Muscle Differentiation in Cell Culture

EFFECTS OF NUCLEOSIDE INHIBITORS AND ROUS SARCOMA VIRUS*

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SUMMARY

We have studied some biochemical aspects of the differentiation of chick embryonic skeletal muscle cells in cell culture. Cultures are initiated in a medium that supports cell fusion and muscle cell differentiation. After 40 to 44 hours of culture, the normal onset of cell fusion is prevented by exchanging the medium for one low in Ca++ (80 μM). After a further 24 hours, high Ca++ (880 μM) standard medium is restored and cell fusion proceeds, followed by synthesis of creatine kinase and myokinase.

Before cell fusion is allowed to proceed, cultures are treated with 5-bromotubercidin or 5-fluorouridine, nucleoside inhibitors of RNA synthesis. 5-Fluorouridine is an irreversible inhibitor of ribosomal RNA synthesis but does not inhibit synthesis of heterogeneous nuclear RNA, or by inference, messenger RNA. 5-Bromotubercidin is a reversible inhibitor of the synthesis of both ribosomal RNA and heterogeneous nuclear RNA and of mRNA. In cultures treated with 5-fluorouridine cell fusion and synthesis of creatine kinase and myokinase proceed as in control cultures. In cultures treated briefly (8 hours) with 5-bromotubercidin, cell fusion and synthesis of creatine kinase and myokinase are delayed and lag about 16 hours behind control cultures. We conclude: (a) continuing synthesis of ribosomal RNA (or ribosomes) is not necessary for expression of differentiated muscle properties, i.e. cell fusion and enzyme synthesis. (b) After cultures are refed high Ca++ medium (permissive for cell fusion) there must be synthesis of one or more messenger type RNA's before differentiated properties are expressed. (c) Synthesis and translation to protein of the messenger RNA(s) needed for the expression of differentiated muscle properties does not require concomitant synthesis of ribosomal RNA (or ribosomes). Cultures of differentiating muscle cells have been infected with the RNA tumor virus Schmidt-Ruppin RSV (SR-RSV). Myotubes in infected cultures become progressively vacuolated and within 3 to 4 days after infection by SR-RSV the differentiated myotubes are destroyed. Cell fusion and synthesis of muscle enzymes continue for 2 to 3 days after cultures are infected with SR-RSV. The vacuolization and destruction of myotubes infected by SR-RSV seems to be a response analogous to the morphological transformation occurring in RSV-infected fibroblasts. The differentiated muscle cell, unlike the fibroblast, is not able to respond in a manner which would balance the deleterious effects of this tumor virus and is subsequently destroyed. Since expression of differentiated properties can begin at least 24 hours after infection by SR-RSV, it does not seem likely that transformation by SR-RSV produces an immediate effect on cell differentiation at the level of mRNA.

The study of embryonic skeletal muscle cells in vitro has demonstrated that many of the properties of differentiating muscle in vivo are reproduced during cell culture. In either case a proliferating pool of mononucleated cells is the precursor of the multinucleated fibers, or myotubes (1). The myotubes produced in culture develop a striated contractile apparatus containing actin and myosin (1), have a system of transverse tubules (3), and show contraction and membrane electrical activity in response to added acetylcholine (4, 5). Myotubes developing in culture differ from fibers in vivo in that they have a higher nucleus to cytoplasm ratio and often show branching (6). Their sensitivity to iontophoretically applied acetylcholine extends over much of their surfaces, implying a distribution of cholinergic receptors like that found in denervated or in embryonic muscle (4).

Tissue culture studies of myogenesis have provided the most convincing evidence that multinucleated fibers arise by fusion of mononucleated myoblasts and that nuclei in myotubes do not replicate their DNA or undergo nuclear division (1, 7, 8). Several studies have shown that increases in muscle-specific proteins in vitro are correlated with the fusion of myoblasts to form myotubes. Coleman and Coleman have shown increases in myosin and creatine kinase as cultures of chick embryonic myoblasts undergo extensive myotube formation (9). Yaffe, using myoblasts of newborn rats, has reported that increases in creatine kinase, myokinase, and glycogen phosphorylase are correlated with cell fusion, and that both cell fusion and enzyme increases can be prevented or interrupted by manipulating the Ca++ concentration in the culture medium (10).

All of these observations suggest that cultures of embryonic muscle cells are a favorable experimental system for investigating the biochemical events comprising cellular differentiation; the experiments reported in this paper explore and confirm the suitability of the material for biochemical analysis. We have used cultures of myogenic cells (a) to investigate the effects of

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the nucleoside inhibitors 5-bromotubercidin and 5-fluorouridine on RNA synthesis during myogenesis and subsequent effects of these inhibitors on cell fission and synthesis of muscle proteins; and (b) to study the consequences of infection with Schmidt-Ruppin Rous sarcoma virus, a nondefective RNA tumor virus, because it is of interest to determine the effects of oncornogenic viruses on differentiation.

The studies described here are the initial results of a program to investigate the biochemical aspects of differentiation in skeletal muscle cells and their control.

Experimental Procedure

Materials—Embryonated hen's eggs are obtained from SPAFAS, Norwich, Conn., Ham's F10, F10 without CaCl₂, and horse serum from Grand Island Biological, and trypsin and hyaluronidase from Nutritional Biochemicals. All other reagents were the best commercially available grades. Schmidt-Ruppin Rous sarcoma virus is a previously described (11) high titer isolate purified from a stock obtained from H. Temin.

Culture Medium—The complete medium is composed of 80% Ham's Nutrient Mixture F10, 15% selected horse serum, and 5% embryo extract. Low Ca++-Mg++ medium is prepared from Ham's F10 without CaCl₂. Horse serum is made 5 InM in ethylene glycol bis(β-aminopropyl ether)-N,N′,N″,N′′-tetraacetic acid and dialyzed at 4° against three changes of NaCl, KCl, MgSO₄, and Na₂HPO₄ in the proportions used in F10 medium. Embryo extract is dialyzed against the same salt's solution.

Embryo Extract—Chick embryos 12 days old are washed thoroughly in Pannett-Compton NaCl solution (12) and homogenized by passage through a 50-mg syringe. After two cycles of freezing on solid CO₂ and thawing in a 37° bath, 1 ml of Pannett-Compton NaCl solution and 6 to 10 μg of hyaluronidase are added per g of homogenate, and the mixture is stirred at 4° for 1 hour. The suspension is centrifuged at 10,000 × g for 15 min and the supernatant saved. The supernatant in a flask is placed in a water bath at 65° for 10 min with occasional stirring then chilled on ice. This suspension is centrifuged at 100,000 × g for 6 to 8 hours. The clear supernatant is retained, while the pellet and the turbid lipid-containing layer at the top of the centrifuge tube are discarded.

Cell Cultures—Cells are prepared essentially as described by Konigsberg et al. (7) and Hauschka and Konigsberg (13). Muscle tissue is dissected from thighs and legs of 12-day chick embryos and washed in Ca⁺⁺-Mg⁺⁺-free saline G (NaCl, 8000 mg per liter; KCl, 400 mg per liter; NaHPO₄·7H₂O, 290 mg per liter; KH₂PO₄, 150 mg per liter; glucose, 1100 mg per liter; tris to pH 7.4 with 0.8 ml of 1 N NaOH (14). Muscle fragments are minced and trypsinized for 10 min at 37° in 0.057%; trypsin (1:300) in Ca⁺⁺-Mg⁺⁺-free saline G, with gentle pipetting at the beginning and midpoint of trypsinization. After 10 min, ½ volume of complete medium is added and the cell suspension is pipetted more vigorously. Cells are collected by centrifugation and freed of tissue fragments by filtration through 20-μm nylon mesh. Selective enrichment of myogenic cells is performed as described by Richter and Yaffe (15).

The cell suspension is diluted in complete medium to give 3 to 4 × 10⁶ cells per ml and is placed in 100-mm Falcon culture dishes in a CO₂ incubator at 37° for 1 hour. After selective attachment of nonmyogenic cells, the unattached cells are transferred in growth medium to Falcon culture dishes coated with 1 μg per cm² of collagen soluble in dilute acetic acid, and maintained at 37°-38° in a water jacketed incubator containing 5% CO₂ in air. Cultures are fed fresh medium after 24 hours. Cultures in 80-mm dishes are initiated with 3 ml of cell suspension, those in 100-mm dishes with 8 ml.

Assays of Creatine Kinase and Myokinase—Cultures are washed three times with cold Dulbecco's phosphate buffered saline, drained thoroughly, and frozen at −80°. Cells are scraped from a dish in 2 ml of cold 50 mm glycyglycine, pH 6.75, using a Teflon policeman and homogenized by 30 strokes of a Dounce homogenizer with close fitting pestle; the homogenate is then assayed within 6 hours or retrofrozen at −80°. Assays of activity are based on those described by Oliver (16) and Shainberg et al. (10) which couple ATP production to TPN reduction. Absorbance at 340 nm is measured in a Zeiss PMQ II spectrophotometer equipped with automatic sample changer and automatic slit unit. Absorbances are recorded manually or by an Esterline-Angus point recorder. All assays are performed at two dilutions of homogenate in the linear range of the assay, and such assays agree in nearly all cases within 10% or better. One unit of activity is defined as reduction of 1 μmole of TPN per min at 30°.

The assay for myokinase contains in a final volume of 1 ml: TPN, 0.8 μmole; glucose-6-phosphate dehydrogenase, 0.5 unit; hexokinase, 2 units; glucose, 20 μmoles; Mg⁺⁺ acetate, 10 μmoles; ADP, 2 μmoles; glycyglycine, 110 μmoles, pH 6.75.

The assay for creatine kinase contains, in addition, 10 μmoles of ATP and 15 μmoles of creatine phosphate but contains only 1 μmole of ADP. Assays are initiated by addition of 100-μl aliquots of substrate (ADP for myokinase, creatine phosphate for creatine kinase) after equilibration and stabilization of A₄₅₀ at 30°. A blank, lacking only substrate, is prepared for each assay and its A₄₅₀ determined at the beginning and end of each assay, shows negligible background activity.

Counts of Cell Nuclei—Cultures are washed in PBS, fixed in ethanol-acetic acid (3:1), and stained with Giemsa. Nuclei are counted using a 40 × objective on a Leitz Ortholux microscope. Photographs are made using a Leitz Orthomat camera.

Protein Estimation—Cultures homogenate in glycyglycine buffer are precipitated in cold 10% trichloroacetic acid, collected on 2.4 cm Whatman GF/C filters, and dried. Protein is dissolved in 1 N NaOH and estimated by the Lowry method (17) using crystalline bovine serum albumin as standard.

Radioisotopes in Cultures—Cultures are labeled continuously in 1 μCi per ml of ³H-P₄. Pulse labeling of cultures is usually for 4 hours in 4 μCi per ml of [³H]guanosine in the presence of 1 μg per ml of unlabeled guanosine.

Isolation of RNA and Polyacrylamide Gel Electrophoresis—RNA is extracted and purified from total cells lysed in 1% sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis exactly as described by Brdar, Rikin, and Reich.²

² The abbreviations used are: BrTu, 5-bromotubercidin; FUr, 5-fluorouridine; SR-RSV, Schmidt-Ruppin strain of Rous sarcoma virus; PBS, Dulbecco's phosphate buffered saline solution; RAV-50, Rous associated virus-50.

³ The systems in the effects of FUr and BrTu have been studied to date are the following: (a) mouse fibroblast cultures (Strain L-2). B. Brdar, E. Reich, and G. Acs, manuscript in preparation. (b) vaccinia virus infection of L-cells. B. Brdar and S. Silverstein, unpublished observations. (c) normal chick embryo fibroblast cultures and cultures transformed by Rous sarcoma virus. B. Brdar, D. B. Rikin, and E. Reich, manuscript submitted for publication. (d) normal cultures of BSC-1 cells and cultures infected with SV-40 virus, and mouse fibroblasts (3T3) transformed by SV-40 virus; see Ossowski and Reich (22). (e) chick embryo and mouse fibroblast cultures infected with one lytic DNA virus and five lytic RNA viruses; B. Brdar and E. Reich, manuscript submitted for publication.
Radioactivity precipitable by cold 5% trichloroacetic acid is determined for cell lysates and for purified RNA. The recovery of RNA radioactivity was at least 80% and, in most experiments, above 90%.

Injection of Cultures with SR-RSV—Purification of virus and infection of cultures are performed essentially as described by Riklin and Reich (11). Cultures are washed with F10 before and after infection. Infection early (approximately 44 hours in culture) is in high Ca++ with 2 to 4 focus-forming units per cell, while infection late is in low Ca++ with 5 to 10 focus-forming units per cell.

RESULTS

Inhibition of RNA Synthesis— Cultures of myogenic cells are initiated in growth medium which contains 1 μCi per ml of [3P]orthophosphate to label cellular RNA to constant specific activity. After 44 hours in high Ca++ medium, cells are changed for the next 24 hours to medium containing 80 μM Ca++. The generation time of myogenic cells grown in culture is 10 to 12 hours (18, 19). Thus, at the end of the incubation period in low Ca++ medium they will have been in culture for 68 hours (at least five generation times) and can be expected to have synthesized at least 95% of their RNA molecules while in culture in 32P.

After 24 hours in low Ca++ medium, high Ca++ medium is restored and [3H]guanosine (4 μCi per ml, 1 μg per ml) added. Following a 4-hour pulse, the cultures are harvested, and RNA is extracted and analyzed on polyacrylamide gels. The profile in Fig. 1A shows RNA extracted from control cultures. The 18 S and 28 S ribosomal RNA's, well represented by 32P activity,

![Fig. 1. Analysis of myogenic cell RNA by polyacrylamide gel electrophoresis. A, control cultures in 32Pi (1 μCi per ml) were fed low Ca++ medium for 24 hours. High Ca++ medium was restored and [3H]guanosine (4 μCi per ml, 1 μg per ml) was added simultaneously. After a 4-hour pulse, cells were harvested, lysed in 1% sodium dodecyl sulfate, and RNA was extracted and analyzed. B, parallel cultures were exposed to FUrd (10 μg per ml) for 4 hours. FUrd was removed when high Ca++ medium was restored and [3H]guanosine added. After the 4-hour pulse, cultures were harvested and processed as in A. C, replicate cultures after FUrd treatment as in B were fed high Ca++ medium without inhibitor. After 24 hours in high Ca++, [3H]guanosine was added for 4 hours, and cultures were processed as in A. D, parallel cultures were exposed to BrTu (5 μg per ml) beginning 4 hours before high Ca++ was restored. [3H]Guanosine was added with high Ca++ in the presence of BrTu for a 4-hour pulse, and cultures were harvested as in A. E, companion BrTu-treated cultures were washed and, after 1-hour incubation, [3H]guanosine was added for a 6-hour pulse.](http://www.jbc.org/)

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are found in Slices 50 to 54 and 23 to 28, respectively. Incorporation of \(^{3}H\)guanosine is extensive in ribosomal RNA as well as in several RNA peaks between 20 and 208 and in heavier nuclear RNA's not resolved in this gel.

5-Fluorouridine—The results of detailed studies show a consistent pattern for the inhibition of eukaryote RNA synthesis by FUrd. In all systems tested, this nucleoside is an irreversible inhibitor of rRNA, but does not inhibit the synthesis of heterogeneous nuclear RNA or mRNA. It is therefore of interest to examine the effects of FUrd on myogenic cells. For this purpose, parallel cultures are exposed to 10 \(\mu\)g per ml of FUrd for 4 hours before high Ca\(^{++}\) medium is restored. Coincident with the restoration of high Ca\(^{++}\) medium the cultures are exposed to a pulse of \(^{3}H\)guanosine as above. The polyacrylamide gel electrophoresis pattern in Fig. 1B shows no peak of \(^{3}H\)-containing RNA corresponding to the peaks of previously labeled (\(^{35}P\)) 28 S and 18 S RNA, indicating that FUrd has inhibited ribosomal RNA synthesis, as expected. Other gels, not presented here, show that 4 S RNA is synthesized at the same rate as in control cultures. In this experiment, over-all RNA synthesis in the FUrd-treated culture was 28% of the controls. When cultures treated with 10 \(\mu\)g per ml of FUrd for 4 hours are subsequently maintained in high Ca\(^{++}\) growth medium for 24 hours in the absence of FUrd, there is no recovery of ribosomal RNA synthesis. This is shown by the RNA profile in Fig. 10 which reveals no recovery of RNA synthesis in 24 hours, although other unidentified RNA's are still being made. This indicates that in myogenic cells, as in mouse and chick fibroblasts, the inhibition of ribosomal RNA synthesis by FUrd is irreversible.

5-Bromotubercidin—From its effects on normal and transformed fibroblasts and BSC-1 cells, 5-bromotubercidin is known to inhibit the synthesis of all cellular RNA species of high molecular weight: these include rRNA, mRNA, and heterogeneous nuclear RNA. However, in contrast to the effects of 5-fluorouridine, those produced by 5-bromotubercidin are fully reversible. To test for its action on myogenic cells, replicates of the preceding cultures were exposed to this analogue. The drug (5 \(\mu\)g per ml) was added to cultures 4 hours prior to restoration of high Ca\(^{++}\) medium and was maintained during the subsequent 4-hour period of exposure to \(^{3}H\)guanosine. Fig. 1D shows the RNA profile obtained from these cultures. During the 1-hour period there is little synthesis of distinct RNA species of sizes 18 S or larger. Other gels not shown here indicate that synthesis of 4 S RNA proceeds at a normal rate, and over-all RNA synthesis is at 18% of the control rate. Recovery of RNA synthesis in 5-bromotubercidin-treated cultures is shown in Fig. 1E. After 8 hours in 5-bromotubercidin, cultures are washed, and following incubation for 1 hour, are pulse labeled with \(^{3}H\)guanosine for 6 hours. The RNA profile in the gel clearly shows rapid recovery of RNA synthesis. Over-all RNA synthesis was 117% of control, after correction for the longer duration of the 6-hour pulse. These results obtained in cultures of myogenic cells are therefore consistent with the more detailed studies performed in chick embryo fibroblasts and in mouse L cells which show that: (a) 5-fluorouridine irreversibly inhibits synthesis of ribosomal RNA's, but not heterogeneous nuclear RNA or mRNA; (b) 5-bromotubercidin reversibly inhibits synthesis of ribosomal RNA's, heterogeneous nuclear RNA, and mRNA but not of 4 S RNA.

1 We assume that RNA synthesis proceeds at linear rates, on the average, over the time periods tested; this assumption has not been tested experimentally.

Cell Fusion in Myogenesis—The normal course of cell fusion in myogenic cell cultures is shown in Fig. 2.

After 44 hours in culture, at the time medium is changed to 80 \(\mu\)M Ca\(^{++}\), most cells are mononuclear. Fig. 24 shows typical bipolar myotubes as well as the spread appearance of fibroblasts (1). Also seen is the linear overlapping association typical of mononuclear myogenic cells. After 24 hours in the 80 \(\mu\)M Ca\(^{++}\) medium, many myotubes have become large and thin (Fig. 2B). Such cells are seen in small numbers in presynthesis cultures maintained in high Ca\(^{++}\) medium. Similar cells have been described by several authors (5, 6, 9, 10) and may be mononuclear cells which already express some differentiataed properties (5).

Within 24 hours after high Ca\(^{++}\) is restored there is extensive cell fusion and myotube formation (Fig. 2C). By 48 hours after high Ca\(^{++}\) is restored, myotubes are larger and more plentiful (Fig. 2D). There are bipolar mononuclear cells remaining between myotubes (Fig. 2E) often in close association with them.

Effect of 5-Fluorouridine Cultures exposed to 5-fluorouridine (10 \(\mu\)g per ml) for 4 hours immediately before restoration of high Ca\(^{++}\) medium no longer synthesize ribosomal RNA, but the myogenic cells proceed to fuse with each other at a rapid rate. Fig. 2, F and G, shows 5-fluorouridine-treated cultures 24 and 48 hours after the drug was removed and the high Ca\(^{++}\) medium restored. These myotubes in the drug-treated cultures are typically less branched than control myotubes, and have a more rough-edged appearance, but the process of cell fusion as such has been completed at normal rates.

Effect of 5-Bromotubercidin—Cultures exposed to 5-bromotubercidin (5 \(\mu\)g per ml) for a total of 8 hours beginning 4 hours before and ending 4 hours after restoration of high Ca\(^{++}\) medium. In the 20 hours following removal of the drug, there is little cell fusion to form myotubes (Fig. 2H); the cells are closely packed together and are nearly all mononuclear. After a further incubation of 24 hours, that is, 44 hours after removal of the analogue, cell fusion to form myotubes has progressed to essentially the extent found earlier in control cultures (Fig. 2I, cf. 2D).

Quantitation of Cell Fusion—By determining the relative proportions of nuclei in myotubes and mononucleated cells as a function of time it is possible to obtain quantitative estimates of the progress of cell fusion. This procedure confirms the conclusions derived from microscopic observation of the culture and indicates that the two analogues differ in their effects on cell fusion. The results of a representative experiment are presented in Fig. 3. These show that following restoration of high Ca\(^{++}\) medium cell fusion proceeds rapidly in both control and 5-fluorouridine-treated cultures. In contrast, in the 5-bromotubercidin-treated cultures there is essentially no cell fusion during the first 24 hours in high Ca\(^{++}\) medium; after this, the inhibition caused by 5-bromotubercidin is reversed and cell fusion proceeds at a rate equivalent to that in control cultures.

For the experiment shown in Fig. 3, cultures were treated with FUrd for 4 hours at the end of the period of Ca\(^{++}\) deficiency. When exposure to FUrd occurs early, that is during the first 4 hours in low Ca\(^{++}\), cell fusion also proceeds normally after the culture is transferred to high Ca\(^{++}\) medium (Table I).

Activity of Creatine Kinase and Myokinase: Effects of Ca\(^{++}\) Restriction and of 5-Fluorouridine and 5-Bromotubercidin—Increases in creatine kinase or myokinase activities in myogenic cell cultures are correlated with cell fusion especially under conditions in which cell fusion is controlled by Ca\(^{++}\) concentrations (9, 10). As seen in Fig. 4 the creatine kinase content of cultures maintained only in high Ca\(^{++}\) medium increases rapidly from approximately 48 hours. In contrast, cultures exposed to low
effects of the nucleoside analogues. For this purpose all cultures were exposed to one or another of the analogues either at the start or at the end of the incubation period in low Ca++. After cell fusion is initiated by restoration of high Ca++, activities increase rapidly in control cultures. However, cultures that are maintained in high Ca++ throughout, the inhibition due to BrTu is reversed, and creatine kinase levels increase linearly after high Ca++ is restored, while in late BrTu cultures there appears to be a 12-hour lag before myokinase increases linearly. However, in this and some other experiments, changes in myokinase activity are not so clear or dramatic as changes in creatine kinase activity. Similar observations on variability of myokinase activity have been noted by Shainberg et al. (10).

The preceding results with FUrd and BrTu were from separate experiments, performed in cultures prepared at the same time the same distinctive results were obtained. 4

The data relating to enzyme activities that are presented in

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**Table I**

Cell fusion in early FUrd-treated cultures

Cultures described here are companion cultures to those described in the legend to Fig. 4.

<table>
<thead>
<tr>
<th>Time after high Ca++ restored</th>
<th>Myotube nuclei/total nuclei</th>
<th>% in myotubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FUrd</td>
<td>No FUrd</td>
</tr>
<tr>
<td>24 hours after high Ca++ was restored</td>
<td></td>
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</tr>
<tr>
<td>-20 hours</td>
<td>39/1251</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>17/1071</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>255/1499</td>
<td>16</td>
</tr>
<tr>
<td>36</td>
<td>355/1370</td>
<td>26</td>
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*a* That is, at the end of the FUrd treatment 20 hours before high Ca++ medium was restored.

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![Diagram](https://via.placeholder.com/150)

**Fig. 3.** Time course of cell fusion. The ordinate shows the percentage of nuclei found in multinucleate cells (i.e., myotubes). The abscissa denotes time and coincides, in this and in subsequent figures, with the explanatory bar at the top, indicating addition and removal of inhibitors and changes of Ca++ concentration in nutrient medium.

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**Fig. 2.** Cell fusion in myogenesis. A, bipolar myoblasts and some fibroblasts from a 44-hour-old culture in high Ca++, just before Ca++ was lowered to 80 µ. B, numerous thin myoblasts in low Ca++, 24 hours after 4 and just before high Ca++ was restored. C, control cultures 24 hours after high Ca++ was restored. There is extensive fusion to form branching myotubes. D and E, control cultures 48 hours after high Ca++ was restored. Myotubes are larger and more numerous. Bipolar myoblasts are closely associated with myotubes (E). F, FUrd-treated culture 24 hours after high Ca++ was replaced and inhibitor removed. Cell fusion in the absence of rRNA synthesis. G, FUrd-treated culture after 48 hours in high Ca++. Cell fusion continues at control rates. H, BrTu-treated culture 24 hours after high Ca++ was restored. No myotube formation up to 20 hours after inhibitor was removed. I, companion BrTu cultures 48 hours after high Ca++ was restored. Rapid cell fusion occurs between 20 and 44 hours after inhibitor was removed.
Effects of Rous Sarcoma Virus on Differentiating Muscle Cells: Morphology—The restricted biosynthetic program of differentiating muscle, and the reproducible kinetics of formation of specialized muscle proteins provide a background of cellular activities that is much simpler than that existing in growing cells such as fibroblasts. The nongrowing muscle cells are therefore more favorable material for exploring the effects of oncogenic viruses on macromolecule synthesis. The susceptibility of muscle cells to infection by the Bryan strain of RSV has already been reported (20), although the consequences for cellular synthetic activities remain to be defined.

We have observed that if cultures of myogenic cells are infected with SR-RSV the myotubes that form are destroyed. In Fig. 8 are presented phase micrographs of myotubes in culture at 24, 48, 72, and 96 hours after high Ca++ medium is restored following a period of 24 hours in Ca++-deficient medium. These myotubes show the typical appearance that is characteristic of those stages of development. In Fig. 9 are phase micrographs of parallel cultures infected with SR-RSV just before high Ca++ was restored. Cytoplasmic vacuolization is evident in some myotubes at 48 hours after infection and is extensive throughout the cultures by 72 hours. At 96 hours after infection most myotubes have retracted from the surface of the culture and have degenerated. As seen in Fig. 10 this process affects the entire culture, and not merely restricted regions. The control dishes fixed and stained at 24-hour intervals after Ca++ is restored develop an extensive network of myotubes. Cultures infected with SR-RSV begin to develop macroscopically visible myotubes but then degenerate. This result differs somewhat from that reported by Lee et al. (20) who were unable to obtain convincing morphological evidence of transformation following RSV infection of myogenic cells. This discrepancy could be due to difference in the virus strains or, more likely, to differences in multiplicity of infection.

This result is of interest since it is in contrast to the effect of the same virus in fibroblast cultures. Whereas the fibroblast is induced to abnormal patterns of growth following infection by SR-RSV, the myotube, which has lost the ability to grow and divide, degenerates. The morphological properties of the myotubes and, in particular, the extensive vacuolization and granular appearance are microscopically very similar to those observed in fibroblasts that have been infected by virulent viruses; however, these degenerative changes in RSV-infected myotubes develop in some myotubes at 48 hours after infection and is extensive throughout the cultures by 72 hours. At 96 hours after infection most myotubes have retracted from the surface of the culture and have degenerated. As seen in Fig. 10 this process affects the entire culture, and not merely restricted regions. The control dishes fixed and stained at 24-hour intervals after Ca++ is restored develop an extensive network of myotubes. Cultures infected with SR-RSV begin to develop macroscopically visible myotubes but then degenerate. This result differs somewhat from that reported by Lee et al. (20) who were unable to obtain convincing morphological evidence of transformation following RSV infection of myogenic cells. This discrepancy could be due to difference in the virus strains or, more likely, to differences in multiplicity of infection.

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somewhat more slowly. Prompted by these observations we have examined the effects of RSV on muscle enzyme synthesis.

Effects of RSV Infection on Creative Kinase and Myokinase in Myotubes—To test the effects of RSV infection on enzyme synthesis in myotubes, replicate cultures of those illustrated in Fig. 8 and 9 were assayed for enzyme content at various times after infection. The data, illustrated in Fig. 11, show that the levels of creative kinase and myokinase are identical in control and infected cultures up to 48 hours following infection and restoration of high Ca++ medium. In the infected cultures enzyme activity reaches a plateau about 72 hours after infection and declines thereafter. The time course of enzyme specific activity in the cultures exactly parallels the total activity. It is of interest to note that the time at which creative kinase and myokinase activities reach a plateau, 72 hours after infection, corresponds to the time at which extensive vacuolization of infected myotubes is seen (Fig. 9C).

If myogenic cells are infected with RSV early, just before they are given low Ca++ medium, the infected cultures have 24 hours before cell fusion in which to express virus-transforming functions. As shown in Fig. 12, infection with SR-RSV at the earlier time seems to have some inhibitory effect on expression of creative kinase and myokinase activities, since the increase of these enzymes in infected cultures is only 60% as fast as in controls. Again, enzyme activities reach a plateau about 72 hours after the time of infection. The extended time between SR-RSV infection and fusion, allowing more time for virus-directed synthesis, may account for the decreased synthesis of creatine kinase and myokinase in this experiment. However, expression of virus-transforming function clearly has had no qualitative effect on expression of myoblast differentiation. If virus transformation did have a qualitative effect on differentiation, one might expect an effect on cell fusion or enzyme synthesis more like that of BrTu, in which macromolecule synthesis required for a step in differentiation is blocked.

DISCUSSION

Because the experiments reported in this paper only constitute an initial survey, the conclusions which may be drawn are necessarily tentative and limited in scope. Nevertheless, several of the present observations deserve comment. The first of these concerns the effects of the nucleoside inhibitors. It is clear from studies of L cells, chick fibroblasts, and SV-40-transformed mouse fibroblasts that FUrd is an irreversible and selective inhibitor of rRNA synthesis; the same appears to be true for chick myoblasts. Thus the fact that BrTu applied late does not much affect creatine kinase or myokinase activity indicates that expression of a differentiated property becomes independent of the synthesis of new ribosomes. It is not so clear how to interpret the inhibition of creatine kinase and myokinase activity when FUrd is applied early, in which case creatine kinase accumulates at 10 to 20% of the control and myokinase at around 30 to 50% of the control rate. This inhibition could reflect a particular biochemical step in myoblast differentiation which is sensitive to FUrd, but when completed is not sensitive to FUrd. Alternatively, the inhibition could result from the general inhibition of protein synthesis in these cells, since it is reasonable to expect that the total cellular complement of ribosomes might become the rate-limiting factor in protein synthesis after 24 hours during which rRNA synthesis is completely blocked by FUrd.

Two observations suggest the second explanation may be true. (a) In cells treated with FUrd after 24 hours in culture, i.e. about 24 hours before fusion would normally begin, creatine kinase synthesis is only 5% of the rate in control cultures; however, the specific activity of creatine kinase in these cultures is exactly the same as in control cultures, and increased at the same rate over a 48-hour period after the FUrd treatment. (b) The rate and extent of cell fusion in cultures exposed to FUrd at the early time, expressed as percentage of nuclei in myotubes, is nearly the same as in control cultures. The actual number of nuclei is less because FUrd has blocked cell multiplication (Table I).

As in the case of FUrd, the patterns of RNA synthesis inhibition produced by BrTu in myoblasts are fully consistent with those observed in previous studies of other cell types; the latter indicate that BrTu reversibly inhibits the synthesis of rRNA, mRNA, and heterogeneous nuclear RNA. We do not have at this time direct evidence that BrTu inhibits myoblast mRNA synthesis. The behavior of BrTu-treated cultures suggests, however, that this interpretation is reasonable for myoblasts as well. Thus, the delay in cell fusion and muscle enzyme synthesis produced by late treatment with the drug in-
Fig. 8. Phase contrast micrographs of control cultures. Cultures were incubated in low Ca++ for a 24-hour period. A, 24 hours after high Ca++ restored; B, 48 hours after high Ca++ restored; C, 72 hours after high Ca++ restored; D, 96 hours after high Ca++ restored. All frames to same scale; bar equals 50 μm.

dicate that both myoblast fusion and the synthesis of specific proteins must be preceded by the synthesis of a nonribosomal RNA. The lack of effect of early BrTu treatment suggests that cells recover from this inhibitor's action in the 16-hour period that precedes the restoration of high Ca++. Three tentative conclusions may be drawn concerning the relationship of RNA synthesis to myoblast differentiation and the phenotypic expression of that differentiation.

8 It has been reported (10) that rat myogenic cells, in low Ca++, treated with actinomycin D (2 μg per ml) can initiate cell fusion if actinomycin was applied 8 hours or less before high Ca++ was restored. The apparent discrepancy with the results presented here is not yet resolved, but it may be due to differences in timing of mRNA synthesis in rat and chick myoblasts. Preliminary observations suggest that there may be limited cell fusion after chick myoblasts are treated with low doses of actinomycin (0.1 μg per ml), although this concentration of actinomycin requires several hours to inhibit RNA synthesis fully.

1. The first conclusion is that continuing synthesis of ribosomal RNA, and hence of ribosomes, is not necessary for the expression of differentiated properties. That is, all of the ribosomes required for synthesis of specialized muscle proteins are present before translation of these proteins in myotubes begins.

2. The second conclusion is that there is an absolute requirement for synthesis of a nonribosomal RNA as a necessary prerequisite to both cell fusion and translation of specialized muscle proteins. The nature of this RNA is unknown. Clearly some type of messenger RNA would be a desirable candidate for this function, but there is no direct evidence that the messenger RNA's coding for muscle proteins are first transcribed at the time cell fusion begins.

3. The third conclusion is that transcription of the RNA required for cell fusion and its subsequent translation do not require concomitant synthesis of ribosomal RNA. That is, the synthesis of an RNA required for a differentiated cell function, cell fusion, is not dependent on continued ribosome synthesis.
FIG. 9. Phase contrast micrographs of SR-RSV-infected cultures. Replicates of cultures described in the legend to Fig. 8 were infected with SR-RSV just before high Ca\textsuperscript{++} was restored. A, 24 hours; B, 48 hours; C, 72 hours; and D, 96 hours after high Ca\textsuperscript{++} was restored. All frames to same scale; bar equals 50 $\mu$m.

Thus it appears that synthesis and transport of the messenger-type RNA required for cell fusion is not necessarily coupled to synthesis of ribosomes.

Taken together, the results of the current studies with FUrd and BrTu suggest that this pair of nucleoside analogues will be useful tools for distinguishing the requirements and timing of RNA synthesis during muscle differentiation. The nucleosides are more selective in their effects on different species of RNA and are less cytotoxic than compounds such as actinomycin; in addition, the effects of BrTu are rapid in onset and fully reversible, thereby permitting precise timing of its effects.

The second aspect of this work that merits comment is the effect of RSV infection on myotube development. In contrast to transformed fibroblasts, which respond to RSV by continuing to infected and control, were then fed low Ca\textsuperscript{++} medium for 24 hours before high Ca\textsuperscript{++} was restored. Top row, left to right: SR-RSV-infected cultures 24, 48, 72, and 96 hours after high Ca\textsuperscript{++} was restored. Bottom row, left to right: companion uninfected cultures 24, 48, 72, and 96 hours after high Ca\textsuperscript{++} was restored. Stained with Giemsa.

Fig. 10. Cultures of control and SR-RSV-infected myotubes. Cultures grown 44 hours in high Ca\textsuperscript{++} medium were infected with SR-RSV and all cultures, infected and control, were then fed low Ca\textsuperscript{++} medium for 24 hours before high Ca\textsuperscript{++} was restored. Top row, left to right: SR-RSV-infected cultures 24, 48, 72, and 96 hours after high Ca\textsuperscript{++} was restored. Bottom row, left to right: companion uninfected cultures 24, 48, 72, and 96 hours after high Ca\textsuperscript{++} was restored. Stained with Giemsa.
Fig. 11. Creatine kinase and myokinase in RSV cultures infected late. All cultures were fed low Ca++ medium at 44 hours. Just before high Ca++ medium was restored, cultures were infected with SR-RSV; control cultures were mock infected. After a 40-min infection period, unabsorbed virus was washed from cultures and high Ca++ medium was restored.

Fig. 12. Creatine kinase and myokinase in RSV cultures infected early. Just before all cultures were changed to low Ca++ medium, some were exposed to SR-RSV for 40 min, as described in the legend to Fig. 11. At 44 hours all cultures were fed low Ca++ medium. After a further 24 hours (i.e. at 68 hours), high Ca++ medium was restored in all cultures.

Although we do not know the basis for the lethal effect of RSV on myotubes, the differentiating muscle cell is an ideal system for attempting to delineate the mechanism. The limited number of specialized proteins and RNA species formed by maturing muscle cells provides an ideal cellular background for detecting new, virus-specific macromolecules that are likely to be synthesized following infection and transformation.

Another advantage of myogenic cells is implied by the work of Heywood and Nwagwu (22) who have isolated a 26 S myosin-specific mRNA using polysomes from developing muscle of chick embryos, and by the observation of Morse et al. (23) that chick embryo muscle cells in culture contain myosin-synthesizing polysomes. We have found that differentiating muscle cells in culture synthesize four RNA’s which are readily identified on polyacrylamide gels as discrete bands migrating at approximately 21 S, 23 S, 24 S, and 26 S (e.g. Fig. 1A). These findings suggest that the timing of synthesis, the metabolic lifetimes, and the translation of mRNA’s in differentiating muscle cells can be clearly defined under different experimental conditions, including RSV infection. These observations will be the subject of a separate communication.

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