Genetic and biochemical characterization of differentiation in a rat myoblast model system using somatic cell hybridization.

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University of Windsor

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GENETIC AND BIOCHEMICAL CHARACTERIZATION
OF DIFFERENTIATION IN A RAT MYOBLAST
MODEL SYSTEM USING SOMATIC CELL
HYBRIDIZATION

by

BRIAN WAYNE LEMIRE

A Thesis
submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the requirements
For the Degree of
Master of Science

Department of Biology
Windsor, Ontario

September, 1980
to my parents
ABSTRACT

Somatic cell hybridization has been extensively used in recent years to probe into the nature of the mechanisms controlling cyto-differentiation. Differentiation of skeletal myoblasts (myogenesis) provides a model system in which phenomena related to cell-cell interactions, membrane fusion and regulation of protein synthesis in eucaryotic systems can be studied. We have used somatic cell hybridization techniques to gain insight into the genetic and biochemical mechanisms involved in myogenesis.

Hybrids obtained from rat myoblast X mouse fibroblast and rat myoblast X chinese hamster ovary fibroblast crosses — all established cell lines — did not express muscle-specific characteristics. Specifically, they were unable to differentiate morphologically into myotubes, and biochemical differentiation, which includes an increased synthesis of muscle-specific creatine phosphokinase, myosin and acetylcholine receptors was also suppressed. The mechanism of this apparent suppression was studied in established rat myoblast X primary human fibroblast hybrids in which human chromosomes are preferentially lost (i.e. original myoblast genome regenerated). Initially, morphological and biochemical functions specific to muscle differentiation were suppressed. With time in culture, however, these hybrids non-coordinately re-expressed one or more muscle-specific differentiated function(s). In all cases re-expression was associated with a concomitant loss of chromosomes.
These results provide new and potentially significant information supporting non-coordinate regulation of muscle-specific phenotypes during differentiation and the involvement of a 'repressor-type' mechanism before differentiation (i.e. repression by the non-myoblast or by analogy, the undifferentiated myoblast, genome product(s)).
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INTRODUCTION

The process of differentiation consists of sequential steps leading from the multipotent embryonic cells to the formation of other variously specialized structures. This process is difficult to study at the cellular level because it has been difficult to isolate embryonic blast cells and get them to differentiate in vitro. However cytodifferentiation, one of the simplest among the phenomena sheltered under the term 'differentiation', is amenable to study with the tools of cell biology. The process being investigated in our laboratory is myogenesis — the cytodifferentiation of myoblasts to myotubes.

Descriptively, the process of myogenesis is simple. If avian or mammalian myoblasts of clonal origin (Konigsberg, 1963; Hauschke et al, 1966) are allowed to grow in a suitable culture medium, they proliferate rapidly and after a few days in culture orient themselves into strings and in the presence of calcium, fuse to form multinucleated myotubes. There is no nuclear fusion however (Lash et al, 1957, Cox et al, 1970) and the nuclei in the myotube do not synthesize any new DNA. The morphological differentiation, i.e. fusion, is accompanied by biochemical differentiation which includes an increased synthesis of several muscle-specific proteins such as myosin (Coleman et al, 1968; O'Neil et al, 1972; Paterson et al, 1972), creatine phosphokinase (Shainberg et al, 1971; Morris et al, 1972), acetylcholinesterase (Fluck et al, 1973), acetylcholine receptors.
(Dryden, 1970; Patrick et al, 1972) and glycogen phosphorylase
(DeLa Haba et al, 1968). These events occur both in primary cul-
tures of avian origin (Coleman, 1968) and in established rat
myoblast lines (Shainberg et al, 1971). Figure 1 illustrates the
fusion-related increase in synthesis of a representative muscle-
specific protein, myosin, in a differentiating rat myoblast cell
line, Azg, used in this study.

Although it is generally agreed that fusion and increased syn-
thesis of muscle-specific proteins are temporally related in myo-
genesis, there is considerable debate as to whether an obligatory
coupling exists between the fusion of myoblasts and the initiation of
the synthesis of muscle-specific proteins. Beginning with the obser-
vations of Holtzer (1975), several workers have demonstrated that
myosin synthesis and accumulation does not require myoblast fusion
(Emerson et al, 1975; Chi et al, 1975). Similarly, Turner et al
(1976) have shown that the accumulation of M subunits of creatine
kinase (isoenzyme specific for muscle) and fructose diphosphate aldolase
begins prior to the onset of myoblast fusion. Several other authors
have reported similar findings for creatine phosphokinase (Morris
et al, 1976; Zalin, 1976). These experiments, conducted under con-
ditions which prevented myoblast fusion by removal of calcium from
the culture medium, suggest that fusion is not a prerequisite for
(i.e. coupled with) the synthesis of muscle-specific proteins. Using
similar procedures, however, other investigators were unable to
detect induction of myosin and creatine phosphokinase in the absence
FIGURE 1

Percent fusion and myosin synthesis in Azg rat myoblast line. Details are described in the text. The symbols represent:

(●) percent fusion

(■) increase in myosin synthesis
of fusion (Paterson et al., 1972; Easton et al., 1972; Yaffe et al., 1973). These observations, if correct, would suggest the fusion event to be the trigger of muscle-specific protein synthesis.

The significance of the experiments just described is limited by one important consideration. The removal of calcium from cultured myoblasts significantly reduces the viability of the cells. This observation suggests that cellular processes (e.g., macromolecular synthesis) other than the process of fusion may also be affected. Under these circumstances, observations concerning the coupling of fusion and initiation of muscle-specific protein synthesis are at best speculative but certainly not conclusive.

To minimize the 'variable effect' problem created by removing calcium, investigators examined the question of coupling of morphological and biochemical differentiation using rat myoblast cell line mutants incapable of fusion (Tarikas et al., 1976; Kaufman et al., 1977; Rogers et al., 1978). Once again, however, the results are at variance with each other. In a non-fusing mutant of established L6 rat myoblasts, an increase in synthesis of the muscle-specific proteins adenylate kinase and creatine phosphokinase was observed without concomitant fusion (Tarikas et al., 1974). On the other hand, in a non-fusing mutant of established L8 rat myoblasts an increase in synthesis of the muscle-specific proteins myosin and creatine phosphokinase was not observed (Kaufman et al., 1977). These results are not hard to reconcile with one another, if one assumes
that the muscle-specific proteins are co-ordinately regulated and non-identical regulatory defects in the variants may lead either to a complete expression or a non-expression of all muscle-specific protein-specifying genes. Indeed, it has been demonstrated (Buckingham et al, 1974; Dufresne et al, 1976) that in rat myoblast X mouse fibroblast hybrids, not only fusion, but all muscle-specific protein synthesis is extinguished simultaneously. For obvious reasons, however, the observation in these hybrids cannot be construed as unequivocal evidence for linkage between fusion and protein synthesis, or even for co-ordinate regulation of muscle-specific protein synthesis.

To gain insight into the regulatory mechanisms in myogenesis attention was focused on two general questions:

1) What is the mechanism of apparent suppression of differentiated functions in non-differentiating cell X differentiating myoblast hybrids?

2) How are fusion and the synthesis of muscle-specific proteins regulated during myoblast differentiation?

To answer these questions it was important to experimentally determine:

1) Whether the apparent 'recessiveness' of myoblast differentiated functions (Dufresne et al, 1976) is
peculiar to the particular cross (rat myoblast X mouse L-cell) and/or the hybridization method used (sendai virus induced fusion),

2) Whether the suppression of the differentiated functions in non-differentiating cell X differentiating myoblast cell hybrids is due to a 'repressor-type' mechanism (e.g. repression by non-differentiating cell genome product(s)),

and

3) Whether these functions are repressed as one unit (i.e. one 'repressor') which would suggest co-ordinate regulation of all muscle-specific functions, or individually (i.e. several 'repressors') which would suggest non-co-ordinate regulation of some or all muscle-specific functions.

The approach to 1) was to repeat the original mouse L-cell X rat myoblast hybridization of Dufresne et al (1976) making use of polyethylene glycol (instead of sendai virus) as the fusion agent. In addition, different hybrids were formed from crosses between rat myoblasts and chinese hamster ovary fibroblasts.

To determine points 2) and 3) a hybrid system had to be established from which the non-myoblast chromosomes could be preferentially eliminated and the original myoblast cell chromosome complement regenerated. This was accomplished by hybridizing rat myo-
blasts with senescing human diploid lung fibroblasts.

This thesis describes the hybridization, selection and characterization of three types of cell hybrids (rat X mouse, rat X hamster, rat X human) and their subsequent application to study the dominance relationship and biochemical regulation of myoblast differentiated functions.
MATERIALS AND METHODS

Biochemicals

Tritiated leucine (111.2 Ci/m mole) was purchased from New England Nuclear. [125I]bungarotoxin was a gift from Dr. B.D Sanwal, University of Western Ontario, London, Canada. All chemicals used throughout this study were analytical reagent (A.R.) grade or better. The reader is directed to Appendix A for information on chemicals and suppliers.

Cell Cultures

i) Established Cell Lines (by definition permanent, aneuploid)

The myoblast cell line used throughout this work was an 8-azaguanine (8-Azg) resistant clonal variant of Yaffe's L6 (Yaffe, 1968) termed Azg (Dufresne et al., 1976). This mutant lacked the enzyme hypoxanthine-guanine phosphoribosyltransferase but had the phenotype of L6 with respect to differentiation and synthesis of muscle-specific proteins. This line has a stable modal chromosome number of 64. Mouse L-cells, generally regarded as fibroblasts (i.e. produce collagen) resistant to 5-bromodeoxyuridine (5-BrdU) and lacking thymidine kinase (Dubbs et al., 1964), were obtained from the Ontario Cancer Institute, Toronto, Canada. A clone, BdU, having a stable modal chromosome number of 54 was used throughout. The Chinese hamster ovary fibroblast cell line, FdU, was a generous gift from Dr. Wayne Flintoff, University of Western Ontario, London, Canada. This line is resistant to 5-fluorodeoxyuridine (5-FrdU)
and lacks the enzyme thymidine kinase. In addition, FdU is auxotrophic for proline (pro) and resistant to alpha amanitin (α-ama) and ouabain (oua). It should be pointed out that resistance, in FdU, to α-ama and oua is expressed (i.e. dominant/co-dominant) in hybrids between drug sensitive X drug resistant cells. These dominant/co-dominant phenotypes were therefore used as genetic markers in somatic cell genetic analysis used in this thesis research. A clone, FdU, having a stable modal chromosome number of 19 was selected and used throughout.

The established cells were routinely grown in 25 cm² Falcon culture flasks in 5 mls of regular medium (Modified Eagle’s Minimal (MEM) alpha (α) medium, supplemented with 10% horse serum (hs) and 50 μg/ml gentamycin). The cells were incubated at 37°C in an atmosphere of 10% CO₂ and 90% air. Cell transfers were routinely made every 3-4 days using 0.125% trypsin to dissociate attached cells.

ii) Primary Cell Strain (by definition, senescing, strictly diploid)

A clone of the primary human lung fibroblast cell strain (WI38) was purchased from American Type Culture Collection, Rockville, Maryland, U.S.A. This cell strain has a modal chromosome number of 46. The cells were routinely grown in Eagle’s Basal Medium (BME) supplemented with 10% fetal calf serum (fcs) and 50 μg/ml gentamycin, and transferred as previously described. Under these culture conditions, WI38 cells senesce (i.e. replication ceases) after approximately 50 generations in culture.
Cloning

To isolate homogeneous populations, cells were cloned in 96-well Linbro microtest plates (6 mm well diameter) as follows. Actively growing cells were dissociated from the flasks with trypsin and adjusted to a concentration of 5 cells per ml of regular medium. In each microtest well 0.2 mls of the cell suspension was added (i.e. 1 cell/well) and the plates were incubated at 37°C (10% CO₂, 90% air). After 2 days of incubation the wells were examined microscopically for colonies in wells receiving 1 cell (i.e. clones). When growth of the clones was well established (≥50 cells/collection/well) the cells were washed with citrate saline (0.4%) and dissociated with 0.125% trypsin. After vigorous pipetting the cells were transferred to 25 cm² Falcon tissue culture flasks and allowed to grow. These clones were characterized and used directly in experiments and/or stored as described below.

Cell Storage

Long term storage of cells was a necessary precaution to minimize possible problems resulting from: a) loss of cells in culture due to factors such as senescence, contamination and incubator malfunction; and b) changes in cell-specific growth parameters, phenotype and genotype, which may occur with prolonged maintenance in culture. In general, trypsinized cells were diluted in regular medium and centrifuged at 1,000 rpm for 5 min to remove trypsin. Cells were then concentrated at 2 x 10⁶ cells/ml in 80% MEM alpha (α) + 10% hs + 10% (v/v) dimethylsulfoxide (DMSO) with the exception of WI38 human
l lung fibroblasts which were concentrated in 85% BME (Basal Medium Eagle) + 10% fetal calf serum (fcs) + 5% glycerol. One ml cell samples in 2 ml capacity silicone capped Nunc cryotubes (Cibco Laboratories) were directly frozen at -80°C in a Revco ultra-low freezer. One week later, samples were checked for contamination and viability. Storage under these conditions yields greater than 60% viability over periods exceeding 12 months.

Fusion of Myoblasts to form Multinucleated Myotubes

A quantitative estimation of myoblast fusion, referred to as fusion index (i.e. percent fusion), was obtained using the procedure described by Morris and Cole (1972). Routinely, to measure fusion index, 5 ml of regular medium containing \(10^5\) cells was placed in 60 mm x 15 mm culture dishes. At various times after incubation at 37°C the medium was removed, cells were washed with 0.4% citrate saline and fixed in 2 ml 95% ethanol. After drying, the cells were stained with Geimsa stain for 20 mins, rinsed with tap water, air dried and examined with an inverted microscope equipped with a grid-marked (12.5 X - mm) objective lens. The total number of nuclei and the number of nuclei within myotubes in 32 fields (0.084 mm²/field) in early stages of fusion and in 10-15 fields in later stages were counted. These represented a total of 400-500 nuclei in early stages of fusion and 800-1000 in later stages. A cell was scored as a myotube if it contained 3 or more nuclei within the same cell membrane. Since binucleate cells were not included in myotube counts, estimates give minimal values.
Formation of Somatic Cell Hybrids

1) Crosses involving Established Cell Lines

Cell hybrids were formed by exposing mixed suspensions of $5 \times 10^5$ fibroblast cells (i.e., mouse L-cells (BdU) or Chinese hamster ovary cells (FdU) and $10^4$ myoblast cells (Azg) to decreasing concentrations of polyethylene glycol 1000 in MEM alpha medium (PEG+$\alpha$) according to a modification of a procedure described by Pontecorvo (1975). Cells were mixed in 2 ml regular medium and plated in 6 well (35 x 10 mm) Linbro trays. [Note: intraparent crosses (i.e. Azg x Azg, BdU x BdU, FdU x FdU) were also made to determine the efficiency of the selection system.] The cells were allowed to attach to the plastic overnight. After attachment, each well was washed once with 0.4% citrate saline and 3-5 ml PEG + $\alpha$ 1:1 (v/v) was added to the cells. The monolayer of cells was covered uniformly by gentle rocking and the PEG + $\alpha$ was thoroughly aspirated within 1 minute of its addition. This procedure was repeated 3 more times, first using PEG + $\alpha$ 1:3, then using PEG + $\alpha$ 1:7 and finally using PEG + $\alpha$ 1:15. Control wells for estimation of spontaneous fusion received regular medium instead of PEG + $\alpha$. The wells were subsequently washed 3 times with regular medium and incubated in regular medium at 37°C. The following day, cells were trypsinized and either plated at a density of $10^4$/ml in 100 mm x 20 mm tissue culture dishes, or cloned out in 96-well microtest plates (see Methods: Cloning) containing MEM alpha supplemented with 10% extensively dialyzed horse serum, $1 \times 10^{-4}$ M hypoxanthine, $4 \times 10^{-7}$ M.
aminopterin and $1 \times 10^{-5}$ M thymidine (HAT medium of Littlefield, 1964).

The selective principle employed is that a mixed culture of parental cells and hybrids are grown under experimental conditions which favour the growth of the hybrids, selecting against both parental types. In the selection system, hybrid cells are isolated after fusion of 8-azaguanine resistant cells (lacking the enzyme hypoxanthine guanine phosphoribosyltransferase ($\text{HGPRT}^+$)) with cells that are resistant to 5-bromodeoxyuridine (lacking thymidine kinase ($\text{TK}^-$)). The genetic defects are of little importance during growth in normal tissue culture media, since these enzymes are only involved in salvage pathways for nucleotide synthesis. When the main biosynthetic pathways for purine and pyrimidine nucleotides are blocked by the folic acid analogue aminopterin, normal (in this case hybrid) cells ($\text{HGPRT}^+$, $\text{TK}^+$) can survive if supplied with exogenous hypoxanthine and thymidine. However, the mutant (in this case parental) cells die because of their inability to synthesize nucleotides from hypoxanthine ($\text{HGPRT}^-$.cells), or from thymidine ($\text{TK}^-$ cells). The hybrid cells, containing one parental chromosome set which is $\text{HGPRT}^-$ but $\text{TK}^+$ and the other parental set which is $\text{HGPRT}^+$ but $\text{TK}^-$, are able to produce enough of the $\text{HGPRT}$ and the $\text{TK}$ enzymes to survive in the HAT medium. Thus, when combined in one cell, the two parental genomes complement each other. A simple biochemical representation of the HAT selection is shown in Figure 2.
FIGURE 2

Biosynthetic pathways for nucleotides. On the selective HAT medium the main biosynthetic pathways are blocked by the folic acid analogue aminopterin. Normal cells survive by utilizing hypoxanthine and thymidine. Cells lacking HGPRT or TK enzymes die on the selective medium (Ringertz, N. and Savage R., 1976).
MAIN BIOSYNTHETIC PATHWAYS FOR:

PURINES  PYRIMIDINES

• • • • • • • • • • AMINOPTERIN

SALVAGE PATHWAYS:

HYPOXANTHINE  GUANINE

THIMIDINE  ATP

HGPRT

TK

RIBONUCLEOTIDES  →  RNA

DEOXYNUCLEOTIDES  →  DNA
ii) Crosses involving Primary Human Lung Fibroblasts

Cell hybrids were formed by exposing mixed suspensions of $5 \times 10^5$ senescing human fibroblast cells (WI38) and $10^4$ rat myoblast cells (Azg) to polyethylene glycol 1000 as described previously. Cells were subsequently plated at a density of $10^4$/ml in 100 mm x 20 mm tissue culture dishes containing MEM alpha medium supplemented with extensively dialyzed horse serum, $10^{-4}$ M hypoxanthine, $4 \times 10^{-7}$ M aminopterin and $1.6 \times 10^{-5}$ M thymidine. In addition to the principle of HAT selection, the fact that the human fibroblasts (WI38) senesce in culture was also employed as an aid for selection. Neither of the parents showed significant growth under these conditions. Colonies which grew up were trypsinized, transferred to 25 cm$^2$ tissue culture flasks, or cloned out in 96-well microtest plates as previously described. A summary of the hybridization procedure is presented in Table 1.

Karyotyping

Putative hybrids growing as monolayer cultures were treated with 0.5 ug/ml colcemid for 3 hours at $37^\circ$C to enrich the population for metaphase cells. Cultures were trypsinized, centrifuged and exposed to 0.075 M KCl for 7 min and recentrifuged. The pellets were fixed in acetic acid: methanol (1:3) solution for 20 min at room temperature. Aliquots of the cell suspension were dropped onto cold slides using pasteur pipettes, hot air dried and stained for five minutes in Giemsa stain. At least 10 metaphase spreads were scored
<table>
<thead>
<tr>
<th>Day</th>
<th>Event Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mix: Azg myoblasts (lack HGPRT) with Bdu, Fdu (lack TK), or WI38 fibroblasts (senesce)</td>
</tr>
<tr>
<td>2</td>
<td>Fusion: treatment with Polyethylene glycol</td>
</tr>
<tr>
<td>3-4</td>
<td>Hybrid selection: plating in selective medium (HAT)</td>
</tr>
<tr>
<td>16</td>
<td>Pick possible hybrids: metal-ring-trypsin treatment of colonies showing significant growth</td>
</tr>
<tr>
<td>30</td>
<td>Initial karyotype: colchicine treatment of cells in exponential growth</td>
</tr>
<tr>
<td>35</td>
<td>Cloning</td>
</tr>
<tr>
<td>60</td>
<td>Final karyotype and check of hybrids before characterization</td>
</tr>
<tr>
<td></td>
<td>Check: chromosome number</td>
</tr>
<tr>
<td></td>
<td>Presence of marker chromosome</td>
</tr>
<tr>
<td></td>
<td>Presence of dominant phenotypes</td>
</tr>
<tr>
<td></td>
<td>Effect on hybrid of antisera against parental lines</td>
</tr>
</tbody>
</table>
for chromosome number, for both parental cells and hybrids.

Assay of Dominant Phenotypes

If the origin of chromosomes in hybrid cells cannot be distinguished by the presence of parental 'marker' chromosomes, identification can be made on the basis of dominant phenotype(s). In this research, the presence of Chinese hamster ovary cell (FdU) chromosomes in putative (Azg x FdU) hybrids was determined by the presence of two dominant phenotypes—resistance to α-amanitin and ouabain—as follows.

The ability of Azg, FdU parents and putative hybrids to survive in the presence of increasing concentrations of each drug (i.e. 0-60 ug/ml 5-FdU and 0-3 mg/ml oua) was tested. (The Chinese hamster ovary cells, FdU, were originally selected for resistance to 40 ug/ml 5-FdU and 2.0 mg/ml oua.) At these drug concentrations FdU demonstrates 100% survival. For each drug concentration, cells at two different densities (10^3 and 2 x 10^3 per ml) were plated in MEM alpha medium supplemented with 10% hs and allowed to attach to the surface overnight (37°C). The next day, the cells were washed with citrate saline and the respective drugs were added in MEM alpha supplemented with 10% extensively dialyzed horse serum. The cells were incubated at 37°C for 4-7 days with a change of medium containing the required amount of drug every 2 days. At the end of the incubation period, the plates were stained with methylene blue dissolved in 50% methanol. Colonies consisting of at least 20 cells were scored as surviving colonies (Thompson and Baker, 1973).
Preparation of Antibodies

Antibodies against the myoblast line A2g, the mouse L-cell line B7U, the chinese hamster ovary fibroblast line FdU and the human lung fibroblast WI38 were obtained from Dr. M. Dufresne, Dept. of Biology, University of Windsor. In brief, the antibodies were prepared by injecting rabbits with whole cell suspensions. The immunization program began with one intra-muscular injection of a saline suspension containing 1 ml of $10^7$ washed cells plus 1 ml of Freund complete adjuvant (Difco). This was followed by 4 weekly intravenous injections of whole cells suspended in phosphate buffered saline (PBS). At the 4th week, the rabbits were bled by heart puncture and the serum collected and stored at $-30^\circ$C. Antisera was heated to 56°C for 30 min to inactivate complement.

Cytotoxicity Assay

The antisera was tested for its specificity by a cytotoxicity assay (Oda and Puck, 1961; Puck and Koa, 1971; Wuthier et al, 1973). Using 60 mm tissue culture dishes, 2 x $10^3$ cells were plated with 1% antisera and 3% complement in MEM alpha + 10% heat inactivated horse serum (hihs). The plates were incubated for 9-11 days, stained with methylene blue and examined for surviving colonies.

Growth Parameters

To determine the growth curves for each cell type, cells were plated in regular medium at a concentration of $10^5$ cells/60 mm tissue culture dish. The cells were incubated at $37^\circ$C. Beginning
with the day after plating (day 1), cells were counted in duplicate (i.e. 2 plates for each cell type), twice daily, for 6 days using a hemacytometer.

To determine efficiency of plating (E.O.P.) each cell type was plated, in duplicate, at a concentration of $2 \times 10^2$ and $8 \times 10^2$ cells per 60 mm tissue culture dish in regular medium. The cells were incubated at 37°C for 5 days after which the growing colonies were stained with methylene blue and counted. The number of colonies counted, divided by the number of cells plated, represents the efficiency of plating and is usually expressed as a percentage.

**Biochemical Characterization**

**Assay of Non-Muscle Specific Proteins**

For assaying lactate dehydrogenase (LDH) and myokinase activities, cells were plated at $10^5$ cells per 100 mm tissue culture dish and allowed to grow in regular medium. Starting on day 2 of growth, the cells were assayed to determine enzyme levels and were subsequently assayed days 3, 4, 5, 6 and day 7. Cells were prepared for assaying as follows. Cells, washed twice with cold citrate saline, were scraped off with a rubber policeman into 2 mls citrate saline. The cell suspension was centrifuged and resuspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 0.63 mM pyruvate. The cells were disrupted by repeated vortexing with glass beads and centrifuged to remove cell debris. The supernatant is referred to as enzyme solution. Standard protein was assayed according to the Bio-Rad procedure.
i) Lactate Dehydrogenase

Lactate dehydrogenase was measured according to an established procedure (Bergmeyer, 1974). A 1 ml assay solution for LDH contained: 50 mM potassium phosphate buffer (pH 7.4), 0.63 mM pyruvate, 0.2 mM NADH (Na salt) and 0.02, 0.05 or 0.10 ml of enzyme solution. The change in extinction was measured at 340 nm and activity expressed as nmoles/min/mg protein.

ii) Myokinase

Myokinase was measured by an assay method similar to that described by Shainberg et al, 1971. The reaction mixture, in a final volume of 1 ml, contained: 200 mM imidazole (pH 6.9), 40 mM glucose, 20 mM magnesium acetate (pH 6.9), 100 mM B-mercaptoethanol, 1.6 mM NADP, 2 mM ADP, 0.5 units/ml each of glucose-6-phosphate dehydrogenase and hexokinase, and 0.05 or 0.10 ml of enzyme solution. The change in extinction was measured at 340 nm and activity expressed as nmoles/min/mg protein.

Assay of Muscle-Specific Proteins

i) Creatine Phosphokinase (CPK)

For CPK, cells were prepared for assaying as described for LDH and myokinase. Each assay mixture contained in a final volume of 1 ml: 200 mM imidazole, 40 mM glucose, 20 mM magnesium acetate (pH 6.9), 0.35 M creatine phosphate, 100 mM B-mercaptoethanol, 1.6 mM NADP, 20 mM AMP, 2.0 mM ADP, 0.5 units/ml each of glucose-6-phosphate
dehydrogenase and hexokinase, and 0.05 or 0.10 ml of enzyme solution. The change in extinction was measured at 340 nm and activity expressed as nmoles/min/mg protein. Standard protein was assayed as described previously.

ii) Heavy Chain Myosin

Myosin synthesis was established by a combined antibody precipitation and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis procedure (Paterson and Strohman, 1970). Antibody was made in rabbits to rat skeletal muscle myosin purified according to minor modifications of the method described by Tonomura et al, (1966). To measure synthesis of myosin heavy chains, monolayers of cells at the required stage of growth were washed 3 times with 37°C citrate saline and exposed to 2 ml of leucine-free MEM alpha medium containing 10% dialyzed horse serum and $[^3H]$-leucine at 37°C for 90 min. The cells were washed with cold phosphate buffered saline (PBS), collected by scraping into a low salt buffer: 0.02 M potassium chloride, 0.002 M potassium phosphate (pH 6.8) (Paterson and Strohman, 1972), and homogenized by 50 strokes of a loose fitting glass Dounce homogenizer. Purified myosin (250 ug/ml) was added as a carrier and the homogenate was incubated for 2 hrs on ice. Following centrifugation (10 min at 3000 xg) the pellet was resuspended in low salt buffer and recentrifuged. Total myosin was extracted in a high salt buffer: 0.4 M potassium chloride, 3 mM magnesium chloride, 3 mM ATP, 0.05 M Tris-HCl (pH 7.9), 50% glycerol; and then incubated 90 min on ice. The supernatant was collected and stored at -20°C.
Myosin was precipitated by adding 0.025 ml of high salt extract to 0.06 ml rabbit anti-myosin antisera plus 0.015 ml high salt buffer. After 90 min incubation on ice, the precipitate was centrifuged down and used directly in the preparation of samples for gel electrophoresis.

Five per cent polyacrylamide gels were prepared using 0.025 M Tris glycine (pH 8.8), 0.05% SDS and 10% glycerol. The gels were pre-run at 5 mA/gel for 10 min. The labelled samples were applied to the gel surfaces and electrophoresed at 1 mA/gel for 90–120 min. Gels were stained with Coomassie blue and destained with 7% acetic acid. The portion of the heavy chain myosin band (subunit) was determined and the top 3 cm of the gels were sliced into 1.5 or 3 mm slices. The radio-activity of the heavy myosin band was determined after solubilization of the gel band in 30% hydrogen peroxide.

iii) Acetylcholine Receptors

Acetylcholine receptors were quantitated by the use of α-bungarotoxin. The toxin was labelled with $^{125}\text{I}_2$ using chloramine T (Greenwood et al., 1963). For the assay of receptors, cells were grown in monolayers in 6-well Linbro plates, washed with citrate saline, flooded with 0.5 ml of $^{125}\text{I}_2$-bungarotoxin and left at 4° for 10 min. The cells were again washed with citrate saline at the end of this period, then 0.5 ml of 10% trichloracetic acid (TCA) was added to the cell layer and left for 10 min at room temperature. After removal of TCA, the cells were scraped with a rubber policeman
and the radioactivity associated with the cells was determined in a liquid scintillation counter.
CHAPTER I

Dominance Relationships in Somatic Cell Hybrids

It is generally assumed that all cells of a higher organism contain the same genetic information and that differentiation is based on the selective expression of different parts of the genome. The mechanism(s) controlling this function of 'controlled selective gene expression' has recently been examined by analyzing the expression/suppression of differentiated phenotypes (i.e. dominance relationship studies) using somatic cell hybridization techniques.

In general, hybridization has been accomplished by exposing mononucleate cells (i.e. parental cells), of two different phenotypes and species, to a fusion promoting agent such as inactivated Sendai virus or polyethylene glycol. The rationale behind using these agents is that they increase the frequency of cell-cell fusion from approximately $10^{-7} - 10^{-6}$ (spontaneous fusion) to approximately $10^{-5} - 10^{-4}$. The initial product of fusion is a binucleate heterokaryon. After a short time in culture the heterokaryon, if viable, gives rise to two mononucleate synkaryons commonly referred to in the literature as 'hybrid' cells. These hybrids contain genetic information originating from both parents and therefore possess the potential to express phenotypes characteristic of each parent.

An important experimental consequence of 'genetic combination' via hybridization is that hybrids can be used to study and compare the expression or suppression of phenotypes specific and/or common to each parent cell. Coon and Weis (1969), for example, crossed
differentiated rat liver epithelial cells with mouse fibroblast cells. They found that greater than 95% of the hybrids selected showed fibroblast morphology. In addition, serum proteins normally synthesized by rat liver epithelial cells were not detected in the hybrids. In terms of dominance relationship analysis these results suggest that the differentiated epithelial cell phenotypes examined were suppressed.

In another study, Minna et al. (1972) crossed differentiated neuroblastoma cells with mouse fibroblast cells. The resulting hybrids retained the ability to synthesize electrically active membranes and high levels of neurites and acetylcholinesterase (phenotypes associated with differentiating neuroblastoma cells). In these experiments, contrary to Coon and Weis' experiments, the differentiated cell phenotypes were expressed in the hybrid.

In terms of research, dominance relationship studies in somatic cell hybrids have been particularly useful toward understanding the mechanisms regulating differentiation. For example, Davidson (1972) examined the regulation of one of the phenotypes in melanoma differentiation—melanin synthesis. Hybrids, between syrian hamster melanoma cells and three different lines of mouse fibroblast cells, were selected by the HAT procedure of Littlefield (1963). The resulting hybrids were unpigmented and did not synthesize melanin. Biochemical analysis indicated that the hybrids, like all the fibroblast parent cells, lacked dopa-oxidase. From these results, Davidson suggested that melanin synthesis was suppressed in the hybrids.
and, in fact, might be 'repressed' by some factor(s). To examine the mechanism of suppression further, Davidson tested the specificity of the 'apparent repression'. Specifically he asked if melanin synthesis alone was 'repressed' in the hybrids or if phenotypes representing other parts of the hamster melanoma genome were also 'repressed'. To answer this question, hybrids were tested for the presence of three enzymes determined by the melanoma cell genome but not related to melanization—malate dehydrogenase, lactate dehydrogenase and thymidine kinase. In brief, the results indicated that all three enzymes were present in the hybrids suggesting that the absence of dopa-oxidase activity and pigmentation (i.e. melanin synthesis) in the hybrids was not due to the non-specific 'repression' of the melanoma cell genome. On the basis of these results, Davidson concluded that the regulation of pigmentation involved the production, by the genome of the undifferentiated cells of repressor molecules which specifically 'repressed', in the hybrid cells, the expression of the pigment forming genes of the melanoma cell. By analogy, Davidson speculated that these repressor products 'repressed' the expression of their own pigment forming genes in undifferentiated melanoma cells. It should be kept in mind that Davidson does not present experimental evidence which clearly demonstrates a 'repressor' function for either the undifferentiated melanoma or undifferentiated non-melanoma genomes in the melanoma X fibroblast hybrids. Thus Davidson's conclusions, although plausible, remain hypothetical.
Using a similar approach—dominance relationship studies involving somatic cell hybridization—Dufresne et al. (1976) examined the process of differentiation in another model system, established rat myoblast cells in culture. As previously mentioned, myoblast (muscle) cells differentiate to produce multinucleated myotubes. Hybrids were formed from crosses between differentiating rat myoblasts and mouse fibroblast cells unable to differentiate into myotubes. The principle objective of these investigators was to determine whether all myoblast-specific properties, such as fusion and the synthesis of various muscle-specific proteins, were expressed or suppressed to the same extent in myoblast × fibroblast hybrids. The cell hybrids were formed by exposing mixed populations of myoblasts and fibroblasts to B-propiolactone-inactivated sendai virus. The synthesis of the muscle-specific proteins, CPK and heavy chain myosin (HCM), along with the two non-specific enzymes, LDH and myokinase, were measured in the parental cells, the hybrids and subclones of the hybrids at various initial cell densities and at various times during growth. LDH and myokinase activity in parental cells and hybrids increased with time and subsequently levelled off. Similarly, the levels of CPK and HCM increased in the myoblast parents reaching maximum levels when 60-80% of the myoblasts had differentiated into myotubes. In the hybrids, however, synthesis of these muscle-specific proteins was not detected. These results suggested that the phenotypic expression of all muscle-specific properties examined, was suppressed.
On the basis of these results, Dufresne postulated the involvement of a 'repressor-type' mechanism in the suppression of muscle-specific phenotypes in the myoblast X fibroblast hybrids. However, this hypothesis, like Davidson's conclusions, was not supported with experimental evidence. Chapter II of this thesis describes the use of specialized somatic cell hybridization techniques to obtain this evidence. However, before attempting to determine the actual mechanism of the observed suppression in the rat myoblast-hybrid system, it was first necessary to determine if the results obtained by Dufresne et al. were 'artifacts' of the particular hybrid system used (i.e. rat myoblast X mouse fibroblast) and/or the methodology used for hybrid formation (i.e. Sendai virus induced fusion). To examine this, the original Dufresne rat myoblast X mouse fibroblast hybridization was repeated using polyethylene glycol as the fusing agent. In addition, hybrids were formed between rat myoblasts and Chinese hamster ovary fibroblasts. All hybrids were characterized and examined for the expression of muscle-specific phenotypes by routine dominance relationship analyses previously discussed. These results are presented in the following section.
RESULTS

Somatic Cell Hybridization Between Established Cell Lines

Somatic cell hybridization techniques were used to study the dominance relationship of muscle-specific fusion and synthesis of muscle proteins. Two crosses were made. The first involved a cross between 8-azaguanine resistant rat myoblast cells (Azg) lacking the enzyme hypoxanthine guanine phosphoribosyltransferase capable of differentiating into myotubes, with non-differentiating, 5-bromodeoxyuridine resistant mouse fibroblast (L)-cells (BdU), lacking the enzyme thymidine kinase. The second cross involved differentiating Azg rat myoblasts with non-differentiating fluoro-deoxyuridine resistant chinese hamster ovary cells (FdU) lacking the enzyme thymidine kinase.

Rat Myoblast (Azg) X Mouse Fibroblast (BdU)

Cells were formed by exposing mixed suspensions of $5 \times 10^5$ BdU mouse fibroblast cells and $10^4$ Azg rat myoblast cells to polyethylene glycol as described in Methods. Cells were subsequently plated at a density of $10^4$ cells per ml in 100 mm x 20 mm tissue culture dishes containing MEM alpha medium supplemented with 10% dialyzed horse serum and HAT (previously described). Neither of the parents show any significant growth under these conditions. The medium was changed every two days for ten days. On the tenth day colonies which showed significant growth were picked using
sterilized metal collars and trypsin. The cells were transferred to 25 cm² tissue culture flasks, allowed to grow, and subsequently cloned in the selective medium. The surviving clones were transferred to 25 cm² tissue culture flasks containing regular medium and karyotyped (see Methods). Out of several putative hybrids obtained, 2 clones of independent origin designated RM-1 and RM-2 (Rat X Mouse) were selected for detailed study.

Rat Myoblast (Azg) X Chinese Hamster Ovary Fibroblast (Fdu)

Cell hybrids were formed by exposing mixed suspensions of 5 x 10⁵ Fdu chinese hamster ovary fibroblast cells and 10⁴ Azg rat myoblast cells to polyethylene glycol as described in Methods. As in the case of the previous hybridization, neither parent showed significant growth when plated at a low cell concentration in HAT selection medium. Colonies growing after 10 days were picked, allowed to grow, subsequently cloned in selective medium and karyotyped. Out of several putative hybrids obtained from this cross, 2 clones of independent origin designated RCHO-2 and RCHO-4 (Rat X Chinese Hamster Ovary) were selected for detailed study.

Characterization of Putative RM and RCHO Hybrids

i) Karyotyping

Putative RM and RCHO hybrids, growing as monolayer cultures were treated with 0.5 μg/ml colcemid for 3 hrs at 37°C to enrich the population for metaphase cells. Cultures were trypsinized, exposed to 0.075 M KCl and the resulting cell suspensions were
fixed with methanol: acetic acid and stained for microscopic analysis (See Methods).

Karyotyping reveals that within experimental error, the RM putative hybrids have an average modal chromosome number of 115, approximately the number one would expect from the number of chromosomes carried by each parent (64 and 54 for rat myoblasts and mouse fibroblast parents, respectively). Moreover, the principle marker chromosome of BdU mouse fibroblast, a long metacentric, and of Azg rat myoblast, distinct acrocentric chromosomes (Buckingham et al, 1974) could be identified in the RM putative hybrids.

Similar karyotype analysis reveals that the RCHO putative hybrids also have the number of chromosomes expected on the basis of the modal number of chromosomes carried by each parent (86, 64, 19 for RCHO putative hybrids, rat myoblasts and chinese hamster ovary fibroblasts, respectively). Although the presence of the Azg rat myoblast principle marker chromosomes (i.e. distinct acrocentric chromosomes) was detected in the RCHO putative hybrids, identification of similar marker chromosomes for FdU chinese hamster ovary fibroblasts was not possible without the use of banding techniques. To circumvent this problem, the presence of FdU chinese hamster ovary fibroblast chromosomes in RCHO putative hybrids was established indirectly by testing for resistance to the drugs ouabain and α-amanitin (two dominant phenotypes expressed by the FdU parental
cell line (see Methods). In brief, both the FdU Chinese hamster ovary fibroblast parental cells and the RCHO putative hybrids are totally resistant to concentrations of each drug which reduced the relative survival of the Azg rat myoblast parental cells to less than 0.01%.

ii) Antigenic Properties of Hybrids

Antisera analysis represents one of the most accepted criteria for the existence of 'true' hybrids. To satisfy this criterion we tested RM and RCHO putative hybrids for their sensitivity to antisera made in rabbit against appropriate parental whole cells.

Table 2 summarizes the antigenic properties of RM hybrids 1 and 2. These results indicate that both rat myoblast and mouse fibroblast antisera had to be present together before hybrid cells were killed. Conversely, the addition of either antisera alone had little effect on the viability of the hybrid cells. As expected, the parental cells were killed only in the presence of their respective antisera. For example, rat myoblasts (Azg) were killed by antisera directed against rat myoblasts while antisera directed against mouse fibroblasts (FdU) had no effect on myoblast viability.

Table 3 summarizes the antigenic properties of RCHO hybrids 2 and 4. The results parallel those presented in Table 2. Again it is clear that both rat myoblast and Chinese hamster ovary fibroblast antisera are needed to effect killing.
### TABLE 2

**Cytotoxicity Test**

<table>
<thead>
<tr>
<th></th>
<th>HIHS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NRS&lt;sup&gt;c&lt;/sup&gt;</th>
<th>1% Azg&lt;sup&gt;b&lt;/sup&gt; Antisera</th>
<th>1% Bdu&lt;sup&gt;b&lt;/sup&gt; Antisera</th>
<th>1% Azg and 1% Bdu Antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azg</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bdu</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RM-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RM-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Heat inactivated horse serum (HIHS). Serum was heated at 56°C for 30 minutes.

<sup>b</sup> All wells contained MEM alpha supplemented with 10% HIHS and 3% guinea pig complement plus the indicated antisera at a concentration of 1%.

<sup>c</sup> Normal rabbit serum (NRS) at a concentration of 1% plus 3% guinea pig complement in MEM alpha supplemented with 10% HIHS.

The symbol (+) indicates normal growth whereas (-) indicates no detectable growth.


**TABLE 3**

**Cytotoxicity Test**

<table>
<thead>
<tr>
<th></th>
<th>HIHS(\text{a})</th>
<th>NRS(\text{c})</th>
<th>1% Azg(\text{b}) Antisera</th>
<th>1% PdU(\text{b}) Antisera</th>
<th>1% Azg and 1% PdU Antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azg</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PdU</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RCHO-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RCHO-4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

\(\text{a}\) Heat inactivated horse serum (HIHS). Serum was heated at 56°C for 30 minutes.

\(\text{b}\) All wells contained MEM alpha supplemented with 10% HIHS and 3% guinea pig complement plus the indicated antisera at a concentration of 1%.

\(\text{c}\) Normal rabbit serum (NRS) at a concentration of 1% plus 3% guinea pig complement in MEM alpha supplemented with 10% HIHS.

The symbol (+) indicates normal growth whereas (-) indicates no detectable growth.
Taken together, the cytotoxicity data presented in Tables 2 and 3 indicate the presence of both appropriate membrane components in the hybrids, thereby providing additional proof that the hybrid cells were indeed 'true' hybrids.

Biology of Established Parental and Hybrid Cells

i) Morphology

The appearance of parental and hybrid cells in early and late stages of growth in culture is photographically described in Figure 3. In general, the hybrids have a larger cytoplasm and nucleus than the cells from which they were formed. This is especially notable in the early stages of growth. One of the most apparent and important results demonstrated in Figure 3 is that all hybrids, like all non-myoblast parental cells, are unable to align and form multinucleated myotubes. This characteristic is stable even after prolonged maintenance in culture.

ii) Growth Parameters

It is well established that some or all of the growth parameters (i.e., growth curve, doubling time (T.D.), saturation density (S.D.) and efficiency of plating (E.O.P.)) of one cell line may vary from those of another cell line cultured under the same conditions. We have made use of this 'inherent growth variability' to determine whether hybrid cells reflect the growth parameters of one, both or neither parental cells. Growth curves for each of the parental and hybrid cell lines were determined as previously described (see Methods) and are presented in Figures 4 and 5. Values for the
FIGURE 3.

Morphology of parental and hybrid cells under the light microscope (mag x.200) at:

a) early stages of growth in culture (1–3 days),
b) late stages of growth in culture (5–7 days).
RAT MYOBLAST (AZG)

(a)

(b)
MOUSE FIBROBLAST (BdU)

(a)

Coloured Paper
Papier de Couleur
RAT MYOBLAST X MOUSE FIBROBLAST (RM)

(a)

(b)

Coloured Paper
Papier de Couleur
HAMSTER FIBROBLAST (Fdu)

(a)

(b)

COLOURED PAPER
PAPIER DE COULEUR
RAT MYOBLAST X HAMSTER FIBROBLAST (RCHO)

(a)

(b)

COLOURED PAPER
PAPIER DE COULEUR
FIGURE 4

Growth curves for the parental and hybrid cell lines. Details are described in the text. The symbols represent:

(●) Azg (rat myoblast)
(▲) Bdu (mouse (L-cell) fibroblast)
(■) RM hybrid (rat myoblast X mouse fibroblast)
FIGURE 5

Growth curves for the parental and hybrid cell lines. Details are described in the text. The symbols represent:

(○) Azg (rat myoblast)
(▲) FdU (Chinese hamster ovary fibroblast)
(■) RCHO hybrid (rat myoblast X CHO fibroblast)
corresponding growth parameters (i.e.: T.D., S.D. and E.O.P.) were also determined and are listed in Table 4.

In both rat myoblast X mouse fibroblast (Fig. 4) and rat myoblast X chinese hamster ovary fibroblast (Fig. 5) crosses, the general growth curve of the hybrid cells more closely resembles that of the non-myoblast parent. This observation is confirmed by the values obtained for doubling time and saturation density (Table 4). No significant difference in efficiency of plating values was observed for hybrid and parental cell lines.

Biochemistry of Established Parental and Hybrid Cells

The specific activities of myosin, CPK and acetylcholine receptors (muscle-specific proteins) as well as LDH and myokinase (non-specific enzymes) were measured in the parents and both hybrid types. Values were recorded at various initial cell densities and at various times during growth. Cells were initially plated at a density of $10^5$ cells per ml. The activity of myosin, CPK and acetylcholine receptors increased with time in Azg myoblasts at which time greater than 60% of the myoblasts had differentiated into myotubes. In the hybrids, as with the non-myoblast parents, there was no detectable increase in specific activity of these muscle-specific proteins. The activities of LDH and myokinase (non-specific enzymes), in both parental and hybrid cells, increased up to day 6 of plating after which the levels slightly decreased. Representative data are presented in Tables 5 and 6. In brief, the data presented is consistent with the dominance relationship studies of
### TABLE 4

Growth Parameters of Parental and Hybrid Cells

<table>
<thead>
<tr>
<th>Cell Line&lt;sup&gt;a&lt;/sup&gt;</th>
<th>E.O.P.&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>T.D.&lt;sup&gt;c&lt;/sup&gt; (hrs)</th>
<th>S.D.&lt;sup&gt;d&lt;/sup&gt; (cells/cm²)</th>
<th>Fusion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azg</td>
<td>88</td>
<td>24</td>
<td>$2.8 \times 10^4$</td>
<td>85</td>
</tr>
<tr>
<td>FdU</td>
<td>82</td>
<td>13</td>
<td>$1.0 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>RCHO-2</td>
<td>87</td>
<td>11</td>
<td>$1.4 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>RCHO-4</td>
<td>86</td>
<td>13</td>
<td>$1.0 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>BdU</td>
<td>84</td>
<td>15</td>
<td>$3.5 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>RM-1</td>
<td>86</td>
<td>13</td>
<td>$4.6 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>RM-2</td>
<td>87</td>
<td>13</td>
<td>$4.6 \times 10^4$</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Azg (rat myoblast), BdU (mouse I-cell fibroblast), RM (Azg X BdU), FdU (Chinese hamster ovary fibroblast), RCHO (Azg X FdU).

<sup>b</sup> Efficiency of plating defined as the number of cells that grow up into colonies as compared to the number of cells plated initially.

<sup>c</sup> T.D. (doubling time) defined as the time it takes for an entire culture to double in number.

<sup>d</sup> Saturation density defined as the maximum number of cells/cm² to which a given cell culture is capable of packing.
### TABLE 5

Protein Synthesis in Parental and Hybrid Cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>LDH(^a)</th>
<th>Myokinase(^a)</th>
<th>CPK(^a)</th>
<th>Myosin(^b)</th>
<th>Acetylcholine(^c) Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azg (pre-fusion)</td>
<td>300</td>
<td>43</td>
<td>&lt;1</td>
<td>914</td>
<td>21</td>
</tr>
<tr>
<td>Azg (post-fusion)</td>
<td>561</td>
<td>51</td>
<td>34</td>
<td>20,146</td>
<td>132</td>
</tr>
<tr>
<td>BdU</td>
<td>737</td>
<td>50</td>
<td>&lt;1</td>
<td>970</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RM-1</td>
<td>1218</td>
<td>107</td>
<td>&lt;1</td>
<td>991</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RM-2</td>
<td>1517</td>
<td>108</td>
<td>&lt;1</td>
<td>987</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

\(^a\) Cells were harvested 5 days after plating. The enzymes (creatine phosphokinase CPK, lactate dehydrogenase LDH and myokinase) are expressed as nmoles product formed per min per mg protein.

\(^b\) Expressed as \(^3\)H-counts per min in the heavy subunit of myosin per mg of cell protein.

\(^c\) Milliunits defined in the text.
### TABLE 6

Protein Synthesis in Parental and Hybrid Cells$^a$

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>LDH$^a$</th>
<th>Myokinase$^a$</th>
<th>CPK$^a$</th>
<th>Myosin$^b$</th>
<th>Acetylcholine$^c$ Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azg (pre-fusion)</td>
<td>285</td>
<td>28</td>
<td>&lt;1</td>
<td>914</td>
<td>21</td>
</tr>
<tr>
<td>Azg (post-fusion)</td>
<td>431</td>
<td>45</td>
<td>28</td>
<td>20,146</td>
<td>132</td>
</tr>
<tr>
<td>FdU</td>
<td>505</td>
<td>30</td>
<td>&lt;1</td>
<td>1,057</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RCHO-2</td>
<td>892</td>
<td>63</td>
<td>&lt;1</td>
<td>2,690</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RCHO-4</td>
<td>1488</td>
<td>80</td>
<td>&lt;1</td>
<td>1,067</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

$^a$ Cells were harvested 5 days after plating. The enzymes (creatine phosphokinase CPK, lactate dehydrogenase LDH and myokinase) are expressed as nmoles product formed per min per mg protein.

$^b$ Expressed as $^3$H-counts per min in the heavy subunit of myosin per mg of cell protein.

$^c$ Millimolar defined in the text.
Dufresne et al (1976) and in addition, indicates that the lack of expression of myoblast differentiated functions in hybrids was not peculiar to the rat X mouse crosses and/or the use of inactivated sendai virus to promote fusion.
CHAPTER II

Regulation of Myoblast Differentiated Functions

Somatic cell hybrids may be functionally classified with respect to the ability of the two parental genomes to co-exist (i.e. stability of hybrid karyotype). For the purposes of this thesis research, two classes of hybrids are important: 1) the class that retain both parental genomes indefinitely, and 2) the class that reject the chromosomes of one parent. The first class is useful in dominance relationship studies (Chapter 1) where the integrity of each parental genome is essential to minimize misleading results concerning the expression (i.e. 'dominance') or suppression (i.e. 'recessiveness') of cell-specific phenotypes. The second class provides a convenient (and at present, perhaps the only) approach to study the molecular mechanism(s) underlying dominance relationships and ultimately their regulation in the model somatic cell system under investigation.

The preferential elimination of chromosomes of one parent in interspecific somatic cell hybrids was reported by Weiss and Green in 1967. In their studies human embryonic lung fibroblast X mouse fibroblast hybrids lost human chromosomes. This result was soon confirmed by other investigators (Migeon and Miller, 1968; Nabholz et al, 1969; Ruddle et al, 1970 b) and by 1970 became the underlying principle for linkage analysis and mapping of the human chromosome. It should be pointed out that prior to 1967 such studies were not feasible since techniques for the segregation of genetic
material in mammalian cells were not available.

In most interspecific crosses which had been examined for chromosome segregation patterns before 1971, the parental cells had been aneuploid cells of established lines. When hybrids involving diploid cells were studies, it became apparent that the direction of chromosome loss depended not only on the species, but on the type of parental cell used. Among hybrids between diploid guinea pig peritoneal cells and human HeLa cells, Colter and Parkman (1972) noted that both parental compliments were reduced but with a preferential loss of guinea pig chromosomes. Reverse chromosome segregation was then observed by Minna and Coon (1974) in a large number of independent hybrids produced by fusing normal diploid mouse or rat cells-freshly liberated from heart, liver, bone marrow, spleen, thymus and the central nervous system—with human cells of an aneuploid line. In these studies the rodent chromosomes were preferentially lost in the hybrid cells. This segregation pattern is the opposite of that observed for interspecific crosses of cells from primary human and established rodent lines. In these crosses, the rodent chromosomes are retained while the human chromosomes are eliminated. [Note: chromosome loss is not predictable and occurs more or less at random. As a result attempts have been made to influence the direction of chromosome elimination using 1) $\alpha$ or $\gamma$ irradiation (Pontecorvo, 1971), 2) BrdU treatment (Pontecorvo, 1971) and 3) back selection with drugs. Unfortunately, the success of these attempts is as unpredictable as spontaneous chromosome loss.]
Although many hybrid systems demonstrate chromosome loss, primary human cell X established rodent cell crosses provide the most satisfactory hybrid system to obtain preferential chromosome loss by one parent. Consequently this has been the system of choice to study regulation of 'differentiation' in somatic cells.

In addition to variation in the direction of chromosome loss, differences also occur in the rate of chromosome loss. The rate at which chromosomes are lost may reflect the phylogenetic distance between the two parental cells in an interspecific fusion. With closely related species, such as two different rodents, the rate is usually slow, whereas with cells from different classes (human and rodent), chromosome segregation is very rapid. Within the life history of a hybrid cell, chromosome elimination may be rapid during an early phase. This phase is followed by a period during which the chromosome number is stabilized and chromosomal segregation is slow (Weiss and Green, 1967; Nabholz et al., 1969). In human X mouse hybrid cells studied by Weiss and Green (1967) all but 2 to 15 human chromosomes were eliminated during the first 20 cell generations after fusion. Following this time the human chromosome count stabilized at 1 to 3 human chromosomes. There may, however, be great variations from one clone to another even within the same hybrid line (Ruddle et al., 1970 a). Thus, Miggiano et al. (1969) found one human X mouse hybrid clone which retained a full chromosome complement for 4 months while at the same time another clone lost 50% of its human chromosomes.
These findings have important consequences for understanding the molecular events bringing about macromolecular changes during myoblast differentiation. As discussed in Chapter 1, rat myoblast X mouse L-cell and rat myoblast X Chinese hamster ovary fibroblast cell hybrids have been generated which do not express myoblast differentiated functions. In order to understand the regulation of these functions, it is important to know whether the suppression of the myoblast differentiated functions is: a) due to a repressor-type mechanism, and b) if these functions are repressed as one unit or individually. To examine these possibilities, a hybrid system was established in our laboratory from which the non-myoblast chromosomes were eliminated. These hybrids, the result of a cross between established rat myoblasts and primary human lung fibroblasts, were subsequently characterized and examined weekly for re-expression of fusion and synthesis of three different muscle-specific proteins (CPK, myosin and acetylcholine receptors) over a 26 week culture period.
RESULTS

Somatic Cell Hybridization (Azg Rat Myoblast X WI38 Human Lung Fibroblast)

Somatic cell hybridization techniques were used, as in Chapter 1, to study dominance relationships (i.e. suppression and possible re-expression) of myoblast differentiated functions in primary human lung fibroblast X established rat myoblast cell hybrids. In brief, polyethylene glycol (PEG)-1000 was used to promote fusion of $5 \times 10^5$ senescing human fibroblast cells (WI38) and $10^4$ rat myoblast cells (Azg). Cells were subsequently plated at a density of $10^3$ per ml in 100 mm x 20 mm tissue culture dishes each containing 5 ml of MEM alpha medium supplemented with 10% extensively dialyzed horse serum and HAT (Methods: Hybridization). The WI38 cells senesce after $50 \pm 10$ generations. This primary human cell characteristic, in combination with the inability of Azg to survive in HAT medium, was used effectively to select against both parental cell types. After approximately 10-14 days, 87 colonies of cells showing significant growth ($\geq 50$ cells per colony) were each isolated, dissociated with trypsin and transferred to 25 cm$^2$ tissue culture flasks containing 5 ml of HAT medium. Of the original 87 transferred colonies, 19 survived. To ensure homogeneity of cell population, near confluent cells (in flasks) were cloned (i.e. 1 cell in 19, 96-well microtest plates containing HAT selective medium (Methods: Cloning). Of the 1,824 wells plated, 36 gave rise to
single colonies showing significant growth. These clones, termed 'putative hybrid clones', were transferred to 25 cm² tissue culture flasks containing selective medium, allowed to grow, karyotyped (Methods: Karyotyping), and tested for the presence of Azg and/or WI38 cell surface antigens (Methods: Preparation of Antibodies, Cytotoxicity Assay). Of the 36 putative hybrid clones tested, 24 appeared to contain both Azg and WI38 cell surface antigens, (i.e. putative hybrids sensitive to either Azg or WI38 antiserum with maximum cytotoxic effect in presence of both). Based on these results, the 24 putative hybrids were established as 'true' hybrids.

Out of the 24 Azg X WI38 Hybrid clones obtained, 6 clones of independent origin, designated RH (Rat X Human)-1 through 6 were randomly selected, characterized and used for further analyses. It is relevant to indicate that appropriate control cell crosses were included in the Azg X WI38 hybridizations. These controls served two purposes: 1) to determine the occurrence of spontaneous hybrid formation (i.e. no PEC treatment) and 2) to determine the effectiveness of the selection system. This latter control also serves as an index for possible backmutations. For example, an Azg TK⁻ to Azg TK⁺ reversion would not be selected against in HAT medium.

In the case of 1), 20, 100 mm x 20 mm tissue culture dishes each containing HAT medium were plated with 10³ cells per ml (5 ml per dish of a Azg X WI38 cell suspension originally obtained from a plated mixture of 10⁴ Azg and 5 x 10⁵ WI38 cells. This original
plated mixture was not treated with PEG (i.e. no fusion promoting agent was used). The selective medium was changed at the same time for both fusion 'induced' and fusion 'non-induced' cells. After approximately 14 days only 3 colonies from 2 of the original 20 fusion 'non-induced' dishes showed growth in selective medium. The 3 colonies, consisting of less than 50 cells – all vacuolated – were each picked and transferred to a 25 cm² tissue culture flask containing HAT medium. After 24 hours, the cells lifted from the flask surface and died. In summary, no 'viable' putative hybrids were obtained spontaneously. For the hybridization experiments described in this thesis, viable hybrids were obtained only when polyethylene glycol 1000 was used as a fusion promoting agent.

In the case of 2), – testing the HAT solution system – intraparent hybridization (i.e. Azg X Azg and WI38 X WI38) were routinely unsuccessful. The result remained invariant regardless of PEG treatment, the density at which cells were exposed to PEG and the density at which cells were plated in HAT medium. In short, neither parent – or its heteroploid hybrid, if formed – showed significant growth in HAT medium.

Characterization of Putative Hybrids

i) Karyotyping

Putative hybrids growing as monolayer cultures were treated with 0.5 ug/ml colcemid for 3 hrs at 37°C to enrich the population for metaphase cells. Cultures were trypsinized, exposed to 0.075 M KCl and the resulting cell suspensions were fixed with methanol: acetic
acid and stained for microscopic analysis (See Methods).

Karyotyping reveals that the RH hybrids have an initial modal chromosome number of 75. This number is somewhat lower than the value one would expect from the modal number of chromosomes carried by each parent (64 and 46 for rat myoblast and human lung fibroblast respectively). This is not unusual since it has been reported that in human X rodent cell hybrids all but 2 to 15 human chromosomes are eliminated during the first 20 cell generations after fusion (Weiss and Green, 1967), and at the time of the first karyotyping the 6 hybrid clones had gone through well over 20 generations—a result of the lengthy but necessary selection methods. Based on the selection procedure, the hybrids would have gone through a minimum of 60 and probably up to 100 cell generations by the time of initial karyotyping. However, the fact that the 6 hybrid clones retained some human chromosomes was surmised from the observation that rabbit antisera to both human lung fibroblasts and rat myoblasts was necessary to effect total killing of the hybrids.

Unlike the chromosome numbers in hybrids between established cell lines (i.e., RM and RCHO hybrids) the chromosome number of the RH hybrids is not stable. As seen in Table 7, the modal chromosome number in all 6 RH hybrids decreased with increased time in culture. Comparisons of parental karyotypes with hybrid karyotypes, at increasing generation times, indicate that the hybrids retain their full complement of rat myoblast chromosomes. Banding studies, although in-
TABLE 7

Modal Chromosome Number in Parental and Hybrid Cells

<table>
<thead>
<tr>
<th>Cells/Generation Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Modal Chromosome Number&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cells/Generation Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Modal Chromosome Number&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axg/ 20</td>
<td>64</td>
<td>WI38/ 20</td>
<td>46</td>
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<tr>
<td>40</td>
<td>64</td>
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<td>46</td>
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<tr>
<td>120</td>
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<td>120</td>
<td>46</td>
</tr>
<tr>
<td>RH-1/ 20</td>
<td>76</td>
<td>RH-4/ 20</td>
<td>74</td>
</tr>
<tr>
<td>40</td>
<td>75</td>
<td>40</td>
<td>73</td>
</tr>
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<td>40</td>
<td>73</td>
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</tr>
<tr>
<td>120</td>
<td>74</td>
<td>120</td>
<td>72</td>
</tr>
<tr>
<td>RH-3/ 20</td>
<td>73</td>
<td>RH-6/ 20</td>
<td>78</td>
</tr>
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<td>105</td>
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</tr>
<tr>
<td>120</td>
<td>72</td>
<td>120</td>
<td>72</td>
</tr>
</tbody>
</table>

<sup>a</sup> Generation time after initial karyotyping

<sup>b</sup> Average of at least 10 metaphase spreads counted independently by three investigators.
complete, support the loss of only human chromosomes. (M. Dufresne, personal communication).

Biology of Parental and Hybrid Cells

i) Growth Parameters

Growth curves for each of the parental and RH hybrid cells were determined (Methods: Growth Curves) and are presented in Figure 6. Values for the corresponding growth parameters (i.e. T.D., S.D., and E.O.) were also determined and are listed in Table 8.

With reference to the growth curves, there appears to be little direct correlation between the growth of RH hybrids and either Azg or WI38 parental cells. One noticeable feature is that both parental cell types demonstrate a characteristic lag period in growth during the initial 24-48 hrs of growth after plating. This period is generally termed, in tissue culture studies, the "trypsin recovery period". Cells which grow in suspension, and not on solid surfaces, do not require trypsin for routine subculture procedures. Cells subcultured without trypsin do not exhibit a marked lag phase during early growth. This period is not observed in any of the 6 RH hybrids although trypsin is used to subculture these cells. The presence of the lag phase in the parental cells results in a shift in the exponential section of their growth curves. Eliminating the parental trypsin recovery period by superimposing the exponential portion of all the growth curves, minimizes any difference between hybrid and parental growth in culture.
FIGURE 6.

Growth curves for the parental and hybrid cells. Details are described in the text. The symbols represent:

- (●) Azg. (rat myoblast)
- (▲) WI38. (human fibroblast)
- (■) RH hybrid (rat myoblast X human fibroblast)
### TABLE 8

Growth Parameters of Parental and Hybrid Cells

<table>
<thead>
<tr>
<th>Cell Line&lt;sup&gt;a&lt;/sup&gt;</th>
<th>E.O.P.&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>T.D.&lt;sup&gt;c&lt;/sup&gt; (hrs)</th>
<th>S.D.&lt;sup&gt;d&lt;/sup&gt; (cells/cm²)</th>
<th>Fusion&lt;sup&gt;e&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azg</td>
<td>88</td>
<td>24</td>
<td>$2.8 \times 10^4$</td>
<td>85</td>
</tr>
<tr>
<td>WI38</td>
<td>76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17</td>
<td>$3.6 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>RH-1</td>
<td>84</td>
<td>22</td>
<td>$5.3 \times 10^4$</td>
<td>47</td>
</tr>
<tr>
<td>RH-2</td>
<td>83</td>
<td>17</td>
<td>$4.1 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>RH-3</td>
<td>83</td>
<td>17</td>
<td>$4.4 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>RH-4</td>
<td>87</td>
<td>17</td>
<td>$5.2 \times 10^4$</td>
<td>38</td>
</tr>
<tr>
<td>RH-5</td>
<td>84</td>
<td>22</td>
<td>$3.5 \times 10^4$</td>
<td>80</td>
</tr>
<tr>
<td>RH-6</td>
<td>87</td>
<td>22</td>
<td>$9.8 \times 10^4$</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Azg (rat myoblast), WI38 (human lung fibroblast), RH (Azg X WI38)

<sup>b</sup> Efficiency of plating defined as the number of cells that grow up into colonies as compared to the number of cells plated initially.

<sup>c</sup> T.D. (double time) defined as the time it takes for an entire culture to double in number.

<sup>d</sup> Saturation density defined as the maximum number of cells/cm² to which a given cell culture is capable of packing.

<sup>e</sup> Percent fusion after 120 generations in culture.
The growth parameters again provide a minimum of comparative information. The results indicate that the E.O.P. of the RH hybrids more closely resembles that of the Azg rat myoblast. On the other hand, the T.D. values are equal to, or greater than, the WI38 human fibroblast parent but always less than the Azg rat myoblast parent. Based on these studies, the RH-hybrids possess characteristics of both parental cell types.

ii) Muscle-Specific Myotube Formation

When RH-1 through 6 were first established in culture, none of the hybrids expressed the fusion phenotype representing morphological differentiation in Azg rat myoblasts. The hybrids, morphologically resembled myoblast cells up to day 3 after plating. After day 3 - the exact day depending on the initial plating density and the hybrid under study, the cells reach confluency (i.e. form a uniform monolayer of cells on the growing surface). At this point in cell growth a characteristic deviation from rat myoblast growth pattern to human fibroblast growth pattern occurred. Instead of lining up in strings (the first evidence for myotube formation) the hybrids packed resulting in a 'net-like' cell pattern characteristic of fibroblast cells. (Figure 7, WI38 and RH-2 photographs). This general pattern of growth was similar for all hybrids in the absence of fusion.

To determine whether re-expression of fusion (indicating initial suppression) could be obtained, RH hybrids were subcultured after every 3-4 doublings and examined for subcultures in which cells aligned them-
FIGURE 7

Morphology of the parental and hybrid cells under the light microscope (Mag x 200) at:

a) early stages of growth in culture (1-3 days),
b) late stages of growth culture (5-7 days).
HUMAN LUNG FIBROBLAST (WI38)

(a)

(b)
NON-FUSING (RAT MYOBLAST X HUMAN FIBROBLAST) RH

(a)

(b)
FUSING (RAT MYOBLAST X HUMAN FIBROBLAST) RH

(a)

(b)
selves into strings and subsequently fused. Sixty generations after initial karyotyping, RH-1 began fusing. Thirty generations later RH-4 and 5 began fusing in culture. The pattern of re-expression of myotube formation was similar in all three RH hybrids, 1, 4 and 5. First the cells, upon reaching confluency, lined up and then fused to form myotubes which could not be distinguished from those formed by the Azg myoblast parent (Figure 7, RH-5 photographs; Figure 4, Azg photographs). It should be pointed out that initial re-expression of fusion in the RH hybrids did not result in immediate, maximum % fusion. Furthermore, once reached, the maximum % fusion was not the same for all 3 RH hybrids. The maximum % fusion values – achieved within 10 subcultures of fusion and – were 80%, RH-5; 47%, RH-4; and 38%, RH-1. For comparison, the Azg rat myoblast parent has a fusion index of 85-90%. Re-expression of the muscle-specific fusion phenotype and the level of fusion index for RH-1, 4 and 5 is stable. The fusion property and the fusion index have demonstrated slight, if any, variation for (at least) 200 generations in culture. Although re-expression of fusion in RH-1, 4 and 5 has been the focus of this particular section, the results concerning the remaining hybrids, RH-2, 3 and 6 are ultimately just as significant. The lack of re-expression of fusion in RH-2, 3 and 6 has thus far remained unchanged.

Biochemistry of Parental and Hybrid Cells

Creatine phosphokinase (CPK) activity, myosin synthesis (muscle heavy chain) and acetylcholine receptor presence were determined in the parental cells and the 6, RH hybrid clones of increasing generation
times in culture to examine the re-expression, if any, of these muscle-specific phenotypes (Methods: Assay of Muscle-Specific Proteins). This data, including re-expression of fusion results, is presented in Table 9. The significance of these combined results to the mechanism of suppression of muscle-specific functions in hybrids and ultimately to the regulation of myogenesis in myoblasts is discussed in the following section.
<table>
<thead>
<tr>
<th>Cells</th>
<th>Fusion&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CPK&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Myosin&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Acetylcholine&lt;sup&gt;d&lt;/sup&gt; Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azg(pre-fusion)</td>
<td>-</td>
<td>&lt;1</td>
<td>1327</td>
<td>21</td>
</tr>
<tr>
<td>Azg(post-fusion)</td>
<td>+++</td>
<td>30</td>
<td>19,214</td>
<td>139</td>
</tr>
<tr>
<td>WI38</td>
<td>-</td>
<td>-</td>
<td>801</td>
<td>-</td>
</tr>
<tr>
<td>RH-1/20</td>
<td>-</td>
<td>-</td>
<td>1321</td>
<td>87</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>-</td>
<td>1100</td>
<td>73</td>
</tr>
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<td>60</td>
<td>+</td>
<td>-</td>
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<sup>a</sup> (-) not detectable (+) < 25% (++) 25-50% (+++) ≥ 50%
<sup>b</sup> measured as nmoles/min/mg protein
<sup>c</sup> expressed as wU counts per minute in the heavy subunit of myosin per mg of cell protein
<sup>d</sup> millimolar defined in the text
TABLE 9

1) To test the specificity of the possible repression mechanism (i.e., repression of all functions as opposed to muscle-specific functions) two non-specific enzymes, LDH and myokinase were routinely assayed. Both enzymes, at approximately 2X specific activity at either parent, were detected in parental and hybrid cells at all generation times assayed.

2) Enzymatic reactions were linear with respect to time and the amounts of enzyme solution used.

3) To establish the effectiveness of the reaction mixtures and the enzyme solutions (prepared from cells), various concentrations of commercial CPK, LDH and myokinase were assayed with and without known volumes (protein concentrations) of enzyme solutions prepared from Azg, WI38 and RH hybrid cells.

4) To assay for the presence of acetylcholine receptors, saturating levels of $^{125}$I-bungarotoxin were used. Binding could be specifically inhibited by pretreating cells with unlabelled bungarotoxin.
SUMMARY

CHARACTERISTICS OF SOMATIC CELL HYBRIDS OBTAINED AND STUDIED IN THIS THESIS RESEARCH
**TABLE 10**

Characteristics of RM (Rat Myoblast × Mouse Fibroblast) Hybrids

1. **Culture** - high efficiency of plating
   - grows on solid surface
   - generation time of 13 hours
   - have large nuclei, fibroblastic appearance

2. **Karyotype** - stable, no loss in modal chromosome number
   - detected after 200 generations
   - Azg (myoblast), 64
   - B6U (mouse fibroblast), 54
   - RM-2, 115
   - has parental chromosome markers

3. **Phenotypes Not Specific to Myoblast Differentiation**
   - has common enzymes LDH and myokinase

4. **Phenotypes Specific to Myoblast Differentiation**
   - fusion negative
   - CPK negative
   - myosin negative
   - acetylcholine receptors negative
### TABLE II

**Characteristics of RCHO (Rat Myoblast X Hamster Fibroblast) Hybrids**

1. **Culture**
   - high efficiency of plating
   - grows on solid surface
   - generation time of 11-13 hours
   - have large nuclei, fibroblastic appearance

2. **Karyotype**
   - stable, no loss in modal chromosome number
   - detected after 200 generations
   - Azg (myoblast), 64
   - FdU (Chinese hamster ovary fibroblast) 19
   - RCHO-2, 86
   - has parental chromosome markers

3. **Phenotypes Not Specific to Myoblast Differentiation**
   - has common enzymes LDH and myokinase

4. **Phenotypes Specific to Myoblast Differentiation**
   - fusion negative
   - CPK negative
   - myosin negative
   - acetylcholine receptors negative
TABLE 12

Characteristics of RH (Rat Myoblast X Human Fibroblast) Hybrids

1. **Culture**
   - high efficiency of plating
   - grows on solid surface
   - generation time of 17-22 hours
   - have large nuclei

2. **Karyotype**
   - not stable
   - chromosome number decrease with time (increased generations)

3. **Phenotypes Not Specific to Myoblast Differentiation**
   - has common enzymes LDH and myokinase

4. **Phenotypes Specific to Myoblast Differentiation**
   - clones vary with respect to re-expression of myoblast differentiating properties
   - 3 clones re-express fusion
   - 3 clones re-express increased CPK synthesis
   - 2 clones re-express increased myosin synthesis
   - 3 clones re-express detectable acetylcholine receptors

(see Table 9 for specifics)
DISCUSSION

This thesis describes the combined application of biochemical and somatic cell techniques to study the mechanisms regulating gene expression of differentiated characteristics in cultured rat myoblast cells—a model differentiating system. In a previous related study concerning the molecular basis for myogenesis (Dufresne et al., 1976) it was shown that differentiated characteristics of myoblasts, such as fusion and synthesis of muscle-specific creatine phosphokinase (CPK) and myosin, were not expressed in rat myoblast X mouse L-cell hybrids. On the basis of these results, Dufresne and co-workers postulated suppression of muscle-specific phenotypes in the myoblast X fibroblast hybrids by a 'repressor-type' mechanism. The questions asked in this thesis are based on these considerations. For uniformity and clarity, the results are discussed in terms of the original questions and experimental approaches presented in the introduction.

i) Is the extinction of myoblast differentiated functions observed by Dufresne et al. peculiar to the method used (sendai virus induced fusion) and/or the particular cross made (rat myoblast X mouse L-cell)?

The results presented in Chapter I demonstrate that both components of myogenesis, morphological differentiation (i.e. fusion/myotube formation) and biochemical differentiation (i.e. increased synthesis of muscle-specific proteins) were not expressed in established rat myoblast X established mouse (L-cell) fibroblast (RM) hybrids obtained
using polyethylene glycol as a fusing agent. These findings confirm and extend the earlier work of Dufresne et al., (1976) with similar hybrids obtained using inactivated sendai virus as the fusing agent. In addition, RCHO hybrids obtained from crosses between established rat myoblasts X Chinese hamster ovary fibroblasts, using polyethylene glycol, did not express the myoblast differentiated characteristics studied (i.e. fusion and synthesis of muscle-specific proteins: CPK, myosin and acetylcholine receptors). Combined, these results indicate that the lack of expression (i.e. extinction) of myoblast differentiated functions demonstrated by Dufresne et al is not due to the cell lines used and/or the fusing agent. These results are supported by the successful isolation of spontaneous rat myoblast X mouse fibroblast hybrids (i.e. no fusion-promoting agent used). The spontaneous hybrids are similar to the fusion induced hybrids with respect to extinction of all differentiated characteristics studied. (J. Rogers, unpublished results). In general, the extinction results obtained in the RM and RCHO hybrids are similar to those obtained by other investigators in crosses of differentiated cells with non-differentiating ones (Coon and Weis, 1969; Davidson et al, 1972). In a majority of cases the differentiated functions are extinguished in hybrids, although more cases are being reported where tissue or cell-specific functions are retained (Minna et al, 1972).

It should be noted that in terms of dominance relationship analyses, the loss of these properties in RM and RCHO hybrids would indicate that myoblast differentiation is "recessive" and its events,
such as fusion, are "recessive" traits. Rather than classify the loss of fusion or the loss in production of muscle-specific proteins as "recessive" traits it would be more appropriate to use the term extinction of function. It is very unlikely that the extinction of differentiation in these hybrid cells is the result of gene mutation to which the terms dominant and recessive are applied.

In contrast to the loss of both morphological and biochemical differentiated properties in RM and RCHO hybrids it would appear that the amounts of non muscle-specific enzymes, LDH and myokinase produced by these hybrids is approximately the sum of activity found in the myoblast and fibroblast (i.e. mouse or chinese hamster ovary) parent cell lines individually. Thus 'dominance' of the mouse and chinese hamster ovary fibroblast genotype over that of the myoblasts may extend only to the muscle-specific properties.

ii) Is the lack of expression of the differentiated myoblast functions observed in RM and RCHO hybrids due to a 'repressor-type' mechanism (e.g. repression by non-differentiating cell genome product(s))? The extinction of myoblast differentiated functions in RM and RCHO hybrids, without concomitant extinction of enzymes not specific to myoblast differentiation (Chapter 1) suggests that suppression or inhibition of the myoblast genome occurs. Furthermore, assuming this is correct, it is reasonable to suggest that inhibition of the myoblast genome is due to the production of (a) diffusible component(s) produced by the non-myoblast genome. Regarded in this manner
it is not difficult to postulate the involvement of a 'repressor-type' mechanism of myoblast differentiated characteristics in hybrids.

The concept of gene regulation by 'repressor-type' mechanisms has been applied to differentiating cell systems. As previously indicated, Davidson (1972) postulated that the regulation of differentiated functions in the cells of higher organisms involved the production of diffusible, intracellular regulatory substances. This model was tested by hybridization techniques and used to analyze the regulation of melanin synthesis in hamster melanoma cells. Hybrids between syrian hamster melanoma cells and mouse fibroblast cells were unpigmented and did not synthesize melanin. Davidson concluded that the regulation of pigmentation (melanin synthesis) involved the production of repressor molecules by the genome of the fibroblast cells which specifically 'repressed' the expression of melanin synthesis in the hybrid cells. However, Davidson does not present any conclusive experimental evidence which clearly demonstrates the involvement of a 'repressor-type' mechanism.

Lack of conclusive evidence is representative of research progress dealing with the regulation of differentiated functions in cell culture systems. Although somatic cell hybridization techniques have been used extensively in this research, the analyses of hybrids obtained from crosses involving only established cell lines has provided little significant information. Unfortunately, at least for
these analyses, established cell-line X established cell-line hybrids have a stable karyotype so that the chromosome complement of the non-differentiating cell is retained. As a result, established cell hybrids cannot be used to examine questions dealing with repression, such as: 1) are the non-differentiating cell chromosomes involved in repression of differentiated characteristics in RM and RCHO hybrids; and 2) is repression of fusion and muscle-specific protein synthesis in the hybrids dependent upon the continued presence of certain genes of the undifferentiated cells?

Although RM and RCHO hybrids display a stable karyotype, the phenomenon of re-expression of differentiated functions which were extinguished in hybrids has been shown in hybrids where one parent is of the human cell type (Weiss and Green, 1967; Migeon and Miller, 1968; Nabholz et al., 1969; Ruddle et al, 1970b). These hybrids show a preferential loss of human chromosomes. This characteristic, most prevalent in rodent X human crosses, has been instrumental in proving that lack of expression of differentiated functions is not due to the loss of the structural genes coding for the corresponding proteins. Based on these considerations, the mechanism of loss (suppression) of muscle-specific characteristics in RM and RCHO hybrids was analyzed in rodent X human cell hybrids.

Hybrids were obtained by crossing established rat myoblasts with primary human lung fibroblasts. When first isolated, the RH hybrids—like the RM and RCHO hybrids—do not express any of the myoblast differentiated characteristics studied. (i.e. fusion and synthesis of
muscle-specific CPK, myosin and acetylcholine receptors).

The initial modal chromosome number of these hybrids, 75, is substantially lower than the value one would expect from the modal number of chromosomes carried by each parent (64 for rat myoblast and 46 for diploid primary human lung fibroblasts). However, as explained in Chapter II, there is significant evidence demonstrating that all but 2 to 15 human chromosomes are eliminated during the first 20 generations in culture. In the case of the RH hybrids, all clones must have divided at least 60-100 times in culture prior to the first karyotype. (i.e. during selection). The low, modal chromosome number, therefore, was not a surprise, although it presented a potential problem in terms of the number of hybrid clones to be examined for myoblast differentiated properties. Segregation of human chromosomes is random; therefore, not all hybrid clones can be expected to contain the same complement of human chromosomes. A low number of human chromosomes segregating out in the hybrids theoretically necessitates analysis of a large number of hybrid clones for any given phenotype. For this reason, 36 putative hybrid clones - of which 24 contained both rat myoblast and human fibroblast cell surface antigens — were isolated. Since it was experimentally feasible to analyze a maximum of 6 hybrid clones simultaneously, six hybrid clones were randomly selected and designated RH-1 through 6. Cells from all 36 clones were stored in Liquid Nitrogen. In addition, RH-1 through 6 were subcultured every 3-4 generation times in culture. Some subculture samples were stored in Liquid Nitrogen, while others
were used directly for karyotype, morphological and biochemical analyses.

The possible involvement of a 'repressor-type' mechanism — in the extinction of myoblast differentiated properties — was approached by monitoring each of the 6 RH hybrids with respect to modal chromosome number and re-expression of morphological and/or biochemical differentiation at increasing generation times in culture.

Of the 6 hybrid clones examined, 2 demonstrated re-expression of both fusion and increased muscle-specific protein synthesis. The specifics of this re-expression will be discussed in connection with question iii). It is sufficient for the purpose of question ii) to note that re-expression was accompanied by a concomitant decrease in chromosome number. The significance of this observation ultimately resides in the absolute identification of the lost chromosomes. This has not been accomplished to date; however, the results presented in Chapter II, and preliminary banding analyses currently in progress, suggests that the RH hybrids lose human chromosomes. This is in agreement with previous experimental evidence (Weiss and Green, 1967; Ruddle et al, 1970b) and current consensus concerning the direction of chromosome loss in rodent X human hybrids.

The observed re-expression of myoblast differentiated functions in some of the RH hybrid clones demonstrates that the rat chromosome responsible for one (or more) functions was not lost, however, it also was not expressed (i.e. genotype present however corresponding phenotype not expressed).
These data support a 'repressor-type' mechanism.

Two additional observations support the hypothesis that repression by the undifferentiating parent cell genome occurs in the RH hybrids. First, re-expression of morphological and/or biochemical differentiation in RH hybrids is stable (i.e. once re-expressed, the muscle-specific characteristics remain expressed). Second, re-expression in RH hybrids is accompanied by a concomitant loss in chromosome(s), most likely human in origin. It should be mentioned that additional experimental evidence (not included in this thesis) supporting loss of human chromosomes in RH hybrids has recently been obtained in this laboratory. Preliminary antibody analyses of RH hybrids 1 through 6 at 20 generations (after initial karyotype) and at 120 generations in culture, clearly demonstrate the loss of human cell surface antigens from RH-1 and RH-5 (i.e. hybrids lose their sensitivity to WI38 human cell antiserum). On the other hand, these hybrids remain sensitive to rat myoblast antiserum. This data suggests that the human chromosome(s) specifying cell surface antigens on RH-1 and RH-5 have been preferentially lost at some time between initial karyotype analyses and 120 generations in culture. Conversely, rat myoblast cell surface antigens remain present in RH-1 and RH-5 suggesting the retention of rat myoblast chromosome(s) which determine the myoblast antigens.

It would be shortsighted not to admit that there are several mechanisms by which differentiated characteristics may be suppressed
in differentiating X undifferentiating cell hybrids. Suppression could easily involve the inactivation or alteration of the differentiated component in the hybrid. An altered CPK, for example, could rule out detection of the enzyme by activity alone. Another mechanism could be related to the synthesis of an altered membrane in hybrid cells. Since fusion is primarily a membrane event and an increased synthesis of muscle-specific proteins is, at least temporally, fusion-related in vivo and in vitro, suppression of fusion by an altered membrane could result in extinction of all muscle-specific characteristics. A third kind of mechanism for suppression—and that supported by the results obtained in Chapter II— involves repression. Here, control may be exerted at the transcriptional level where procaryotic type 'repressors' may be produced by the genetic complement of the undifferentiated cell in the hybrids. Strong experimental support for this type of mechanism lies in the recovery of the suppressed component (i.e. re-expression) in RH hybrids which have lost some, but not all, of the chromosomes of the undifferentiated parental cell.

Additional information concerning the source and nature of repression could also be obtained from the established RM and RCHO hybrids (Chapter I). These hybrids display a stable karyotype and should provide a good system for long term in vitro analyses of repression without significant change in genotype. These analyses could include determining the presence or absence of muscle-specific mRNA in hybrids. (If, for example, control is at transcriptional
level, as discussed for the third mechanism of suppression, messenger RNA for myosin should not be present in the RM and RCHO hybrids.)

iii) Are these functions repressed (assuming a repression mechanism) as one unit (i.e. one 'repressor') which would suggest coordinate regulation of all muscle-specific functions, or individually (i.e. several 'repressors') which would suggest non-coordinate regulation of some or all muscle-specific functions?

In addressing this question, the specifics of the re-expression data (Table 9) will be discussed. Since the interpretation of these data is relatively complex, it is appropriate to describe, in general, the basis for correlating the data with the question (i.e. question iii) asked. As previously indicated, one of the properties of hybrids which have human chromosomes is that these chromosomes are gradually eliminated from hybrids with the regeneration, in favourable cases, of the original non-human cell type. This property, introduced in discussing question ii), is fundamental to the analysis of Table 9 re-expression results. Theoretically, if recovery of the fusion property of myoblasts is concurrent with the recovery of synthesis of all muscle-specific proteins in RM hybrids which have lost one or more human chromosomes, then the involvement of a 'one-repressor' mechanism would be supported. Furthermore, this result would suggest the coordinate regulation of both fusion and all muscle-specific proteins. Similar chromosome loss(es) resulting in the recovery of either fusion or all muscle-specific protein synthesis would suggest a 'double-repressor' mechanism and provide evidence that morphological and biochemical differentiation are not coregulated.
If individual proteins appear in partial segregants without the simultaneous appearance of others then a 'multi-repressor' mechanism in which the synthesis of muscle-specific proteins are not coordinately regulated would be supported. Other combinations are possible, however these three adequately demonstrate the application of the data to answer question iii).

a) **Independent Re-expression of Fusion, CPK Activity and Acetylcholine Receptors**

A time course morphological and biochemical analysis of the RH hybrid clones (1 through 6) showed independent re-expression of fusion, CPK activity and acetylcholine receptors.

Specifically, re-expression of CPK activity was observed in RH-3 without re-expression of fusion. Conversely, RH-1, re-expressed fusion without a re-expression of CPK. These results clearly support the hypothesis that fusion and increased synthesis of CPK are not coordinately regulated.

Evidence for the independent re-expression of acetylcholine receptors and fusion is supported by the results obtained with RH-1 and RH-2. In the latter, increased synthesis of acetylcholine receptors is observed without re-expression of fusion. In the former both fusion and the presence of acetylcholine receptors are detected, however, the onset of re-expression is clearly different. In RH-1 fusion is first detected after 60 generations in culture, whereas acetylcholine receptors are present throughout.
Evidence for the independent regulation of fusion and myosin synthesis was not observed. Fusion is re-expressed in the absence of increased myosin synthesis (RH-4), however re-expression of significant increased myosin synthesis was not observed in the absence of fusion (RH-2, 3 and 6). The results, in fact, suggest that the increase in myosin synthesis is related to fusion with respect to the time of onset of detectable re-expression as well as with respect to the extent of re-expression observed (i.e. increased fusion is accompanied by increased myosin detected) (RH-1 and RH-5).

The above combination of results support the following conclusions:

1) With respect to fusion and the muscle-specific proteins, CPK and Acetylcholine receptors, morphological and biochemical differentiation are not coregulated.

2) Increased myosin synthesis appears to be dependent on the fusion process although the reverse does not appear to be the case. [Conclusive evidence for the coregulation (or independent regulation) of fusion and myosin synthesis was not obtained.]

3) Each of the muscle-specific functions—fusion, CPK activity and acetylcholine receptor presence—is independently regulated (i.e. not coregulated).

b) Independent Re-expression of the Muscle-Specific Proteins:

CPK, Myosin and Acetylcholine Receptors

The re-expression of acetylcholine receptors in the absence of increased myosin synthesis is observed in RH-2. Whether or not this indicates independent regulation cannot be determined since neither the reverse re-expression (i.e. increased myosin in absence
of acetylcholine receptors) nor a differential time of onset of re-expression of each was observed.

Evidence for the independent regulation of increased myosin synthesis and CPK is supported by the results obtained with RH-1, 3, 5 and 6. For example, RH-1 shows the re-expression of increased myosin synthesis without re-expression of CPK. On the other hand, RH 3 and 6 show expression of CPK in the absence of increased myosin synthesis. In RH-5, a hybrid re-expressing fusion after 90 generations in culture, both CPK and myosin are re-expressed. However, CPK synthesis is first detected after 60 generations in culture, whereas a significant increase in myosin synthesis occurs between 90 and 105 generations in culture.

The above combination of results, together with those discussed in part a) concerning re-expression of CPK activity and acetylcholine receptor presence, supports the following conclusion:

1. Each of the muscle-specific proteins studied – CPK, myosin and acetylcholine receptors – appear to be independently regulated (i.e. not coregulated).

c) Mechanism of Suppression of Muscle-Specific Fusion and Synthesis of CPK, Myosin and Acetylcholine Receptors

The results presented thus far concerning re-expression of fusion, CPK activity, myosin synthesis and acetylcholine receptors support the involvement of a 'repressor-type' mechanism in the suppression of myoblast differentiated characteristics in differentiating
X undifferentiating cell hybrids. This is supported by the fact that re-expression was accompanied by a loss in human chromosome(s). The exact identification and correlation of specific chromosomes lost with re-expressed myoblast characteristics is the subject of another investigation — the assignment of muscle differentiated characteristics to human chromosomes. For the purposes of this thesis it is necessary and sufficient to indicate that chromosomes were gradually lost in all the RH hybrids and this loss, to some extent, was found to be associated with the re-expression of differentiated functions in hybrids. These results support the involvement of the undifferentiating parental cell chromosomes in the repression mechanism.

Since the majority of the characteristics were re-expressed independently (the exception being increased myosin synthesis and fusion) it would appear that repression does not involve a 'one-repressor' mechanism. It is more likely, on the basis of the results presented in this thesis, that a 'multi-repressor' mechanism involving the undifferentiating parental cell chromosomes is involved in the suppression of myoblast differentiated characteristics in undifferentiating X differentiating cell hybrids. This mechanism is not unlikely since the structural and functional determinants of myogenesis are diverse in vivo. Because of this diversity, it is reasonable to assume that there will be many factors regulating myogenesis rather than one.
Before the importance of the results presented in this thesis can be assessed it is necessary to understand the limitations set by the system studied and the experimental procedures used. There is little doubt that the results are incomplete. Application of extensive banding analyses, examination of more hybrid clones, the use of more refined biochemical techniques, the isolation of repressor molecules, etc., would have been desirable. However, when working in tissue culture, it is necessary to separate the ideal approach from the feasible approach.

Despite the need for more research, the results presented in this thesis should help in understanding the regulatory mechanisms involved in normal myogenesis as well as defining the molecular determinants of these mechanisms.
APPENDIX A

Chemicals and Suppliers

Adenosine - 5' - diphosphate - Boehringer Mannheim
Adenosine - 5' - monophosphate - Boehringer Mannheim

Acetic acid - Fisher
Aminopterin - Sigma
8-Azaguanine - Sigma

Carbowax PEG 1000 - Fisher
Colcemid - Gibco

Coomassie blue - Fisher
Creatine phosphate - Sigma
Creatine phosphokinase - Sigma

Dimethylsulphoxide (DMSO) - Fisher

Gentamicin - Schering Corporation

Giemsa - Fisher
Glucose - Sigma

Glucose - 6 phosphate dehydrogenase - Sigma

Hexokinase - Sigma
Horse serum - Flow Laboratories

Hydrochloric acid - Fisher
Hydrogen peroxide - Fisher

Hypoxanthine - Sigma

Imidazole - Sigma

Magnesium acetate - BDH chemicals
B-Mercaptoethanol - Eastman Kodak

Methanol - Fisher
Methylene blue - Fisher

Modified Eagle’s Medium (alpha) - Gibco

Nicotinamide-adenine dinucleotide, reduced - Sigma

Nicotinamide-adenine dinucleotide phosphate - Sigma

Ouabain - Sigma

Potassium acetate - Sargent Welch

Potassium chloride - Fisher

Potassium phosphate (K₂HPO₄) - Fisher

Potassium phosphate (KH₂PO₄) - Fisher

Pyruvate - Sigma

Sodium acetate - Fisher

Sodium bicarbonate - Fisher

Sodium chloride - Fisher

Sodium citrate - Fisher

Sodium hydroxide - Fisher

Sodium phosphate (Na₂HPO₄) - Fisher

THAM (Tris Hydroxymethyl Aminomethane) - Fisher

Thymidine - Sigma

Trypsin (1:250) - Difco

Addresses

BDH Chemicals - Toronto, Canada

Boehringer Mannheim - Dorval, Quebec

Difco Laboratories - Detroit, Michigan

Eastman Kodak - Rochester, N.Y.

Fisher Scientific Co. - Toronto, Canada

Flow Laboratories - Toronto, Canada

Gibco Laboratories - Grand Island, N.Y.

Sargent Welch Laboratories - Toronto, Canada

Schering Corporation - Grand Island, N.Y.

Sigma Chemical Co. - St. Louis, Missouri
REFERENCES


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SCHOLARSHIPS: ONTARIO GRADUATE SCHOLARSHIP 1978

UNIVERSITY OF WINDSOR SCHOLARSHIP 1979