Cytoplasmic Activation of Human Nuclear Genes in Stable Heterocaryons

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Summary

We have induced the stable expression of muscle-specific genes in human nonmuscle cells. Normal diploid human amniocytes were fused with differentiated mouse muscle cells by using polyethylene glycol. The fusion product, a stable heterocaryon in which the parental cell nuclei remained distinct, did not undergo division and retained a full complement of chromosomes. This is in contrast with typical interspecific hybrids (syncaryons), in which the parental nuclei are combined and chromosomes are progressively lost during cell division. The human muscle proteins, myosin light chains 1 and 2, MB and MM creatine kinase and a functional mouse-human hybrid MM enzyme molecule were detected in the heterocaryons. Synthesis of these proteins was evident 24 hr after fusion and increased in a time-dependent manner thereafter. Our results indicate that differentiated mouse muscle nuclei can activate human muscle genes in the nuclei of a cell type in which they are not normally expressed, and that this activation occurs via the cytoplasm. The activators are still present in cells which have already initiated differentiation, are recognized by nuclei of another species, and do not diffuse between unfused cells. The reprogrammed amniocyte nuclei of stable heterocaryons provide a unique system in which to study the mechanisms regulating gene expression during cell specialization.

Introduction

Muscle cells provide an advantageous system in which to examine biochemical and molecular changes associated with differentiation. Mononucleated myoblasts spontaneously fuse to form multinucleated myotubes, which become visibly striated and rhythmically contract in tissue culture. These distinct morphological changes are accompanied by the cessation of DNA synthesis and the initiation of synthesis of several muscle enzymes, structural proteins of the contractile apparatus and specialized membrane receptors (Shainberg et al., 1971; Perriard et al., 1978; Garrels and Gibson, 1978; Devlin and Emerson, 1979; DeVreotes et al., 1977; Weinberg et al., 1981; for review, see Pearson, 1980). Although this sequence of biological events has been well characterized, the regulatory mechanisms underlying the transition to the differentiated state remain poorly understood. In addition, no muscle ton cell, or undetermined myoblast precursor, has yet been identified in which to examine the initial commitment to cell specialization.

Studies of differentiation using somatic cell hybrids, or syncaryons, have demonstrated the existence of regulatory factors capable of repressing or inducing the expression of differentiated functions (Ephrussi, 1972; Davidson and de la Cruz, 1974; Davis and Adelberg, 1973; Ringertz and Savage, 1976; Weiss, 1977). Extinction of a differentiated protein was typically observed upon fusion of a cell that expressed the protein with a cell that did not (Davidson et al., 1976; Davidson and Benda, 1970; Bertolotti and Weiss, 1972; Mevel-Ninio and Weiss, 1981). Evidence that extinction was due to the product of a gene encoding a repressor was best demonstrated by the re-expression of the extinguished function following chromosome loss (Kieble et al., 1970; Weiss and Chaplain, 1971). Activation of a gene was occasionally observed when the genome of the differentiated cell type was present in increased dosage (Peterson and Weiss, 1972; Darlington et al., 1974; Brown and Weiss, 1975; Darlington et al., 1982). During the propagation of such interspecific hybrid cells, however, chromosomes were frequently lost so that the genetic composition of the hybrids was unstable and highly variable. As a result, syncaryons became invaluable for gene mapping, but were of limited utility for controlled studies of gene regulation.

We have developed a stable heterocaryon system that overcomes many of the problems experienced with syncaryons and is ideal for characterizing the role of regulatory factors in cell differentiation. Our approach takes advantage of the fact that the differentiated muscle cell is naturally a nonreplicating multinucleated cell. Thus, after fusion of muscle with other cell types, the parental cell nuclei do not divide, but remain distinct and intact, with the entire chromosome complement of each present. Previous attempts to induce novel gene expression in heterocaryons have failed (Carlsson et al., 1970, 1974a, 1974h; Zeuthen et al., 1976; Mevel-Ninio and Weiss, 1981). Here we demonstrate that heterocaryons between mouse myotubes (differentiated muscle cells) and human amniotic cells (nonmuscle cells) can be produced with high efficiency and that in these stable fusion products, silent human genes encoding muscle M-creatine kinase and three myosin light chains are activated. The induction of gene expression in these heterocaryons demonstrates conclusively that factors capable of activating genes are not limited to the nucleus, but also exist in the cell cytoplasm. These factors act rapidly and can gain access to intact foreign nuclei. Heterocaryons of this kind provide a stable and reproducible model system in which to study biochemical and molecular aspects of regulation of gene expression during cell specialization.

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Results

Production of Heterocaryons

The mouse muscle cells used were a permanent cell line, which predictably differentiated to form contractile fibers in culture (Figure 1a). The human nonmuscle cells were normal diploid amniotic fluid cells of the F, or fibroblastic, cell type (Figure 1b). When more than 90% of the muscle nuclei were contained in actively contracting myotubes, sufficient amniocytes were added to achieve confluence (Figure 1c). Cells were fused by using polyethylene glycol according to the method of Davidson et al. (1976). Unfused cells were eliminated by treatment of the cultures with selective

![Figure 1. Formation of Heterocaryons by Fusion of Mouse Muscle Cells and Human Amniocytes with PEG.](image)
agents (Figure 1d). Cytosine arabinoside, which inhibits DNA synthesis (Cohen, 1966; Yooh and Holtzer, 1977), was used to eliminate unfused proliferating myoblasts. Residual amniocytes were removed by using ouabain (10^{-6} M), an inhibitor of sodium potassium-ATPase with a 1000-fold greater affinity for the human than for the rodent enzyme (Thompson and Baker, 1973). The resulting mixture of multinucleated muscle-amniocyte heterocaryons and muscle homocaryons survived a minimum of 6 to 7 days, were striated and exhibited typical muscle contractile behavior.

To distinguish heterocaryons from homocaryons, a means for identifying the nuclei of the two parental cell types was required. For this purpose, species-specific differences were used to advantage. As shown in Figure 2, the nuclear composition of heterocaryons formed between cells of mouse and human was easily determined by using Hoechst 33258. This compound fluoresces with greater intensity in the presence of DNA with a high content of adenine and thymine (Weisblum and Haenssler, 1974). Satellite DNA in the centromeric regions of mouse chromosomes is rich in poly(dAT), and mouse interphase nuclei stained with the fluorochrome appear punctate. On the other hand, human nuclei, whose DNA does not contain AT-rich regions, are characterized by a generalized low level fluorescence (Moser et al., 1975). This method for identifying nuclei is rapid and unambiguous.

Efficiency of Heterocaryon Production
The efficiency of heterocaryon production and the average ratio of mouse to human nuclei contained in heterocaryons were determined by visually scoring the nuclei stained by Hoechst 33258. The results of a sample experiment, in which heterocaryons were followed as a function of time after fusion, are shown in Table 1. Column 2 shows that at each time point, an average of 73% of all multinucleated myotubes were heterocaryons, or contained a mixture of mouse and human nuclei. Thus only one quarter of the myotubes present were homocaryons, composed solely of mouse muscle nuclei. Thirty heterocaryons at each time point were randomly selected for detailed analysis. Heterocaryon size (column 3), or the total number of nuclei per heterocaryon, ranged from 7 to 15, but did not appear to increase systematically with time after fusion. The proportion of human nuclei in heterocaryons shown as percentage of human nuclei (column 4) and as the ratio of mouse to human nuclei (column 5) also remained constant over this period of time. These results were confirmed in five separate experiments in which heterocaryons were analyzed on a daily basis. They suggest that after PEG treatment, heterocaryon composition remains relatively constant, that myotubes do not fuse with one another and that the fusion of any residual myoblasts to existing myotubes is minimal, if it occurs at all.

Synthesis of Human Muscle Proteins by Heterocaryons
The data presented below represent the results of 18 fusion experiments in which multiple replicate dishes were analyzed at several different time points. The amniocytes used in these experiments were fibroblastic F amniocytes isolated from three sources. All gave consistent and highly reproducible results.

Creatine Kinase
Creatine kinase (CK) is of central importance in generating the energy required for vertebrate muscle contraction. The enzyme is a homodimer or heterodimer of two subunits, B and M; consequently, three isoenzymes are possible—BB, MB and MM. The syntho-
### Table 1. Heterocaryons of Mouse Muscle Cells and Human Amniocytes

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<thead>
<tr>
<th>Efficiency of Fusion&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nuclear Composition&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Days after PEG Fusion</td>
<td>% Heterocaryons per Total Myotubes</td>
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<td>1</td>
<td>67</td>
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<td>2</td>
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<td>Mean</td>
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<sup>a</sup> On each day, 40 myotubes were randomly selected and scored for nuclear heterogeneity.

<sup>b</sup> Thirty heterocaryons were scored each day for the total number of mouse and human nuclei in each and for the proportion of total nuclei that were human. Data are presented as the mean ± SEM.

<sup>c</sup> The total number of nuclei scored each day is indicated in parentheses.

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Figure 3. Expression of Human M-CK in Mouse–Human Heterocaryons

Whole cell extracts were subjected to electrophoresis on non-denaturing polyacrylamide gels for 4 hr (A) and 7 hr (B). CK isozymes (BB, MB and MM) were detected with UV illumination using a coupled enzyme reaction with NADPH as its end product. (A) Isozymes from amniocytes alone (lane 1); amniocytes plus PEG (lane 2); cultured mouse muscle (lane 3); cultured human muscle (lane 4); and cultured mouse and human muscle extracts mixed in vitro (lane 5). (B) Isozymes from cultured mouse muscle and human muscle extracts mixed in vitro (lane 1); coculture of mouse muscle and human muscle mixed in vitro (lane 2); heterocaryons of mouse muscle and human amniocytes after 6 days without PEG (lane 3); heterocaryons of mouse muscle and human amniocytes at the following time intervals after PEG fusion—1 day (lane 3), 3 days (lane 4), 4 days (lane 5), 5 days (lane 6); 6 days (lane 7); heterocaryons of mouse muscle and human muscle formed spontaneously (lane 8). Arrows indicate CK isozymes containing human subunits.

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The three isozymes of CK are well separated by electrophoresis of whole cell extracts on non-denaturing polyacrylamide gels, as shown in Figure 3. The BB, MB and MM isozymes can be specifically visual-
Differences in the mobility of both the MM and MB isozyme reaction with NADPH as its end product. Myokinase (adenylate kinase), which frequently complicates the analysis of CK activity in other gel systems, does not comigrate with the CK isozymes under our electrophoretic conditions, and has been specifically inhibited by the presence of p,p'-d(adenosine-5') pentaphosphate in the reaction mixture (Lienhard and Socoli, 1973). The limit of detection is 1 mU of enzyme activity, that produced by approximately 10^7 well differentiated mouse muscle cells. As shown in Figure 3A, human and mouse CK isozymes can be readily distinguished by this method (lanes 3 and 4). Differences in the mobility of both the MM and MB isozymes of the two species are especially clear when mouse and human muscle extracts are combined (lane 5).

Human muscle CK is not normally synthesized by either amniocytes or mouse muscle cells. Human amniocytes, like undifferentiated myoblasts (Blau et al., 1982, 1983), contain only a small amount of CK, all of which is the BB isozyme. There is no evidence of M subunit synthesis in amniocytes alone (Figure 3A, lane 1) or in amniocyte homocaryons formed by treatment of the cells with PEG (lane 2). Homocaryons of C2 muscle cells produced with PEG and exposed to the selective agents cytosine arabinoside and ouabain also do not synthesize an enzyme with the electrophoretic mobility of the human MM isozyme (data not shown). Thus neither the fusion process per se nor exposure to the selective agents results in altered synthesis of this enzyme by either parental cell type alone.

The expression of creatine kinase by amniocytes and mouse muscle cells fused in heterocaryons was examined at time intervals after PEG treatment (Figure 3B). For accurate comparison, cell extracts containing equivalent CK activities were layered on gels and subjected to electrophoresis. Within 1 day after fusion, novel isozymes are detectable in heterocaryons (lane 3). After 3 days, human MB-CK and a band intermediate between mouse and human MM-CK are clearly evident (lane 4). The intensity of these two bands continues to increase with time (lanes 4 to 6). By day 6, a third isozyme, which comigrates with pure human MM-CK, is distinguishable (lane 7). By contrast, when mouse muscle cells and amniocytes not treated with PEG are grown together for 6 days the pattern of CK isozymes resembles that of mouse muscle alone (lane 2).

We have identified the intermediate band between human MM-CK and mouse MM-CK as a functional mouse-human hybrid enzyme by examining the isozyme composition in intertocicpic muscle heterocaryons. Human muscle cells and mouse muscle cells plated together in culture spontaneously fused in the absence of PEG to form multinucleated contracting myotubes. The heterocaryon nature of the myotubes was verified with Hoechst 33258, and the average ratio of mouse to human nuclei determined to be 2:1. Like the synthetic muscle–amniocyte heterocaryons described above, the muscle–muscle heterocaryons contained isozymes that comigrated with the mouse and human MM standards and a third form intermediate between the two (Figure 3B, lane 5). This hybrid enzyme was present only in synthetic or spontaneously formed heterocaryons in which the cells shared a common cytoplasm. It was not present in mouse and human muscle cell extracts mixed in vitro (lane 1) or in cocultures of mouse muscle and human amniocytes not exposed to PEG (lane 2). This interspecific hybrid molecule was a functional enzyme, since the method for visualization was based on enzyme activity. Although the ratios of mouse to human nuclei of both the synthetic and spontaneous heterocaryons were similar, the relative intensity of the human MM-CK band differs between lanes 7 and 8. This difference is likely to be due to the presence of human muscle homocaryons in the spontaneously fused cultures (lane 8), which contributed pure human MM isozyme.

An analysis of nuclear ratios and MM-CK isozymes in amniocyte–muscle heterocaryons reveals that the efficiency of gene activation is high. Since residual mouse myoblasts and human amniocytes synthesize only BB-CK, their presence does not influence the analysis. The only other source of MM-CK in these experiments is homocaryon mouse myotubes, which comprise approximately one quarter of the total myotubes (Table 1, column 2). As shown in columns 4 and 5, one third of the nuclei in heterocaryons were human. Thus one quarter of all nuclei in myotubes (homocaryons and heterocaryons) were human, and the relative contribution of human M subunits to the observed MM isozymes approximates this proportion (lane 7). Within the limits of the assay, these results suggest that almost every amniocyte nucleus incorporated into a myotube is activated.

**Myosin Light Chains**

The myosin light chains, together with the myosin heavy chains, interact with actin to form the major components of the muscle contractile machinery. Myosin light chains are not synthesized by cultured myoblasts, but appear once muscle differentiation is initiated. We partially purified contractile proteins from differentiated cultures of human and mouse muscle, fractionated a mixture of the proteins of both species on two-dimensional gels and visualized the accumulated proteins by staining with Coomassie brilliant blue (Figure 4). The actins, tropomyosins and myosin light chain 3f of the two species comigrated and could not be distinguished by this method, but four of the myosin light chains were distinct. The first fast myosin light chains (1f) differ in molecular weight, the second fast and slow myosin light chains (2s and 2f) differ in isoelectric points, and the first slow myosin light
The synthesis of the human myosin light chains is induced following fusion of amniocytes with muscle. The synthesis of these proteins is evident in either parental cell type and ouabain also did not synthesize human myosin light chains (data not shown).

To determine whether human myosin light chain genes were activated in heterocaryons, we examined the proteins synthesized in amniocytes, in mouse muscle cells and in heterocaryons made between the two cell types (Figure 5, right). Cultures of these cells were incubated with $^{35}$S-methionine, and the labeled contractile proteins were partially purified and fractionated on two-dimensional gels. Autoradiograms of the gels revealed that in heterocaryons (Figure 5, right, bottom), human myosin light chains 1s and 2f are synthesized in substantial amounts, 2s is present in trace amounts and 1f is not detectable. None of these proteins is evident in either parental cell type alone (top and middle). To ensure precipitation of these proteins was confirmed by using a monoclonal antibody that recognizes a determinant on myosin light chains (1s) of both mouse and human. Contractile proteins of mouse muscle and human muscle cultures were separated on two-dimensional gels, and the myosin light chains were detected on immunoblots according to the method of Burnette (1981), as shown in Figure 5, left. Four of the myosin light chains exhibit species-specific differences and are useful markers of human muscle gene expression in heterocaryons.

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Gene Activation in Stable Heterocaryons

Figure 5. Expression of Human Myosin Light Chains in Mouse-Human Heterocaryons

(Left) Identification of myosin light chains on immunoblots. The region of the gel in the inset of Figure 4 is shown for cultured human muscle (top), cultured mouse muscle (middle) and cultured mouse and human muscle mixed in vitro (bottom). Contractile proteins were partially purified from differentiated cultured muscle cells and electrophoresed on two-dimensional gels. Myosin light chains of human and mouse species were identified on Western blots with a monoclonal antibody to myosin light chains and a second antibody coupled to biotin and reacted with an avidin–biotin horseradish peroxidase. (Right) Myosin light chain synthesis in heterocaryons. Human amniocytes plus PEG (top); mouse muscle cells plus PEG (middle); heterocaryons 4 days after fusion with PEG (bottom). Cultured cells were labeled with 35S-methionine, and the contractile proteins were partially purified, fractionated on two-dimensional gels and processed for autoradiography. Proteins were identified by comigration with unlabeled contractile protein standards. Amniocyte proteins were coextracted with unlabeled mouse muscle proteins to ensure precipitation of contractile proteins. Mouse muscle cells and heterocaryons were grown in the presence of ouabain and cytosine arabinoside. Electrophoresis of gels at left and right was performed at separate times. The positions of the human myosin light chains are indicated by arrows. Myosin light chains 1s and 2f are clearly induced in heterocaryons; a trace of 2s is also evident.

Figure 6. Expression of Human Myosin Light Chains in Mouse-Human Heterocaryons

Another advantage of the heterocaryon system we have developed is that gene expression can be examined immediately after fusion. Numerous cell divisions are usually required to select for and identify hybrids or cybrids and to obtain sufficient numbers of these cells for analysis of gene products. By contrast, heterocaryons can be identified histochemically at any time after fusion. In our experiments, more than two thirds of all multinucleated myotubes consistently contained nuclei of both species. This high efficiency of heterocaryon production permitted detection of a novel gene product in cell extracts 24 hr after fusion. In addition, the frequency of amniocyte activation was high: when one quarter of all nuclei in myotubes were of amniocyte origin, a comparable proportion of the M subunits present in the CK isozymes of heterocaryons were human (Figure 3, lane 6). We therefore think that the time course and efficiency of gene activation in
this system is favorable for an attempt to identify the regulatory factors involved.

In all cases in which we observed activation, muscle cell nuclei were present in excess. Similar gene dosage effects have been reported in other systems, suggesting that an alteration in the balance of repressor and activator regulatory molecules can influence gene expression (Peterson and Weiss, 1972; Darlington et al., 1974, 1982; Brown and Weiss, 1975). Apparently, these regulatory molecules are not restricted to the nucleus, but exist also in the cytoplasm. This is clear from the fact that in our heterocaryons, activation of gene expression was possible, although the nuclei of the two cell types remained distinct. Moreover, since the heterocaryons were formed by fusing amniocytes with myotubes, the activator(s) were still present in muscle cells in which differentiation was already under way. The continued presence of activator molecule(s) suggests that they may be required not only to initiate differentiation but also to maintain the differentiated state.

In summary, the reprogrammed amniocyte of the muscle heterocaryon provides a novel approach to the examination of gene regulation during cell differentiation. The extent to which the expressed potential of a determined cell is heritable and stable as a result of changes in the genome itself and the extent to which it is reversible and subject to regulation by cytoplasmic factors can be usefully explored in stable heterocaryons. This system should be helpful in determining the mechanisms and characterizing the molecules responsible for induction and maintenance of the differentiated state in mammalian cells.

Experimental Procedures

Cell Types, Culture and Fusion Conditions

Muscle cells were a diploid continuous cell line (C2C12) originally isolated by Yaffe from the thigh muscle of a 2-month-old C3H mouse (Yaffe and Sael, 1977) and subcloned and karyotyped in our laboratory. C2C12 cells are maintained as undifferentiated myoblasts in a nutrient-rich growth medium containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum and 0.5% chicken embryo extract. To induce myoblast fusion and the formation of multinucleated fibers, DMEM supplemented with 2% horse serum and determination of a normal euploid karyotype. Amniocytes consist of three individuals following amniocentesis for prenatal diagnosis of three distinct cell types, AF, F and E, distinguishable in culture by their morphology and by their collagen synthetic pattern (Hoehn et al., 1974; Crouch and Bornstein, 1978). Using both these criteria (data not shown), we identified the fibroblastic F amniocytes of three individuals for use in heterocaryon experiments. Prior to fusion with myotubes, amniocytes were grown in the C2 growth medium described above.

Polyethylene glycol (PEG) was chosen as the fusion agent because of its simplicity and reproducibility relative to other agents. Cultures were treated with PEG 1000 at a concentration of 50% (w/v) in DMEM, pH 7.4, for 60 sec at 37°C, according to the method of Davidson et al. (1976). Activated Sendai virus was not used as a fusion agent, in order to eliminate the possibility that the virus might in some way induce gene expression. The addition of dimethylsulfoxide to PEG increased neither the viability of muscle-amniocyte heterocaryons nor the frequency of cell fusion, as it appears to do with other cell types (Norwood et al., 1976).

All media were obtained from Grand Island Biologicals. The horse serum was from Kanose City Biologicals, and fetal calf serum from Sterile Systems, Logan, Utah. Tissue culture plasticware was from Falcon. Cells were grown at 37°C in a humidified Forma incubator with 10% CO2 in air.

Nuclear Identification

Heterocaryons grown on collagen coated (cat skin collagen, Labochem, 1.4 mg/ml dH2O, autoclaved) 35 mm dishes were washed three times with Hanks’ balanced salt solution and fixed first in 1% paraformaldehyde in Hanks’ solution for 20 min at 37°C and then in 100% methanol for 20 min at −20°C. Dishes were then rinsed extensively with 3H2O and stained with Hoechst 33258 (Riedel-de Haen, Hannover, Germany), 0.12 µg/ml 0.9% NaCl for 15 min at 37°C. Stained nuclei were visualized in a Leitz fluorescence microscope with illumination at 340–380 nm.

Extraction and Identification of Contractile Proteins

Contractile proteins were partially purified from tissues and from cell cultures by repeated cycles of high salt solubilization and low salt precipitation according to a modification of the method of Kielley and Harrington (1991). Newly synthesized contractile proteins were labeled in cultured cells for 3 hr with 250 µCi/ml 35S-methionine (Amersham) prior to extraction, fractionated on two-dimensional gels (O’Farrell, 1975) and visualized by autoradiography.

Two-dimensional polyacrylamide gel electrophoresis was performed as described by O’Farrell (1975), with the exception that the second-dimension gel contained 1% acrylamide. Electrophoretic transfer of proteins from SDS gels to unmodified nitrocellulose was performed overnight at 250 mamp, as described by Burnette (1981). For immunodetection of the transferred proteins, a modified procedure of Burnette (1981) was followed. The first antibody was a monoclonal antibody to myosin light chains, designated T14 (Crow et al., 1983), generously provided by Drs. Crow and Stockdale. The monoclonal antibody to myosin light chains, designated T14 (Crow et al., 1983), generously provided by Drs. Crow and Stockdale. The second antibody was linked to biotin and reacted with an avidin-biotin horseradish peroxidase (Vecstain, Vector Labs). Antibody complexes were visualized by reacting the bound horseradish peroxidase with diaminobenzidine and H2O2 according to the method of Graham and Karnovsky (1966).

CK Activity Assay and Gel Electrophoresis

Cell cultures were washed three times with PBS at 4°C and harvested into glycol-glycine buffer (0.05 M glycolglycine, pH 7.5, 1% NP40, 0.1% BME at 4°C) by scraping with a rubber policeman. The cell suspensions were disrupted by sonication, and the supernatants obtained after centrifugation were used for analysis of CK activity and isozyme composition. Total CK activity was determined spectrophotometrically by a coupled enzyme reaction as previously described (Blau and Webster, 1981) using reagents from Boehringer Mannheimer. One unit of CK is the amount that catalyzes the formation of 1 µmol/min of NADPH at 30°C. All measurements of CK activity were corrected for myokinase (adenylate kinase) activity by subtracting the enzyme activity observed in the absence of the substrate, creatine phosphate. Enzymic activity was expressed as a function of total protein determined for the same samples by the method of Lowry et al. (1961).

CK isozymes were visualized by a modification of the method of Perring et al. (1978). Cell extracts were electrophoresed under nondenaturing conditions using a 5% polyacrylamide slab gel, pH 8.9 (0.05 V, 0 in., 4°C). CK isozymes were detected in gels with an agarose overlay (0.5%) impregnated with the same reaction mixture.
as described above, and visualized by ultraviolet illumination (366 nm).

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