Multiple Controls for the Synthesis of Muscle-specific Proteins in BC₃H₁ Cells

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ABSTRACT The regulation of the synthesis of muscle-specific proteins has been examined in BC₃H₁ cells, a smooth muscle-like cell line isolated by Schubert et al. (J. Cell Biol., 1974, 61: 398–413). The synthesis of both creatine kinase and the acetylcholine receptor appear to be under dual control, a positive control due to cell-cell contact which increases the rate of synthesis of this protein, and a negative signal, elicited by serum components, that decreases the rate of synthesis of these proteins. Induction of muscle-specific proteins in BC₃H₁ cells is a reversible process and can be arrested after partial induction has taken place by the addition of serum or high-molecular-weight protein fraction from serum to these cells. The high-molecular-weight protein fraction from serum is not by itself mitogenic for BC₃H₁ cells and cannot be replaced by a variety of known hormones (mitogenic factors).

The control of the synthesis of specific proteins during myogenesis has been the subject of intensive investigation in various tissue culture systems using myoblasts or established cell lines such as L-6 (1, 17, 20). Most of these systems use fusing cells, where the events related to cell fusion cannot always be readily separated from the cell recognition events necessary for the induction of muscle-specific proteins (such as creatine kinase and the acetylcholine receptor) (2, 7, 19, 21). Less information is available regarding the induction of muscle-specific proteins in smooth muscle cells (5). BC₃H₁ is a smooth-muscle-like cell line isolated several years ago by Schubert et al. (18). These cells do not fuse but induction of the synthesis of the muscle isoenzyme of creatine kinase and the acetylcholine receptor can be demonstrated to take place under a variety of conditions (13, 18).

In this communication we present evidence to show that in these cells the synthesis of muscle-specific proteins is regulated by the balance between two antagonistic signals: cell contact brings about the induction of the synthesis of these proteins whereas serum factors suppress the induction of these proteins. Induction of these muscle-specific proteins is a reversible phenomenon. While induction of these muscle-specific proteins only occurs in quiescent cells, repression (inhibition of induction) is not obligatorily coupled to the resumption of cell growth.

MATERIALS AND METHODS

BC₃H₁ cells, a nonfusing muscle cell line, was obtained from Dr. D. Schubert at the Salk Institute. The cells were grown at 37°C in a 10% CO₂-enriched atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented, where indicated, with 15% nutrient medium F12 and the specified concentration of dialyzed fetal calf serum or dialyzed horse serum and 1-glutamine (0.1 mg/ ml), penicillin (0 U/ml), and streptomycin (100 μg/ml). Cells were transferred from log phase cultures by trypsinization with crystalline trypsin, and replated. Fresh cultures were started from frozen stock at 4- to 6-wk intervals. Media were obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY) or the Washington University Basic Cancer Center. Sera were obtained from Gibco Laboratories and K. C. Biologicals.

In some experiments cells were grown on collagen-coated dishes. Dishes were coated by NH₂ vapor precipitation of acid-soluble calf skin collagen (Sigma Chemical Co., St. Louis, MO)(14). The dishes were stored dry after sterilization and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% dialyzed fetal calf serum or dialyzed horse serum and L-glutamine (0.1 mg/ml), penicillin (0 U/ml), and streptomycin (100 μg/ml). Cells were transferred from log phase cultures by trypsinization with crystalline trypsin, and replated. Fresh cultures were started from frozen stock at 4- to 6-wk intervals. Media were obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY) or the Washington University Basic Cancer Center. Sera were obtained from Gibco Laboratories and K. C. Biologicals.

Creatine kinase was determined by the following modification of published procedures (11, 20). Cultures in 35-mm dishes were washed once with 2 ml of phosphate-buffered saline (PBS) and the cell layer was suspended in 0.5 ml of 1% nonidet P-40 (NP-40) in 50 glycyl-glycine, pH 6.75. The cells were homogenized (10 to 15 strokes) in a 1-ml Dounce homogenizer. For assay, the extract was appropriately diluted in the same buffer containing 1 mg/ml bovine serum albumin (BSA).

A two-stage assay for creatine kinase is used. In the first stage, the reaction mixture contains 1 mM ADP, 10 mM MgCl₂, 0.2% BSA, 0.1 mM P₃P₃ diadenosine, 5' pentaphosphate (Boehringer Mannheim Biochemicals, Indianapolis, IN), 2.5 mM dihydrothreitol, 25 mM phosphocreatine, 3 mM d-glucose and 20 U/ ml hexokinase, and extract in a total volume of 40 μl. Controls lack phosphocreatine. The reaction is initiated by the addition of extract. After incubation at 37°C for 30 min, the reaction is stopped by the addition of 10 μl of 1 N NaOH. After incubation at 25°C for 10 min, the reaction mixture is diluted to 0.8 ml with 50 mM imidazole buffer, pH 7.0, followed by the addition of NADP and glucose-6-P-dehydrogenase to a final concentration of 0.82 mM and 1.07 U/ml, respectively, in a total volume of 1 ml. After incubation at 25°C for 10 min, the fluorescence due to NADPH is determined in a Farrand Fluorimeter (11), and values obtained in the absence of phosphocreatine were subtracted. The assay is linear with time, and up to 10 nmol of NADPH formed during the assay period.
At least two concentrations of each extract were tested to insure linearity, and all results are the average of duplicate dishes. Creatine kinase was normalized to cell numbers determined in duplicate dishes with a Coulter counter or to cell protein obtained by growing cells for a minimum of two generations in medium supplemented with radioactive amino acid usually with 1 μCi/ml of [15N]-leucine, and measuring the radioactivity in protein by precipitation with trichloroacetic acid in the presence of deoxycholate (15) in the same extracts as were used for the assay of creatine kinase. Although both protein and cell number were determined in each experiment, the results were always identical; therefore, in most experiments we present only the data based on cell numbers. All reagents, unless otherwise indicated, were obtained from Sigma Chemical Co. It is important that the ADP used be as free of ATP as possible.

The acetylcholine receptor was determined by measuring the binding of [125I]a-bungarotoxin (13). a-Bungarotoxin (Sigma Chemical Co.) was iodinated using immobilized lactoperoxidase (Bio-Rad Enzymoabads; Bio-Rad Laboratories, Richmond, CA); the specific activity of the labeled toxin preparations used averaged 25 μCi/μg. Nonspecific binding was determined in the presence of 1 carbamylcholine. 0.1 μg of [125I]a-bungarotoxin was added directly to 35-mm culture dishes, which were then incubated for 50 min at 37°C. Control dishes received carbamylcholine (1 mM final concentration) 10 min before the addition of a-bungarotoxin. At the end of the incubation, the dishes were subjected to six rapid washes with 2 ml of DME containing 2% horse serum. The cell layer was solubilized in 2 ml of 1% SDS (in PBS) and counted in a Beckman gamma counter (Beckman Instruments, Inc., Fullerton, CA).

Fetal calf serum (Gibco Laboratories, Lot C88289) was fractionated at 4°C as follows. The serum was dialyzed overnight against 10 vol of 0.1 M Tris-Cl, pH 7.4. 50 ml of dialyzed serum was applied to a 2.4 x 20-cm column of CM-Sephadex C-50 equilibrated with the same buffer. Serum proteins were eluted from the column with the same buffer until the OD280 was below 0.6 (16). The eluate was concentrated to the original volume, using a Pellicon PTGC 10,000 molecular weight exclusion filter (Millipore Corp., Bedford, MA) in an Amicon concentrator (Amicon Corp., Lexington, MA), and dialyzed against PBS. 20-ml aliquots were applied to a 2.5 x 137-cm Sephacryl S-200 column equilibrated with PBS. The sample was eluted at a flow rate of 0.53 ml/min. 5.3-ml fractions were collected and pooled and concentrated as indicated in Fig. 9 (see below).

All experiments described below have been done at least three times with results similar or identical to those illustrated.

RESULTS

Creatine kinase can be induced in BC3H1 cells under several conditions. As shown in Fig. 1, if the cells are allowed to grow in medium containing 20% serum without medium changes (13), they cease to grow at ~1.8 x 10^5 cells/cm^2. After the cessation of growth, the level of creatine kinase in these cells rises. If the medium is changed daily (an experiment which can only be carried out on collagen-coated dishes to which confluent cells adhere firmly), then the cells grow to slightly higher density but the rate of induction of creatine kinase after cessation of growth is almost unaffected. Compare, in Fig. 1, the open squares (nonfed culture) with the closed squares (culture fed daily). If late in the logarithmic phase of growth the medium is changed to 0.5% serum (day 3 in the experiment in Fig. 1), cell growth ceases within 24 h, and within 48 h, one observes a rapid increase in the level of creatine kinase (closed circles in Fig. 1). Note the reproducibility of these observations in the experiments in which we compare cells on collagen-treated dishes and cells on plastic (open squares and open circles in Fig. 1).

These experiments raise the question whether the induction of creatine kinase at high cell density is due to the depletion by the cells of medium components required for growth, or is due to an event related to cell contact. If depletion of the medium is the cause of the induction of creatine kinase, it must be extremely rapid, because daily changes of medium delay but do not abolish the induction of creatine kinase. To answer this question, we have used medium obtained from confluent cells actively synthesizing creatine kinase (such as those indicated by filled squares in Fig. 1) and added this medium to sparse growing cells, to ascertain whether these sparse cells would continue to grow in this medium or whether they would cease to grow and rapidly initiate the synthesis of creatine kinase. If the former occurred, then depletion of the medium components would be considered as a likely cause of the induction of creatine kinase at high cell density, but if sparse cells continued to grow in medium conditioned by cells at high density, then depletion of medium components cannot by itself amount for the induction of creatine kinase at high density.

The data in Fig. 2 show that cells transferred to conditioned medium continue to grow for several days and do not immediately initiate the synthesis of creatine kinase; thus, medium depletion cannot account for the induction of creatine kinase at high cell density. In contrast, sparse cells at the same density, transferred to medium containing 1% serum, rapidly induce creatine kinase.

The induction of creatine kinase in sparse cells, after a change to medium containing 1% serum, is also dependent on cell density. As shown in Fig. 3, no induction is observed at very low cell density, where 1% serum will support cell growth and will not lead to the induction of creatine kinase. In contrast, daily changes of medium containing 1% serum at higher cell densities (triangles in Fig. 3) do not prevent the induction of creatine kinase. Similar observations have been made with cells plated at 2 x 10^5 cells/35-mm dish, corresponding to the open squares in Fig. 3, where daily feeding of the cultures does not alter the kinetics of induction of creatine kinase (see Fig. 5 below). The data suggest that cell contact is required for the cessation of growth and the induction of creatine kinase. Fig. 4 illustrates typical areas in cultures at densities in which creatine kinase can (panels d-f) and cannot (panels a-c) be induced by transfer of the culture to medium containing 1% serum. BC3H1 cells tend to aggregate in patches, and even at modest cell densities the cells show extensive cell-to-cell contact and a spindly morphology under conditions where creatine kinase is induced. Panels a, b, d, and e represent cultures at the time they were transferred to 1% serum (day 2). Note that the cell densities are such that cell-cell contact is very limited in

![FIGURE 1 Induction of creatine kinase in BC3H1 cells. Cells were plated in 60-mm Falcon dishes at a density of 7 x 10^4 cells/dish in DME - 20% fetal calf serum - 0.1 mg/ml glutamine, 100 U/ml penicillin and 100 μg/ml Streptomycin. Cells were plated on collagen-coated dishes (■), or directly on plastic (□). In the series designated (■), the medium was changed daily; in all other cultures the medium was not changed. On day 3 (designated by the arrow), one set of cultures was transferred to medium containing 0.5% fetal calf serum (□). Cell number and creatine kinase concentration were measured colorimetrically (6). All determinations are the average of duplicate determinations.](https://example.com/figure1.png)
induced cultures, consistent with the biochemical data (open
ance of these cultures at the end of the experiment (day 6). The
cultures, except in culture indicated by (0) where the medium was
changed to medium containing 1% serum. Panel A documents the rate of cell growth in these cultures. Under both conditions, synthesis of the acetylcholine receptor is induced, and in each case its appearance precedes the high cell density results in localized change in the cell environment which affects enzyme induction.

If BC3H1 cells are plated at densities of ~ 2 x 10^4 cells/dish and if after 2 d the medium is changed to 1% serum, induction of creatine kinase can be detected by day 4; the kinetics of induction are the same whether medium is changed daily or every second day (Fig. 5). If the medium recovered from these cultures is added to cells growing in 1% serum at lower cell density, the kinetics of growth or induction of creatine kinase are the same in conditioned medium as in fresh medium containing 1% serum. Thus, induction of creatine kinase in these cells cannot be due to depletion of a medium component, or secretion by the cells of "inducing component" which is stable in the growth medium.

The experiments presented can be rationalized by the assumption that the induction of creatine kinase in BC3H1 is determined by a balance between induction signals derived from cell-to-cell contact and repression due to one or more components present in serum.

To further test this model, we have asked whether the induction of creatine kinase once initiated can be arrested by the addition of serum. The observations in Fig. 6 show that addition of serum (or serum fractions, see below) to BC3H1 cells that have initiated the induction of creatine kinase will prevent further synthesis of this enzyme; compare, in Fig. 6, closed circles and dashed line (inducing conditions) with open circles and solid line (repressing conditions). The induction process thus appears to be reversible. The reversibility of the induction of a protein characteristic of differentiated cells is a unique phenomenon, and it prompted us to examine whether the synthesis of other muscle-specific proteins by BC3H1 cells was subject to similar control mechanism.

Fig. 7 illustrates the induction of the acetylcholine receptor (defined by its ability to bind [125I]-α-bungarotoxin) in BC3H1 cells under two sets of conditions, high cell density in 20% serum and low cell density in 1% serum. For comparison, the induction of creatine kinase has also been measured in parallel cultures. Under both conditions, synthesis of the acetylcholine receptor is induced, and in each case its appearance precedes the low-density cultures. Panels c and f represent the appearance of these cultures at the end of the experiment (day 6). The high-density cells show the morphology characteristic of such induced cultures, consistent with the biochemical data (open triangles, Fig. 3). The low-density cultures remain fibroblastic in appearance. Moreover, they have continued to proliferate during the 4-d period without any significant increase in creatine kinase activity or [125I]-α-bungarotoxin binding. By day 6, they have reached a density where cell islands have formed, and they are beginning to show the biochemical differentiation (open circles, Fig. 3).

Although we prefer to interpret the density effects on enzyme induction to reflect cell-to-cell contact, we cannot rigorously exclude that high cell density results in localized change in the cell environment which affects enzyme induction.

1 The induction of creatine kinase can be arrested by the addition of either fetal calf serum or horse serum. All the initial experiments were carried out with fetal calf serum, but later experiments used horse serum with identical results. Both of these sera support active growth of BC3H1 cells.

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by at least one day the appearance of high levels of creatine kinase activity in the same cultures. Thus, for example, when a culture that has been maintained in 20% serum for 2 d and has reached $10^5$ cells/dish in 20% serum is transferred to 1% serum, an immediate induction of the synthesis of the acetylcholine receptor is observed (open triangles in Fig. 7) where the rise in the activity of creatine kinase does not occur until day 4. Thus, if transfer to 1% serum initiates a developmental program in these cells, then the program is one in which induction of the acetylcholine receptor occurs significantly earlier than the induction of creatine kinase activity. Similar temporal differences in the induction of these two muscle-specific proteins have been observed with myoblasts (see, for example, 18).

If BC3H1 cells in which a partial induction of the acetylcholine receptor has taken place in 1% serum are transferred to 20% serum, then we observe a decrease in the concentration of the acetylcholine receptor present on the surface of these cells with a half-life of ~ 40 h (Fig. 8). In control experiments using cycloheximide to inhibit protein synthesis, we observe a half-life for the acetylcholine receptor of 32 h (data not shown). The decrease in the level of the acetylcholine receptor in these cells after addition of serum strongly suggests that serum prevents further synthesis (or surface expression) of this differentiated protein in partially induced cells and does not simply prevent the recruitment of new cells into the differentiation program, because if the latter were the case, the level of the surface acetylcholine receptor in the culture would either increase slowly or remain constant.

The addition of 20% serum to partially induced cells results in a slow reinitiation of growth (Fig. 6); it becomes important to consider whether the reinitiation of cell growth is required to repress the synthesis of muscle-specific proteins. We have in

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Figure 4  Morphology of BC3H1 at different densities. The cells shown in this figure are from a parallel experiment to that shown in Fig. 3. Cells shown in panels a–c are plated at $3 \times 10^3$ cells/35-mm dish, corresponding to open circles in Fig. 3. Panels a and b show, at two different magnifications, cells on day 2, the day on which the medium is changed to 1% serum. Panel c shows the culture at the higher magnification on day 6. In Panels d–f the cells were plated at $5 \times 10^5$ cells/35-mm dish corresponding to the open triangles in Fig. 3. panels d and e are on day 2 and panel f on day 6. Note, in panel f, the spindly morphology characteristic of the cells that are differentiating and the fact that, even on day 2 (panel e), cells plated at this density are primarily present as cell clusters rather than isolated cells. In contrast, the cells shown in panels b and c remain flat, are well separated, and remain undifferentiated almost until the end of the culture period. Cultures were photographed with an inverted Nikon phase microscope. Bar, 100 μm.
FIGURE 5 Induction of BC3H1 cells in medium conditioned by sparse cells. BC3H1 cells were plated in 35-mm dishes as described. On day 2, the medium was changed to 1% serum (---) and changed either daily (----) or every 2 d (---). The time scale for this culture is shown by the upper numbers on the abscissa. Medium was recovered from the cultures fed daily and used to grow a second series of cultures shown by the open symbols. Three sets of cultures were prepared and grown after day 2 (lower numbers on abscissa) either in 1% serum (O---O), or in conditioned medium as above (O---O) or in control conditioned medium, which had been exposed to plastic dishes with no cells for 24 h. All of these cultures show a low induction of creatine kinase characteristic of these sparse cultures, but no difference was detected between the three media.

FIGURE 6 Effect of serum addition on partially induced cells. BC3H1 cells were plated in 20% serum on day 1. On day 2, some dishes were transferred to medium containing 1% serum (---) while others (O---O) remained in 20% serum. On day 4, some dishes in 1% serum were transferred back to 20% serum (O---O). In this particular experiment, reinitiation of cell growth in 20% serum was extremely rapid. In cultures maintained longer in 1% serum, a variable lag period is observed before reinitiation of growth (see, for example, Fig. 10).

preliminary experiments fractionated serum as described in Materials and Methods by removal of proteins that bind to CM-Sephadex followed by gel exclusion chromatography (Fig. 9). The high molecular weight fraction from serum will repress the synthesis of creatine kinase, without reinitiating cell growth, whereas the lower molecular weight fractions have no effect (Figs. 10 and 11). Compare, in Fig. 11, panel B, the high

FIGURE 7 Induction of acetylcholine receptor in BC3H1 cells. BC3H1 cells were plated in 35-mm collagen-coated dishes in 20% serum. On day 2, some of the dishes were transferred to medium containing 1% serum. Panel A, cell density: (O), 20% serum, (O), 1% serum. Panel B, creatine kinase (A) and specific α-bungarotoxin binding (Δ) to cells in 1% serum. Panel C, creatine kinase (■) and specific α-bungarotoxin binding (□) to cells in 20% serum. Nonspecific binding was <0.01 pg/10^6 cells. Note that both in 1% serum (Panel B) and at high cell density (Panel C) the induction of the acetylcholine receptor precedes the onset of creatine kinase induction by at least 1 day.

FIGURE 8 Effect of serum addition on the level of the acetylcholine receptor. BC3H1 cells were plated in 20% horse serum; after 24 h the medium was changed to 1% serum. On day 4, the medium was changed to 20% serum, and at the times indicated the ability of the cells to bind [³²P]-α-bungarotoxin was determined as described in Materials and Methods. The level of specific α-bungarotoxin binding at the time of the change of the medium to 20% serum was 0.2 pg/10^6 cells.

FIGURE 9 Fractionation of fetal calf serum. Fetal calf serum was fractionated as described under Materials and Methods, first by removal of proteins that are retained on CM-Sephadex, and next by gel exclusion chromatography on Sephacryl S-200. Fractions were pooled as indicated in the figure and assayed for their ability to block the induction of creatine kinase when added to cells in 1% serum at a level equivalent to that present in 20% serum. The results of these experiments are shown in Figs. 10 and 11.
molecular weight fraction, with panel D, the low molecular weight fraction. The addition of the high molecular weight fraction from serum (Fraction I, Fig. 9) does not result in reinitiation of cell growth, not only as determined by cell number (Fig. 10), but also as determined by the incorporation of [3H]-thymidine into DNA, a much more sensitive measurement of the movement of cells out of the G1 phase of the cell cycle and into S phase (Table I).

We conclude from these preliminary experiments that reinitiation of cell growth is not necessary for the repression of differentiation by serum components. By contrast, a variety of known mitogenic compounds including the platelet-derived growth factor, epidermal growth factor, dexamethasone and insulin do not repress differentiation. Neither fibronectin nor cold-insoluble globulin can replace the high molecular weight fraction from serum as inhibition of the induction of creatine kinase.

**DISCUSSION**

The observations presented are interpreted to indicate that, under our culture conditions, the induction of the creatine kinase and of the acetylcholine receptor in BC3H1 cells is under dual control. Serum contains one or more components which inhibit differentiation whereas cell-to-cell contact1 induces differentiation. For the purpose of this discussion, differentiation is defined as the synthesis of creatine kinase and the acetylcholine receptor. In other systems, plasma membranes added to cells have been successfully used to mimic cell contact phenomena (22); experiments are in progress to test whether membranes, when added to BC3H1 cells at low density, will induce the synthesis of muscle-specific proteins.

Inhibition of differentiation can take place in the absence of cell growth (Fig. 10), and the proteins present in the high molecular weight fraction of serum are not by themselves mitogenic for BC3H1 cells. Whether the functional molecules are truly high molecular weight serum proteins or whether they are bound to proteins present in the high molecular weight fraction in serum remains to be determined. The inhibition by serum or serum fractions of the induction of creatine kinase is particularly striking in light of the observations in Fig. 7. These

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**TABLE 1**

| DNA Synthesis in BC3H1 Cells | Rate of DNA synthesis |
|-----------------------------|----------------------|
|                             | Day 5-6 | Day 6-7 | Day 7-8 |
| Exponentially growing cells in 20% serum |          |          |          |
| A) 1% Serum                 | 0.08    | 0.058   | 0.08    |
| B) Cells in A changed to 20% serum on day 5 | 0.13    | 0.33    | 0.46    |
| C) Cells in A changed to 1% serum + serum fraction 1 on day 5 | 0.058   | 0.069   | 0.13    |

The experiment was carried out in parallel to that shown in Fig. 10. Appropriate cultures were incubated for the 24-h periods indicated with 1 μCi/ml (0.67 mCi/mM) [3H]-thymidine to determine the rate of DNA synthesis. The results are expressed as a ratio of [3H]/[35S], the latter derived from [35S]-methionine incorporated into protein. Because the cells had been chronically labeled with [35S]-methionine, the latter is a measure of total cell protein (22).
cells would not differentiate at low density, and would do so at
observations reported with BC3H1 cells, that smooth muscle
cesses (3, 4). It is not always clear to what extent observations made with
established cell lines can be applied to more "normal" primary
cells in culture and ultimately to cells in the whole animal.
Nevertheless the observations with BC3H1 cells represent an
ideal model to study the role of cell-cell interactions and of
serum components in controlling certain aspects of cellular
differentiation. BC3H1 cells are an established cell line with
stable properties. Because the cells do not fuse, it has been
possible to show that, at least for those cells, differentiation
is not an irreversible phenomenon. Although partially differen-
ciated cells can reinitiate growth, repression of differentiation
can take place without reinitiation of cell growth. These obser-
vations are in contrast to those made by many laboratories
with myoblasts, where cessation of growth is a requirement for
differentiation, but where the process of differentiation, once
initiated, is irreversible (see for example, reference 10).
An extensive series of investigations have been carried out
with primary cultures of smooth muscle cells (for review, see
Chamley-Campbell et al., reference 5). The main observation
relevant to this communication is that cell division can occur
at least in some differentiated smooth muscle cells, and that, in
culture, growing cells will lose many characteristics of smooth
muscle cells but regain these at high cell density when growth
cesses (3, 4).6

These observations are fully compatible with the observa-
tions on BC3H1 cells and suggest that BC3H1 cells may be a
good model for the study of the synthesis of muscle-specific
protein in smooth muscle cells. One would expect, from the
observations reported with BC3H1 cells, that smooth muscle
cells would not differentiate at low density, and would do so at

5 Prolonged culture of smooth muscle cells at low density results in irreversible differentiation or modulation (Chamley-Campbell et al. [5]). This may reflect the selection by prolonged growth of cells which can no longer differentiate at high cell density. That the origin of these cells in smooth muscle rather than fibroblasts can be shown in the antibodies specific for smooth muscle actin, which indicate that cells that have lost the ability to differentiate at high cell density so as to show the morphological characteristics of smooth muscle cells, still synthesize smooth muscle actin.

6 Confluence as a result of cell-to-cell contact. Whether serum components influence differentiation of primary smooth muscle cells is not known at the present time. BC3H1 cells differ from primary cultures of smooth muscle cells in their ability to maintain the capacity to differentiate after repeated subculture and in the fact that the platelet-derived growth factor is not a mitogen for these cells. A more extensive comparison of the differentiation of BC3H1 cells and smooth muscle cells in culture is clearly warranted, including the assessment of the role of serum components in the differentiation of both types of cells. Because serum lots may differ in the concentration of molecules that inhibit cell differentiation, side-by-side compari-
on of the effects of different serum lots on smooth muscle
cells and BC3H1 cells will have to be carried out.

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