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# Analysis of Creatine Kinase Isozymes During Muscle Differentiation

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## I. INTRODUCTION

The terminal differentiation of skeletal muscle cells is characterized by high rates of synthesis of myofibrillar contractile proteins and by the assembly of the myofibrillar contractile apparatus. A number of isoprotein switches from ubiquitously synthesized isoproteins to muscle-specific isoproteins occur, making this system well suited for the study of the regulatory processes controlling tissue-specific gene expression.

 

Isoprotein transitions can be observed during embryonic development as well as during myogenesis in cell culture. Mononucleated skeletal myoblasts in vivo and in vitro become postmitotic, ie, stop dividing and start to fuse to form multinucleated myotubes in which the contractile organelles of the muscle cell, the myofibrils, are assembled. Frequently, the embryonic and/or ubiquitous isoprotein forms are replaced by muscle-specific isoproteins during terminal differentiation. Isoprotein transitions have been described, among other proteins, for myosin where a whole sequence of isoprotein forms of the heavy- and light-chain proteins appears during differentiation. A similar transition from an ubiquitous and/or embryonic isozyme to a muscle-specific form can be observed for one of the key enzymes of muscle energy metabolism, namely creatine kinase (CK). The regulation of the activity of the genes involved, along with the possible significance of the CK-isozyme switch for myofibrillar function, and the general usefulness of CK as a marker protein of muscle differentiation will be discussed here.

## II. CREATINE KINASES

CK, the enzyme which catalyzes the reversible reaction

$$\text{ATP} + \text{creatine} \rightleftharpoons \text{ADP} + \text{P-creatine}, \qquad (1)$$

is found in abundance in skeletal and heart muscle but also occurs in brain [Eppenberger et al, 1967; Watts, 1973] in relatively high concentrations. Presence of high amounts of CK in muscle and brain tissues may allow maintenance of a constant adenosine triphosphate (ATP) level and ensures an immediate rephosphorylation of adenosine diphosphate (ADP). Moreover, using more sensitive detection methods, low amounts of CK have also been discovered in a number of other tissues [Jockers-Wretou and Pfleiderer, 1975; Smith, 1972; Yasmineh and Hanson, 1975].

CK has been known to exist in multimolecular form since the early Sixties when the occurrence of three isozymes with different electrophoretic mobilities was described in a number of species [Burger et al, 1963; Eppenberger et al, 1964]. These isozymes had a pronounced tissue specificity and were named at that time M and B according to their predominant occurrence in skeletal muscle and brain, respectively. The enzymatically active protein is a dimer formed by combination of either two homologous (MM- and BB-CK) or heterologous (MB-CK) subunits. Both subunit types have molecular weights of about 40,000 [Dawson et al, 1967]; they can, however, be separated on SDS acrylamide gels, which indicates slightly different molecular weights [Perriard et al, 1978b; Caravatti et al, 1979]. Antibodies against M-CK do not cross-react with the B-CK subunit and vice versa, but both

antibodies react with the heterodimer MB-CK [Eppenberger et al, 1967; Perriard et al, 1978a]. These facts facilitate CK analysis.

In chicken, MM-CK is almost exclusively found in skeletal muscle, thymus, and thyroid gland [Perriard et al, 1982]. Other muscles like heart, gizzard, or stomach contain the BB-CK isozyme as the most abundant type; in addition BB-CK appears to be widely distributed and thus may be the ubiquitous form of CK [Perriard et al, 1982; Specker, 1975]. While a clear-cut pattern of distribution can be observed in chicken, a more complex situation is found in other birds and in mammals. Mammalian heart contains rather large amounts of MM-CK and MB-CK and smaller amounts of BB-CK [Eppenberger et al, 1967; Jockers-Wretou and Pfleiderer, 1975]; in addition to BB-CK, M-subunit-containing isozymes are also found occasionally in other tissues like kidney or spleen [Yasmineh and Hanson, 1975]. However, it seems quite clear that MM-CK is the CK species accumulating in adult skeletal muscle, and therefore it serves as an excellent muscle marker.

Peptide mapping [Eppenberger et al, 1967; Rosenberg et al, 1981], amino acid composition [Eppenberger et al, 1967], and isolectric point differences indicate that the M- and B-CK subunits are products of at least two genes. In a more detailed study using two-dimensional gel analysis Rosenberg et al [1981] found that M-CK, as well as B-CK itself, shows a distinct heterogeneity which was interpreted as resulting from the expression of multiple genes for each subunit. Microheterogeneity of the MM-CK isozyme has been described in various species including man [Wevers et al, 1977], rabbit [Williamson et al, 1977], and trout [Eppenberger et al, 1971; Perriard et al, 1972]. A random combination of the two observed subspecies of M-CK could well result in additional dimeric M-type isozymes as has been demonstrated by Wevers et al [1977] for human MM-CK. An interesting observation has also been made by Perriard et al [1972], namely, the occurrence of a further (presumably independent) gene locus for CK in stomach tissue of the trout. This was later substantiated by Whitt and his group [Champion and Whitt, 1976; Fisher and Whitt, 1978], who subsequently discussed the interesting fact that advanced teleosts generally have more active CK gene loci than land vertebrates [Fisher et al, 1980].

The existence of a particular mitochondrial form of CK (Mi-CK) has been known since 1964 [Jacobs et al, 1964]. Whereas heart, skeletal muscle, and brain mitochondria show rather high Mi-CK activity, mitochondria from liver, kidney, and testis contain very little or no Mi-CK [Jacobus and Lehninger, 1973; Saks et al, 1980]. Owing to the reported biochemical differences between Mi-CK on one hand and M-CK and B-CK on the other hand, the existence of an additional gene locus for Mi-CK was suggested [Wevers et al, 1977]. In this review only the M- and B-CK-subunits, predominantly from chicken, will be considered.

## III. REGULATION OF THE CREATINE KINASE ISOZYME TRANSITION DURING DEVELOPMENT

### A. Accumulation of Creatine Kinase Isozymes

A rather dramatic change in the isozyme pattern of CK during the ontogeny of rat and chick was observed by Eppenberger et al [1964] in a number of tissues. Particularly striking was the transition in differentiating skeletal muscle tissue, from predominantly BB-CK in early embryos to exclusively MM-CK in adult tissue. During this transition period MB-CK was also observed, indicating that at some time both types of subunits were synthesized and accumulated within the same cell and randomly assembled into the MB-dimer. It was further shown that the developmental profile of CK activity not only included a qualitative change but also a quantitative one, ie, an enhancement of the accumulated enzyme activity. The availability of muscle cell cultures [Morris et al, 1972; Morris and Cole, 1972; Turner et al, 1974] and eventually cell lines [Dym et al, 1978] facilitated studies of these changes during myogenesis. The early stages of the CK isozyme transition in embryonic muscle and myogenic cell cultures undergoing differentiation (Fig. 1) are indistinguishable [Perriard et al, 1978a,]. There is, however, a discrepancy in the completeness of the transition in vivo as compared to the situation in vitro; even after maintaining the cells in culture for more than ten days a significant amount of BB and MB activity is still found in cells. This has been observed by many authors [Morris et al, 1972; Turner et al,

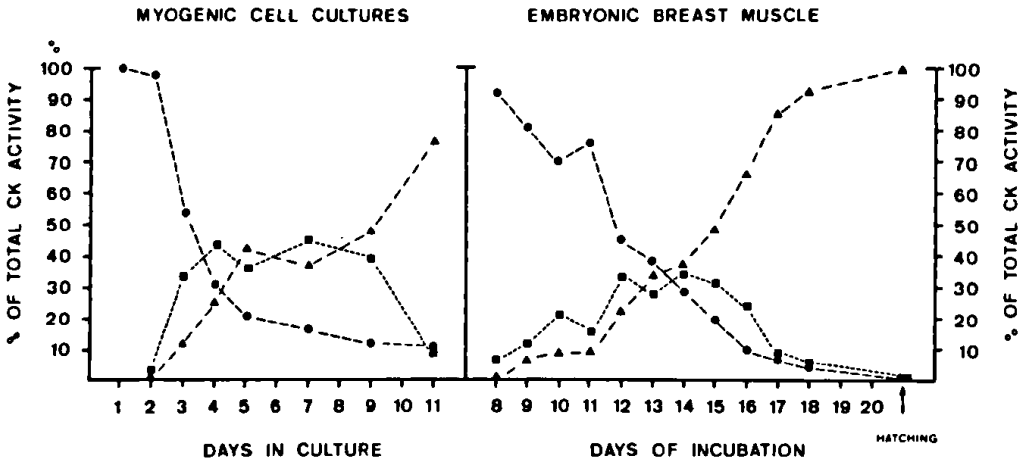


Fig. 1. Comparison of the relative amounts of CK isozymes during the transitions in embryonic muscle and in myogenic cell cultures. ●---●, BB-CK; ■· · ·■, MB-CK; ▲--▲, MM-CK. Isozyme activities are given as percent of total CK activity. (From Perriard et al [1978a], with permission).

1976; Lough and Bischoff, 1977; Dym et al, 1978; Perriard et al, 1978a; Caravatti et al, 1979]. It has been shown [Lough and Bischoff, 1977], at least for chicken muscle cell cultures, that contaminating fibroblasts are not the main source of the significant amounts of BB- and MB-CK activity in these cultures. No satisfactory explanation has been found so far. The phenomenon may be related to some conditions of the cell culture system. The possible control of this isoprotein switch was studied at the level of protein biosynthesis and accumulation of translatable mRNA [Caravatti et al, 1979; Perriard, 1979]. The study of stage-specific expression and structure of the genes involved in the CK switch will become possible as the experiments now in progress with cloned cDNA probes of CK gene sequences are completed.

### B. Biosynthesis

Incorporation of radioactively labeled aminoacids into the subunits of M- and B-CK was measured by immunoprecipitation methods [Perriard et al, 1978b; Caravatti et al, 1979]. The cells were exposed to two-hour pulses of $^{3}$H-labeled leucine. A significant increase in B-CK synthesis with a peak at 72 hours, followed by a decrease to the initial values, was observed, whereas no significant M-CK synthesis could be measured in 24-hour cultures; however, a dramatic increase of M-CK synthesis occurred thereafter, and continued throughout the duration of the experiment (144 hours). When differentiation of myogenic cells was blocked by growing them in a medium containing the thymidine analogue, bromodeoxyuridine (BrdU) [Caravatti et al, 1979], a reduced incorporation of $^{3}$H-leucine into B-CK was observed whereas no synthesis of M-CK subunits could be seen, even after prolonged culturing. BrdU has been shown to block fusion of myoblasts into myotubes [O'Neill and Stockdale, 1974; Merill et al, 1980] and to inhibit biochemical differentiation including the CK-transition from B to M [Turner et al, 1976; Caravatti et al, 1979]. However, the regulation of CKs might be controlled not only by the rate of synthesis, but also by the rate of differential degradation. To solve this question, turnover kinetics for both subunit types were determined in cultured myogenic cells [Caravatti and Perriard, 1981]. No great differences were found; thus, differential degradation appears not to be a major factor in the control of the isozyme transition during myogenesis.

### C. mRNAs for Creatine Kinase Subunits

The rates of synthesis of the CK subunits during development may be controlled either by changing amounts of newly transcribed mRNAs for B- or M-CK, or by activation of preexisting and possibly unprocessed or sequestered mRNAs. In order to test these possibilities and further elucidate the mechanisms underlying the CK-isozyme switch in cell culture, relative

amounts of translatable mRNA for the CK subunits were determined by means of a cell-free translation system [Perriard et al, 1978b]. The availability of translatable mRNA in total cellular RNA or in polysomal RNA is comparable to the rates of biosynthesis of B-CK and M-CK subunits. Only insignificant amounts of mRNA for the M-CK subunit were found in 48-hour cultures, but one day later a strong increase in translatable mRNA for the muscle-specific M-CK could be demonstrated, which paralleled the observed increase of $^{3}$H-leucine incorporation into M-CK subunits [Caravatti et al, 1979]. In contrast, translatable mRNA for the ubiquitous B-CK subunit was detectable in cultures at the time of plating, suggesting the presence of enough mRNA for B-CK in proliferating presumptive myoblasts [Perriard, 1979]. The possibility that the CK transition is subject to transcriptional control was supported by further experiments employing BrdU. If myogenic cultures were grown for three days in BrdU-containing medium and subcultured under the same conditions, no mRNA for M-CK was present that could be translated in the cell-free system; in contrast, mRNA for B-CK could readily be detected by translation of RNA from BrdU-treated cells (Fig. 2). The inhibition is reversible by subculturing into BrdU-free medium after which translatable mRNA for M-CK appears. It is still unclear at which level BrdU acts. It is conceivable that the M-CK gene and other muscle genes are transcribed in BrdU-treated cells but that the mRNA precursors are not properly processed. There are, however, indications from several laboratories that proliferating, presumptive myoblasts contain only little if any mRNA for muscle-specific proteins [Paterson and Bishop, 1977; Dym et al, 1979; Devlin and Emerson, 1978; Affara et al, 1980]. The reported data for the CK isozyme transition, both at the levels of protein biosynthesis and accumulation of translatable mRNAs, suggest that M-CK is not additionally controlled at the level of translation as has been found for myosin heavy chain in chicken skeletal muscle [O'Loughlin et al, 1981; Havaranis and Heywood, 1981]. Progress in the evaluation of the control of protein synthesis in muscle cells has lately been made by using cDNA clones corresponding to the major contractile proteins, actin, myosin light and heavy chains in mammalian [Katcoff et al, 1980; Medford et al, 1980; Minty et al, 1981] and avian [Hastings and Emerson, 1982] systems. An overall abundance of poly-$A^{+}$ RNA in mRNAs for these contractile proteins has been found in multinucleated myotubes as compared to myoblasts; by gel transfer hybridization analysis a 20- to 30-fold increase in cellular content of these mRNAs during differentiation could be determined [Shani et al, 1981; Hastings and Emerson, 1982]. Conclusive answers concerning the control of the CK switch clearly require a similar strategy, and the construction of cDNA sequences from CK mRNA has therefore become a primary goal.

### D. Molecular Cloning of M-Creatine Kinase Sequences

By the use of recombinant DNA technology a cDNA library containing muscle sequences was constructed [Rosenberg et al, 1982]. The clones were screened for plasmids yielding positive hybridization signals with radioactively labeled cDNA synthesized from the poly-$A^+$ RNA fraction of RNA isolated from chicken leg muscle, but not hybridizing with cDNA derived from chicken gizzard (smooth muscle) RNA. Since gizzard does not contain measurable amounts of MM-CK or mRNA for M-CK a first selection could

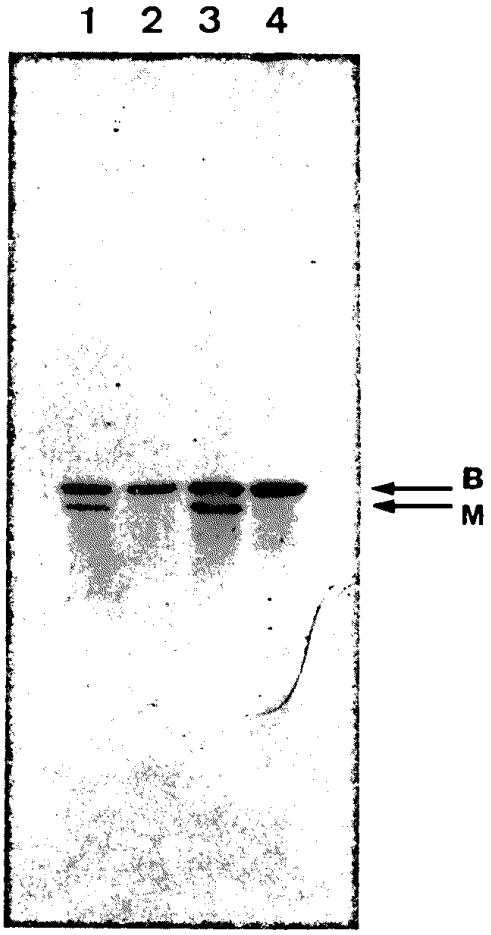


Fig. 2. Fluorography of a 10% SDS-polyacrylamide gel of the CK subunits synthesized in an in vitro cell-free system (rabbit reticulocyte lysate) primed by RNA from BrdU-treated cells. (1) Total cell RNA from cells cultured for 72 hours in BrdU-containing medium and subcultured into standard medium for 72 hours; (2) total cell RNA from cells cultured for 72 hours in BrdU-containing medium and subcultured into the same medium for 72 hours; (3) polysomal RNA from cells as described in 1; (4) polysomal RNA from cells as described in 2. B, M indicate the positions of the B-CK and M-CK subunits, respectively.

be achieved in that way. Plasmids which preferentially hybridized with cDNA from poly-$A^+$ RNA, enriched for M-CK mRNA on a density gradient, were further selected and characterized by transfer of nucleic acids onto membranes [Thomas, 1980]. The DNA from positive clones was purified and immobilized on nitrocellulose membranes. The matrix-bound DNA was hybridized with poly-$A^+$ RNA from leg muscle. The DNA from one clone (later named $pMCK_1$) retained specifically mRNA for M-CK, as demonstrated by in vitro cell-free translation of the eluted mRNA. A protein band with the same mobility as purified M-CK was obtained. This protein band was also pre-

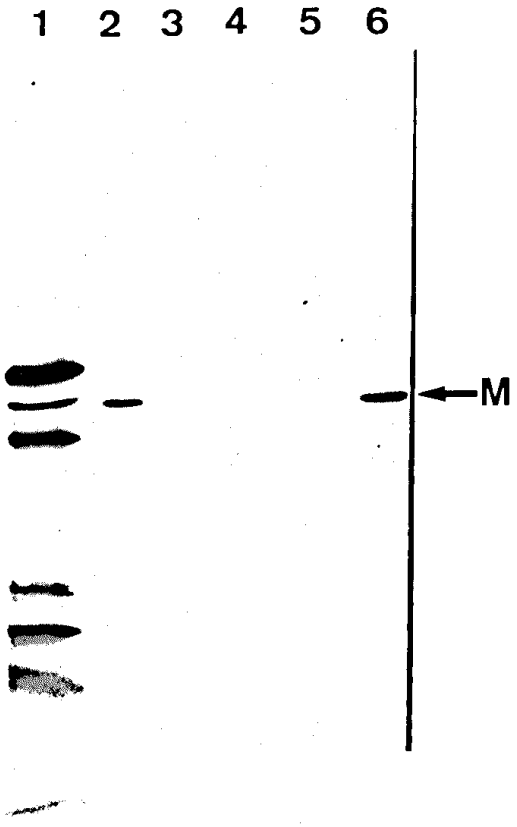


Fig. 3. Identification of cloned M-CK sequences by positive hybrid-selected translation. Fluorography of in vitro translation products separated on 14% polyacrylamide-SDS gels after immunoprecipitation with anti-M-CK antibody (lanes 2–6). The RNA used in the translation assays on lanes 2 and 3 was selected by hybridization to membrane-bound plasmid DNA. Lane 1: 1 μg poly-$A^+$ RNA from chicken leg; lane 2: RNA eluted from a membrane containing bound $pMCK_1$ DNA; lane 3: RNA eluted from a membrane containing bound pBR322 DNA; lane 4: 50 μg of rabbit reticulocyte RNA enriched for globin mRNA; lane 5: no RNA added (control); lane 6: 1 μg poly-$A^+$ RNA from chicken leg. M indicates the position of the M-CK subunit.

cipitable with specific anti-M-CK antibody (Fig. 3). Analysis by two-dimensional electrophoresis revealed a double spot pattern of snythesized protein identical to that of both purified M-CK and translated M-CK mRNA [Rosenberg et al, 1982]. The $pMCK_1$ DNA was further characterized by restriction mapping. Thus the sequence homology between $pMCK_1$ and M-CK mRNA was established. When poly-$A^+$ RNA from adult chicken muscle or from gizzard was denatured and separated on agarose gels, hybridization of $pMCK_1$ with an RNA fraction slightly smaller than 18S was observed in the case of skeletal muscle RNA, but no hybridization was detected with poly-$A^+$ RNA from chicken gizzard. This was another indication that smooth muscle does not contain MM-CK or translatable mRNA for the M-CK subunit. Additional information on possible levels of control for M-CK expression was obtained by experiments in which radioactive $pMCK_1$ DNA was hybridized with RNAs from differentiating myogenic cell cultures (Fig. 4). In cells from 24-hour cultures (mainly proliferating presumptive myoblasts) no hybridizing RNA could be detected, whereas mRNA for M-CK was readily found in cells from 42 or 72-hour cultures (myotubes) by using the specific $pMCK_1$ probe. Similar experiments were carried out with RNA derived from embryonic muscle and showed that muscle from 5-day old embryos or older contained mRNA sequences homologous to $pMCK_1$ (Fig. 4). These data support the assumptions made earlier on the basis of synthesis and accumulation of M-CK subunits and of synthesis and accumulation of translatable mRNA for M-CK.

With the probes now available another cDNA library was screened and the clone $pMCK_2$ was detected, which carried an insert of about 640 base pairs (bp) of CK cDNA. This DNA contains an internal PstI restriction site and was subcloned as two fragments of 350 and 290 bp into the PstI site of the phage M 13 mp8. Determination of the nucleotide sequence of the single-stranded $pMCK_2$ probe will provide a substantial part of the mRNA length and allow screening genomic DNA libraries for CK gene sequences.

## IV. FUNCTIONAL SIGNIFICANCE OF CREATINE KINASE TRANSITION

### A. M-Line-bound Creatine Kinase in Myofibrils

An important question is whether the newly appearing MM-type isozyme of CK has a functional role in the differentiated cell that cannot be fulfilled by the isozyme found in the precursor cells. A number of biochemical characteristics of MM- and BB-CK have been studied, such as specific activities, turnover rates, pH- and temperature optima, etc [Dawson et al, 1965; Dawson et al, 1967; Eppenberger et al, 1967; Caravatti and Perriard, 1981], but no striking differences have been found between the two isozymes. There is,

however, a significant difference between MM-CK and BB-CK with respect to the ability to bind to the contractile organelles of myogenic cells. One component of the electron-dense M-line, which traverses the center of the sarcomere of myofibrils and consists mostly of so-called m-bridges, has been identified as MM-CK [Turner et al, 1973; Eppenberger et al, 1975]; ap-

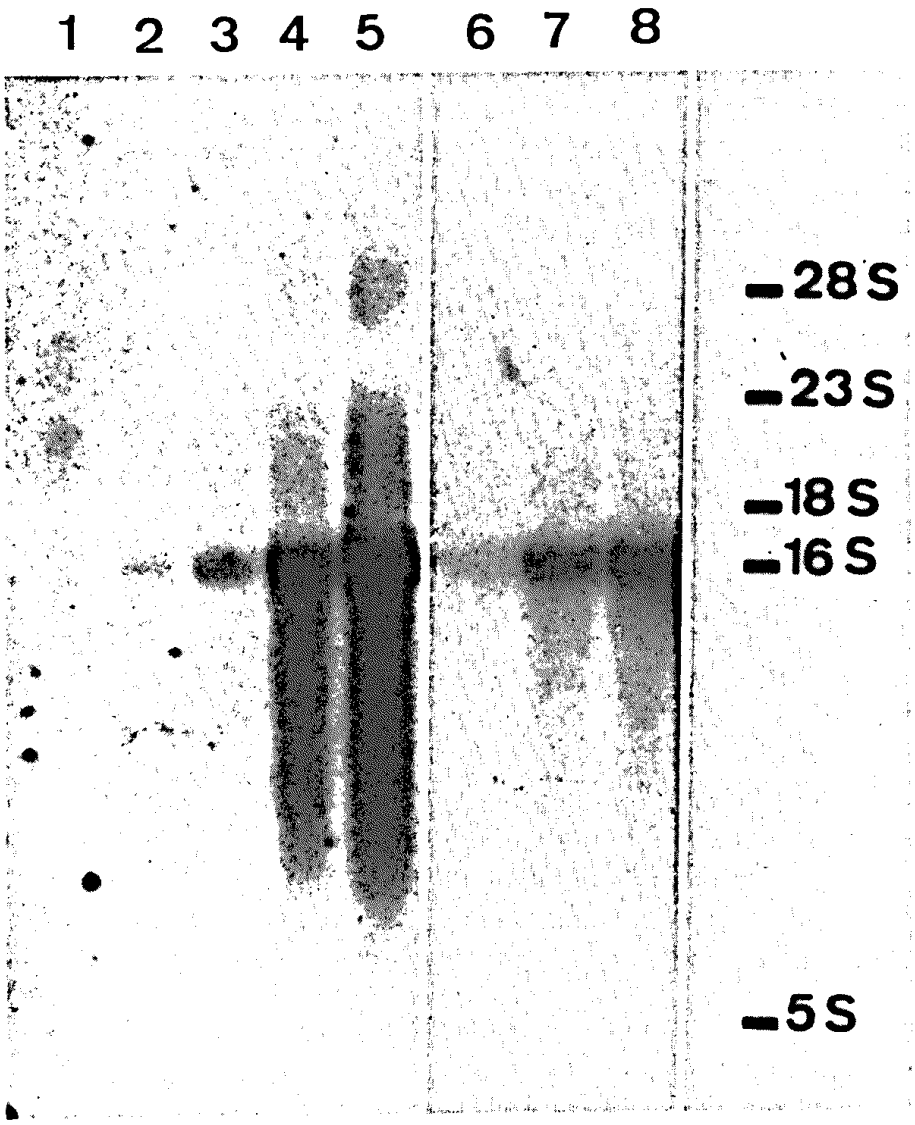


Fig. 4. Detection of mRNA for M-CK in RNA isolated from embryonic tissue and differentiating myogenic cells; 50 μg denatured total RNA was separated on a 1.2% agarose gel, transferred to "gene screen" membrane, and hybridized with $^{32}$P-labeled pMCK$_1$ DNA as a probe. Lanes 1–5: total RNA from leg muscle of 5-, 8-, 11-, 15-, 19-day-old embryos respectively; lanes 6–8: total RNA from 24-, 42-, 72-hour myogenic cell cultures, respectively. By courtesy of Dr. U. Rosenberg.

proximately 5% of the total CK of adult chicken skeletal muscle is specifically bound to this structure under physiological conditions [Wallimann et al, 1977a]. Isozyme identification was achieved by employing anti-CK antibodies. As judged by either immunofluorescence staining or by heavy IgG decoration of the M-line, only the anti-MM-CK antibodies reacted with the M-line-bound antigen (Fig. 5), whereas anti-BB-CK antibody only gave a weak reaction in the I-band region of somitic myofibrils [Wallimann et al, 1977b] or of myofibrils in cell cultures. Incubation with an excess of monovalent Fab fragment of anti-M-CK antibody, on the other hand, led to either a complete removal of fluorescence or the removal of the electron-dense material, or both [Wallimann et al, 1978]. MM-CK was assigned to the primary m-bridges which form part of the electron-dense M-line structure. This is based on the observation that most of the electron-dense material can be removed either by anti-MM-CK Fab fragment incubation or by low ionic

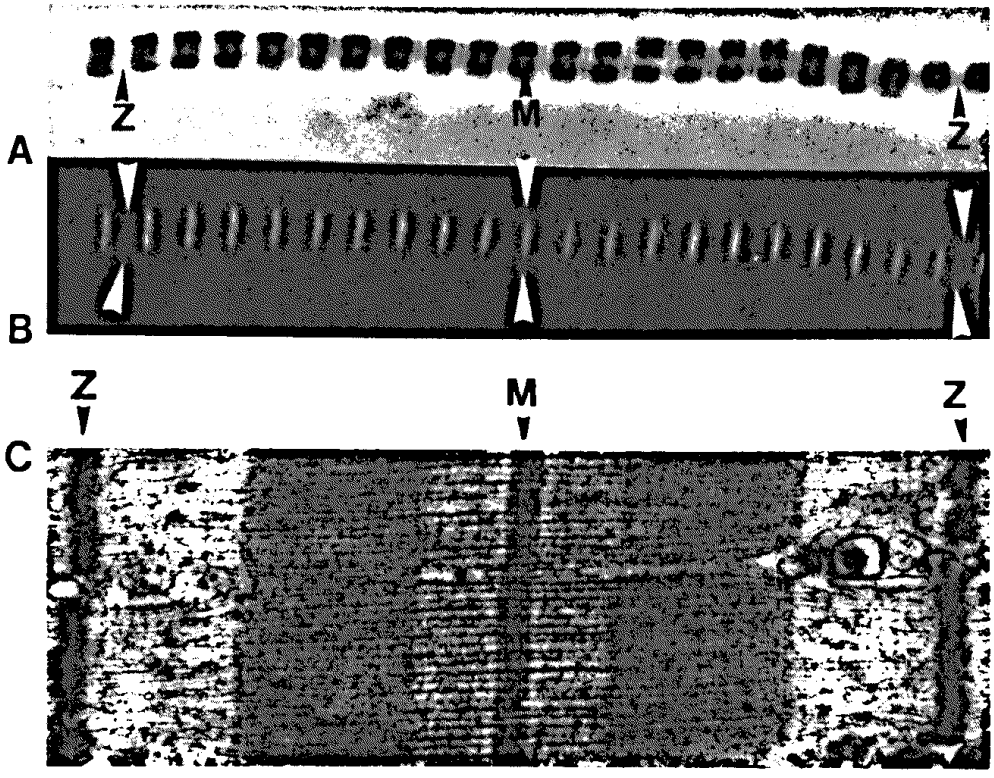


Fig. 5. Reaction of M-line-bound M-CK in chicken skeletal myofibril with anti-M-CK IgG. B: Indirect immunofluorescence staining of M-line region. A (top): Corresponding phase-contrast picture. C (bottom): Electron micrograph of myofibrillar sarcomer IgG decoration of M-line region. × 45,000. M, M-line; Z, Z-line. By courtesy of Dr. E. Strehler.

strength buffer treatment [Wallimann et al, 1977a, 1978] concomitantly with the removal of all the measurable CK activity. The identity of MM-CK and primary m-bridges is also supported by the fact that the amount of the bound MM-CK and the molecular dimensions of the CK dimer are consistent with the number and dimensions of the primary m-bridges [Wallimann et al, 1977a; Woodhead and Lowey, 1982; Arps and Harrington, 1982]. However, it is unlikely that MM-CK has only a structural function within the myofibrillar structure because the protein can be removed rather easily, in contrast to myomesin [Eppenberger et al, 1981], another M-line component, which has been shown to be much more intrinsically bound to the structure [Strehler et al, 1980].

### B. Role of Bound Creatine Kinase in ATP Regeneration

A dual role as a structural element and as an enzyme involved in the energy metabolism of the myofibril has been suggested for CK, analogous to the dual role of myosin, which functions as a major component of the contractile structure and has ATPase activity. Recent data indeed suggest a physiological role of M-line-bound CK as an intramyofibrillar ATP regenerating system [Schlösser et al, 1982]. Using isolated and washed skeletal muscle myofibrils in an in vitro coupled system, in which the combined activities of myofibrillar CK and myofibrillar actin-activated $Mg^{++}$-ATPase were measured, it was possible to show that addition of exogenous CK had no influence on the steady-state velocity of the ATPase activity measured by creatine phosphate hydrolysis. This indicates that the M-line-bound MM-CK was sufficient for rephosphorylating all the ADP which had been formed by the myofibrillar ATPase (Table I). The ATP concentration has been kept constant under in vitro conditions by the endogenous CK and creatine phosphate, exactly as had been observed under in vivo conditions [Carlson and Siger, 1960]. However, when myofibrils were extracted by low ionic strength buffer or incubated with an excess of anti-MM-CK Fab in order to remove all bound MM-CK from the M-line, no more rephosphorylation of ADP to ATP took place, but the ATPase was still fully active and resulted in depletion of ATP (Fig. 6). A similar effect could be produced by blocking CK activity either by anti-MM-CK antibody (IgG), or by iodoacetic acid treatment of the myofibrils, which inactivates CK but not ATPase activity (Table I). It was estimated that under the most unfavorable conditions enough CK is present in vivo to regenerate a significant portion of the hydrolized ATP [Wallimann et al, 1977a].

### C. Developmental Appearance of Creatine Kinase in the M-Line

M-line-associated MM-CK can be found rather early in development. In somitic cells from 4-day-old chicken embryos MM-CK is detectable within the M-line structure of early myofibrils only by immunofluorescence staining.

BB-CK, on the other hand, representing the major isozyme at that developmental stage, is never found in the M-line region [Wallimann et al, 1977b]. A similar isozyme-specific association of MM-CK with the M-line has recently been shown to occur in myofibrils of differentiating muscle cells in culture [Wallimann et al, 1982]. Unlike differentiated muscle these cells contain all 3 CK isozymes (MM-CK, MB-CK, BB-CK) and therefore allow a better demonstration of the isozyme-specific association of CK with subcellular organelles. After permeabilization of the cells, the bulk of the cytoplasmic CK was removed, thus leaving behind only myofibrillar-bound CK. Upon immunofluorescent staining of such extracted cells with anti-MM-CK antibodies, MM-CK could be detected in the M-line (Fig. 7). If, however, staining was done with anti-BB-CK antibodies, only a weak fluorescence

**TABLE I. Effects of Inactivation and Removal of M-Line-Bound MM-CK on ATP Regeneration**

| Myofibril treatment | Actin-activated $Mg^{++}$ ATPase activity[a] (μmoles CP/min/mg myofibril) | | M-line-bound CK activity (μmoles CP/min/mg myofibril) | % |
|---|---|---|---|---|
| | − exogenous CK | + exogenous CK | | |
| None | 0.37 | 0.37 | 0.800 | 100.00 |
| 10 mM IAA[b] (inactivation) | 0.00 | 0.33 | 0.001 | 0.13 |
| 45-min incubation in 5 mM TRIS[c] pH 7.8 (removal) | 0.00 | 0.29 | 0.03 | 3.80 |
| Anti-M-CK IgG (inactivation) | 0.00 | 0.34 | 0.15 | 19.00 |
| Anti-M-CK Fab (removal) | 0.00 | 0.36 | 0.06 | 7.60 |
| Preimmune IgG or Fab | 0.35 | 0.35 | 0.79 | 100.00 |

[a]Combined pH-stat assay measuring myofibrillar ATPase via P-creatine (CP) hydrolysis:

(1) myofibrils + ATP $\xrightarrow[Mg^{++}\ ATPase\ (+Ca^{++})]{\text{actin-activated}}$ myofibrils + ADP + $P_i$ + $H^+$

(2) ADP + CP + $H^+$ $\xrightarrow{CK}$ ATP + C

At pH 7.0 the net consumption of $H^+$ per CP hydrolyzed is 0.33, by which the steady-state rate of the myofibrillar actin-activated $Mg^{++}$—ATPase is measured, provided that CK is present in excess [Schlösser et al., 1982].

[b]Iodoacetic acid.

[c]Low ionic strength conditions [Wallimann et al, 1977a].

was observed in the Z-region. Since no B-CK-specific staining was apparent in the M-line all the MB-CK appeared to be soluble and had been removed previously (Fig. 7). It is apparent from these results that only the MM-CK isozyme which appears during terminal differentiation, but not the other CK isozymes also present in these cells, is able to associate with the M-region of the myofibril, even under culture conditions in which CK-isozyme transition has not yet gone to completion.

No conclusive results have been obtained as yet concerning the developmental timing of MM-CK incorporation into the M-line. The appearance of MM-CK within the M-line can be compared to that of the M-line protein, myomesin, which is present in nascent myofibrils [Eppenberger et al, 1981]. After incubation with antimyomesin antibody, nascent myofibrils showed an

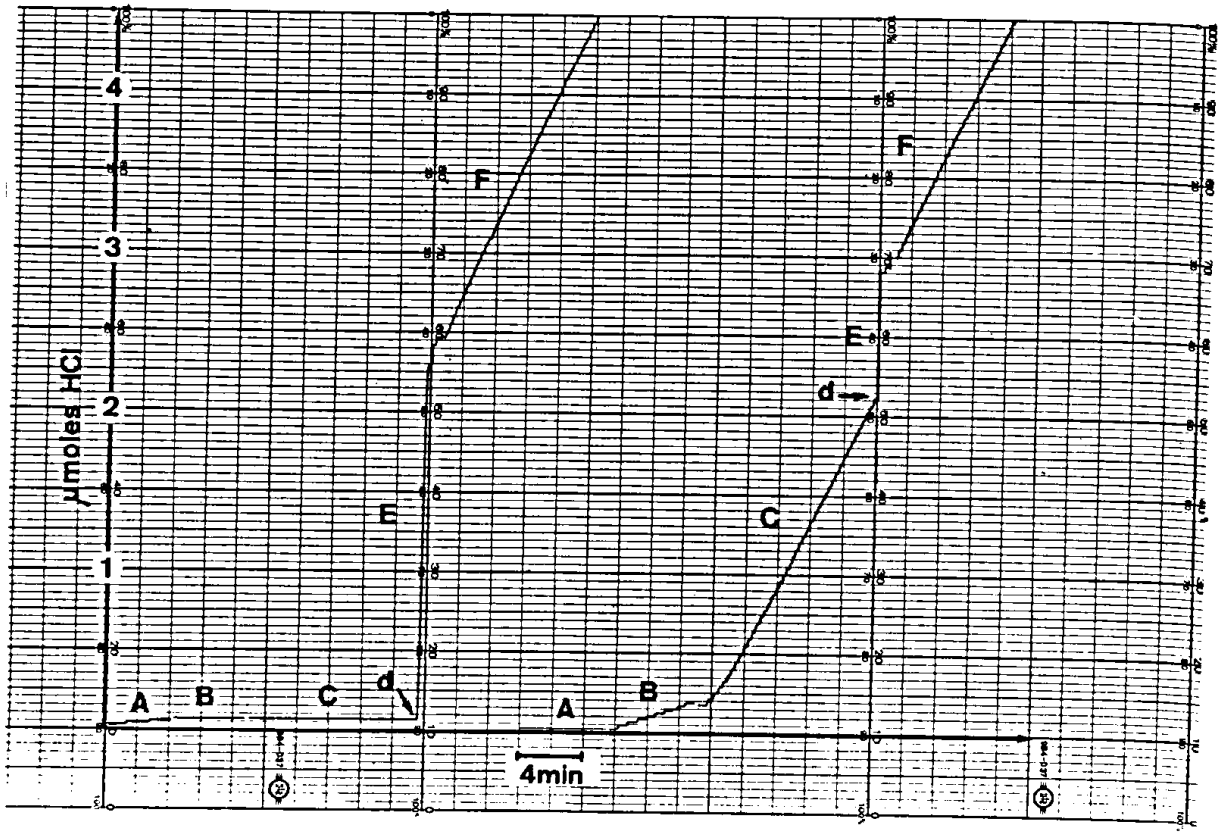


Fig. 6. pH-stat titration recording of coupled actin-activated $Mg^{++}$-ATPase/CK assay. Left, anti-MCK Fab-extracted myofibrils; right, preimmune Fab-treated myofibril (no extraction of M-line-bound CK). A, reaction blank (buffer + 4 mM ATP + 10 mM CP); B, addition of 2 mg myofibrils; C, addition of 0.4 mM $CaCl_2$; E, compensatory reaction after addition of 20 EU of exogenous CK (d); F, slope after stabilization of compensatory reaction. For more details see also Table I. By courtesy of T. Schlösser.

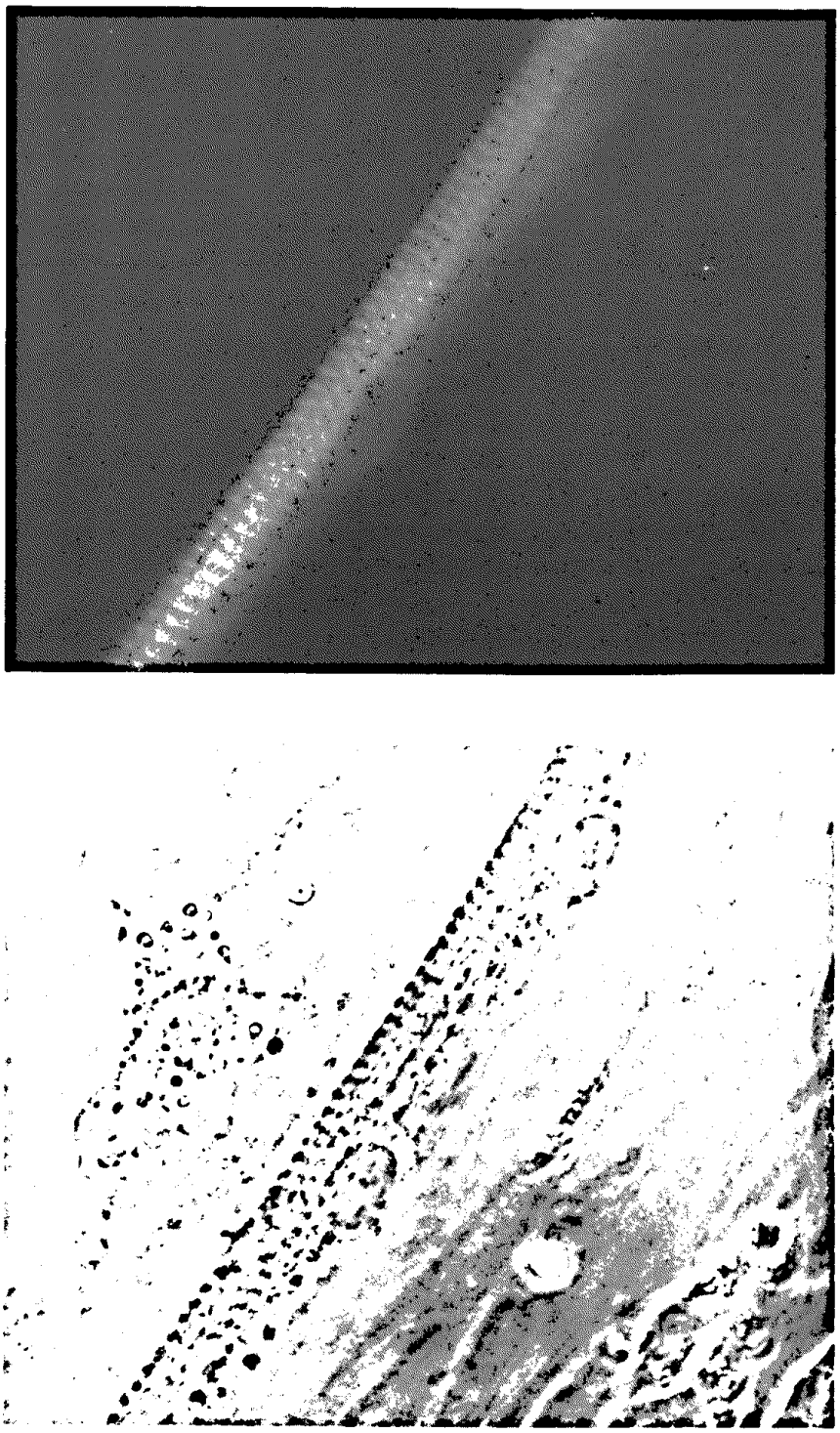

Fig. 7. Eight-day-old skeletal muscle cell culture (myotubes). Fixed and permeabilized cells (extraction of soluble M-CK) were incubated with anti-M-CK IgG. A. Indirect immunofluorescence staining of M-line; B. Corresponding phase-contrast picture. By courtesy of Dr. T. Doetschman.

antimyomesin IgG decoration in the M-line region whereas only traces of an electron-dense M-line were observed in untreated nascent myofibrils, indicating a somewhat delayed incorporation of MM-CK in skeletal muscle myofibrils [Eppenberger et al, 1981]. Thus an involvement of the structural fraction of MM-CK in the assembly of myofibrils is unlikely, but a role for an improvement of muscular efficiency might be assumed. Attached to a strategically important site, MM-CK might provide, on the one hand, an enzyme involved in ATP regeneration, and on the other hand, a structural reinforcement of myofibrils by means of the primary m-bridges in such muscle types where a higher degree of maintenance of filament-order is required. The variations observed in connection with the timing as well as with the appearance of a visible electron-dense M-line can be considered to support such a scheme. There are fast and slow muscle types differing in the number of primary m-bridge arrays but having the same type of MM-CK [Rosenberg et al, 1981]; and there are muscles which lack a distinct electron-dense M-line like chicken heart muscle, which also does not show synthesis or accumulation of M-CK subunits [Eppenberger et al, 1967]. In chicken cardiac muscle the B-CK gene is expressed throughout life. In mammalian heart, on the other hand, a transition from B- to M-CK takes place during differentiation and a delayed incorporation of MM-CK into the M-line of cardiac myofibrils has also been observed [Anversa et al, 1981; Carlsson et al, 1982]. It has been suggested in this case that MM-CK appearance within the M-line parallels the improvement of contractile properties. The association of CK with myofibrils may indeed represent an important step in their physiological maturation.

## V. CONCLUSIONS

Many isozyme or isoprotein transitions are known to occur during muscle differentiation. They characterize the changing phenotypes of cells throughout development, but little is known about their functional significance, or about their advantage for the differentiating and finally differentiated cells. In contrast, the isozyme transition from BB- to MM-CK described above is accompanied by the acquisition of an additional functional feature that can only be fulfilled by the muscle-specific form of CK (MM-CK) but not by the ubiquitous BB-CK. The isozyme-specific incorporation into the myofibrillar M-line structure of an enzyme directly involved in energy provision may represent a functional advantage restricted to cells which express the M-CK gene. As more knowledge about the structure and organization of genes becomes available it should also be possible to investigate the levels of control of muscle-specific protein expression. The CK-isozyme system is certainly well suited for studying those fundamental questions of regulation which might also be common to many other muscle isoprotein transitions.

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