells per Capsule:	# Viable Cells per Capsule:
0	89 ± 5	113 ± 22	~ 101
3	75 ± 6	N/D	N/D
5	73 ± 3	86 ± 15	~ 63
9	63 ± 7	69 ± 14	~ 43
11	61 ± 11	49 ± 9	~ 30
14	60 ± 10	75 ± 16	~ 45

3.5.2 Photography of Microcapsules

Microcapsules containing pNMG3-pool myoblasts were photographed via the use of a dark-field microscope on days 0, 3, 5, 9, 11 and 14 of differentiation (Figure 15). The capsules are approximately 500-700 um in diameter and are magnified approximately 1000 X. It should be noted that although there does not appear to be much difference between day 0 and day 14 of differentiation with respect to the percentage of cells occupying the capsule space, many of the encapsulated cells died over the course of growth in differentiation media (i.e. there was a decrease in cell viability of ~ 30% by day 14).

3.6 Effects of Purified hGH on C2C12 Proliferation

In order to determine whether or not the human growth hormone (hGH) protein being produced by the transfected C2C12 myoblasts (pNMG3-pool cells) had any autocrine and/or paracrine mitogenic effects on the transfected cell population, purified hGH was added to untransfected C2C12 cells and the rate of cell proliferation was measured. The basis for this query includes studies done which suggest that human growth hormone may, in fact, play a role in stimulating the growth of C2C12 myoblasts (Ewton and Florini, 1980; Palmiter et al., 1983). This mitogenic effect of hGH could possibly circumvent the action of the differentiation media on the myoblasts, thereby suppressing the myogenic differentiation to some degree.

C2C12 cells were seeded onto 60-mm tissue culture dishes at a density of 60,000 cells per dish. The cells were grown in regular growth media and were exposed to

Figures 15a, 15b & 15c.

Dark-field photomicroscopy of encapsulated pNMG3-pool myoblasts on days 0, 3, 5, 9, 11 and 14 of differentiation. A sample of capsules (approximately 200 ul in volume) were placed into a 35-mm tissue culture dish with most of the excess media drawn away prior to photography. The cells appear as white clumps within the alginate-polylysine microcapsules, which are approximately 500 to 700 um in diameter. The following images are magnified approximately 1000 X:

Figure 15a-A: Day 0 of differentiation.

Figure 15a-B: Day 3 of differentiation.

Figure 15b-C: Day 5 of differentiation.

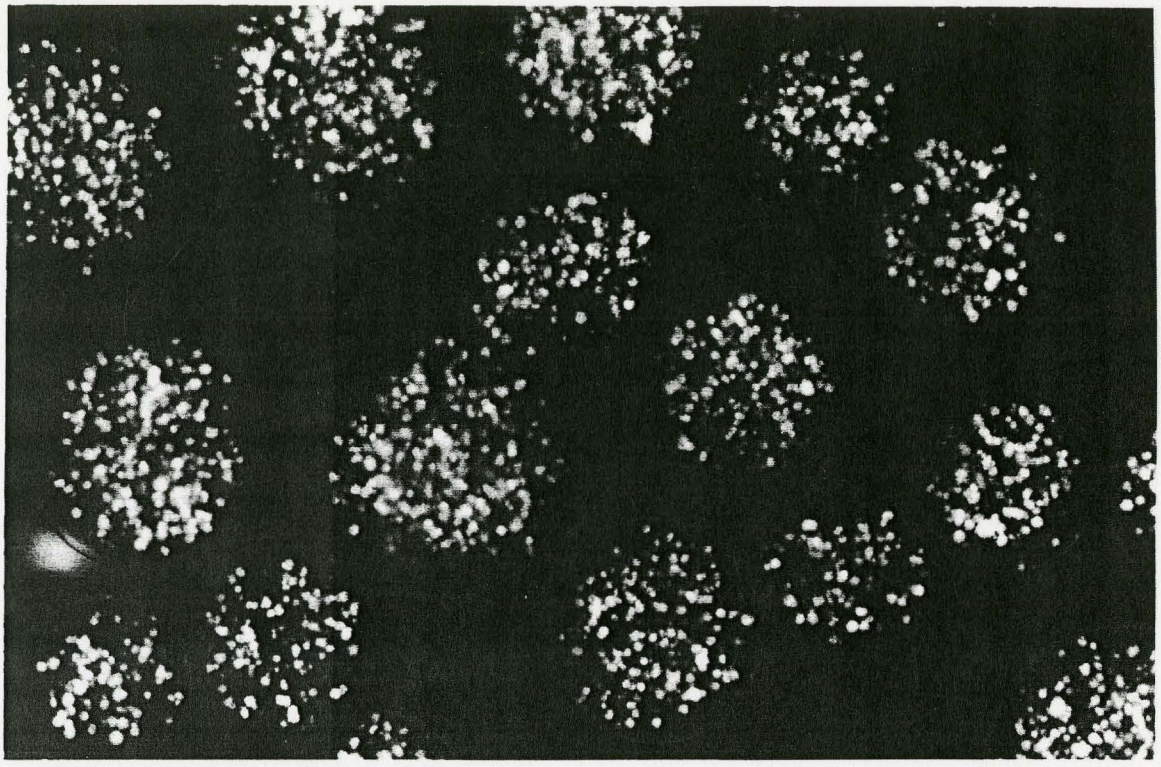
Figure 15b-D: Day 9 of differentiation.

Figure 15c-E: Day 11 of differentiation.

Figure 15c-F: Day 14 of differentiation.

Figure 15a

A



B

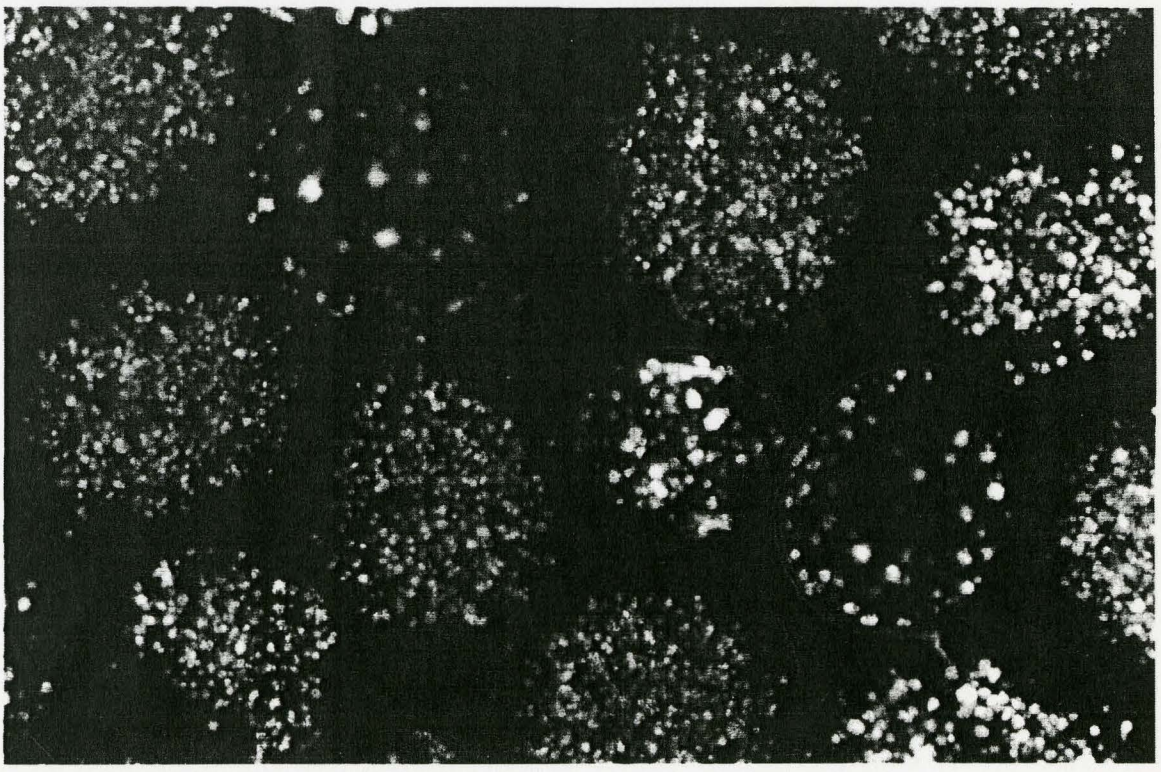
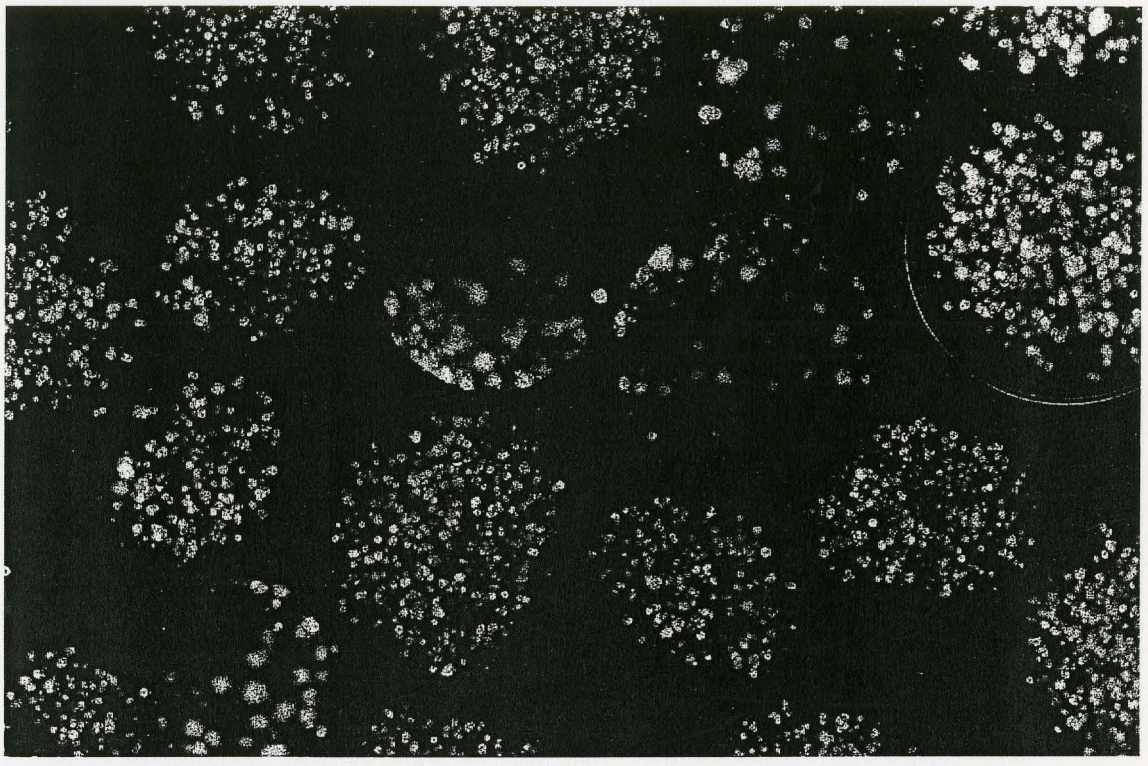


Figure 15b

C



D

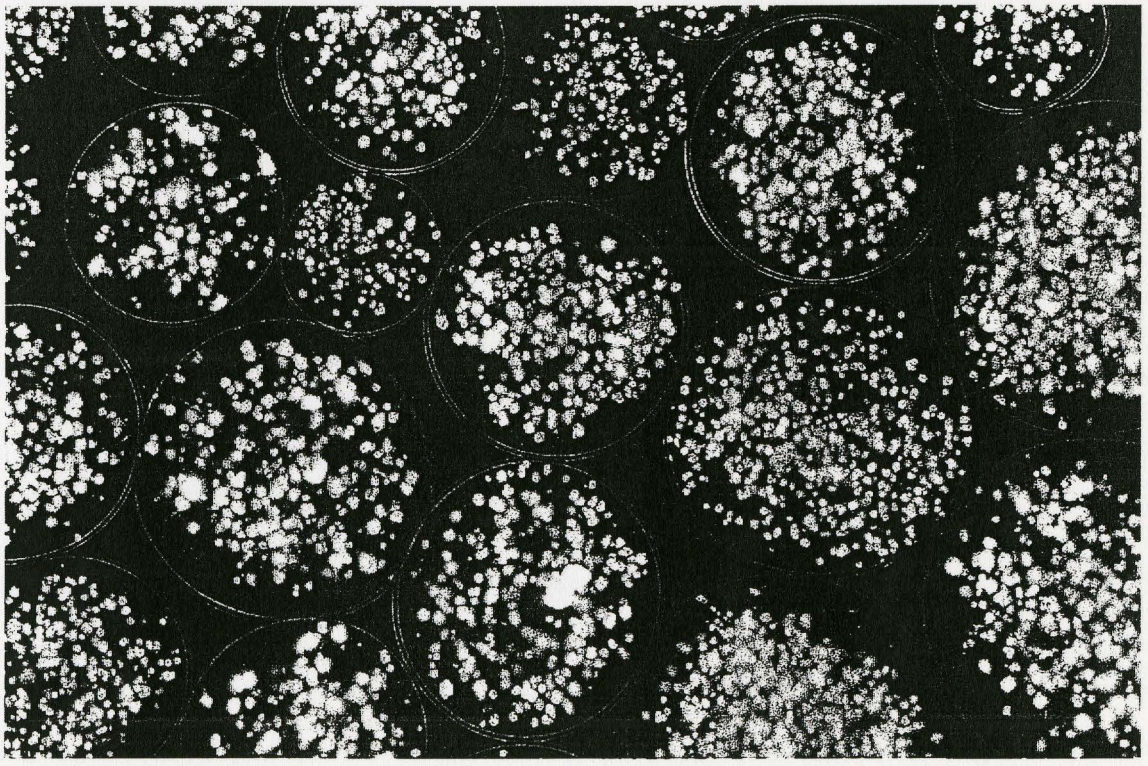
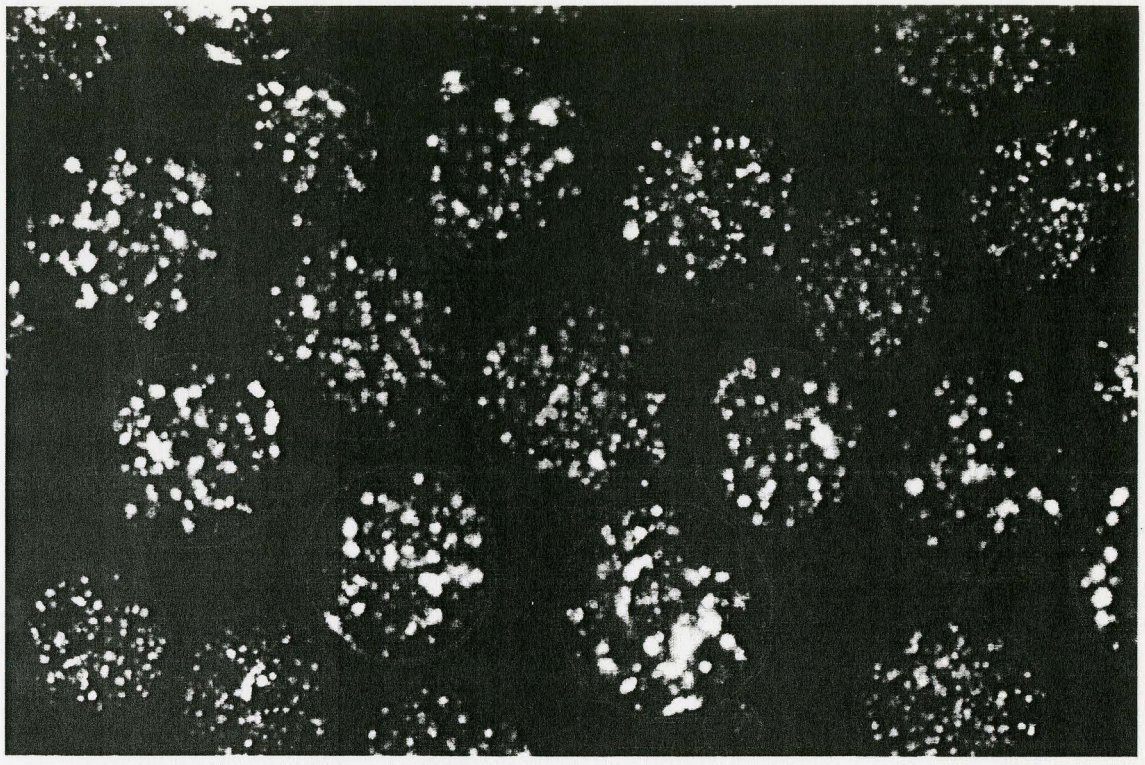
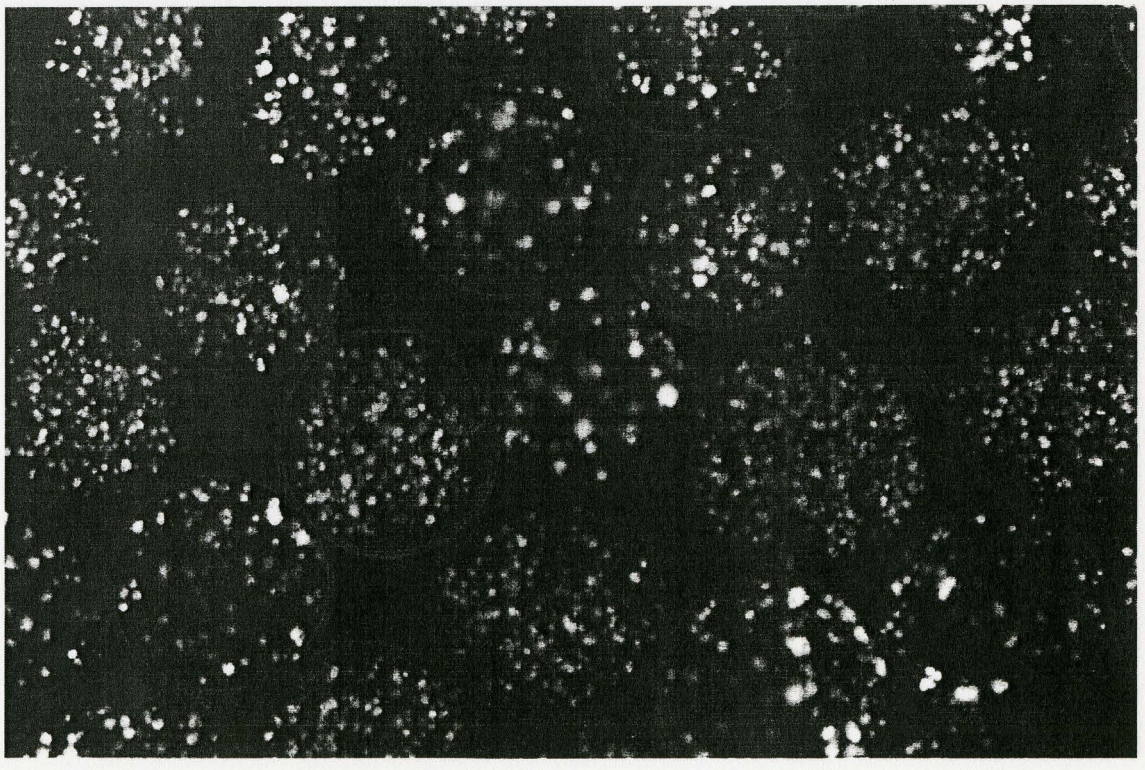


Figure 15c

E



F

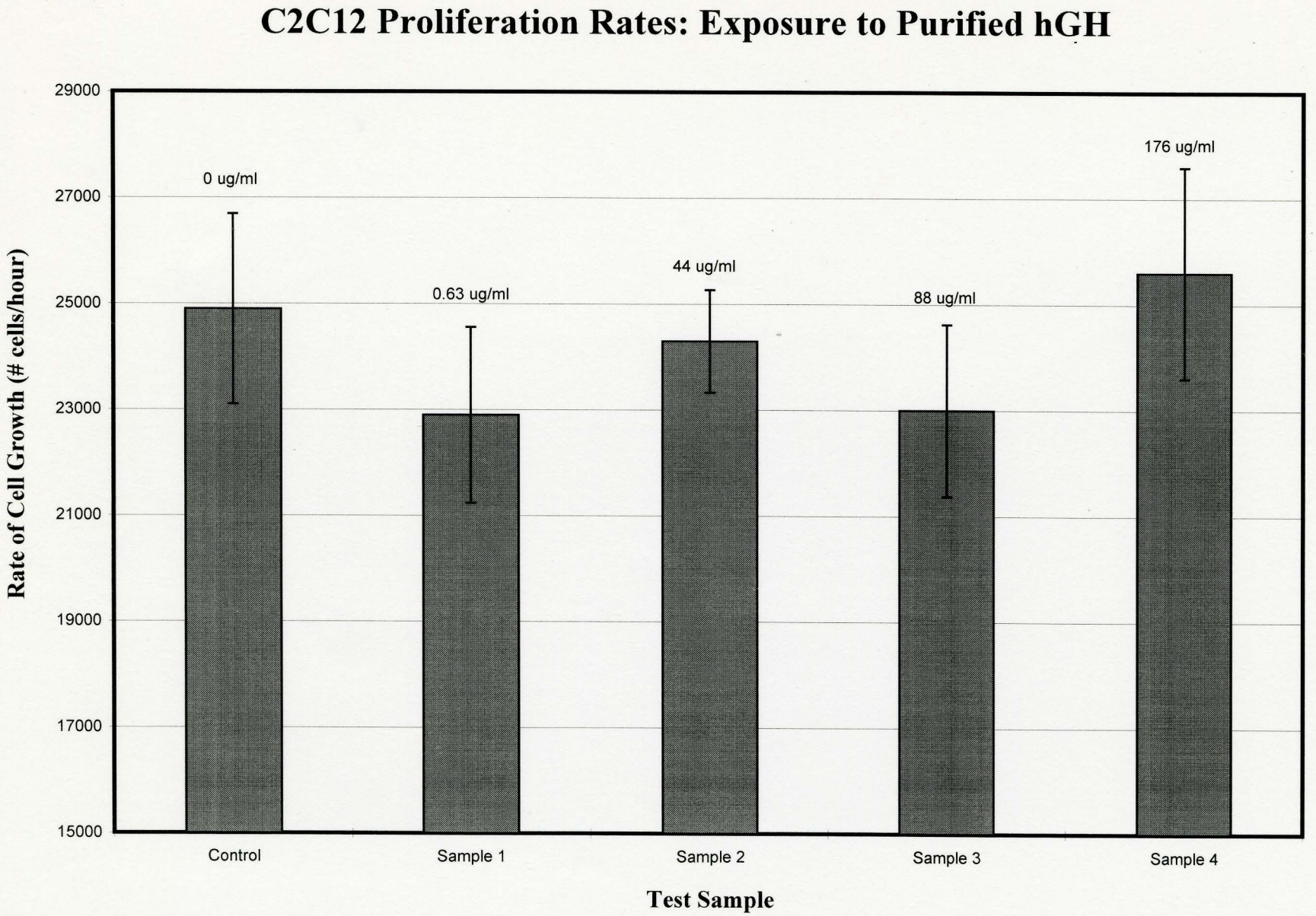


various concentrations of purified hGH (0 μg/ml, 0.63 μg/ml, 44 μg/ml, 88 μg/ml and 176 μg/ml), for a period of 48 hours (Figure 16). The rate of C2C12 cell proliferation over the 48-hour time period was calculated for each treatment group. The average rate of cell proliferation for the control group (i.e. those cells receiving no hGH) was 2.49 x 10^4 cells/hr, and the average rate of cell proliferation among the treatment groups (i.e. Samples 1, 2, 3 and 4 receiving purified hGH) was 2.40 x 10^4 cells/hr. It was determined that there was no significant difference ($p>0.05$) between the control group and the treatment groups. These results suggest that hGH does not play a role in stimulating the growth of C2C12 myoblasts in culture.

Figure 16.

Comparison of untransfected myoblast cell proliferation of C2C12 cells grown in: (a) growth media only; (b) growth media supplemented with purified hGH. The rate of cell proliferation (# cells/hour) over a time period of 48 hours was determined for the following samples: (i) Control (receiving no purified hGH); (ii) Sample 1 (0.63 μg/ml); (iii) Sample 2 (44 μg/ml); (iv) Sample 3 (88 μg/ml); (v) Sample 4 (176 μg/ml). The data represents the average of triplicate samples ± SD.

Figure 16


C2C12 Proliferation Rates: Exposure to Purified hGH
Rate of Cell Growth (# cells/hour)
29000
27000
25000
23000
21000
19000
17000
15000
0 ug/ml
0.63 ug/ml
44 ug/ml
88 ug/ml
176 ug/ml
Control
Sample 1
Sample 2
Sample 3
Sample 4
Test Sample

4.0 DISCUSSION

The use of alginate-polylysine microcapsules for the delivery of various gene products offers a relatively safe and efficient alternative for the purposes of somatic gene therapy. Alginate-polylysine microcapsules have been well described and characterized with respect to the immunoisolation of a variety of recombinant cell types, both *in vitro* (Tai and Sun, 1993; Chang et al., 1993; Liu et al., 1993; Hughes et al., 1994) and *in vivo* (Al-Hendy et al., 1995; Hortelano et al., 1996). Recombinant fibroblasts have been widely employed in encapsulation studies due to their stable nature and excellent proliferation (Tai and Sun, 1993; Chang et al., 1994). It has been observed, however, that the uncontrolled growth of the fibroblasts within the capsule space results in decreased cell viability and sub-optimal efficiency with respect to recombinant gene product delivery. Myoblast cell lines offer an attractive solution to this problem as a result of their long-term stability upon terminal differentiation into multinucleate myotubes. In fact, prolonged cell survival and persistent secretion of a recombinant gene product *in vivo* has been documented in a study employing an encapsulated myoblast cell line secreting human factor IX (Hortelano et al., 1996). In order to manipulate the myoblast for the purposes of gene delivery, so as to maximize cell proliferation and protein secretion within alginate-polylysine capsules, the events surrounding myoblast growth and differentiation in an encapsulated environment must first be examined and understood. This study looked at various parameters of C2C12 myoblast growth and proliferation, both in encapsulated and unencapsulated conditions, which are outlined in the following discussion.

4.1 Secretion of Human Growth Hormone

Human growth hormone was employed as a marker protein in the study of recombinant gene product secretion from naked and encapsulated myoblasts since it is known to be readily secreted from alginate-polylysine microcapsules (Tai and Sun, 1993; Chang et al., 1994). This is supported by the estimated pore size of alginate-polylysine capsules, which is ~ 60 to 100 kD (Ma et al., 1994), through which the human growth hormone protein [22 kD (Albertsson-Wikland et al., 1993)] can easily diffuse. C2C12 myoblasts transfected with a metallothionein / human growth hormone fusion construct (pNMG3-pool cells) were observed to secrete ~51 ng hGH/hr/10^6 cells (Appendix G). Given the fact that one million cells contains approximately 1000 ug of protein (C. Ross, unpublished observations), the secretion rates observed in the experiment which compared naked and encapsulated pNMG3-pool myoblasts (Figures 1 & 2) were comparable to the rate of hGH secretion observed upon transfection (i.e. ~ 80 ng hGH/hr/10^6 cells compared with 51 ng hGH/hr/10^6 cells).

Due to some concerns with respect to the possibility that the secreted hGH may be influencing the rate of proliferation of the recombinant pNMG3-pool myoblasts via an autocrine mechanism, an experiment which tested the growth rates of untransfected C2C12 cells upon exposure to purified hGH (Figure 16) was performed. It was determined that the exogenous purified hGH did not exert any biological effects on the C2C12 cells in culture, at least in terms of cell proliferation, as there was no significant difference in growth rates between the control group and the treatment group. Other studies have shown that hGH exerts no such effects on myoblasts or myotubes in culture (Ewton and Florini, 1980), and that it does not interfere with gene expression via an

autocrine pathway (Selden et al., 1986). It is suggested that the responsiveness of skeletal muscle to GH occurs at a much later stage of differentiation and/or muscle development that is not observable in cell culture studies (Ewton and Florini, 1980). This "delayed" effect of GH is supported by a study which demonstrated increased growth of transgenic mice which had incorporated a metallothionein-hGH fusion construct, thereby indicating an *in vivo* biological effect of hGH upon muscle tissue (Palmiter et al., 1983).

The hGH secretion rate observed in naked myoblasts appeared to remain relatively stable over the two week period of differentiation (Figure 1), which suggests that the process of myogenic differentiation did not alter the production of hGH. This was not surprising given the fact that the human growth hormone gene was under the control of the ubiquitous mouse metallothionein I promoter which regulated hGH expression at a relatively constant level since it was not induced by extraneous sources of heavy metals and glucocorticoids (Pavlakis and Hamer, 1983).

The levels of hGH secretion from the pNMG3-pool cells were significantly different from the secretion levels demonstrated by the encapsulated myoblasts on days 11 and 14 of differentiation (Figure 1). The ~ 40 % decrease in hGH secretion observed in the encapsulated cell sample over the period of two weeks in differentiation media is notable, but it is in contrast to the hGH secretion values obtained, primarily on day 14 of differentiation, when the encapsulated cell viability is taken into account (Figure 2). In this case, the hGH secretion rate of the encapsulated cells on day 14 of differentiation is not significantly different from the naked cell samples. There is likely some error inherent in calculating an accurate rate of secretion based upon the total protein present in the cell samples as it is assumed that each dead cell would contribute the same amount of

total protein as would each live cell. This would undoubtedly result in an inflated level of encapsulated cell hGH secretion that is not representative of the true level of hGH secretion. The true level of secreted hGH from the encapsulated myoblasts probably lies between the two different measurements of 3.72×10^{-2} ng/hr/μg (cell viability not taken into account) and 7.01×10^{-2} ng/hr/μg (cell viability accounted for). Nevertheless, the hGH secretion from the encapsulated myoblasts is somewhat repressed over the two week period of growth in differentiation media which may be due to the harsh conditions posed by the serum withdrawal in addition to the unnatural capsule environment, both of which may compromise the efficiency with which the cells secrete hGH. The effects of these sub-optimal conditions are reflected in the continued decrease in cell viability over the two-week period from ~ 90% to ~ 53%. The decline in the number of viable hGH-expressing cells is also evident upon immunofluorescence staining of differentiating encapsulated myoblasts (Figure 3), which shows that by day 14 of differentiation, only a portion (~ 50%) of the cells are demonstrating a positive signal for hGH. One must consider the possibility that the presence of certain toxic waste products, accumulating in the capsule space as a result of the on-going cell death, may contribute to the decreased levels of hGH produced by the remaining viable cells. Previous studies have, in fact, linked a decline in cell viability with a concomitant decrease in recombinant gene product secretion (Chang et al., 1994).

The levels of hGH secretion are of importance, however, when considering the *in vivo* use of such encapsulated recombinant myoblasts for therapeutic purposes, in which case, increased levels of hGH or other recombinant gene products may be required in order to produce a biological effect. Several studies have incorporated a variety of

muscle-specific enhancers and promoters into their expression vectors and have thereby demonstrated increased expression of the corresponding gene product. Examples of such muscle-specific elements include the muscle creatine kinase enhancer (Dai et al., 1992; Wang et al., 1996), the desmin enhancer/promoter regulatory system (Naffakh et al., 1996) and the α-actin, β-actin and myogenin promoters (Wang et al., 1996). These enhancer and promoters may be employed in further studies to create constructs containing hGH under the control of such muscle-specific regulatory elements. The construction of vectors which result in the up-regulation of hGH upon myogenic differentiation could circumvent the problem of declining rates of secretion upon encapsulation and may produce higher levels of secreted gene product overall.

4.2 Myogenic Index / Myosin Heavy Chain Production

Several muscle-specific genes encoding structural and contractile proteins are up-regulated during myogenic differentiation. These changes in gene expression are followed by the final step towards terminal differentiation, that being cell fusion. In this study, the detection of myosin heavy chain (MHC) in differentiating myoblasts, via the use of an anti-MHC primary antibody and a horseradish peroxidase-conjugated secondary antibody, demonstrated the onset of biochemical myogenic differentiation upon exposure to differentiation media. The percentage of cells expressing MHC in naked myoblast samples on day 14 of differentiation (~ 61%) was much greater than the percentage of encapsulated myoblasts expressing MHC on day 14 of differentiation (~ 13%). This

indicates that the differentiation of pNMG3-pool myoblasts, on a molecular level, is significantly delayed upon encapsulation.

The delay in MHC production in differentiating encapsulated myoblasts would preclude the process of cell fusion, and, in fact, no evidence of multinucleate cells was found. The naked pNMG3-pool myoblasts, however, demonstrated a dramatic increase in cell fusion over the course of two weeks in differentiation media, reaching a myogenic index (i.e. percentage of multinucleate cells) of ~ 50% by day 14 of differentiation. The small percentage of myoblasts which are differentiating upon encapsulation may be a result of the sub-optimal conditions posed by the capsule environment itself which is supported by the rapidly declining cell viability during the two weeks of growth in differentiation media, from ~ 90% to ~ 53% by day 14 of differentiation. Although both the naked myoblasts and the encapsulated myoblasts were subjected to the same culture conditions (i.e. D-MEM media supplemented with 2% horse serum), the major difference existing between the two relates to cell adhesion. The encapsulated myoblasts are essentially "free-floating" within the capsule space, as little, if any, of the inner alginate remains crosslinked or gelled following the liquefaction of the capsule core with sodium citrate. This "suspension-type" growth is in sharp contrast to the typical growth patterns observed with cultured myoblasts which involves the adhesion of the myoblasts to some type of extracellular matrix. It is possible that this non-adhesive growth within the microcapsules, accompanied by the fact that the cells are rather sparsely distributed upon encapsulation, may contribute to the delay in, or inhibition of, myogenic differentiation. Studies have demonstrated, in fact, that one of the important factors essential to the process of terminal myogenic differentiation is the interaction of integrin (a family of cell

surface receptors) with the extracellular matrix (Menko and Boettiger, 1987). Integrin appears to function in both cell attachment and cell migration, in addition to possessing a signal transduction role which may play a part in influencing gene expression. Furthermore, the interaction between integrin and the extracellular matrix is tightly linked to the cell population itself (i.e. the induction of myogenic differentiation by integrin is dependent upon an interaction with the extracellular matrix, which, in turn, requires that a sufficient population of myogenic cells, in the proper positions, are present in the immediate environment) (Menko and Boettiger, 1987). Hence, the sparse distribution of the cells within the capsules, accompanied by the decrease in cell viability and the lack of any available attachment matrix, would not appear to be a very permissive environment for the promotion of myogenic differentiation and more specifically, myoblast fusion.

4.3 CPK Activity During Myoblast Differentiation

Measurements of creatine phosphokinase (CPK) activity have been widely used as an index of myogenic differentiation in cell cultures. Upon exposure to mitogen-depleted medium, cultured myoblasts cease to proliferate and subsequently demonstrate an up-regulation of muscle-specific proteins, one of which is CPK, a metabolically critical enzyme in muscle (Chamberlain et al., 1985). It was shown that the up-regulation of CPK in differentiating pNMG3-pool myoblasts was relatively linear (Figures 7 and 8), at least until the rate of cell fusion, as evidenced by the myogenic index, decreased. The levels of CPK activity continued to rise linearly until about day 9 of differentiation, when the levels began to plateau. This "levelling-off" of CPK activity was concomitant with a

significant decrease in the rate of cell fusion, which was demonstrated by the plateau effect observed in Figure 4 (graph of Myogenic Index) after day 9 of differentiation. It has been suggested previously that, in fact, little enzyme synthesis takes place after myoblast fusion is terminated (Shainberg et al., 1971). Similar studies have also reported a linear rate of increase in CPK activity starting close to the onset of cell fusion (Shainberg et al., 1971), which is not unlike this study, which demonstrated an increase in CPK levels and cell fusion beginning sometime within the first 24 hours after mitogen-rich media withdrawal (Figures 8 and 4, respectively).

CPK activity levels demonstrated a maximum increase of 23-fold in differentiating naked pNMG3-pool myoblasts (over a two-week period of growth in differentiation media). This is much lower than the CPK increases observed in a study which reported a 150-fold increase in CPK activity in differentiating MM14 mouse myoblasts (Chamberlain et al., 1985). It is important to note, however, that the MM14 myoblasts are a rapidly differentiating cell line which demonstrate almost 100% cell fusion, unlike the pNMG3-pool myoblasts used in this study, of which only ~ 50% of the cell population demonstrated cell fusion upon exposure to mitogen-depleted medium. Upon comparison of CPK activity in two populations of differentiating pNMG3-pool cells (Figure 8), a marked difference in the rates of increase of CPK activity were found between the two experiments, although both demonstrated similar increases in activity overall (i.e. Experiment #1 demonstrated a 19-fold increase in CPK levels within a 5-day period, while Experiment #2 demonstrated a 23-fold increase in CPK levels over a 14-day period). The most reasonable explanation for this discrepancy is that the different rates of

CPK accumulation are a result of the differential passaging of the pNMG3-pool cell line prior to the start of each experiment (i.e. Experiment #1: cell passaged ~ 3 times; Experiment #2: cells passaged ~ 7 times). It has been observed that C2C12 cells may differentiate less efficiently, in that the degree of cell fusion is much reduced, when the cells are passaged a number of times (personal observations, data not shown). The variability in myogenic differentiation among different experiments may not be as marked if clonally-derived cells from the pNMG3-pool line were used. In addition, the pNMG3-pool clones could be tested for their capacity to differentiate prior to experimentation.

The pNMG3-pool cells, once encapsulated, demonstrated a great degree of variability with respect to the induction of CPK activity upon exposure to differentiation media (Figure 9), with CPK increases of ~ 0 (Experiment #2), ~ 8-fold (Experiment #1b) and ~ 63-fold (Experiment #1a). The variability appears to relate to the encapsulated cell viability as well as the number of cell passages prior to the start of the experiment. Experiments #1a and #1b demonstrated similar decreases in cell viability during their growth in differentiation media (~ 10 %; data not shown) while Experiment #2 experienced a more dramatic loss of viable cells (~ 30 %) which was correlated with no significant rise in CPK activity. Similarly, the comparison of Experiments #1a and #1b point to increased cell passages as a predictor for decreases in the rate of CPK induction.

Also of interest was the apparent "lag" in the induction of CPK activity which refers to the virtual absence of any increase in CPK activity during the first 48 to 72 hours following serum withdrawal (Figure 9). This "lag phase" was observed in all three experiments which examined CPK induction in differentiating encapsulated myoblasts and may be explained, at least in part, by the combined effects of serum withdrawal in

conjunction with the abnormal capsule environment itself (which, as discussed previously, may be less than ideal for the progression of myogenic differentiation at rates typically observed with naked myoblast samples). In order to rule out the possibility that the pre-treatment with bFGF (during the growth period in regular serum-rich media) was not exerting any lasting effects upon the encapsulated myoblasts, thereby resulting in a prolonged inhibition of differentiation, an experiment was carried out which compared CPK levels in capsules exposed to bFGF and capsules not exposed to bFGF (Figure 10). The results demonstrated that no significant difference in CPK levels existed between the two groups, therefore eliminating any causal relationship between the exogenously supplied bFGF and the lag in the onset of CPK induction. Thus, it would seem appropriate and necessary to improve the cell viability upon encapsulation, as well as to ensure that the myoblasts have retained their differentiation potential throughout the experiment (which could be accomplished by harvesting encapsulated cell samples at various time points during the experiment and seeding them onto tissue culture dishes to monitor their proliferation and differentiation under normal culture conditions). Although the level of CPK activity provides a good measure of the extent to which a cell population has begun to commit to terminal differentiation, it must be recognized that this system of measurement merely provides a narrow picture of what is truly occurring on a molecular level with respect to the complex regulation inherent in myogenic differentiation.

4.4 RNA Transcript Levels in Differentiating Myoblasts

In order to examine the differentiation of the pNMG3-pool myoblasts on a molecular level, levels of muscle-specific gene expression were studied over a five-day period of differentiation (Figure 12c). All RNA transcript levels were expressed in "volume" or "signal intensity" units relative to the corresponding level of PGK1 transcript. It was observed that myogenin, TnIslow, LHC3, LC2 and TnT gene expression was significantly up-regulated upon exposure to differentiation media, the onset of which began within the first 24 hours following mitogen withdrawal. MyoD1 gene expression was present in proliferating myoblasts (i.e. "day 0" samples) and was only slightly up-regulated upon differentiation in comparison to the other muscle-specific genes mentioned. This relatively constant level of MyoD1 expression in differentiating C2C12 myoblasts has also been reported in the literature, where the amount of MyoD1 mRNA stayed constant or decreased upon exposure to differentiation media for 3 days (Miller, 1990). This same study reported large increases in the amounts of myogenin mRNA after 3 days of differentiation, which corresponds to the dramatic increase in myogenin transcript levels observed in this study by day 4 of differentiation.

The levels of LC2 and LHC3 gene expression also correspond to what has been observed in other studies. It was shown that differentiating quail myoblasts demonstrated a dramatic increase in LC2 mRNA by day 2 of differentiation, while the levels of LHC3 mRNA increased at a much slower rate upon the onset of differentiation (Devlin and Emerson, 1979). In this study, pNMG3-pool cells demonstrated a sharp increase in LC2

mRNA which also began to plateau at about day 2 of differentiation, while the LHC3 mRNA levels increased much more slowly and at lower levels of expression overall.

Studies of differentiating quail myoblasts demonstrated the coordinate expression of TnI and TnT genes as evidenced by the similar levels of TnI and TnT mRNA accumulation observed over a period of approximately 3 days of differentiation (Bucher et al., 1988). This trend is also reflected in this study which noted similar levels of TnT and TnI mRNA throughout the five days of differentiation.

The levels of hGH mRNA fluctuated over the five days in differentiation media. It is not known whether this variation is accurate (the levels may be somewhat suspect due to the non-specific binding of the hGH probe to the untransfected C2C12 cell samples) or whether the fluctuations are an artefact of some kind. It is interesting to note, however, that increases in the levels of hGH mRNA seem to loosely reflect the increases in hGH secretion rates observed throughout the first five days of differentiation (Figure 1).

The encapsulated myoblasts (Experiment #2), which were passaged to a greater extent than the naked myoblasts, did not demonstrate any significant up-regulation of MyoD1 or myogenin throughout 14 days of growth in differentiation media (Figure 14b). The overall levels of MyoD1 and myogenin are, in fact, significantly lower than what is seen in the naked differentiating myoblasts from the same blot (Figure 14a) when one compares the levels to the PGK1 reference point on each graph (Figures 14a and 14b). These comparisons must be accepted with some trepidation due to the fact that the encapsulated myoblast samples were not done in triplicate. It would appear that the encapsulated myoblasts were not committing to terminal differentiation on a molecular

level, at least in this case (Experiment #2). This assumption is supported by the fact that there was essentially no increase in CPK activity observed throughout the 14-day period either (Figure 7). It is evident, however, that the encapsulated cells are capable of muscle-specific gene up-regulation, typical of differentiating myoblasts, due to the large increase in CPK activity observed in differentiating pNMG3-pool cells in Experiment #1a (which showed a 63-fold increase in CPK activity).

Regardless, the differentiation program outlined in this study, with respect to the induction of the various muscle-specific genes in naked differentiating C2C12 cells, appears to approximate the trends observed in the literature and can be used as a template for further studies examining the molecular differentiation of C2C12 cells.

4.5 Improving the Capsule Environment

It is clear that the microcapsule environment needs to be greatly improved in order to optimize the proliferation and differentiation of the myoblasts within the capsule space. Previous studies utilizing encapsulated C2C12 myoblasts have encountered similar problems to those demonstrated in this study, namely, decreasing cell viability and retarded cell growth (Hughes et al., 1994; Al-Hendy et al., 1995; Hortelano et al., 1996). Although this study did not show any evidence of myoblast fusion within the capsule environment, a previous study has reported the presence of differentiated myotubes in alginate capsules retrieved from mice approximately 6 months following implantation (Al-Hendy et al., 1995). This finding suggests that the encapsulated myoblasts are in an environment which cultivates myogenic differentiation when placed *in vivo*, and that one

or more factors involved in terminal differentiation are lacking in the *in vitro* surroundings.

Upon consideration of the differentiation-promoting elements which are lacking in the *in vitro* system, two basic solutions are apparent: (i) to provide an exogenous supply of one or more soluble growth factors to enhance proliferation and differentiation; (ii) to create some type of extracellular matrix within the capsule space to allow for myoblast attachment / adhesion. The effects of specific growth factors on myogenic proliferation and differentiation have been well documented, a few of which include insulin-like growth factors (IGFs) which stimulate differentiation (Allen and Boxhorn, 1989; Florini et al., 1991), basic fibroblast growth factor (bFGF) which is mitogenic and differentiation-inhibiting (Florini et al., 1991; Rao and Kohtz, 1995), transforming growth factor beta (TGF-β) which inhibits differentiation (Allen and Boxhorn, 1989; Florini et al., 1991; Rao and Kohtz, 1995) and epidermal growth factor (EGF) which inhibits differentiation on its own and acts synergistically with FGF to promote myoblast proliferation (Kelvin et al., 1989). It has been demonstrated that bFGF, IGF-1 and TGF-β, in combination, have successfully improved rat primary myoblast proliferation (Allen and Boxhorn, 1989) and have also stimulated a 250% increase in proliferation in encapsulated C2C12 myoblasts expressing human factor IX, while simultaneously inhibiting myogenic differentiation (Hortelano et al., unpublished results). Aprotinin, a serine protease inhibitor, has also been shown to positively influence C2C12 myoblast differentiation by inhibiting proteolytic activation of latent growth factors, such as TGF-β (Wells and Strickland, 1994). A final example of the effects of exogenous growth factors on the proliferation and fusion of myogenic cells involves the coculture of activated

macrophages with primary rat myoblasts (Cantini and Carraro, 1995). Although the nature of the mitogenic element(s) present in the vast array of growth factors, cytokines and enzymes secreted by macrophages is not yet known, it is clear that the macrophages produce one or more soluble factors which greatly stimulate the growth of primary myoblasts and increases the myotube yield *in vitro*.

To address the need for some type of extracellular matrix within the capsule space, there exist several studies which outline the use of a variety of compounds that appear to provide a growth-stimulatory and/or differentiation-promoting surface for myogenic cell lines to adhere to. The positive effect of collagen on the development of muscle clones was first identified in 1966 (Hauschka and Konigsberg) and collagen has since been employed as a matrix for myoblast seeding, in which the seeded cells demonstrated reasonable cell survival, cell spreading and differentiation capabilities (van Wachem et al., 1996). Recent studies using collagen as a matrix for encapsulated C2C12 myoblasts have demonstrated a significant increase in cell proliferation over time (~1000% more than controls) when used in addition to media supplemented with bFGF, TGF-β and IGF-1 (G. Hortelano, personal communication). Other macromolecules which support myoblast adhesion, spreading and/or differentiation include laminin, a major glycoprotein of basement membranes (Kostrominova and Tanzer, 1995), Thrombospondin-1, an extracellular matrix glycoprotein (Adams and Lawler, 1994) and perlecan, a large basement membrane heparan sulfate proteoglycan (Aviezer et al., 1994). A combination of two or more of these extracellular molecules has also been effective in promoting the attachment and differentiation of anchorage dependent cell types, including

myoblasts. One example is Matrigel, which is solubilized basement membrane containing laminin, collagen IV, heparan sulfate proteoglycans and entactin (Kleinman et al., 1982). A similar product offered by Upstate Biotechnology Incorporated (Lake Placid, New York) is termed "E-C-L Cell Attachment Matrix", which is composed of entactin, collagen IV and laminin.

It is, therefore, evident that a variety of growth factors and extracellular matrices exist which can play a role in the improvement of encapsulated cell proliferation and differentiation. As mentioned previously, collagen used in conjunction with a growth factor cocktail (i.e. bFGF, TGF-β and IGF-1) successfully promoted increased myoblast proliferation within alginate capsules, however, the possibility that other combinations of growth factors and matrices would be as effective, if not more so, remains to be seen.

4.6 Conclusions and Future Considerations

The use of encapsulated myoblasts as universal gene delivery vehicles has been documented (Al-Hendy et al., 1995; Hortelano et al., 1996) and the stability of the capsules, accompanied by the sustained cell viability *in vivo* for a period of up to two years in mice, holds great promise in using this method of gene therapy for a wide variety of diseases. The prospect of utilizing the microcapsule space to its full capacity, thereby delivering higher levels of recombinant gene products *in vivo*, remains a challenge. The solution lies not only in the construction of better plasmid vectors in order to increase gene expression, but also in creating a microcapsule which is optimized to promote myoblast growth and differentiation. The alginate-polylysine microcapsule, in and of itself, is not seemingly ideal for the growth and differentiation of myoblasts, as

demonstrated in this study by the decrease in cell viability, the lag in CPK induction (signalling a delay in the onset of molecular differentiation) and the lack of cell fusion. It is evident, however, that the encapsulated myoblasts do possess the ability to differentiate on a molecular level as evidenced by the dramatic increases in CPK activity observed in preliminary experiments (Experiment #1a). The microcapsule environment is easily manipulated and can be altered to suit the containment of myoblasts by adding exogenous growth factors to the growth media which promote myogenic proliferation and differentiation and by providing an extracellular matrix within the capsule space for the purposes of cell attachment. Further studies aimed at examining the differentiation profile of transformed myoblast cell lines and primary myoblasts under improved encapsulation conditions will hopefully produce results which more closely resemble the biochemical and morphological events that take place in cultured cells, which would undoubtedly be indicative of a more effective gene delivery system overall.

APPENDIX A

SOLUTIONS

2M $CaCl_2$

$CaCl_2 6H_2O$	10.8 g
H_2O	to 20 ml
Filter sterilize	

2% CHES stock solution

2-(N-Cyclohexylamino)ethanesulfonic acid (CHES)	10 g
0.9 % NaCl	to 500 ml
pH to 8.2 with NaOH	
Filter sterilize	

10 X Citrate Saline

Potassium chloride	50 g
Sodium citrate	22 g
H_2O	to 500 ml

50X Denhardt's Solution

Ficoll	1 g
Polyvinylpyrrolidone	1 g
Bovine serum albumin	1 g
ddH_2O	up to 100 ml

2X HEPES-Buffered Saline (HEBS)

NaCl	8 g
KCl	0.37 g
$Na_2HPO_4 \cdot 7H_2O$	0.376 g
Dextrose	1 g
Hepes	5 g
ddH_2O	up to 500 ml
Adjust pH to 6.2-7.1 with 1 N NaOH	
Sterilize by filtration	

Luria Broth

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
H_2O	to 1 L
Autoclave	

LB-Ampicillin Plates

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar Bacteriological (Gibco)	15 g
H_2O	to 1 L
Autoclave	
Ampicillin (added at 55°C)	50 μg/ml

5X MOPS Buffer, pH 7.0

Morpholinopropanesulfonic acid (MOPS)	10.464 g
3 M sodium acetate	4.167 ml
0.5 M EDTA (pH 8.0)	2.5 ml
ddH_2O (sterile)	up to 200 ml
Adjust pH to 7.0 with 10N NaOH	
Make up to 250 ml with ddH_2O	
Store at 4°C	

4 % Paraformaldehyde

Paraformaldehyde	20 g
ddH_2O at 60°C	110 ml
Stir for 10 minutes	
Add 1 N NaOH while stirring until solution is cleared	
ddH_2O	up to 125 ml
0.2 M phosphate buffer	up to 250 ml
Adjust pH to 7.3-7.4	
Filter solution	

10X PBS

NaCl	36 g
KCl	0.9 g
$Na_2HPO_4.7H_2O$	5.175 g
KH_2PO_4	0.382 g
Dextrose	4.5 g
ddH_2O	450 ml
Adjust pH to 7.4	

0.1 M Phosphate Buffer

(A) Sodium phosphate monobasic . H_2O	27.6 g
(B) Sodium phosphate dibasic (anhydrous)	28.4 g
Bring each up to 1 L with ddH_2O separately	
Mix 1 part (A) with 4 parts (B)	
Adjust pH to 7.3-7.4	
Dilute buffer with an equal volume of ddH_2O to reach 0.1 M	

0.05 % PLL solution

Poly-L-lysine (PLL) (Sigma; MW of 15-30,000)	2.5 g
0.9 % NaCl	to 500 ml
Filter sterilize	

1.5 % Potassium-Alginate

Potassium alginate (Kelco, San Diego, CA)	3 g
0.9 % NaCl	to 200 ml
Stir slowly overnight	
Filter sterilize	

Prehybridization Buffer

Deionized formamide (Sigma)	5.0 ml
20X SSC	3.0 ml
50X Denhardt's solution	1.0 ml
ddH_2O	0.3 ml
20% SDS	0.5 ml
Sonicated herring sperm DNA (10 mg/ml)	0.1 ml

Reagent A

Sodium carbonate (Na_2CO_3)	20 g
0.1 M NaOH	to 1 L

Reagent B

Cupric sulphate ($CuSO_4 \cdot 5H_2O$)	0.5 g
NaOH	1-2 pellets
1.0 % sodium tartrate	to 100 ml

Reagent C

Reagent A	50 ml
Reagent B	1 ml

0.055 M Sodium Citrate

Sodium citrate dihydrate	8.08 g
0.9 % NaCl	to 500 ml

20 % SDS

Sodium laurylsulfate	20 g
ddH_2O	90 ml
Heat to 68°C to dissolve	
Adjust pH to 7.2 with concentrated HCl	
Make up to 100 ml with ddH_2O	

20X SSC

NaCl	87.66 g
Sodium citrate	44.12 g
ddH_2O	to 500 ml
Adjust pH to 7.0 with glacial acetic acid	
Autoclave	

TFBI

KoAc	0.736 g
$MnCl_2$	2.47 g
KCl	1.86 g
$CaCl_2$	0.367 g
Glycerol	37.5 ml
ddH_2O	up to 250 ml
Filter sterilize	

TBFII

50 mM Na-MOPS (pH 7.0)	20 ml
$CaCl_2$	1.1 g
KCl	0.0745 g
Glycerol	15 ml
ddH_2O	up to 100 ml
Filter sterilize	

TYM Broth

Bactotryptone	20 g
Yeast extract	5 g
NaCl	5.84 g
$MgSO_4$	2.46 g
ddH_2O	up to 1 L
Autoclave	

1X TE buffer (pH 8.0)

1 M Tris Cl (pH 8.0)	1 ml
0.5 M EDTA (pH 8.0)	0.2 ml
H_2O	to 100 ml

TENS

10 N NaOH	200 ul
20 % SDS	0.5 ml
TE	up to 20 ml

0.125 % Trypsin

10X citrate saline	10 ml
Trypsin (Sigma)	0.125 g
H_2O	to 100 ml
Filter sterilize	

APPENDIX B

Cell Trypsinization

Cells were trypsinized in order to harvest the cells or to split and re-seed the cells. The media was removed from the dishes/flasks containing the cells to be trypsinized and the cells were washed with 5-10 mls of 1X PBS. The PBS was aspirated, after which approximately 0.5 mls of a 0.125% trypsin solution was added to the dish and swirled over the cells. Following a 2 to 5 minute incubation period, the trypsinization reaction was stopped by the addition of 10 mls of growth media. The cells were then resuspended in the media and harvested or seeded appropriately.

APPENDIX C

Probe Labelling

All probes were labelled via the use of the T7 QuickPrime kit (Pharmacia, Uppsala, Sweden; catalogue # 27-9252-01) according to kit instructions. The double-stranded insert DNA to be labelled (25 ng in a volume of 34 ul of TE) was denatured by boiling at 95-100°C for 3 minutes, after which the following were added: 10 µl of Reagent mix (Pharmacia), 5 µl (50 µCi) of [α-^{32}P]dCTP (3,000 Ci/mmol) (Amersham), and 1 µl of T7 DNA Polymerase (Pharmacia). The resulting mixture was incubated at 37°C for approximately 15-20 minutes in order to allow the "oligolabelling" reaction to proceed. The labelled DNA was then spun through a sephadex G-50 column (see Preparation of Sephadex G-50 Column for details) in order to remove any unincorporated nucleotides. The "clean" probe preparation was then denatured once again, immediately cooled on ice, and added to 2 mls of prehybridization buffer to allow for hybridization to the RNA blots overnight.

Preparation of Sephadex G-50 Column

Probe purification columns were assembled by plugging a 1cc syringe (Becton Dickinson) with a wisp of glass wool, and filling the barrel of the syringe with sephadex G-50 (Pharmacia) slurry. The syringes were placed into conical 15ml tubes (Sarstedt) containing an eppendorf tube at the bottom for fluid collection, and were spun at 1500 rpm for 2.5 minutes. The syringes were filled with G-50 slurry a second time and spun as previously, such that the G-50 beads formed a packed column (0.9 ml in volume). The

column was subsequently rinsed three times with a solution of TE (10 ml) and 5 M NaCl (0.2 ml) via centrifugation as before. 50 µl of the TE / NaCl buffer was run through the column by centrifugation prior to the addition of the labelled probe (50 µl), which was collected into a clean 1.5 ml eppendorf tube.

APPENDIX D

Plasmid Vectors Containing Insert DNA for Use as Probes:

(1) **pLC2-18** -contains sequences homologous to **rat fast muscle myosin light chain 2 (LC2)** RNA, (Garfinkel et al., 1982).
- found in embryonic / adult muscle.
- insert represents approx. 50% of the mRNA sequence.
- insert size: approx. 500 bp.
- 500 bp insert is released upon digestion with Pst1.
- contains sequence coding for Tetracyline resistance.

(2) **pLC84** - contains sequences homologous to **rat fast muscle myosin light chain 1 or 3 (LHC3)**, (Garfinkel et al., 1982).
- adult-muscle specific.
- insert represents 72% of light chain 1 or 92% of light chain 3 mRNA sequence.
- insert size: approx. 900 bp.
- 900 bp insert is released upon digestion with Pst1.
- contains sequence coding for Tetracycline resistance.

(3) **pTNT-15** - contains sequences homologous to **fast muscle troponin T (TnT)**, (Garfinkel et al., 1982).
- found in embryonic / adult muscle.
- insert represents approx. 80% of the mRNA sequence.
- insert size: approx. 1000 bp.
- 1000 bp insert is released upon digestion with Pst1.
- contains sequence coding for Tetracycline resistance.

(4) **cR165R** - cDNA clone homologous to **rat troponin I, slow skeletal muscle isoform (TnIslow)**, (inserted into pEMBL18+), (Koppe et al., 1989).
- size of mRNA: approx. 1200 bp.
- insert size: approx. 700 bp.
- 700 bp insert is released upon digestion with EcoR1.
- contains sequence coding for ampicillin resistance.

(5) **pEMC11s** - cDNA encoding **mouse MyoD1**, (Davis et al., 1987).
- regulatory gene involved in the induction of skeletal muscle differentiation.
- expressed in embryonic and adult muscle.
- size of mRNA: approx. 1700 bp.
- insert size: approx. 1.8 Kb.
- 1.8 Kb insert released from vector upon digestion with EcoR1.
- contains sequence coding for ampicillin resistance.

(6) **pEMSV-myogenin**
- cDNA encoding **mouse myogenin**, (Wright et al., 1989).
- regulatory gene involved in skeletal muscle differentiation.
- expressed in embryonic and adult muscle.
- size of mRNA: approx. 1500 bp.
- insert size: approx. 1.6 Kb.
- 1.6 Kb insert released from vector upon digestion with EcoR1.
- contains sequence coding for ampicillin resistance.

(7) **pCA-*pgk*3'**
-cDNA encoding **mouse *pgk*-1**, (Adra et al., 1987).
-phosphoglycerate kinase (*pgk*) is a constitutively expressed gene.
-3' UTR of the *pgk*-1 gene cloned into pSP64 parent vector.
-700 bp insert released from vector upon digestion with Pst1.
-contains sequence coding for ampicillin resistance.

(8) **pNMG3**
-cDNA encoding **human growth hormone (hGH)**, (Chang et al., 1990).
-hGH gene is placed under the control of the mouse metallothionein (mMT) promoter.
-1.3 Kb insert released upon digestion with EcoR1.
-contains sequence coding for neomycin (G418) resistance.

APPENDIX E

Cytocentrifugation of Cells onto Slides

Cells were firstly harvested from the microcapsules by exposure to sodium citrate and passage through a 26 guage needle (see Methods section for specific details), after which the cells were spun down at 1500 rpm for 10 minutes. The washed cell pellet was then resuspended in 50-100 μl of 1X PBS and a small volume (approximately 20 μl) was used to perform a cell count via the use of a haemocytometer. The cell suspension was kept on ice at all times prior to cytocentrifugation. Plain, coated glass microscope slides (Corning Glass Works, N.Y.) were placed into a steel holder, along with a plastic funnel, the latter of which was necessary to direct the cell sample onto a small region of the coated slide. Approximately 70 μl of 1X PBS was added to the funnel to premoisten the slide, accompanied by a 3 minute spin. The cell sample, in a total volume of 100 μl, was then added to the funnel and the sample was centrifuged for 5 minutes. The slides were then removed and allowed to dry for approximately 5 minutes prior to the subsequent cell fixing procedure.

APPENDIX F

Microencapsulation: Order of Wash Solutions:

Once the alginate / cell mixture was extruded into a 1.1% $CaCl_2$ (w/v) bath, thereby crosslinking the alginate, the resulting capsules were subjected to the following solutions (note: the capsules were allowed to settle in between each wash, after which the supernatant was removed):

(1) 30 ml of cold 0.55% (w/v) $CaCl_2$ in 0.85% NaCl

(2) 30 ml of cold 0.28% (w/v) $CaCl_2$ in 0.85% NaCl

(3) 30 ml of cold 0.1% (w/v) CHES (5 ml of 2% CHES stock solution in 95 ml of 1.1% $CaCl_2$) (gentle mixing for 1-2 minutes)

(4) 30 ml of cold 1.1% (w/v) $CaCl_2$

(5) 30 ml of cold 0.05% (w/v) poly-L-lysine (MW 15,000-30,000) in 0.85% (w/v) NaCl (gentle mixing for 4 minutes)

(6) 30 ml of cold 0.1% (w/v) CHES

(7) 30 ml of cold 1.1% (w/v) $CaCl_2$

(8) 30 ml of cold 0.85% (w/v) NaCl

(9) 30 ml of cold 0.03% (w/v) sodium alginate in 0.85% (w/v) NaCl (gentle mixing for 3 minutes)

(10) 30 ml of cold 0.85% (w/v) NaCl

(11) 30 ml of cold 55 mM sodium citrate in 0.425% (w/v) NaCl (gentle mixing for 4 minutes)

(12) 30 ml of cold serum-free Dulbecco's Minimal Essential Media (D-MEM)

(13) repeat step 12

(14) capsules maintained in growth media (D-MEM supplemented with 10% FBS) (approximately 10 mls of media per 2 mls of capsules)

APPENDIX G

C2C12 myoblasts were transfected with the pNMG3 vector, which contains the human growth hormone (hGH) gene under the control of the mouse metallothionein (mMT) promoter, as well as the gene for neomycin (G418) resistance. Transfected cells were grown in G418 selection media, the resistant clones pooled together, and thereby designated "pNMG3-pool". The rate of hGH secretion from the pNMG3-pool cells was analyzed in triplicate, as shown in Figure A (42.8 ng hGH/hour/10^6 cells), Figure B (55.6 ng hGH/hour/10^6 cells), and Figure C (53 ng hGH/hour/10^6 cells). The rates of hGH secretion were determined for each data set by calculating the slope of the best fit line. An average rate of secretion was calculated to be 50.5 ± 6.8 ng hGH/hour/10^6 cells.

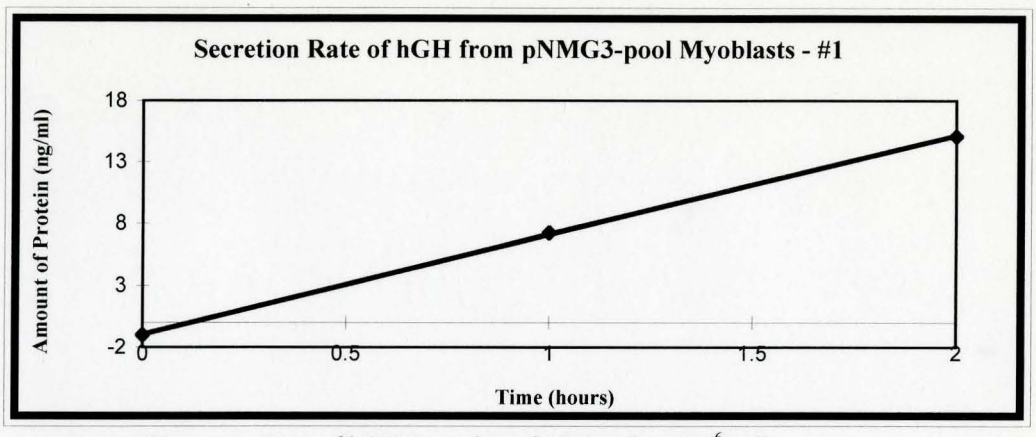


Figure A. Rate of hGH Secretion of 42.8 ng/hour/10^6 cells

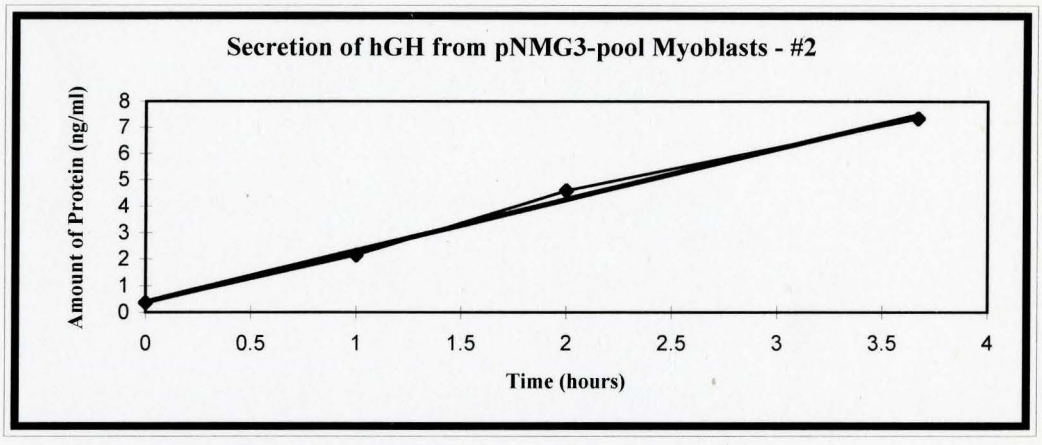


Figure B. Rate of hGH Secretion of 55.6 ng/hour/10^6 cells

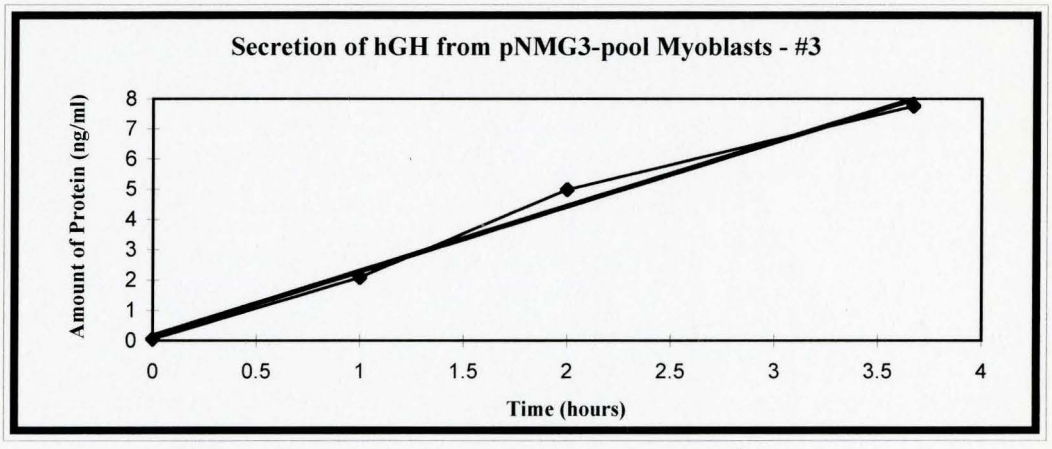


Figure C. Rate of hGH Secretion of 53.0 ng/hour/10^6 cells

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