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STIMULATION AND INHIBITION OF MYOBLAST DIFFERENTIATION BY HORMONES

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SUMMARY

The growth and differentiation of L6 myoblasts are subject to control by two proteins secreted by cells of the Buffalo rat liver line. The first of these, rat insulinlike growth factor-II (formerly designated multiplication stimulating activity) is a potent stimulator of myoblast proliferation and differentiation, as well as associated processes such as amino acid uptake and incorporation into protein, RNA synthesis, and thymidine incorporation into DNA. In addition, this hormone causes a significant decrease in the rate of protein degradation. All of these actions seem to be attributable to a single molecular species, although their time courses and sensitivity to the hormone differ substantially. The second protein, the differentiation inhibitor (DI), is a nonmitogenic inhibitor of all tested aspects of myoblast differentiation, including fusion and the elevation of creatine kinase. Indirect immunofluorescence experiments demonstrated that DI also blocks accumulation of myosin heavy chain and myomesin. Upon removal of DI after 72 h incubation, all of these effects were reversed and normal myotubes containing the usual complement of muscle-specific proteins were formed. Thus, this system makes it possible to achieve specific stimulation or inhibition of muscle cell differentiation by addition of purified proteins to cloned cells in serum-free medium.

Key words: muscle; myoblasts; differentiation; somatomedin; insulinlike growth factor; immunofluorescence.

INTRODUCTION

For more than 20 yr, the senior author and his colleagues have investigated the control of muscle growth and development, with emphasis on the role of hormones in those processes. In recent years, virtually all of this work has been done with cultured cells, so it is appropriate that this summary of recent results be presented to the Tissue Culture Association, which has done so much to make this kind of work possible. We are particularly grateful to the TCA for introducing us to Temin's multiplication stimulating activity (MSA) (6) and its source, the Buffalo rat liver (BRL) cell line, which we first heard described at the TCA Annual Meetings in Boston in 1972.

Recently, the principal mitogen in these preparations has been designated rat insulinlike growth factor-II (rIGF-II) as a result of sequence determinations (24) that demonstrated its very close similarity to human insulinlike growth factor-II. This corresponds to one of the two major somatomedins (the other is designated IGF-I) that are believed by many investigators to mediate the growth-promoting actions of growth hormone. We have found rIGF-II active in stimulating proliferation (13–15) and differentiation (9,11,12) as well as some related processes (8,10,27,28). Although these experiments consistently indicated that the somatomedins play a major role in mediating the growth-promoting actions of growth hormone in muscle, there were practical limitations to the conclusions that could be reached with the available techniques and preparations, and questions worthy of careful attention have arisen. In our view, the most important of these are the following: (a) Is the same molecule responsible for all of the observed effects of rIGF-II on myoblasts and is it identical to the form characterized by Marquardt et al.? (b) What is the relative potency of rIGF-II in stimulating proliferation, differentiation, and related processes

in myoblasts? (c) Is the apparent stimulation of proliferation and differentiation of myoblasts by rIGF-II in serum-free medium simply a result of enhanced metabolism or survival of the cells? (d) What is the temporal order in which the various actions occur? Our relatively large-scale preparations of rIGF-II, coupled with the use of high performance liquid chromatography techniques, have made it possible for us to obtain reasonably complete answers to these questions. The results of these determinations are presented in this paper.

In addition to rIGF-II, BRL cells also secrete another protein we find very interesting. An earlier report (9) described the discovery and partial purification of a protein, the differentiation inhibitor (DI), from the conditioned medium of BRL cells; DI inhibits fusion and creatine kinase (CK) accumulation in cultured skeletal myoblasts. To complete this description of the actions of BRL cell products on muscle cells, we report the effects of DI on the extent of fusion and on the accumulation of CK, myosin heavy chain, and myomesin and the reversibility of these effects in skeletal myoblasts. The data presented here demonstrate that DI blocks all measured aspects of myoblast differentiation in myoblasts from two species (quail and rat), that the inhibition persists as long as fresh DI is added to the cultures, and that all effects are completely reversible upon removal of DI from the culture medium.

Thus, with the systems described here, it is possible to stimulate or inhibit the differentiation of cloned muscle cells by addition of purified proteins to serum-free medium and thereby dissect the process in much greater detail than was previously possible.

Materials and Methods

Materials. Tissue culture supplies were purchased from GIBCO Laboratories (Grand Island, NY). Tissue culture plates were purchased from Corning (Elmira, NY) and Costar (Cambridge, MA). Insulin, bovine serum albumin (RIA grade), and reagents for the CK assay were purchased from Sigma Chemical Co. (St. Louis, MO). Tritiated thymidine was from Moravek Biochemicals (City of Industry, CA), and [^{3}H]AIB from New England Nuclear (Boston, MA). [^{125}I]NaI was also purchased from New England Nuclear, and iodobeads were from Pierce Chemicals (Rockford, IL). Fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) conjugated goat antibodies to IgGs were obtained from Cappel Laboratories (Cochranville, PA). Conditioned medium from Buffalo rat liver cells was from two sources: large scale cultures grown at the Cell Culture Center, MIT, as described by Dulak and Shing (5), and smaller cultures (in 125-ml T-flasks) grown in this laboratory; rIGF-II and DI content of the conditioned media were similar in the two preparations.

Antibodies. The mouse monoclonal antibody, MF-20, directed against chicken skeletal muscle myosin was a generous gift from Donald A. Fischman, Cornell Univ. Med. College, NY, NY. Isolation and characterization of this antibody have been described (25). Guinea pig polyclonal antibodies specific for Type I collagen were prepared and characterized previously (40). Polyclonal antibodies against myomesin were raised in rabbits and were the generous gift of Emanuel Strehler, Harvard University Children's Hospital, Boston, MA. The specificity of these antibodies has been documented by Strehler, et al. (37).

Purification of rIGF-II. Initial purification of the rIGF-II was by batchwise absorption-elution from Dowex 50 followed by molecular exclusion chromatography on Fractogel TSK-55(F) (EM Reagents, Gibstown, NJ) by a variation (9) of the original procedure of Dulak and Temin (6). For the experiments reported here, pooled rIGF-II fractions from several preparations were rechromatographed on Fractogel as described in the legends of the figures and tables. The DI-containing fractions were combined as described earlier (9); some experiments were done with material further purified by a variety of techniques, but we have not yet been successful in devising a consistent and reliable method for the complete purification of the DI.

For the dose-response experiments reported here, the rIGF-II fractions (containing 7500 M_r material that appeared homogeneous on sodium dodecyl sulfate (SDS) gel electrophoresis) from a number of routine preparations were combined, freeze-dried, and chromatographed on a Pharmacia PRO-RPC 5/10 column using the Pharmacia FPLC apparatus. Initial chromatography using a rather steep gradient (0 to 80% acetonitrile in 15 ml of 1% acetic acid at 0.5 ml/min) indicated the presence of a single major peak. The peak fractions were pooled, diluted with an equal volume of 1% acetic acid, and reapplied to the column. Rechromatography in a shallow gradient (28 to 44% acetonitrile in 20 ml of 1% acetic acid) gave a single peak. This material was extremely active in the chick embryo fibroblast assay, and it exhibited a single band

with a M_r of approximately 7500 on SDS polyacrylamide gel electrophoresis (PAGE) (12.5% gel) using the BioRad silver stain kit.

Cell cultures. Two clones of Yaffe's L6 cell line (43) were used in these experiments. For most studies, the rapidly fusing L6-A1 cells were employed; for some measurements of proliferation and rIGF-II binding, the much more slowly fusing parental L6 cells were used. Initial cell plating densities were varied to suit the different experimental determinations and are specified accordingly. In most cases, cells were plated in Dulbecco's modified Eagle's medium (DMEM) containing 5% horse serum, 1% chick embryo extract, and 1% antibiotic-antimycotic solution. After 18 to 24 h at 37° C, the cultures were washed once (at least 15 min at 37° C) with serum-free DMEM and the specified additions made in DMEM containing 0.5 mg/ml bovine serum albumin (BSA), which had been tested and shown free of detectable mitogens or materials that competed with rIGF-binding (both have been found in some batches of BSA). In all cases, the volume of DMEM used was adjusted for the size of the chamber so that there was 1.0 ml medium/ 4.0 cm^2 of culture surface.

Quail primary myoblasts were prepared from breast muscle by differential trypsinization as described by Konigsberg (21). Cells were plated on gelatinized carbon-coated cover slips at 4 $\times 10^4$ cells/ml in 15% horse serum and 10% chick embryo extract. Sephadex-purified DI (10 μg/ml) was added at plating time to some cultures. We consistently found that a higher concentration of DI (3.3 times that used with L6 cells) was required for inhibition of differentiation in primary myoblasts than was needed for L6 cells. After 24 h the plates were washed with DMEM and cells treated with DMEM supplemented with 5% horse serum, 3% chick embryo extract, and DI as specified. Fresh medium was added daily.

Measurement of multiplication stimulating activity (MSA) in chick embryo fibroblasts. Our standard MSA assay is a modification of the procedure of Dulak and Temin (6). Third or fourth passage CEF cells were plated in 24-well (2 cm^2) multiwell plates in 0.5 ml DMEM containing 0.4% fetal bovine serum (FBS) and allowed to grow with no medium changes for 4 or 5 d. On the day of the assay, medium was changed to fresh serum-free DMEM containing the fractions being tested in a final volume of 0.5 ml. To this was added 0.25 μCi of [^{3}H]thymidine (sp act 25 Ci/mmol) in 0.05 ml of DMEM, and the cultures were incubated 18 h. At the end of this time, the cultures were washed rapidly three times with cold phosphate buffered saline (PBS), and treated with cold 10% trichloroacetic acid (TCA) for at least 10 min; this was followed by three rapid washings with 1% trichloroacetic acid. The plates were allowed to drain and the precipitated cellular material then dissolved in 0.4 ml 0.5 *M* NaOH. After at least 2 h, 0.3 ml of this solution was added to a minivial, acidified (to suppress fluorescence) with 0.1 ml of 5 *M* HCl, and suspended in 4 ml of Budget-Solve (RPI, Inc., Mount Prospect, IL). Samples were counted in a Beckman Model 7500 liquid scintillation counter; counting efficiency was monitored by the channels ratio technique. Results were expressed as percent of the incorporation by untreated control cells. Cells treated with 1% horse serum served as positive controls; their incorporation was normally 300 to 400% of control values.

Measurement of amino acid uptake. These determinations were done in 2-cm^2 wells (24-well plates) in which cells were plated at 2.5 $\times 10^4$ cells/well. At the specified times after addition of the materials being assayed, medium was removed, 0.3 ml of [^{3}H]AIB (0.2 m*M*, 3 μCi/ml) in Earle's balanced salt solution was added, and the cultures were incubated for 5 min at 37° C. Uptake of the amino acid was terminated by removing the labeling medium and washing the cultures rapidly three times with cold PBS. The cells were dried, solubilized, and counted as described under the thymidine incorporation assay.

Measurement of myoblast proliferation. In some experiments, slow-fusing L6 cells were plated at 1×10^5 cells/8 cm^2 (35 mm diam) tissue culture dishes. The cells were incubated and washed as described above and the specified materials added to the cultures at zero time. Cell number was determined 48 h later, using a Model ZBI Coulter counter and the trypsinization method detailed by Florini and Roberts (14). Where indicated, the more rapidly fusing L6-A1 clone was used; in these cases, cells were counted 24 to 28 h after hormone administration.

Assay of creatine kinase activity and DNA content. For this assay, L6-A1 myoblasts were plated at 1×10^5 cells/8 cm^2 tissue culture plate. After the indicated incubation periods, myoblast cultures were rinsed twice with phosphate buffered saline (pH 7.2), covered with 250

μl of glycylglycine buffer (0.05 *M*, pH 6.8), and stored at −20° C. On the day the assays were done, cells were thawed, scraped from the dishes with a rubber policeman, and vortexed. Aliquots (33 μl) were assayed for CK activity by the NADP-coupled method of Shainberg et al. (35) and 100 μl of the remaining suspension was taken for analysis of DNA (17).

Quantitation of myoblast fusion. In cultures prepared as described in the preceding paragraph, percent nuclei in myotubes was determined to evaluate the extent of fusion. Cultures were fixed in 100% methanol and stained with Wright's stain followed by Giemsa and nuclei in random fields were counted until a total of at least 800 nuclei was reached for each sample. Structures containing three or more nuclei were scored as myotubes.

Immunofluorescence. Cell monolayers were washed several times in Hanks' balanced salt solution (HBSS), fixed in 75% ethanol, treated for 2 min with 98% ethanol/ether (1:1 vol/vol) and air-dried (41). Permeabilized cells were then reacted for 15 min at room temperature with the specified antibodies. After washes in HBSS, the cells were reacted with the appropriate FITC or TRITC coupled goat anti-antibodies. After further washes in HBSS, the cultures were mounted in glycerol and viewed and photographed using a Leitz Ortholux microscope equipped with phase and epifluorescence optics using specific filters for the visualization of fluorescein and rhodamine fluorescence.

Measurement of [^{125}I]rIGF-II binding. For these determinations, 10^5 cells/2 cm^2 well were plated in 24-well plates. Labeled rIGF-II was prepared by treating 2 μg of rIGF-II with 2 mCi [^{125}I]NaI using the Pierce Iodobead procedure, followed by purification by gel filtration on Sephadex G-25. The labeled rIGF-II fractions were combined and stored at −20° C. Just before use, an aliquot was diluted with binding buffer (DMEM containing 3 mg/ml HEPES and 6 mg/ml BSA, adjusted to pH 7.4) to 9 ml and treated with approximately 90 mg of Dowex-1 anion exchange resin (200 to 400 mesh) to remove free ^{125}I. This solution was vortexed and centrifuged in a IEC Centra-7R centrifuge at 2000 rpm for 15 min and the supernatant fluid used for binding studies. To each well, the following additions were made: 225 μl of binding buffer containing the test sample followed by 3×10^{-2} μCi of [^{125}I]rIGF-II in 75 μl of fresh binding buffer. Incubation was at 37° C for 1 h. At the end of that time, the labeled medium was removed and the cell surfaces washed four times with cold PBS. Cells were solubilized as described under the thymidine assay and samples were counted in a Beckman Model 4000 gamma counter. Displacement was compared to a standard curve at concentrations from 10 to 300 ng/ml, and results are plotted as relative amounts of rIGF-II present.

Measurement of protein degradation. Effects of rIGF-II on L6 myoblast protein degradation were measured by a technique derived from that of Ballard (1). The L6 myoblast cultures were established in 24-well plates, washed once with serum-free DMEM, and then incubated with DMEM containing 1% horse serum and 10 μCi [^{3}H]leucine for 20 h to label intracellular proteins. The surface was washed three times with DMEM containing 2 m*M* unlabeled leucine, and the cells were then incubated for 3.5 h at 37° C in 0.5 ml of DMEM containing 2 m*M* leucine to allow decay of rapidly degraded proteins. At the end of this period, cell surfaces were washed with 1.0 ml DMEM and the medium was replaced with DMEM containing 2 m*M* leucine, 0.5 mg/ml BSA, and rIGF-II as specified.

At the end of the 4 h incubation, medium from each well (0.5 ml) was transferred to a conical centrifuge tube, mixed with 0.25 ml of H_2O containing 2.5 mg of carrier BSA, and treated with 0.25 ml of 50% (vol/vol) TCA. After at least 1 h at 4° C, the precipitates were removed by centrifugation, and 0.5 ml aliquots of the supernatant fluid were counted in 4.0 ml of Budget-Solve.

The cell layer was treated with 1.0 ml of 10% TCA immediately after removal of the supernatant fluid. After at least 1 h at 4° C, the plates were washed three times with 1% TCA, shaken dry, and then solubilized and counted as described under the CEF assay above. Counting efficiencies of representative samples were determined by the internal standards procedure; they averaged 33.3% for the solubilized cells and 18.0% for the TCA-containing supernatant fluid. With appropriate calculations for differences in counting efficiency and portion of the total sample counted, the portion of protein radioactivity released during the 4 h incubation was calculated for each well as described by Ballard (1).

Incorporation of leucine and uridine into macromolecules. The L6-A1 cells were plated at 2.5 × 10^4 cells/2 cm^2 well (24-well plates) in DMEM containing 5% horse serum and 1% chicken embryo extract (CEE). After 24 h, the cells were

washed with 1 ml DMEM containing 0.5 mg/ml BSA and then incubated with or without rIGF-II at 1 μg/ml. At various specified times, either [^{3}H]leucine (3 μCi/well) or [^{3}H]uridine (2 μCi/well) was added in 25 μl of DMEM. One hour later the wells were washed and the culture processed as described under the assay for MSA activity described above.

RESULTS

Multiple effects of rIGF-II on myoblasts can be attributed to molecules of identical M_r. In our initial purification procedures, we routinely pooled all fractions that showed mitogenic activity in the chick embryo fibroblast assay and eluted after the DI peak. When this pool of active materials was rechromatographed on a long (3.2 × 185 cm) column of Fractogel TSK-55(F), a broad A_{280} peak was observed (in the molecular weight range of 32 000 to 7 000), and the mitogenic activity closely followed UV absorbancy throughout the peak (Fig. 1). Analysis of the fractions by SDS gel electrophoresis indicated the presence of four major bands, with M_r's approximately 31 500, 20 000, 13 500, and 7500. The larger forms seemed likely to be multimeric forms of rIGF-II held together by disulfide (or other covalent) bonds that were not dissociated by SDS, and some initial experiments with mercaptoethanol indicate that this may be the case. These very large forms were not reported in previous characterizations of BRL cell media (6,31); possibly they form under the conditions in which we handle BRL cell-conditioned medium.

For detailed comparisons of a number of the

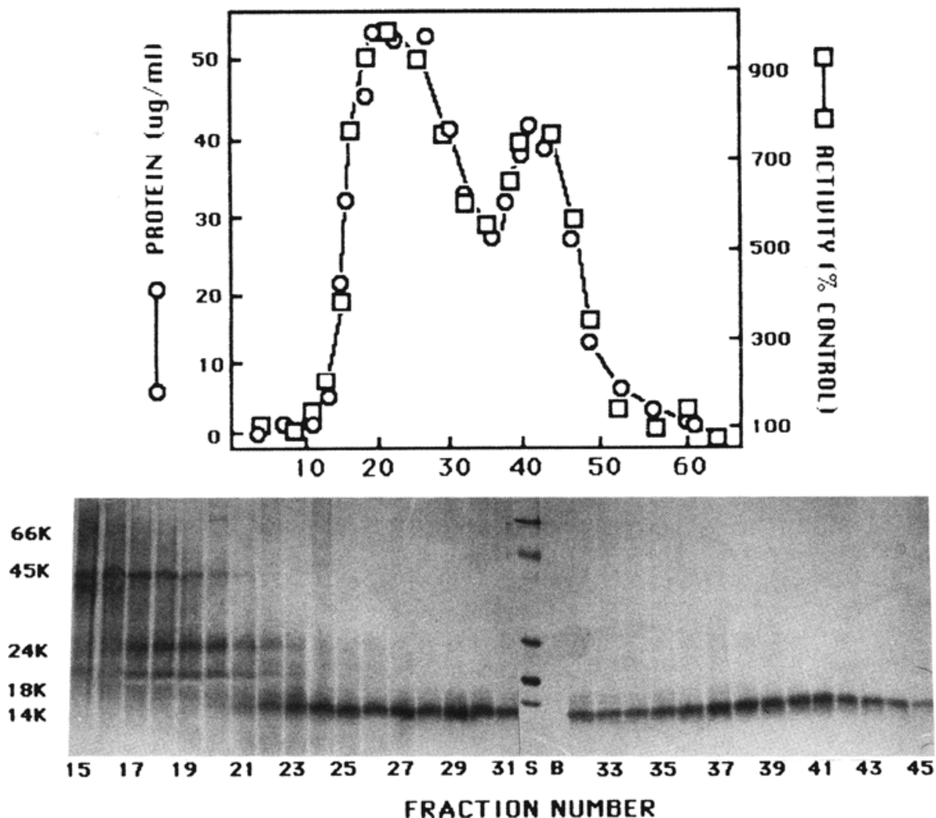

FIG. 1. Sodium dodecyl sulfate gel electrophoresis and mitogenic activity of Fractogel fractions or rIGF-II. Mitogenically active fractions from 12 routine rIGF-II preparations were combined and rechromatographed on a large (3.2 × 185 cm) column of Fractogel TSK HW-55(F). Fractions were assayed for mitogenic activity and analyzed by SDS gel electrophoresis as described under Materials and Methods. Staining was done using the BioRad silver staining kit. "S" and "B" label standards and a blank lane on the gel.

actions of rIGF-II on muscle cells, we concentrated on the putative 7 500 mol wt monomer. To do this, we pooled the most slowly eluting fractions, which exhibited a single band on SDS gel electrophoresis and a single peak on high performance liquid chromatography (HPLC) under the conditions used by Marquardt et al. (24). The material was rerun on the Fractogel column, and the UV absorption presented in Fig. 2 was recorded during elution. Fractions throughout the peak area were analyzed for activity in five assays as summarized in Fig. 2. All of these activities

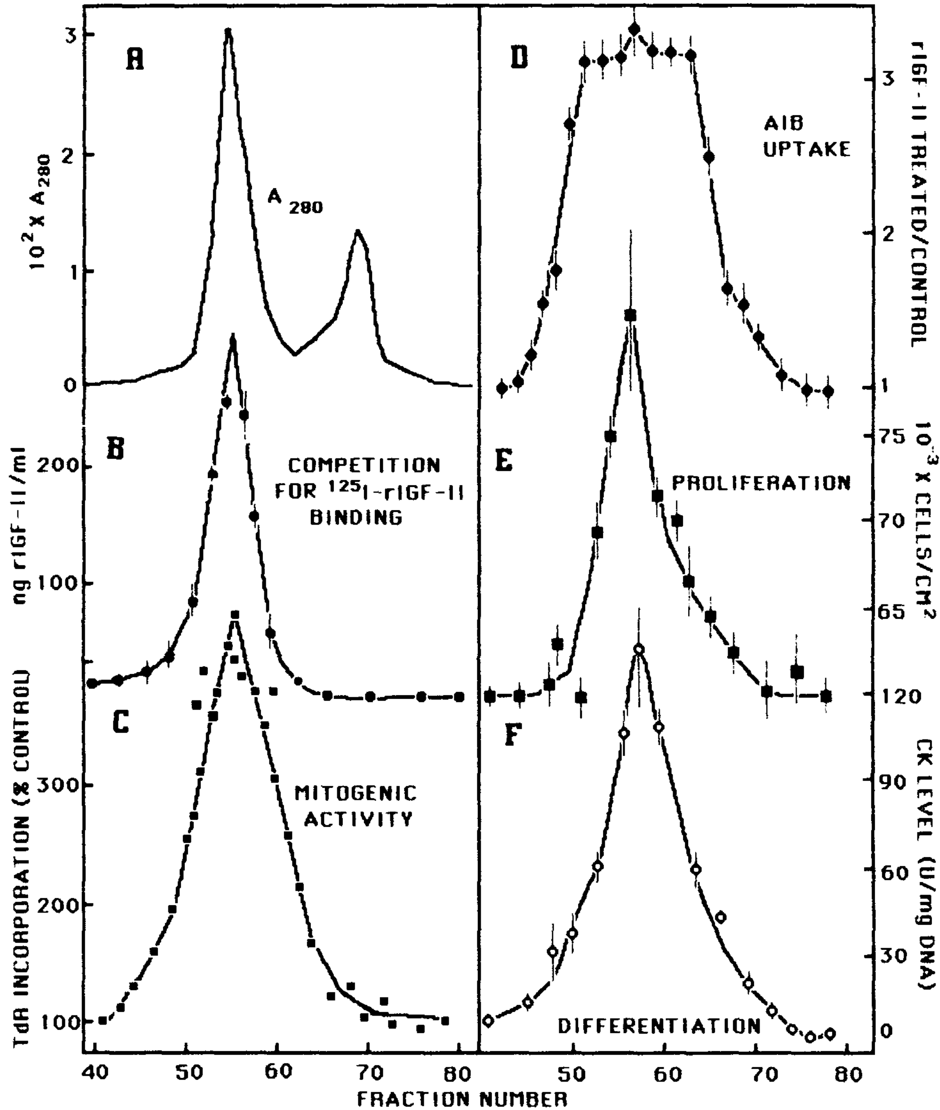

FIG. 2. Activity in various assays of rIGF-II fractions from a Fractogel column. Fractions 35 to 50 from the column shown in Fig. 1 were combined and rerun on the large Fractogel column. From even numbered, 10-ml fractions 25-μl aliquots were taken for each of the assays presented; the assays were done as described under Materials and Methods. The Radioreceptor Assay (RRA) and proliferation determinations were done using slow-fusing parental L6 cultures, AIB uptake and differentiation were measured in the fast-fusing L6-A1 clone, and thymidine incorporation was measured in chick embryo fibroblasts. *Points* are means and *bars* are SEM of triplicate determinations in all except the A_{280} and mitogenic activity; the former is a Pharmacia UV-1 monitor trace, and the latter are the averages of duplicate determinations.

closely followed UV absorption in the major peak (Tube 58) and the smaller material in the secondary peak (breakdown product?) was not active in any of the assays used. Thus it seems that all of these processes are stimulated by molecules that have identical mobilities upon repeated chromatography on Fractogel and seem to be homogeneous by two rather stringent criteria. The greater width of the alpha-amino-isobutyric acid (AIB) uptake curve suggests that this process was more sensitive to stimulation by rIGF-II than were cell proliferation and differentiation, but this was not confirmed in more direct experiments (*see below*).

Concentration dependence of somatomedin actions. These indications of differing concentration dependencies of rIGF-II effects were further investigated in a more direct experiment in which four major actions of somatomedins were compared. As shown in Fig. 3, there is a substantial difference in sensitivity of some of these actions, although AIB uptake is not as sensitive as we inferred from the results in Fig. 2. Suppression of proteolysis is most sensitive, followed by stimulation of cell proliferation and AIB uptake,

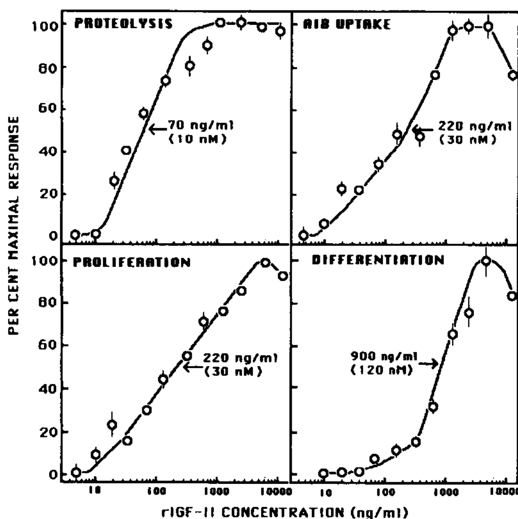

FIG. 3. Concentration dependence of several rIGF-II actions on L6 myoblasts. Highly purified rIGF-II (*see* Materials and Methods) was used in a series of parallel assays of the indicated activities, all of which were done on the same L6-A1 clone in an experiment starting on the same day. Alpha-amino-isobutyric acid uptake was measured as a 5-min pulse 5 h after hormone addition, proteolysis during the first 4 h after rIGF-II addition, proliferation after 28 h, and differentiation after 72 h. Determinations were done as detailed under Materials and Methods. Results are means ± SEM for triplicate determinations.

and the least sensitive process is differentiation. The most surprising observation is the decrease in the stimulation of differentiation at high concentrations of rIGF-II (even lower levels of CK were observed at 5 and 10 μg/ml in other experiments). This is a highly purified preparation, and it is unlikely to be contaminated by DI. We had not previously noted this biphasic response because our supplies of highly purified rIGF-II had been insufficient to allow use of such high concentrations. Initial experiments with highly purifed SM-C/IGF-I have given similar results, and incubation of rIGF-II with myoblasts maximally stimulated to differentiate with 3×10^{-7} *M* insulin gave dose-dependent inhibition of CK formation. This phenomenon is currently under study in this laboratory; it is important to us because it indicates that the insulinlike family of hormones, like other mitogens, inhibits myoblast differentiation as previously suggested by Konigsberg (20) and Linkhart et al. (22), thus removing an apparent disagreement between their results and ours.

Stimulation of proliferation and differentiation by rIGF-II does not result simply from improved cell viability. It has occurred to us—and to others—that the apparent stimulatory effects of rIGF-II on myoblasts in serum-free medium might be tissue culture artifacts resulting from hormonal support of viability or stimulation of metabolism in the hormone-treated cells. In contrast, the serum-free control cells might be dying or enduring some metabolic deprivations. We investigated this possibility in several ways: (a) Measuring effects of rIGF-II in the presence of varying concentrations of serum; (b) counting lifted cells in control and treated cultures; and (c) determining DNA content of the cultures during incubation in the presence or absence of hormone.

It was possible to find levels of serum that supported modest rates of cell proliferation (i.e. maintained cell viability) but did not mask all effects of rIGF-II. At horse serum concentrations up to 8% (vol/vol), there was small but significant stimulation of myoblast proliferation upon addition of saturating quantities of rIGF-II (Fig. 4 *A*). At 10% and higher concentrations of serum, stimulation of proliferation by serum was so great that significant additional stimulation by rIGF-II could not be detected.

The effects on differentiation (Fig. 4 *B*) are more striking and more complex. In this case, the presence in horse serum of a DI-like protein

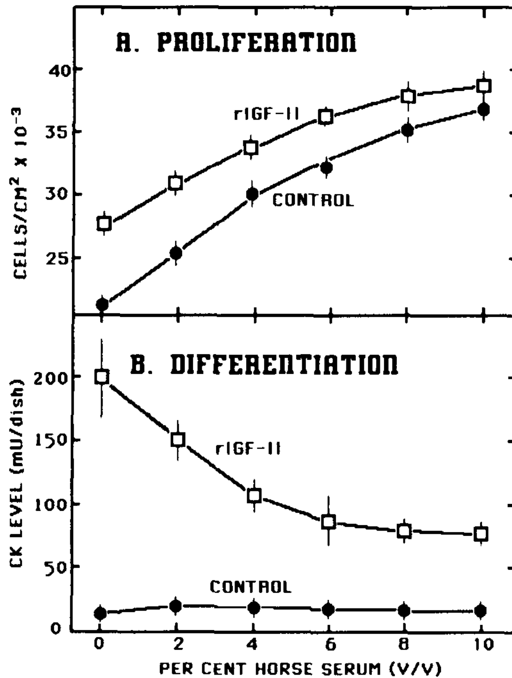

FIG. 4. Effect of horse serum on the stimulation of L6 myoblast proliferation and differentiation by rIGF-II. Cells were plated as described under Materials and Methods and then transferred to medium containing the indicated concentration of horse serum with and without rIGF-II at 1.0 μg/ml. Proliferation was measured at 48 h and differentiation at 72 h by the methods detailed under Materials and Methods.

or mitogens prevented the elevation of creatine kinase activity as serum concentration increased. However, even at the highest serum levels (which support rapid proliferation in L6 cells), there was substantial stimulation of differentiation in all cultures to which rIGF-II was added. It should be noted that addition of 2% serum gave a very slight (and not significant) stimulation of differentiation compared to no serum in control cultures. This small increase may actually reflect improved survival or metabolism, but it is far less than the stimulation of differentiation observed upon addition of rIGF-II. The striking differences between the two parts of Fig. 4 provide additional evidence that the stimulation of differentiation by rIGF-II is not simply a secondary result of the stimulation of cell proliferation and the resultant greater culture density (11).

A second indication that the stimulatory effects of rIGF-II were not due to enhanced myoblast survival was obtained in a series of experiments on the effects of rIGF-II on cell proliferation. At various times, lifted (presumably dead) cells were counted. There were relatively few of them—only 5 to 10% of the total in any case. At 36, 48, and 72 h, the numbers of lifted cells were identical (within 5% of the mean) in all comparisons of control and rIGF-II treated L6 myoblasts. Under the conditions of these experiments, L6 myoblasts (quite unlike primary myoblasts from rats or chickens) survive well in serum-free medium even in the absence of rIGF-II.

A third piece of supporting data is provided by the determinations of DNA content we do routinely to provide a basis for expression of creatine kinase levels. As shown in Fig. 5 *C*, the levels of DNA increased during the initial parts of the experiments, but both treated and control cultures reached their final DNA levels at 24 h, and neither exhibited any subsequent decrease. Taken together, all of these observations eliminate differential cell loss or impaired metabolism of control cultures as likely explanations for the stimulatory effects of rIGF-II on myoblasts. We also point out that rIGF-II does not act simply as an inducer of CK; all measured aspects of myoblast differentiation are stimulated in response to this hormone, and addition of the hormone to preformed myotubes had no effect on levels of the enzyme (11).

Time course of actions of rIGF-II on myoblasts. Elucidation of the mechanisms of IGF-II actions requires knowledge of the time course of various effects of the hormone. To get results that were directly comparable, we did a large, coordinated experiment in which the time course of rIGF-II effects was measured in the same L6 clone, at the same time, with a highly purified rIGF-II preparation. The results (summarized in Fig. 5) show an early stimulation of uptake and incorporation of amino acids into protein and incorporation of uridine into RNA plus subsequent accumulation of protein and DNA. Increases in cell number were detectable at 24 to 36 h, and stimulation of fusion and elevation of creatine kinase levels were observed 48 h after addition of rIGF-II to the cells.

These data show that muscle cell differentiation occurred in the normal sequence when L6 myoblasts were stimulated to differentiate by addition of rIGF-II in serum-free medium. It is clear that DNA accumulation ceased as the cells began to differentiate. The number of countable cells decreased strikingly as the myoblasts fused to form postmitotic myotubes, but this does not reflect cell death. Myotubes are apparently disrupted by

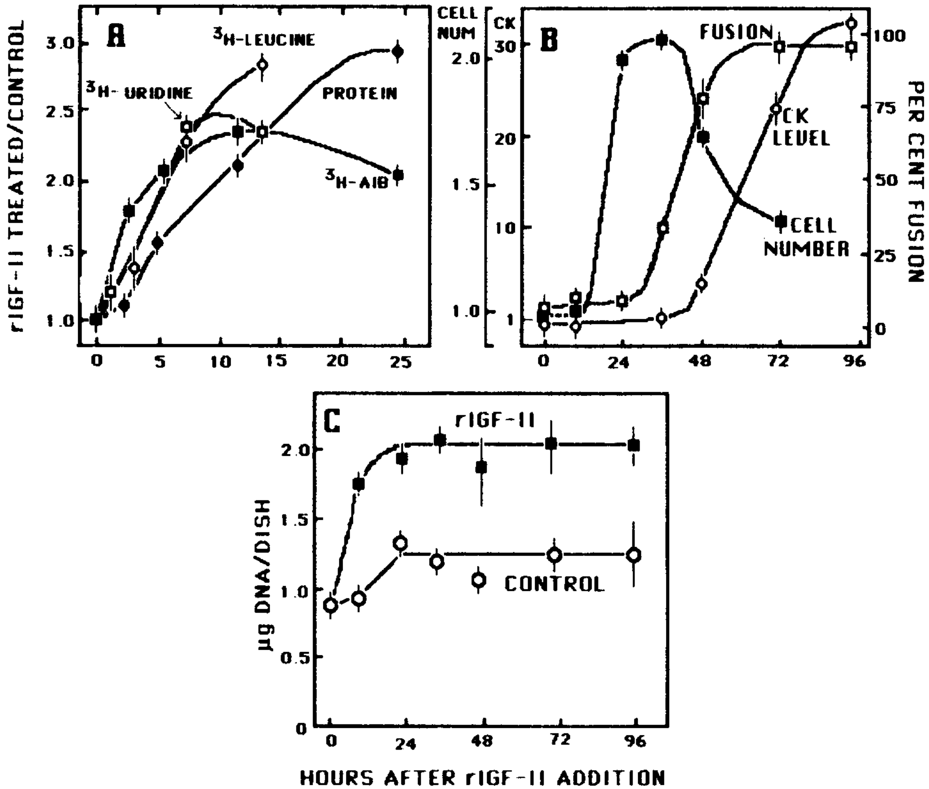

FIG. 5. Time courses of actions of rIGF-II on L6 myoblasts. Cultures were established by overnight plating in 5% serum, rinsed, and treated with rIGF-II at 1.0 µg/ml. The plating and washing conditions are detailed under Materials and Methods, as are methods for measuring each of the specified effects.

the trypsin treatment required for the determination of cell number in the Coulter counter; the constancy of DNA content of the cultures during this time (*Panel C*) indicates that there was no loss of nuclei during the time (36 to 72 h) that the number of countable cells decreased substantially.

Inhibition of myoblast differentiation by the differentiation inhibitor. In addition to rIGF-II, BRL cells secrete another protein, DI, which exhibits potent and reversible inhibition of the fusion of myoblasts to form postmitotic myotubes. Complete purification of this molecule has been elusive, but we have been able to demonstrate the reversibility and generality of DI actions using partially purified preparations.

We normally quantitate DI effects by determining creatine kinase levels; this enzyme shows up to 100-fold elevation as L6 myoblasts differentiate. (In our DI studies, we routinely add insulin at 0.3 *µM* to stimulate L6 cell differentiation in serum-free medium.) In the presence of DI, there was little change in CK activity for at least 96 h, despite the presence of insulin (Fig. 6). In other experiments, we have prevented CK elevation by daily additions of DI until the experiment was terminated at 216 h. This effect of DI was fully reversed by simply washing the cultures to remove DI and adding insulin-containing medium without DI. When DI was removed after 24 to 48 h, the cells subsequently attained CK levels that were similar to those found in normally differentiating myogenic cultures (Fig. 6). These results, and previously reported observations on the effects of the addition of DI at various times [Fig. 5 in (9)], suggest that DI blocks an early event in myoblast differentiation.

The accumulation of myosin heavy chain, the major structural protein of skeletal muscle, was examined using mouse monoclonal antibodies specific for skeletal muscle myosin heavy chain (MHC) in indirect immunofluorescence studies. As illustrated in Fig. 7 and quantified in Table

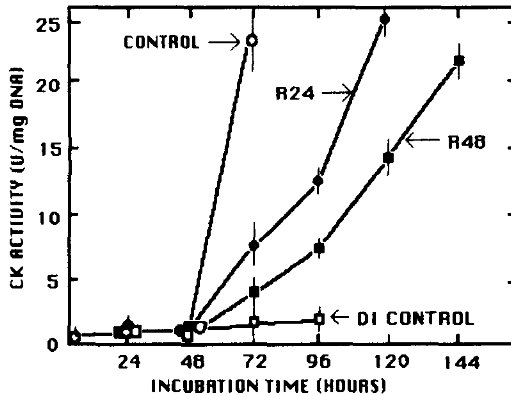

FIG. 6. Reversal of the effects of DI on L6 myoblast differentiation. L6 cells were cultured in medium containing 0.3 μM insulin in the presence and absence of 3 μg/ml DI as described under Materials and Methods. Cultures were maintained continuously in DI-containing medium (DI control), or were incubated initially in DI-containing medium (with insulin) and then transferred to insulin-containing medium without DI at either 24 h (*R24*) or 48 h (*R48*). Myoblasts were cultured in insulin-containing medium as positive controls. Cells were assayed for CK activity as described under Materials and Methods.

1, L6 control cultures (Fig. 7 *A*) fused extensively 72 h after plating. Myosin in the myotubes stained diffusely with an occasional streaking pattern (Fig. 7 *B*) presumably reflecting the formation of myofibrils, and no staining was observed in the regions occupied by nuclei. Consistently, flattened mononucleated cells of the sort shown here did not stain with MHC antibodies.

In contrast, incubation of L6 myoblasts with DI inhibited both fusion (Fig. 7 *C*) and MHC accumulation (Fig. 7 *D*). In cultures incubated with DI, we occasionally observed individual cells (usually binucleated) that stained for MHC; these "escaped" cells comprised a minor portion of the population (less than 2%), which did not increase in number with incubation time. Inhibition of differentiation was reversed upon further incubation of cells in medium lacking DI. After 72 h, fusion (Fig. 7 *E*) and the amount and distribution of accumulated myosin (Fig. 7 *F*) were similar to those properties of control cultures. In both control and DI-reversed cultures a very small percentage of bipolar mononucleated cells contained MHC in apparent intracellular pools (Fig. 8).

Similar effects of DI were observed with primary quail muscle cells (Fig. 9). At 88 h after plating, myoblasts in control cultures had fused to form myotubes that contained well-organized myofibrils (Fig. 9 *A*). Antibodies against MHC demonstrated the presence of myosin, which stained in the characteristic myofibril banding pattern (Fig. 9 *B*). Strongly periodic banding of myomesin, a myofibrillar M-line protein (37), was revealed by the localization of myomesin antibodies (Fig. 10 *A*). The remaining mononucleated cells probably included both myoblasts that had not yet fused and contaminating fibroblasts that synthesized Type I collagen (Fig. 9 *C*). Differentiation inhibitor-treated myoblasts at 88 h remained mononucleated (Fig. 9 *D*), flattened considerably, and contained large amounts of intracellular Type I collagen (Fig. 9 *F*), thus making it impossible to distinguish myoblasts from fibroblasts either by morphology or staining for Type I collagen. Virtually no staining for MHC (Fig. 9 *E* and Table 2) or for myomesin (Fig. 10 *B*) was observed in these cells. The few cells in DI-treated cultures that did stain for MHC or myomesin were mostly bi- or multinucleated.

Differentiation inhibitor did not preferentially select for fibroblast survival in the quail cell cultures, and its effects on myoblasts were fully reversible. When cultures treated with DI for 72 h were switched to medium without DI, cells fused to form myotubes (Fig. 9 *G*) that accumulated myosin (Fig. 9 *H*) and myomesin (Fig. 10 *C*) in a manner similar to that of cells in the control cultures. Immunofluorescent staining revealed that the myofibrils present in myotubes formed after release from DI treatment were as well organized as those formed in control cultures. The distribution of MHC and myomesin in control and DI-reversed cultures reflected this organization. The proportion of the cell population that fused after DI removal was essentially identical to that in the control cultures (Table 2). In contrast, myoblasts kept in DI-containing medium did not undergo phenotypic changes characteristic of the normal program of myogenesis. When these myoblasts were finally released from DI treatment, their behavior was similar to that of untreated myoblasts; they fused, accumulated CK, and formed cross-striated myofibrils characteristic of skeletal muscle.

Occurrence of DI in sera from various species. If we are to refer to DI as a hormone (as is done in the title of this paper), it should be found in the circulation. Using our standard batchwise Dowex-50 purification followed by chromatography on Sephadex G-75, we have found DI activity in parallel fractions in preparations from

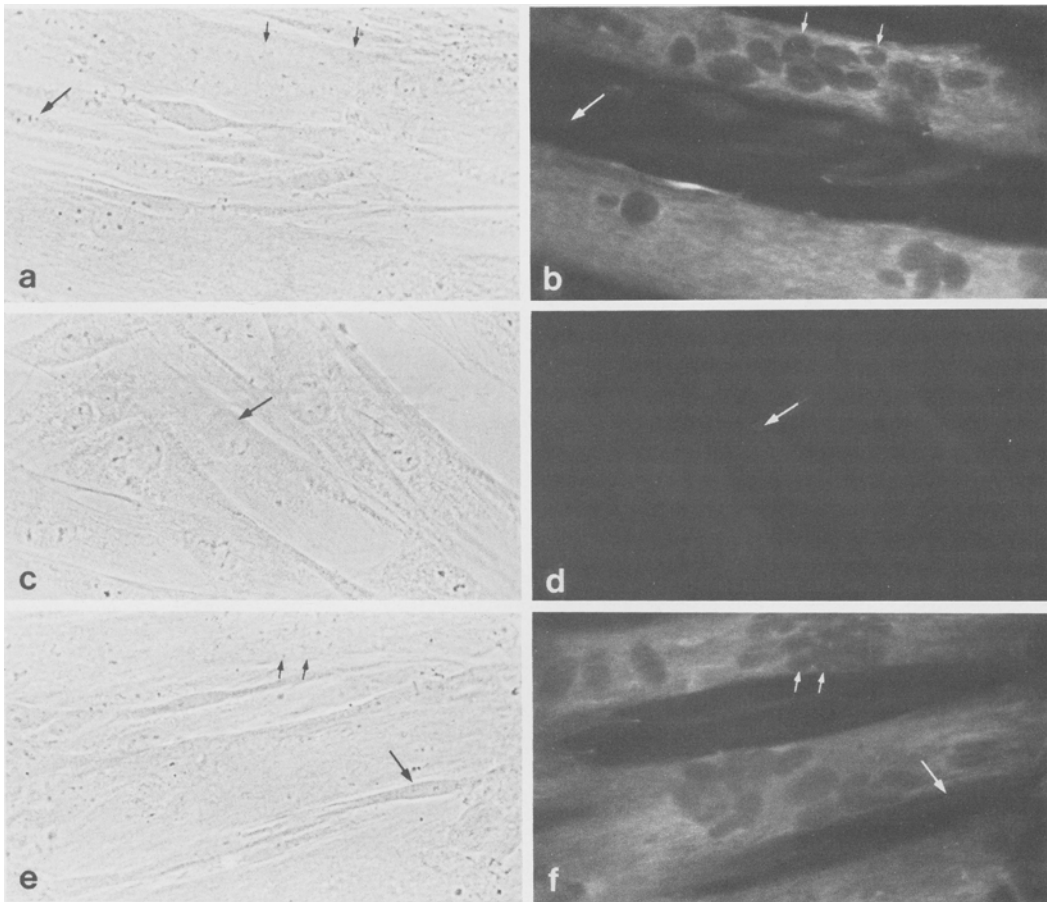

FIG. 7. Effects of DI on fusion and myosin heavy chain accumulation in L6 muscle cells. L6 myoblasts were cultured in medium containing 0.3 μM insulin in the presence or absence of 3 μg/ml DI as described under Materials and Methods. After 72 h the cells were fixed in alcohol, prepared for immunofluorescence, and immunolabeled with mouse monoclonal antibodies to MHC and TRITC goat antimouse IgG as described under Materials and Methods. Phase and fluorescence micrographs of control cultures (*A*, *B*) and DI-reversed cultures (*E*, *F*) show multinucleated myotubes and accumulation of MHC. Corresponding DI-treated cultures (*C*, *D*) did not fuse and did not stain with MHC antibodies. Nuclei in multinucleated myotubes (*small arrows*) and mononucleated myoblasts (*large arrows*) are indicated.

chick embryo extract, FBS, rat hepatocyte conditioned medium, and sera from rats, cows, horses, and humans. Sodium dodecyl sulfate-gel electrophoresis revealed a substantial band at about 32 300 M_r in each of these preparations. Identical heat-denaturation curves were obtained in comparisons of the material from human serum and BRL cell conditioned medium; activities of both were unchanged upon heating for 15 min at a series of temperatures up to 80° C; they were about 50% inactivated at 90° C and totally inactivated at 100° C. Obviously, these indications of identity require confirmation by immunological methods; we will pursue this point when purification of the DI has been achieved.

DISCUSSION

In recent years, members of this group have published a number of papers documenting the effects of rIGF-II (previously designated MSA) on proliferation and differentiation of muscle cells. However, the length of time elapsed and the number of separate individuals (both investigators and cultures) involved made it difficult to be certain that the results were directly comparable. In this paper, we report a series of

TABLE 1

FUSION AND MYOSIN HEAVY CHAIN ACCUMULATION IN L6 CULTURES[a]

Condition	Fusion, %	MHC		
		Number Positive	Number Negative	Percent Positive
Control	90.0	965	174	84.7
DI treated	3.0	20	1230	1.6
DI reversed	89.1	1012	150	87.1

[a] Cells were plated as described under Fig. 7. Percent fusion was determined as described under Materials and Methods. The percent positive for MHC was determined by counting the number of nuclei present in cells that stained for MHC and dividing that number by the total number of nuclei counted. At least 1000 nuclei were counted for each determination.

closely coordinated experiments (using cloned cells and highly purified rIGF-II) that were designed to provide direct comparisons of the various actions of rIGF-II on L6 myoblasts. We have investigated the molecular specificity, concentration dependence, and some aspects of the mechanism(s) of these actions. We have also eliminated a possible trivial explanation for some of the actions of rIGF-II on myoblasts.

We conclude from the experiments reported here that the smallest detectable mitogen secreted by BRL cells [apparent M_r 7500, presumably corresponding to the 7484 dalton molecule sequenced by Marquardt et al., (24)] is capable of eliciting all of the responses we have described in this and previous papers (Fig. 2). There were, however, substantial differences in the sensitivity of the various responses (Fig. 3). Inhibition of proteolysis exhibited a half-maximal response at about 10 n*M*, stimulation of proliferation and AIB uptake were nearly as sensitive (30 n*M*), whereas 120 n*M* rIGF-II was required for half-maximal stimulation of differentiation. In contrast, Klapper et al. (18) found a greater sensitivity of AIB uptake compared to thymidine incorporation in studies of the effect of somatomedin-C/IGF-I on fibroblasts. It is tempting to attribute highly sensitive effects to interaction of rIGF-II with homologous Type II receptors (26) and to assign the less sensitive actions to cross-reaction with the Type I receptor. Initial experiments (Ewton and Florini, work in progress) have demonstrated the presence of a Type II receptor on L6 myoblasts, and we expect soon to test for the Type I receptor.

There were large differences in the time course of the various effects (Fig. 5). Stimulation of amino acid uptake and incorporation into protein occur early, and net accumulation of protein follows. As might be expected for a hormone initially isolated as the primary mitogen in serum (29), net accumulation of DNA was observed, and this was followed by an increase in cell number. Finally, there was a substantial increase in differentiation of the myoblasts to form postmitotic myotubes, with large increases in marker proteins such as creatine kinase,

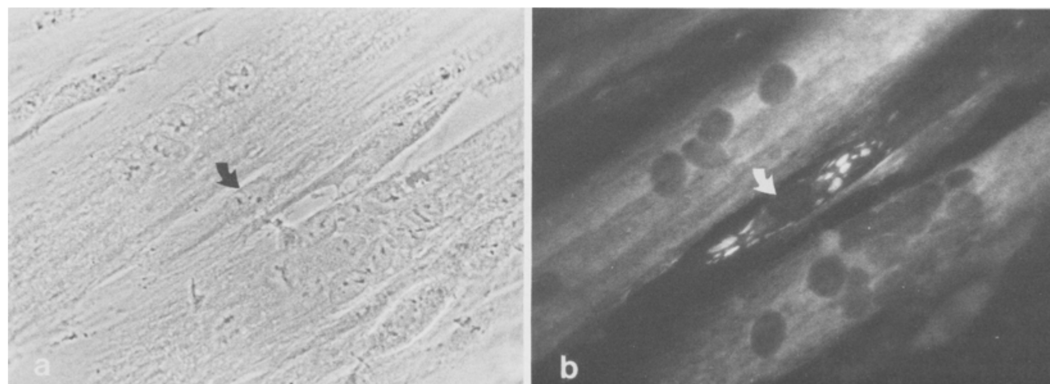

FIG. 8. Myosin accumulation in an unfused L6 myoblast. L6 cells were plated as described under Materials and Methods. Seventy-two hours after removal of DI, the cells were prepared for immunofluorescence and stained with mouse monoclonal anti-MHC followed by TRITC goat antimouse IgG as described under Materials and Methods. Occasionally we observed a mononucleated myoblast (*arrow*) that contained accumulated myosin in apparent cytoplasmic pools and not in organized myofibrils.

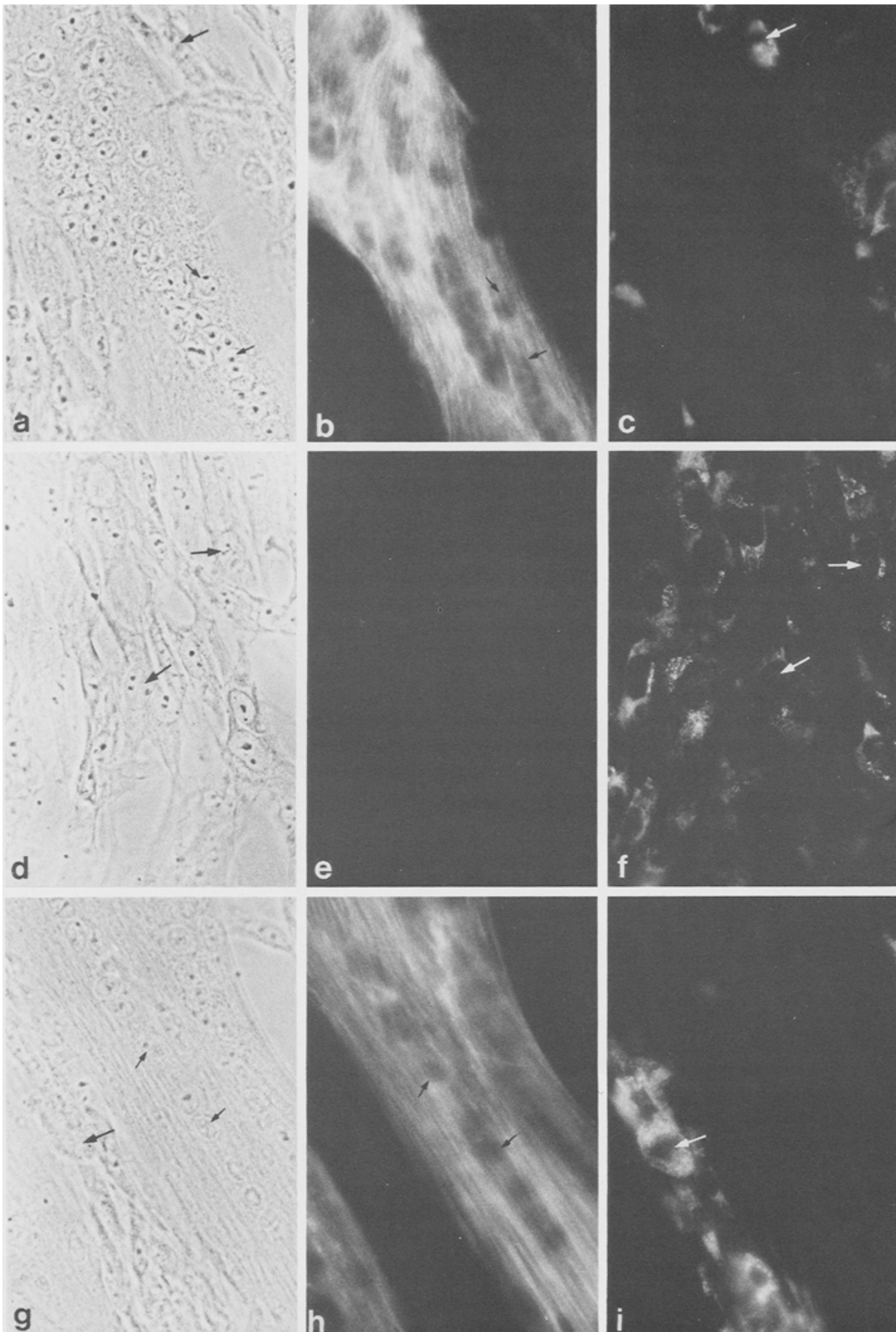

FIG. 9. Effects of DI on fusion, myosin heavy chain and Type I collagen accumulation in quail muscle cultures. Quail muscle cells were cultured in the presence or absence of DI as described under Materials and Methods. After 88 h in culture, the cells were fixed in alcohol and prepared for immunofluorescent staining. Samples were incubated with antibodies in the following order: (1) mouse monoclonal anti-MHC, (2) TRITC-coupled goat antimouse IgG, (3) guinea pig anti-Type I collagen, (4) FITC-coupled goat antiguinea pig IgG. Immunolocalization of MHC (*B, E, H*) and Type I collagen (C, F, I) is presented. Phase contrast micrographs (*A, D, G*) are shown to the left for each series. Myotubes in both the control (*A, B*) and DI-reversed (*G, H*) cultures stain heavily for MHC, whereas the DI-treated cells do not fuse (*D*) and show no accumulation of MHC (*E*). Nuclei present in myotubes are indicated by *small arrows*. Mononucleated cells (*large arrows*) present in all the cultures stain for Type I collagen.

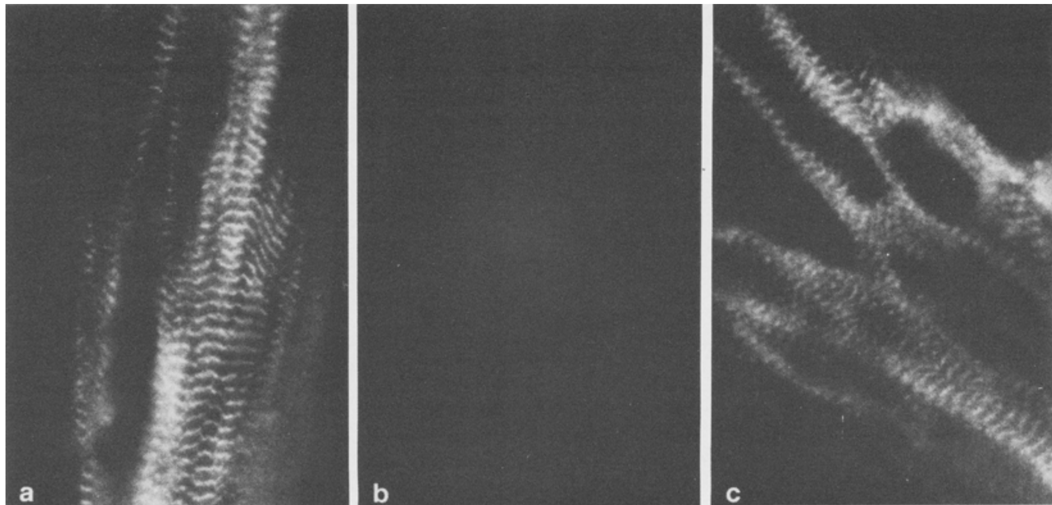

FIG. 10. Effect of DI on myomesin accumulation in quail muscle cultures. Quail muscle cells were cultured for 88 h in the presence or absence of 10 μg/ml of DI, fixed with ethanol, and prepared for immunofluorescent staining as described in Materials and Methods. Samples were incubated in rabbit antimyomesin, washed in HBSS, and incubated in TRITC-coupled goat antirabbit IgG. A periodic banding pattern was observed for myomesin staining of myofibrils in both control (*A*) and DI-reversed (*C*) cultures. In contrast, muscle cells treated with DI did not stain with myomesin antibodies (*B*).

myosin heavy chain (Figs. 7 and 9), and myomesin (Fig. 10). We have previously shown by two distinct approaches (11,39) that the stimulation of differentiation is not simply secondary to the larger number of cells resulting from the mitogenic action of rIGF-II. A very recent paper (12) demonstrates that a part of the mechanism by which rIGF-II stimulates differentiation is the induction of ornithine decarboxylase and the consequent elevation of cellular polyamine levels.

TABLE 2

FUSION, MYOSIN HEAVY CHAIN, AND TYPE I COLLAGEN ACCUMULATION IN QUAIL SKELETAL MUSCLE CULTURES[a]

Condition	Fusion, %	MHC Positive Number	MHC Positive %	Type I Collagen Positive Number	Type I Collagen Positive %	Total Counted
Control	75.6	1256	83.5	252	16.8	1508
DI treated	0.2	11	0.8	1358	99.2	1369
DI reversed	81.3	1018	86.3	161	13.7	1179

[a] Experimental details are described under Fig. 9. Percent fusion was determined as described under Materials and Methods. The number of cells positive for MHC or Type I collagen staining reflects the number of nuclei present in cells that stained for either of these two proteins divided by the total number of nuclei counted.

This cannot be the entire mechanism, because addition of exogenous polyamines does not induce differentiation in the absence of rIGF-II.

Relatively little work has been done in other laboratories on effects of somatomedins on muscle. Schmid et al. (33) have recently reported stimulation of myoblast differentiation by human IGF-I and IGF-II. Unfortunately, the preparation of "myoblasts" (body walls of 10-d chick embryos) was substantially contaminated with fibroblasts, so these investigators could not distinguish between effects on proliferation and on differentiation, and their dose-response data are too limited to reveal any differences in sensitivity of the myoblasts to IGF-I and IGF-II. Using the nonfusing BC_3H-1 myoblast line, De Vroede et al. (4) have shown that these cells possess Types I and II IGF receptors and respond to these hormones with increases in amino acid and glucose uptake. The groups headed by Ballard, Gunn, and Etlinger have all shown that our preparations of rIGF-II exhibit high potency in decreasing the rate of protein breakdown in chick and rat muscle cells. Janeczko's and Etlinger's observations have recently been published (16), and they demonstrate a very high sensitivity to rIGF-II for protein degradation in chick myotubes.

Yu and Czech (44) have reported stimulation by IGF-I of AIB and glucose uptake in isolated

rat soleus muscle and concluded that these effects are mediated by the Type I receptor. Poggi et al. (30) reported a similar stimulation of glucose utilization in mouse soleus muscle. It is somewhat surprising that stimulation could be detected in muscles from normal (rather than hypophysectomized) animals; injected growth hormone usually has little effect on rapidly growing young intact rats. Both of these reports must be viewed with some reservations because it was not possible to eliminate effects on nonmuscle cells in the muscles that were studied and it is possible that connective tissue and blood vessel cells contributed significantly to the observed responses.

In addition to rIGF-II, BRL cells in serum-free medium also secrete a nonmitogenic protein that inhibits myoblast differentiation (9). Differentiation inhibitor is a potent inhibitor of fusion; in our most purified preparations, the addition of 200 ng/ml gave 97% inhibition. Fusion can be inhibited to a similar extent by the incubation of myoblasts in calcium-depleted medium (34,38) or by the addition of cytochalasin B to the medium (19,42). Under some of these conditions, myoblasts synthesize and accumulate muscle-specific proteins even though they do not fuse. In contrast, myoblasts treated with bromodeoxyuridine (BUdR) or dimethyl sulfoxide (DMSO) neither fuse nor synthesize muscle-specific products (2,23,36). Thus, inhibitors of fusion in some cases block and in other cases do not block other changes characteristic of myogenesis. For this reason we also examined several additional and independent parameters of muscle differentiation: elevation of creatine kinase, accumulation of myosin heavy chain and myomesin, and disappearance of Type I collagen.

Addition of DI to the culture medium also prevented the increase in CK activity characteristic of postfusion muscle cells; only very low levels of CK activity were measurable after 96 h in cultures containing Di (Fig. 6). (In other experiments, we have prevented myoblast differentiation—measured both as fusion and CK elevation—by adding fresh DI at 24-h intervals for periods as long as 9 d.) Upon removal of DI from the culture medium, myoblasts fused and exhibited levels of CK about equal to those in control cultures.

Although our enzymatic assay of CK activity was a useful measure of differentiation, it provided only the average of enzyme levels in the entire cell population. To assess possible differences in response of individual cells, we also measured myosin heavy chain, myomesin, and Type I collagen accumulation using indirect immunofluorescence. After 88 to 96 h in control medium, quail skeletal muscle cells were fused and contained well-organized myofibrils that stained for MHC by indirect immunofluorescence; they exhibited clear banding patterns. In L6 myoblasts, staining was more diffuse, suggesting that the myofibrils were not as well organized as in the quail myotubes. In addition, we observed pools of MHC in the cytoplasm of a few mononucleated L6 myoblasts (Fig. 9). These may have been myoblasts in the process of fusing; similarly stained myoblasts have been observed occasionally during reversal of DI actions.

In the presence of DI, very few cells (usually bi- or trinucleated) stained for MHC (quail: 0.8%; L6: 1.6%). These MHC-positive cells probably represent the portion of the population that was already synthesizing myosin before or shortly after the addition of DI. Our previous study of the time course of DI addition demonstrated the necessity that it be added before any detectable differentiation occurred (9). On the other hand, our results do not eliminate the possibility that the MHC-positive cells represent a small DI-resistant subpopulation of myoblasts.

In DI-treated cultures, quail myoblasts were indistinguishable from fibroblasts (Fig. 9). Both myoblasts and the few contaminating fibroblasts in the quail muscle cultures were very flattened, and stained extensively for intracellular Type I collagen. Quail myoblasts in culture have been reported to synthesize Type I collagen before fusion (32) and mononucleated cells in our untreated quail muscle cultures also showed staining for Type I collagen by indirect immunofluorescence. The L6 myoblasts, too, were considerably flattened when incubated in the presence of DI; in this case, it was not possible to assess Type II collagen accumulation because our antibodies to avian Type I collagen do not cross-react with the rat protein. These effects on Type I collagen synthesis and cell morphology remained unchanged as long as DI was present in the medium, yet were completely reversed upon switching to DI-free medium. Apparently, BUdR (23) and DMSO (2) have morphological effects similar to those of DI on myoblasts in culture. In both instances myoblasts flatten after treatment and, in the case of BUdR addition, synthesize large amounts of Type I collagen (23).

Myotubes already formed are not affected by

the addition of DI to the culture medium. Myofibrils remain intact in the presence of DI, and CK levels remain elevated. In contrast, continued exposure of myotubes to the tumor promoter tetradecanylphorbol acetate (3) caused a disassembly of myofibrils and a decrease in CK activity.

Reversal of the effects of DI after its removal from the culture medium was apparently complete, though not immediate (Fig. 6). Differentiation inhibitor-treated myoblasts were not ready to fuse immediately after removal of DI, as has been reported when fusion was inhibited by growth in calcium-depleted medium (7,34). Accumulation of muscle-specific proteins was substantially delayed after removal of DI, further supporting previous indications (9) that DI acts at an early stage of skeletal muscle differentiation. It is also possible that the time lag after DI removal represents the period required for myoblasts to recover from DI treatment.

The data presented in this paper show that BRL cells secrete, and serum from several species contains, two proteins that have major effects on muscle cell differentiation. The somatomedins are potent stimulators of differentiation (measured as fusion and CK accumulation), whereas the DI prevents several changes characteristic of differentiating muscle.

REFERENCES

1. Ballard, F. J. Regulation of protein accumulation in cultured cells. Biochem. J. 208: 275–287; 1982.
2. Blau, H. M.; Epstein, C. J. Manipulation of myogenesis in vitro: Reversible inhibition by DMSO. Cell 17: 95–108; 1979.
3. Croop, J.; Toyama, Y.; Dlugosz, A. A.; Holtzer, H. Selective effects of phorbol 12-myristate 13-acetate on myofibrils and 10-nm filaments. Proc. Natl. Acad Sci. USA 77: 5273–5277; 1982.
4. De Vroede, M. A.; Romanus, J. A.; Standaert, M. L.; Pollett, R. J.; Nissley, S. P.; Rechler, M. M. Interaction of insulin-like growth factors with a nonfusing mouse muscle cell line: binding, action, and receptor down-regulation. Endocrinology 114: 1917–1929; 1984.
5. Dulak, N. C.; Shing, Y. W. Large scale purification and further characterization of a rat liver cell conditioned medium multiplication stimulating activity. J. Cell. Physiol. 90: 127–138; 1977.
6. Dulak, N. C.; Temin, H. M. Multiplication-stimulating activity for chick embryo fibroblasts from rat liver cell conditioned medium: A family of small polypeptides. J. Cell. Physiol. 81: 161–170; 1973.
7. Emerson, C. P.; Beckner, S. K. Activation of myosin synthesis in fusing and mononucleated myoblasts. J. Mol. Biol. 93: 431–447; 1975.
8. Erwin, B. G.; Ewton, D. Z.; Florini, J. R.; Pegg, A. E. Polyamine depletion inhibits the differentiation of L6 myoblast cells. Biochem. Biophys. Res. Comm. 114: 944–949; 1983.
9. Evinger-Hodges, M. J.; Ewton, D. Z.; Seifert, S. C.; Florini, J. R. Inhibition of myoblast differentiation in vitro by a protein isolated from liver cell medium. J. Cell Biol. 93: 395–401; 1982.
10. Ewton, D. Z.; Erwin, B. G.; Pegg, A. E.; Florini, J. R. The role of polyamines in somatomedin-stimulated differentiation of L6 myoblasts. J. Cell. Physiol. 120: 263–270; 1984.
11. Ewton, D. Z.; Florini, J. R. Relative effects of the somatomedins, multiplication-stimulating activity, and growth hormone on myoblasts and myotubes in culture. Endocrinology 106: 577–583; 1980.
12. Ewton, D. Z.; Florini, J. R. Effects of the somatomedins and insulin on myoblast differentiation in vitro. Dev. Biol. 86: 31–39; 1981.
13. Florini, J. R.; Ewton, D. Z. Insulin acts as a somatomedin analog in stimulating myoblast growth in serum-free medium. In Vitro 17: 763–768; 1981.
14. Florini, J. R.; Roberts, S. B. A serum-free medium for the growth of muscle cells in culture. In Vitro 15: 983–992; 1979.
15. Florini, J. R.; Nicholson, M. L.; Dulak, N. C. Effects of peptide anabolic hormones on growth of myoblasts in culture. Endocrinology 101: 32–41; 1977.
16. Janeczko, R. A.; Etlinger, J. D. Inhibition of intracellular proteolysis in muscle cultures by multiplication stimulating activity (MSA): Comparison of effects of MSA and insulin on proteolysis, protein synthesis, amino acid uptake and sugar transport. J. Biol. Chem. 259: 6292–6297; 1984.
17. Kissane, J. M.; Robins, E. J. The fluorometric measurement of deoxyribonucleic acid in animal tissues with specific reference to the central nervous system. J. Biol. Chem. 233: 184–190; 1958.
18. Klapper, D. G.; Svoboda, M. E.; Van Wyk, J. J. Sequence analysis of somatomedin-C: confirmation of identity with insulin-like growth factor I. Endocrinology 112: 2215–2217; 1983.
19. Koneiczny, S. F.; McKay, J.; Coleman, J. R. Isolation and characterization of terminally differentiated chicken and rat skeletal muscle myoblasts. Dev. Biol. 91: 11–26; 1982.
20. Konigsberg, I. R. Diffusion-mediated control of myoblast fusion. Dev. Biol. 26: 133–152; 1971.
21. Konigsberg, I. R. Skeletal myoblasts in culture. In: Methods in enzymology, vol. LVIII. Jacoby, W. B.; Pastan, I. H., eds. New York: Academic Press; 1979: 511–526.
22. Linkhart, T. A.; Clegg, C. H.; Hauschka, S. D. Myogenic differentiation in permanent clonal mouse myoblast cell lines: Regulation by macromolecular growth factors in culture medium. Dev. Biol. 86: 19–30; 1981.

23. Lipton, B. H. Collagen synthesis by normal and bromodeoxyuridine-modulated cells in myogenic culture. Dev. Biol. 61: 153–165; 1977.
24. Marquardt, H.; Todaro, G. J.; Henderson, L. E.; Oroszlan, S. Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures. Homology with human insulin-like growth factor II. J. Biol. Chem. 256: 6859–6865; 1981.
25. Masaki, T.; Bader, D. M.; Reinach, F. C.; Shimizu, T.; Obinata, T.; Shafiq, S. A.; Fischman, D. A. Monoclonal antibody analysis of myosin heavy chain and C-protein isoforms during myogenesis. In: *Muscle development: Molecular and cellular control.* Pearson, M. L.; Epstein, H. F. eds. New York: Cold Spring Harbor Lab.; 1982: 405–418.
26. Massague, J.; Czech, M. P. The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. J. Biol. Chem. 257: 5038–5045; 1982.
27. Merrill, G. F.; Florini, J. R.; Dulak, N. C. Effects of multiplication stimulating activity (MSA) on AIB transport into myoblast and myotube cultures. J. Cell. Physiol. 93: 173–182; 1977.
28. Merrill, G. F.; Dulak, N. C.; Florini, J. R. Stimulation of AIB transport is independent of K^+ accumulation in myoblasts. J. Cell. Physiol. 100: 343–350; 1979.
29. Pierson, R. W., Jr.; Temin, H. M. Partial purification from calf serum of a fraction with multiplication-stimulating activity for chicken fibroblasts in cell culture and with non-suppressible insulin-like activity. J. Cell. Physiol. 79: 319–330; 1972.
30. Poggi, C.; le Marchand-Brustel, Y.; Zapf, J.; Froesch, E. R.; Frechet, P. Effects and binding of insulin-like growth factor I in the isolated soleus muscle of lean and obese mice: Comparison with insulin. Endocrinology 105: 723–730; 1979.
31. Rechler, M. M.; Nissley, S. P.; King, G. L.; Moses, A. C.; Van Obberghen, E.; Romanus, J. A.; Knight, A. B.; Short, P. A.; White, R. M. Multiplication stimulating activity (MSA) from the BRL 3A rat liver cell line: relation to human somatomedins and insulin. J. Supramol. Struct. Cell Biochem. 15: 253–286; 1981.
32. Sasse, J.; von der Mark, H.; Kuhl, U.; Dessau, W.; von der Mark, K. Origin of collagen types I, III, and V in cultures of avian skeletal muscle. Dev. Biol. 83: 79–89; 1983.
33. Schmid, C.; Steiner, T.; Froesch, E. R. Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF-I and IGF-II) in primary cultures of chicken embryonic cells. FEBS Lett. 161: 117–121; 1983.
34. Shainberg, A.; Yagil, G.; Yaffe, D. Control of myogenesis in vitro by Ca^{++} concentration in nutritional medium. Exp. Cell Res. 58: 163–167; 1969.
35. Shainberg, A.; Yagil, G.; Yaffe, D. Alterations of enzymatic activities during muscle differentiation *in vitro.* Dev. Biol. 25: 1–29; 1971.
36. Stockdale, F. E.; Okazaki, K.; Nameroff, M.; Holtzer, H. 5-bromouridine: effect on myogenesis in vitro. Science 146: 533–535; 1964.
37. Strehler, E. E.; Pelloni, C. S.; Heizmann, C. W.; Eppenberger, H. M. Biochemical and ultrastructural aspects of M_r 165 000 M-protein in cross-striated chicken muscle. J. Cell Biol. 86: 775–784; 1980.
38. Sutherland, W. M.; Konigsberg, I. R. CPK accumulation in fusion-blocked quail myocytes. Dev. Biol. 99: 287–297; 1983.
39. Turo, K. A.; Florini, J. R. Hormonal stimulation of myoblast differentiation in the absence of DNA synthesis. Am. J. Physiol. 243: 278–284; 1982.
40. Vertel, B. M.; Dorfman, A. Simultaneous localization of type II collagen and core protein of chondroitin sulfate proteoglycan in individual chondrocytes. Proc. Natl. Acad. Sci. USA 76: 1261–1264; 1979.
41. von der Mark, K.; Gauss, V.; von der Mark, H.; Muller, P. K. Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. Nature 267: 531–532; 1977.
42. Wahrmann, J. P.; Drugeon, G.; Delain, E.; Delain, D. Gene expression during the differentiation of myogenic cells of the L6 line. Biochemie 58: 554–562; 1976.
43. Yaffe, D. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc. Natl. Acad. Sci. USA 61: 477–483; 1968.
44. Yu, K.-T.; Czech, M. P. The type I insulin-like growth factor receptor mediates the rapid effects of multiplication-stimulating activity on membrane transport systems in rat soleus muscle. J. Biol. Chem. 259: 3090–3095; 1984.

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