Developmental Regulation of Creatine Kinase Isoenzymes in Myogenic Cell Cultures from Chicken

BIOSYNTHESIS OF CREATINE KINASE SUBUNITS M AND B*

Mario Caravatti, Jean-Claude Perriard, and Hans M. Eppenberger

From the Institute for Cell Biology, Swiss Federal Institute of Technology, Honggerberg, CH-8093 Zurich, Switzerland

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At various intervals after plating, cultures were exposed to 2-h pulses of tritium-labeled leucine. For determination of incorporated radioactivity, the creatine kinases were isolated by specific immunoprecipitation. Sodium dodecyl sulfate-gel electrophoresis was used to separate the two types of creatine kinase subunits M-CK and B-CK from each other and their radioactivity was determined in the respective gel slices.

In cells cultured in standard medium, B-CK synthesis increased sharply up to a maximal incorporation at 72 h and then it decreased. Synthesis of M-CK could not be detected in 24-h cultures, it increased dramatically during the 3rd and 4th day of culture, and its synthesis was maintained at a high level afterwards. Depletion of Ca** in such cultures blocked fusion and creatine kinase synthesis still took place but was reduced 2-fold for B-CK and 3.5-fold for M-CK. Differentiation was inhibited by growth in 5-bromo-2'-deoxyuridine-containing media and incorporation into B-CK was reduced; however, the synthesis of M-CK could not be measured even after prolonged culturing. Rates of synthesis for B-CK in fibroblasts 4 days after subculturing were comparable to the ones measured in myogenic cells at 24 h but much lower than in cells grown in 5-bromo-2'-deoxyuridine for the same period.

Changing rates of synthesis of the two types of creatine kinase subunits seem to govern the creatine kinase isoenzyme transition. The early increase in B-CK synthesis could be a property of differentiating myogenic cells, whereas a high rate of M-CK synthesis is characteristic for myotubes.

One of the outstanding morphological features of terminally differentiating embryonic skeletal muscle cells is the fusion of mononucleated myogenic cells to the formation of multinucleated myotubes (reviewed in Ref. 1). Biochemical changes, such as the enhanced synthesis and accumulation of myofibrillar proteins (2-8) and membrane proteins (9-12), as well as increases of a number of different enzymatic activities (1, 2, 13, 14), have been described in differentiating cultures regardless of whether fusion occurs or is blocked (6, 15-21). In addition to these quantitative alterations, transitions from isoenzymatic forms typical for embryonic tissue to the species characteristic for differentiated cells have been observed.

Creatine kinases, aldolases, and other muscle-specific enzymes (21-25) have been shown to undergo such isoenzymatic transitions. Recent work has suggested that, among the different forms of the myofibrillar structural proteins like, for example, myosin (26-29) or actin (30-32), similar transitions take place.

In view of the increasing attention that these processes gained, we decided to explore the isoenzyme transition of creatine kinase as a representative example. Creatine kinases are composed of two types of subunits designated M-CK and B-CK, presumably products of at least two different genes, which form three different dimeric isoenzymes, namely MM-CK, MB-CK, and BB-CK (33). Electrophoretic analysis of extracts from differentiating embryonic muscle tissue revealed a transition from BB-CK to MM-CK (34, 35) which was also found in myogenic cells undergoing differentiation in vitro (21, 23, 25). The distribution of creatine kinase isoenzymes demonstrated that BB-CK occurs in a variety of tissues, including among others brain, smooth muscle, and heart, as well as embryonic, not terminally differentiated, cells (34, 36). MM-CK, however, appears to be restricted to skeletal muscle and to terminally differentiated myogenic cells in culture. Determination of the three species of creatine kinase in extracts from differentiating myogenic cultures demonstrated an initial, but rather slight, increase of RR-CK and MR-CK activity (38). At later stages, however, the major increase was shown to be due to enhanced accumulation of isoenzymes containing M-CK subunits (39).

The regulatory events leading to such a complex pattern of accumulation are at present poorly understood. It is not clear whether the increasing enzymatic activities represent enhanced de novo synthesis or whether they are due to modification of the enzymatic activity or to stabilization of the protein, reflecting altered rates of degradation. The mechanisms that are important in regulating levels of cellular proteins have been discussed by Schimke and Doyle (40).

Therefore, we developed methods that allow the determination of the synthesis of creatine kinase subunits and thus enable us to relate the already described patterns of accumulation of enzymatic activity with the changes in the rates of synthesis of creatine kinase subunits. Both kinds of subunits were found to be regulated at the level of protein biosynthesis. Therefore, this system will be useful in the future to study the regulation of changing creatine kinase expression in detail as an example of developmentally regulated genes.

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1 The abbreviations used are: M-CK and B-CK, subunits of creatine kinase; BB-CK and MM-CK, homomeric creatine kinases; MB-CK, heterodimeric creatine kinase; EGTA, ethylene glycol bis[beta-amino-ethyl] ether-N,N'-tetraacetic acid; SDS, sodium dodecyl sulfite; BrdUrd, 5-bromo-2'-deoxyuridine.

EXPERIMENTAL PROCEDURES

Animals—Fertilized white Leghorn eggs were obtained from Gefugel Wolf, Volkenet, Switzerland.

Chemicals—Primary antibodies against MM-CK and BB-CK were prepared and characterized as described by Perriard et al. (21). Cells were cultured at a density of 1.5 x 10^6 cells/ml in 100-mm gelatine-coated Falcon tissue culture dish in standard medium containing 10 parts horse serum, 3.5 parts embryo extract, and 86.5 parts minimal essential medium with Earle's salts. Enriched myotube cultures were obtained by subculturing old secondary fibroblast cultures. Secondary fibroblast cultures were cultured from cell homogenates were precipitated with 10% trichloroacetic acid and the upper part of the sucrose cushion was transferred to a clean gelatine-coated Falcon tissue culture dish in standard medium containing 10 parts horse serum, 3.5 parts embryo extract, and 86.5 parts minimal essential medium with Earle's salts. Enriched myotube cultures were prepared from primary myogenic cultures by adding cytosine arabinoside, an inhibitor of DNA synthesis, at a concentration of 5 x 10^-5 M to the culture medium on Day 2 until Day 6 (44). If fusion of myogenic cells was to be blocked, EGTA was added at 24 h of culture at a final concentration of 1.72 x 10^-3 M (3). BrUrd (2.5 µg/ml), a thymidine analogue known to suppress myogenic differentiation (45), was added to the initial inoculum as indicated.

Labeling Conditions—For the determination of relative rates of synthesis of the creatine kinase subunits at intervals after plating, cultures were labeled for 2 h with 30 µCi of [3H]leucine in 3 ml of fresh medium. The rates of incorporation within the time span of the pulse was constant, as demonstrated by the linear increase of acid-precipitable radioactivity, from 15 min after the start of labeling up to at least 4 h (data not shown). Long term labeling was performed using the same concentration of [3H]leucine or [35S]methionine (10 µCi/ml), a thymidine analogue known to suppress myogenic differentiation (45), was added to the initial inoculum as indicated.

Determination of Total Acid-precipitable Radioactivity—Aliquots of cell homogenates were precipitated with 10% trichloroacetic acid on paper disc and washed batchwise with 5% trichloroacetic acid, 95% ethanol (42). The pellets were then dried and counted in a toluene-based scintillation mixture containing 0.4% (w/v) Omnifluor, 3% (v/v) Protosol, and 0.75% (v/v) H2O (48). Radioactivity was measured in a Packard Tri-Carb 2600 scintillation spectrometer and the results were standardized automatically by the external standard channels ratio method. Counting efficiency for 3H was usually 36% for paper discs and 51% for gel fragments.

RESULTS

The proteins synthesized in cultures of differentiating muscle cells were labeled by pulses of [3H]leucine given immediately before the cultures were used. The soluble fraction then was extracted and the creatine kinase enzyme was isolated by immunoprecipitation. The resulting immunoprecipitate was analyzed by SDS-polyacrylamide slab gel electrophoresis. The gel was stained for protein (Fig. 1A), as well as subjected to autoradiography (Fig. 1B). It demonstrates not only that the antisemum to creatine kinase antibodies used in the experiments described by Showe et al. (51) using a dilution of 1:3 to 1:6 of crude antisemum against creatine kinases in the supernatant and about 25% of the sucrose cushion were removed by suction. The walls of the tubes were rinsed with phosphate buffer which finally was removed carefully along with the remainder of the sucrose cushion. The immunoprecipitates were dissolved in 30 µl of sample buffer (50), heated at 95°C for 4 min, and then were ready for electrophoresis.

The amount of specific antibody used had been shown to precipitate 3 to 4 µg of purified antigen in preliminary immunotitration experiments exceeding the maximal amounts of total antigen (enoughous creatine kinase + carrier creatine kinase) used in the experiments. The creatine kinases were precipitated quantitatively by the procedure used and no radioactivity could be detected co-migrating with creatine kinase if the specific immunoprecipitation step was repeated (data not shown).
purified creatine kinases or MM-CK alone (Fig. 2, A, Lanes a and b). A faint band could be detected in the anti-BB-CK replica of the SDS gel loaded with an identical sample (Fig. 2, C and D, Lanes d). Anti-BB-CK immunoprecipitates produced, on the other hand, a heavy band in the anti-BB-CK replica (Fig. 2, C and F, Lanes e) as did BB-CK in the mixture of both purified creatine kinases or BB-CK alone (Fig. 2, C, Lanes a and c) and a faint band in the anti-MM-CK replica (Fig. 2, A and D, Lanes e). The faint bands produced by the anti-MM- and anti-BB-CK immunoprecipitates give further evidence for the assumption made above that these bands are due to the precipitation of the heterodimeric isoenzyme MB-CK from the cell extracts. No reaction, however, was found in gels containing separated unspecific anti-guinea pig immunoprecipitates (Fig. 2, A, C, D, and F, Lanes f) or in controls with immunoglobulin fraction of preimmune serum (Fig. 2, A, C, D, and F, Lanes g). Since the sensitivity of the immune replica technique is high (0.02 to 0.04 μg of either creatine kinase could be detected, data not shown), we concluded that precipitates obtained from treatment with anti-guinea pig serum or preimmune serum do not contain significant amounts of creatine kinase.

It was of great importance to demonstrate the purity of the two antigens precipitated by the antibodies because, for example, actins, a group of proteins precipitating in controls, were found to co-migrate with B-CK under the conditions used (Fig. 1B, Lane f). We therefore designed an experiment to compete out the radioactivity in the creatine kinase bands by adding either unlabeled MM-CK, BB-CK, or actin in increasing amounts to the incubation mixture containing a radioactive cell extract from 4-day-old myogenic cultures. Within the range of up to 40 μg of added unlabeled creatine kinase, the amounts of creatine kinase precipitated by the antibody were equal. Radioactivity co-migrating with M-CK from anti-MM-CK immunoprecipitates was competed out by the addition of MM-CK but remained constant if increasing amounts of BB-CK or actin had been added (Fig. 3A). If BB-CK was used as competitor during the immunoprecipitation with anti-BB-CK, there was a reduction of radioactivity co-migrating with B-CK but addition of MM-CK or actin had no effect (Fig. 3B). The radioactivity due to the precipitation of the MB-CK isoenzyme was observed to follow the same

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**Fig. 1.** Ten per cent SDS-polyacrylamide gel of immunoprecipitates stained for protein (A) and autoradiographed (B). Myogenic cells grown in standard medium were labeled with [35S]methionine for 72 to 88 h in culture. Preparation of cellular extract and immunoprecipitation were carried out as described under "Experimental Procedures." a, molecular weight markers: rabbit muscle phosphorylase b, 94,000; bovine serum albumin, 68,000; rabbit muscle pyruvate kinase, 57,000; chicken M-CK, 40,000; bovine chymotrypsinogen, 25,000. b, purified chicken BB-CK and MM-CK; stained protein bands in a and b were marked with dots of radioactive ink before autoradiography. c, specific immunoprecipitate with anti-MM-CK made of supernatant of an identical sample as in f, d, specific immunoprecipitate with anti-BB-CK. e, specific immunoprecipitate with anti-MM- and anti-BB-CK; f, nonspecific immunoprecipitate from an aliquot of cell extract with rabbit antibody against guinea pig serum and guinea pig serum. g, reprecipitation of supernatant from an aliquot of cell extract with rabbit antibody against guinea pig serum and guinea pig serum. h, control carried out with a sample as in f with preimmune serum. i, sample of labeled cell extract.

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**Fig. 2.** Immunoreplica of SDS-polyacrylamide gel. Analogous immunoprecipitates as those used in Fig. 1 were done in the absence of carrier creatine kinase and were electrophoresed on a 10% SDS-polyacrylamide gel. The part of the gel containing the creatine kinase bands was overlaid with an agarose gel containing antisera. A, immunoprecipitate with anti-MM-CK stained for protein; B, immunoprecipitate with both anti-MM-CK and anti-BB-CK; C, immunoprecipitate with anti-BB-CK; D, E, and F, corresponding autoradiographs. a, purified chicken BB-CK and MM-CK; b, purified chicken MM-CK; c, purified chicken BB-CK; d, specific immunoprecipitate with anti-MM-CK; e, specific immunoprecipitate with anti-BB-CK; f, specific immunoprecipitate with rabbit anti-guinea pig serum and guinea pig serum; g, control with preimmune serum.
Radioactivity co-migrating with MM-CK is assumed to derive from the heterodimeric enzyme in culture, myogenic cells were labeled for 24 h with [3H]leucine. Purified BB-CK or MM-CK during immunoprecipitation. After acid-insoluble material varied with time in culture or the synthesis, however, were not big enough to explain the enzymatic activity if the dimers were assembled from the same subunit.

By the antibody is creatine kinase and only very little, if any, is precipitated. Since almost all the radioactivity precipitated labeled peptide can be found on the gel, but no creatine kinase species of creatine kinase subunits can be separated on SDS gels into a faster migrating species representing M type subunits and a slower migrating species made up of B type subunits. The MB isoenzyme is precipitated by both antibodies. If an unrelated antibody-antigen combination is used to carry out the immunoprecipitation with the cell extract, some labeled peptide can be found on the gel, but no creatine kinase is precipitated. Since almost all the radioactivity precipitated by the antibody is creatine kinase and only very little, if any, radioactivity is due to unspecific precipitation, this procedure is adequate for measuring incorporation of radioactivity into the creatine kinase subunits.

These experiments allow the following conclusions: the two species of creatine kinase subunits can be separated on SDS gels into a faster migrating species representing M type subunits and a slower migrating species made up of B type subunits. The MB isoenzyme is precipitated by both antibodies. If an unrelated antibody-antigen combination is used to carry out the immunoprecipitation with the cell extract, some labeled peptide can be found on the gel, but no creatine kinase is precipitated. Since almost all the radioactivity precipitated by the antibody is creatine kinase and only very little, if any, radioactivity is due to unspecific precipitation, this procedure is adequate for measuring incorporation of radioactivity into the creatine kinase subunits.

Incorporation of Radioactivity into Total Protein—Generally, an increase of total protein synthesis was observed during the first 3 days of culture followed by a slight decrease as shown in Fig. 4. Cultures grown in standard medium or in medium containing cytosine arabinoside reached maximal values of leucine incorporation 1.3 to 1.4 times higher than those observed in cultures grown in the presence of EGTA, BrdUrd, or in fibroblast cultures. The ratio of minimal value observed to the maximal rate, however, did not exceed 2.5 times. The changes of [3H]leucine incorporation into protein in standard and EGTA cultures during such a cultivation period reflect alterations in the rates of protein synthesis since it was shown that the pool sizes for [3H]leucine remained constant (3, 5). Overall incorporation of tritiated leucine into acid-insoluble material varied with time in culture or the culture conditions used. The observed stimulations of protein synthesis, however, were not big enough to explain the enhanced synthesis of creatine kinases which are described in the following sections.

Synthesis of B-CK and M-CK in Standard and Ara-C Cultures—The synthesis of B-CK and M-CK changed greatly during in vitro myoblast differentiation (Fig. 5). In cultures grown in standard medium, B-CK was synthesized at low but significant levels at 24 h. Its synthesis increased rapidly after the first day, reached a maximum at 72 h, and dropped then to about 30% of the maximum at 144 h. There was no significant synthesis of M-CK at 24 h. A slight increase, however, in...
M-CK synthesis was observed during the 2nd day in culture but the pronounced increase in M-CK synthesis occurred during the 3rd day and maximal values were reached at 96 h. Cells cultured for longer periods showed a slight decrease in M-CK synthesis. In such cultures, the fused cells were cross-striated and spontaneous contractions were observed. Later, myotubes began to detach from the plates while fibroblasts synthesizing only B-CK at low levels (Fig. 5) continued to grow and, therefore, a decline of M-CK synthesis per nuclear DNA equivalent was to be expected.

A series of cultures were kept in the presence of cytosine arabinoside. This treatment resulted in the elimination of replicating mononucleated cells and in enrichment of myotubes. The synthesis of B-CK reached similar values as in standard cultures and showed a slightly greater decrease after 72 h than standard cultures. M-CK synthesis attained a level 40 to 50% higher than observed in standard cultures after 96 h (Fig. 5).

The values obtained for M-CK synthesis per myotube nucleus increased during cultivation and similar maximal values after 96 h in culture were attained by cells kept in standard medium in the presence or absence of cytosine arabinoside (Table I). A similar increase was shown for myosin synthesis when expressed in this manner (3). In contrast to these findings in chicken cells, the rate of myosin synthesis normalized per fiber nucleus was shown to be relatively constant throughout the fusion period in myogenic cells from quail embryo (5).

**Synthesis of B-CK and M-CK in EGTA Cultures**—The synthesis of B-CK and M-CK was examined in cultures in which the fusion of myogenic cells had been blocked by the addition of EGTA (3) during the first 3 days and then these cultures were switched to standard medium. In cultures where fusion was blocked during the whole experiment, synthesis of B-CK was observed to increase until 72 h and then fall off to values comparable to the ones for standard cultures (Fig. 5). Synthesis of M-CK increased after 24 h and reached a plateau after 96 h. EGTA cultures showed an about 2-fold reduction of the maximal [3H]leucine incorporation in B-CK and a 3.5-fold reduction in M-CK synthesis compared to the values obtained for standard cultures. Since some fused cells could be observed in cultures grown in Ca2+-depleted media, M-CK synthesis in these cultures might conceivably be attributed solely to those cells. This is, however, unlikely since antibody staining demonstrated also that mononucleated cells in EGTA contain M-CK (37) and calculation of M-CK synthesis per fiber nucleus yields values exceeding the ones obtained for standard cultures (Table I).

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Table I

The synthesis of M-CK as measured (Fig. 5) normalized to the number of nuclei within multinucleated fibers

Therefore, it is likely that these mononucleated cells blocked in fusion not only accumulate but also actively synthesize M-CK.

Parallel cultures were released from the fusion block at 72 h replacing the EGTA medium by standard medium. Within the next 24 h, cells achieved the same extent of fusion as standard cultures. The synthesis of M-CK increased almost to the levels obtained in standard cultures. The synthesis of B-CK showed only a slight increase after the medium switch and reached, at 144 h, a level of synthesis similar to that observed in standard cultures (Fig. 5).

**Synthesis of M-CK and B-CK in BrdUrd Cultures**—Synthesis of M-CK and B-CK was also determined in cultures grown in the presence of BrdUrd (Fig. 5). According to several authors (2, 21, 45, 55), BrdUrd blocks terminal differentiation. Incorporation into B-CK increased during the first 72 h to about 30% of the synthesis of B-CK in standard cultures and decreased thereafter. Synthesis of M-CK remained at very low levels during cultivation, showed only a minor increase after long culture periods, and is probably due to the presence of some contaminating myotubes (37, 56).

It is known that the effect of BrdUrd is reversible if the cells are allowed to undergo at least one round of cell division in the absence of BrdUrd (45). Cultures grown in BrdUrd for 72 h were transferred to standard medium. The extent of fusion observed upon switching was low; probably the high density of these cultures impeded further cell divisions and, thus, not all cells were allowed the replications necessary to dilute out the BrdUrd in the cultures and to express differentiated functions. A slight increase in M-CK synthesis was observed in cultures switched from BrdUrd-containing medium to standard medium and the level of B-CK synthesis was comparable at the end of the culture period to the level observed in standard cultures (Fig. 5).

**DISCUSSION**

Highly sensitive procedures were necessary to determine the synthesis of B-CK and M-CK subunits in myogenic and fibrogenic cultures since incorporation into creatine kinase subunits represented only 0.01 to 0.3% of total radioactivity incorporated into cellular protein. Separation of two types of creatine kinase subunits from each other and from contaminating myotubes could be accomplished on SDS-acrylamide gel electrophoresis despite the identical or very similar molecular weights that were reported for B-CK and M-CK (53). Whether these differences in mobility on denaturing gels are due to different molecular weights, an artifact induced during sample preparation, or properties of the peptides other than size remains to be elucidated.

Many studies of protein metabolism in differentiating myogenic cell cultures have focused on the changes of accumulated muscle-specific enzymes and in some cases on de novo synthesis of the most abundant proteins like myosin and actin (reviewed in Refs. 14 and 57). Most determinations of myosin heavy chain synthesis have shown about an 10-fold increase compared to levels observed in unfused cells (3, 58, 59), but a 50-fold increase in the rate of myosin heavy chain synthesis reaching a constant level immediately after fusion was observed in cultures of quail myogenic cells (5). Synthesis of skeletal muscle myosin light chains LC, and LC2, were shown to increase during the period of fusion, whereas the synthesis of LC1 was detectable only at later periods (7). The increases in total actin synthesis, however, are less striking than in the case of myosin and, thus, could be explained by the observation that an even greater portion of cellular protein synthesis is devoted to actin in unfused cells or non-muscle cells (30, 32). Overall actin synthesis increases about 8-fold in myogenic...
chick cultures from Day 2 to Day 3 in culture (4).

One problem that has not always been considered in measuring the synthesis of myosin or actin during differentiation of muscle cells is the fact that there are different isoproteins for each of these; and the molecules typical for muscle are synthesized in differentiated myogenic cells (36, 38, 39, 40). Since the methods used did not distinguish in all cases between non-muscle and muscle-specific forms, it is likely that the increases of the muscle-specific proteins may be more pronounced than the ones reported for the synthesis of the whole fraction.

Results of creatine kinase biosynthesis in myogenic cell cultures published earlier indicated a significant level of creatine kinase synthesis only in cultures older than 2 days (60) and led to the assumption that the early increases in BB-CK enzymatic activity reported by the same authors (38) may not involve de novo synthesis of B-CK prior to the 2nd day of culture. We found a 10-fold increase of B-CK synthesis during the first 3 days in standard cultures, explaining the early increases in enzymatic activity (38, 39). The rate of synthesis of the muscle-specific M-CK in differentiated cultures enriched for myotubes attained a 25-fold increase from Day 2 to Day 4 even without correcting for the background synthesis of M-CK before Day 3. The synthesis of M-CK detected during the first 2 days is probably due to the formation of myotubes containing up to 10% of the nuclei (data not shown) derived from fusion-competent cells in the initial inoculum (66).

The rates of synthesis measured for the creatine kinase subunits correspond well with the pattern of accumulation of creatine kinase isoenzyme activity during myogenesis (38, 39). This suggests that alteration in the rates of synthesis of creatine kinase subunits are principally involved in the regulation of creatine kinase levels. Relative amounts of translatable mRNA for the creatine kinase subunits M-CK and R-CK also correlate well with the observed rates of synthesis. Other authors have postulated different possibilities for the regulation of creatine kinases, e.g. in adult rabbit muscle as in the production of enzymatically inactive BB-CK (61), a BB-CK which is turning over very rapidly (62). Experiments in progress have shown no significant difference in the rates of turnover of the two types of creatine kinase subunits.

The investigation of the expression of the differentiated muscle phenotype in the absence of cell fusion has been the subject of many reports. It was shown that cultures grown in the presence of EGTA, or, in medium containing low Ca2+ concentrations, were blocked in fusion but expression of differentiated characters, such as myosin synthesis (5, 15, 16), the appearance of acetylcholine receptor (15, 17), or CK activity (18, 19), took place at levels comparable to those in cultures grown at standard Ca2+ concentrations. Other authors have reported accumulation of, for example, myosin (19, 20), actin (4), or M-CK activity (21, 37, 46) in low Ca2+ cultures but with lower levels than in standard cultures. Our results are in agreement with the latter data. If cultures were grown in the presence of EGTA, synthesis of both B-CK and M-CK was lower when compared to parallel standard cultures.

There are indications that small variations in free Ca2+ concentration in culture media may also affect protein synthesis (63-65) and explain the observed discrepancies.

If myogenesis is blocked by the addition of BrdUrd to the medium, the creatine kinase transition fails to occur (21). The data presented here indicate insignificant synthesis of M-CK as expected; synthesis of R-CK, however, could easily be measured and it displayed the same type of curve as observed in standard cultures with maximal synthesis of B-CK in 72-h old cultures. In comparison, subcultured homogenous fibroblast cultures synthesize B-CK at much lower levels than both standard cultures or BrdUrd- arrested cells. Although synthesis of B-CK may occur in a variety of non-muscle cells (36), we favor the interpretation that substantial synthesis of B-CK may not be only a response to culture conditions but characteristic for myogenic cells and could serve as a diagnostic for a penultimate stage of myogenic differentiation.

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M Caravatti, J C Perriard and H M Eppenberger


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