*Journal of Muscle Research and Cell Motility* 4, 429–441 (1983)

# Isoenzyme-specific localization of M-line bound creatine kinase in myogenic cells

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Received 26 October 1982 and in revised form 10 December 1982

## Summary

Experiments using isolated fibre bundles or myofibrils of chicken skeletal muscle have shown that a relatively small portion of the muscle-specific MM-type* of creatine kinase (CK) (EC 2.7.3.2) is specifically bound to the M-line and yet greatly contributes to the electron-dense M-line structure. Here we demonstrate the presence of M-line bound CK in cultured myogenic cells by removing the unbound sarcoplasmic CK through permeabilization with Triton X-100 and extensive washing of the cells prior to immunofluorescence staining. When stained with antibodies specific for M-CK subunits these cells exhibit bright fluorescence within the M-line region of myofibrils. Occasionally this cross-striated pattern is also observed in mononucleated presumably postmitotic myoblasts. Anti-B-CK incubation, in contrast, results in a weak, diffuse fluorescence at the Z-band. Even though these cells contain appreciable amounts of B-type CK, specific fluorescence at the M-line is never observed with anti-B-CK antibody thus ruling out the presence of BB-type or MB-type CK at this location. Therefore the presence of CK within the M-line structure of myogenic cells which contain all three CK isoenzymes seems to be restricted to the MM-type isoenzyme.

## Introduction

Myogenic cells derived from developing skeletal muscle of avian and mammalian embryos are known to proliferate and differentiate readily *in vitro*. Fusion of mononucleated, postmitotic myoblasts leads to the formation of multinucleated, cross-striated, contractile muscle fibres (Konigsberg, 1960; Stockdale & Holtzer, 1961; Yaffe & Feldmann, 1964). The process of terminal differentiation of muscle is accompanied by a number of biochemical changes such as initiation of synthesis and accumulation of muscle-specific proteins which subsequently form the specialized

*MM-CK and BB-CK refer to the muscle and brain type of creatine kinase, respectively. MB-CK represents the hybrid form.

0142-4319/83 $03.00 + .12 

subcellular structures characteristic of functional muscle, for example, myofibrils (for review, see Buckingham, 1977, and Merlie *et al.*, 1977). During muscle cell differentiation quantitative alterations of enzyme levels are frequently accompanied by isoenzyme transitions indicating differential gene activation.

The ubiquitous form of creatine kinase, BB-CK, which is found in brain, smooth muscle and heart is the predominant form in embryonic skeletal muscle. During muscle cell differentiation MM-CK is induced and becomes the prominent form of CK, both *in vivo* (Eppenberger *et al.*, 1964) and *in vitro* (Turner *et al.*, 1974, 1976a,b). Since the two subunits of CK are at times simultaneously synthesized within myogenic cells the transition from BB-CK to MM-CK proceeds via the transitory MB-CK hybrid so that at intermediate stages of development all three isoenzymes of CK are found within a single cell (Perriard *et al.*, 1978a). As shown by *in vitro* translation experiments (Perriard *et al.*, 1978b) and protein turnover studies (Caravatti & Perriard, 1981) the transition is principally regulated by differential rates of synthesis of B and M subunits (Perriard, 1979). As a consequence, in fully differentiated chicken skeletal muscle the predominant form of CK is the MM type which is present at high levels (approx. 5 mg of CK per gram wet weight of muscle) while MB or BB are undetectable (Eppenberger *et al.*, 1964; Wallimann *et al.*, 1977b). In chickens a small but significant amount of this otherwise mostly soluble MM-CK (approx. 5% of the total CK present) is specifically bound to the M-line of skeletal myofibrils (Morimoto & Harrington, 1972) representing the major part of the electron-dense material within the M-line structure (Wallimann *et al.*, 1975, 1977b, 1978). Based on the finding that M-line fluorescence of myofibrils isolated from muscle tissues of different developmental states is observed only with anti-M-CK but not with anti-B-CK antibodies, it has been concluded that the incorporation of CK into myofibrils is isoenzyme specific (Wallimann *et al.*, 1977a,b). This is supported by the observation that in chicken heart muscle where BB-CK is the predominant form of CK the M-region is devoid of a distinct electron-dense M-line (Sommer & Johnson, 1969; Strehler *et al.*, 1979; Wallimann *et al.*, 1977b). On the other hand, using myofibrils isolated from embryonic tissue for immunofluorescence staining one cannot conclusively prove that these myofibrils, though staining exclusively at the M-line with anti-M-CK, are actually derived from muscle cells which still contain all three CK isoenzymes. Consequently it has not been possible to draw definitive conclusions on the subcellular localization of both CK isoenzymes within the myofibrils during differentiation. Here we investigate the M-line bound CK within myogenic cells and demonstrate the isoenzyme-specific binding of CK to myofibrillar structures within cells which actually contain all three isoenzymes of creatine kinase.

## Materials and methods

### PREPARATION OF MYOGENIC CULTURES

Primary cultures of chicken skeletal muscle were prepared from breast muscles of 11–12-day-old chick embryos essentially according to the methods described by Turner *et al.* (1974). Nominal plating density was kept at $4 \times 10^5$ cells per 4 ml standard medium and 50 mm gelatinized culture

dish (Lux). The standard medium consisted of 86.5 parts of MEM (Earle, Seromed), 10 parts of heat inactivated horse serum (Seromed), 3.5 parts of chicken embryo extract prepared in an equal volume of Simm's balanced salt solution (BSS), 2.5 parts of 200 mM L-glutamin (Seromed) and 1.5 parts of a mixture of penicillin and streptomycin (10 000 units $ml^{-1}$ Gibco No. 54). Cells were maintained at 37° C in a high humidity incubator with 5% $CO_2$. Usually the medium was not changed.

## FIXATION AND PERMEABILIZATION OF CELLS

### *Standard technique*

After rinsing the plates with phosphate-buffered physiological salt solution (PBS) cells were fully fixed *in situ* using 3% paraformaldehyde in PBS (this solution was stored frozen) at pH 7.2–7.5 for 15 min. After three washes with PBS traces of paraformaldehyde were quenched by three changes of 0.1 M glycine in PBS pH 7.5 for 30 min. Permeabilization of cells for subsequent staining with antibodies was achieved by incubation of cells for 4 min with 0.2% Triton X-100 in PBS. After three washes with PBS the fully fixed cells were ready for immunofluorescence staining.

### *Modified technique*

Superficial prefixation of cells to prevent cell detachment was achieved by addition of 3% paraformaldehyde in PBS for 20–30 s. Cells were immediately washed with PBS followed by gentle permeabilization using 0.05% freshly prepared Triton X-100 in PBS for 3 min. Soluble intracellular material was removed by three washes with PBS and the remaining cellular constituents postfixed by treatment with 3% paraformaldehyde in PBS for 15 min. After quenching with three changes of 0.1 M glycine in PBS, followed by three washes with PBS, the cells were ready for immunofluorescence staining. Postfixation can be omitted though cells detach from the plate more easily without it. To avoid detachment, liquids were added and removed carefully from the side of the dish using a Pasteur pipette.

## INDIRECT IMMUNOFLUORESCENCE STAINING

After PBS was aspirated from the culture plates 100–200 $\mu$l of affinity purified anti-B-CK or anti-M-CK IgG or control IgG diluted with PBS to 1–5 $\mu g\ ml^{-1}$ containing 1 mg $ml^{-1}$ of bovine serum albumin was placed on selected areas on the culture dish. After incubation for 30 min followed by three washings with PBS 100–200 $\mu$l of 1:100 diluted FITC-conjugated goat anti-rabbit antibody (Cappel) was placed on the same area and the cells incubated for another 30 min. After three washings with PBS and removal of excess PBS, a drop of 50% glycerol in 0.1 M glycine–NaOH at pH 9.0 was placed onto the cells which then were covered by a coverslip, examined and photographed with a Zeiss fluorescence microscope essentially as described (Turner *et al.*, 1976b). The same antisera and affinity purified antibodies specific for B and M subunits of CK previously characterized by immunodiffusion, immunoprecipitation, immunoelectrophoresis, microcomplement fixation and affinity chromatography in this laboratory (Turner *et al.*, 1976a; Wallimann *et al.*, 1977a; Perriard *et al.*, 1978b; Caravatti *et al.*, 1979) and in addition by immunoblotting on nitrocellulose were used in this study. Anti-M-CK and anti-B-CK both react with the heterodimer, MB, but not with the heterologous homodimers.

## IMMUNOBLOTS

Tissue extracts (1:5 diluted w/v) and concentrated extracts from cell cultures were subjected to PAGE under native conditions which were best suited to separate CK isoenzymes (Wallimann *et al.*, 1977a,b). Subsequently the protein bands were electrophoretically transferred onto nitrocellulose paper (Towbin *et al.*, 1979) and then stained by aliquots of the same anti-M-CK and anti-B-CK antibody preparations that were used for indirect immunofluorescence staining of cells.

Bound antibody was visualized by incubation with FITC-conjugated goat anti-rabbit IgG (1:150) similar to a method described for myosin light chains (Wallimann *et al.*, 1982).

## Results

*Permeabilization of muscle cells*

Myotubes stained for CK using indirect immunofluorescence staining techniques following complete fixation of cells (Turner *et al.*, 1976b) exhibit strong fluorescence due to staining of soluble sarcoplasmic CK throughout the cell without displaying subcellular details. By this technique the presence of both, M-CK and B-CK subunits is demonstrated within myotubes of six-day-old myogenic cultures as shown in Figs. 1a and 2a where cells have been stained with anti-M-CK and anti-B-CK antibodies, respectively. Staining with preimmune IgG following fixation is minimal (not shown). If such myotubes are permeabilized and freed of soluble sarcoplasmic constituents by the modified technique (Materials and methods) and then stained by immunofluorescence with anti-B-CK or anti-M-CK antibodies, CK is detected bound to myofibrils in a cross-striated pattern (Figs. 1c,e, 2c). The short prefixation is sufficient, probably affecting only the cell surface and cell attachment sites, to reduce significantly the cell detachment frequently observed after treatment with Triton X-100, without rendering soluble cell components unextractable. Note the low background fluorescence of permeabilized cells (Figs. 1c, 2c). Permeabilization of cells by using Triton X-100 or 50% glycerol on unfixed cells, and by using concentrated methanol at $-25^{\circ}$ C results in considerable cell loss and significant background fluorescence, respectively, and therefore does not give the clarity and reproducibility of results of the modified procedure. Nonetheless, myofibril-bound M-CK can also be seen under these conditions, suggesting that fixation artefacts are unlikely to be involved. It is important to re-emphasize that in order to prevent cell loss due to detachment the steps following permeabilization by Triton X-100 were performed with great care and that the Triton concentration was lowered to 0.05% (v/v). Gelatinized Lux culture dishes proved to be most satisfactory in this respect.

*Subcellular localization of MM-CK*

Myotubes stained with anti-MM-CK antibody following prefixation, permeabilization and removal of soluble CK display bright fluorescence concentrated at the M-line in the

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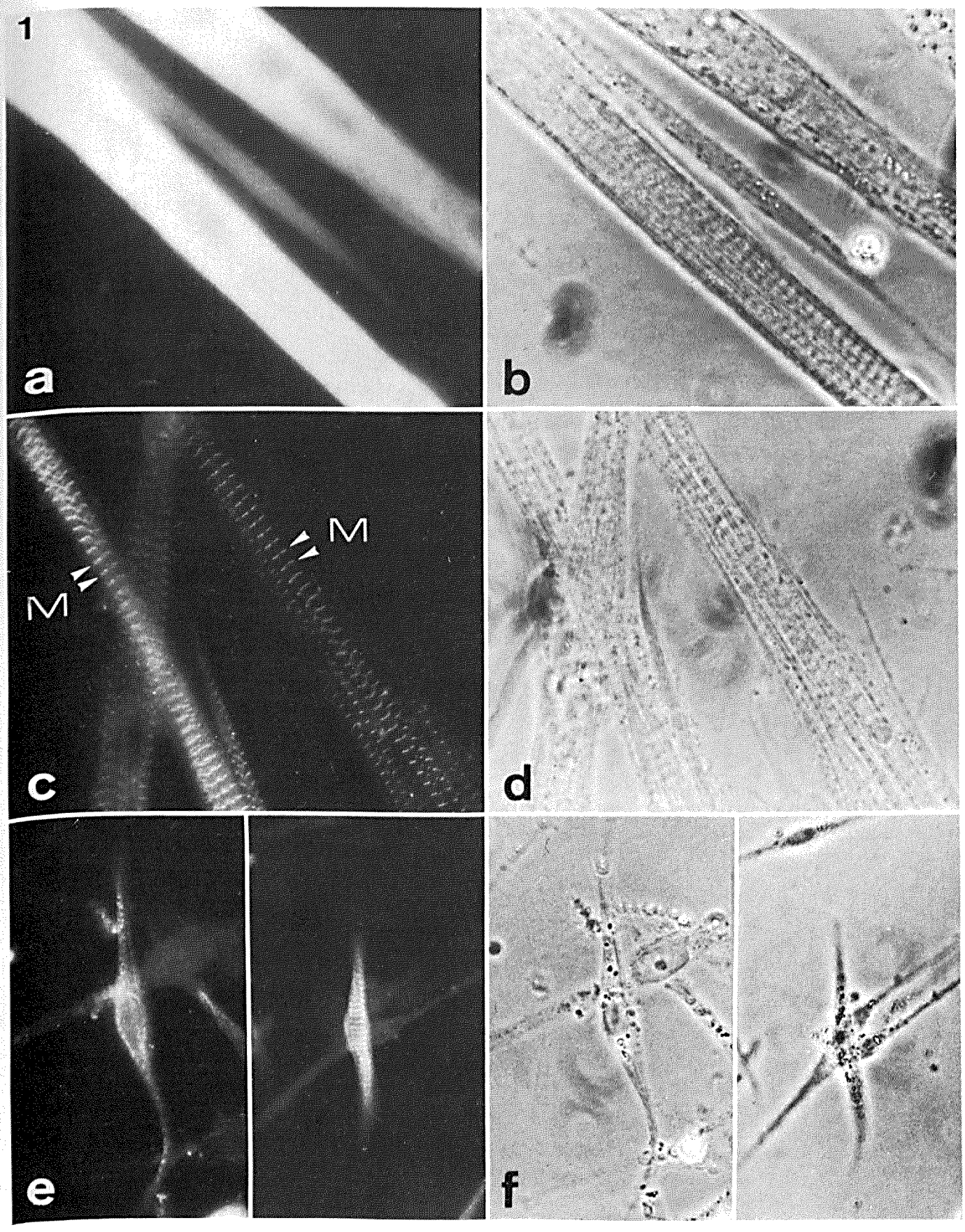
**Fig. 1.** Fluorescence (a, c, e) and phase-contrast pictures (b, d, f) of myogenic cells stained by indirect immunofluorescence using anti-M-CK antibodies after complete fixation of six-day-old cultured cells (a, b), and after superficial fixation of cells of the same age followed by permeabilization and subsequent removal of soluble intracellular constituents by washing (c, d). Two-day-old myogenic cells pretreated as in c and d (e, f). M, M-line. (For details see Materials and methods.)


1
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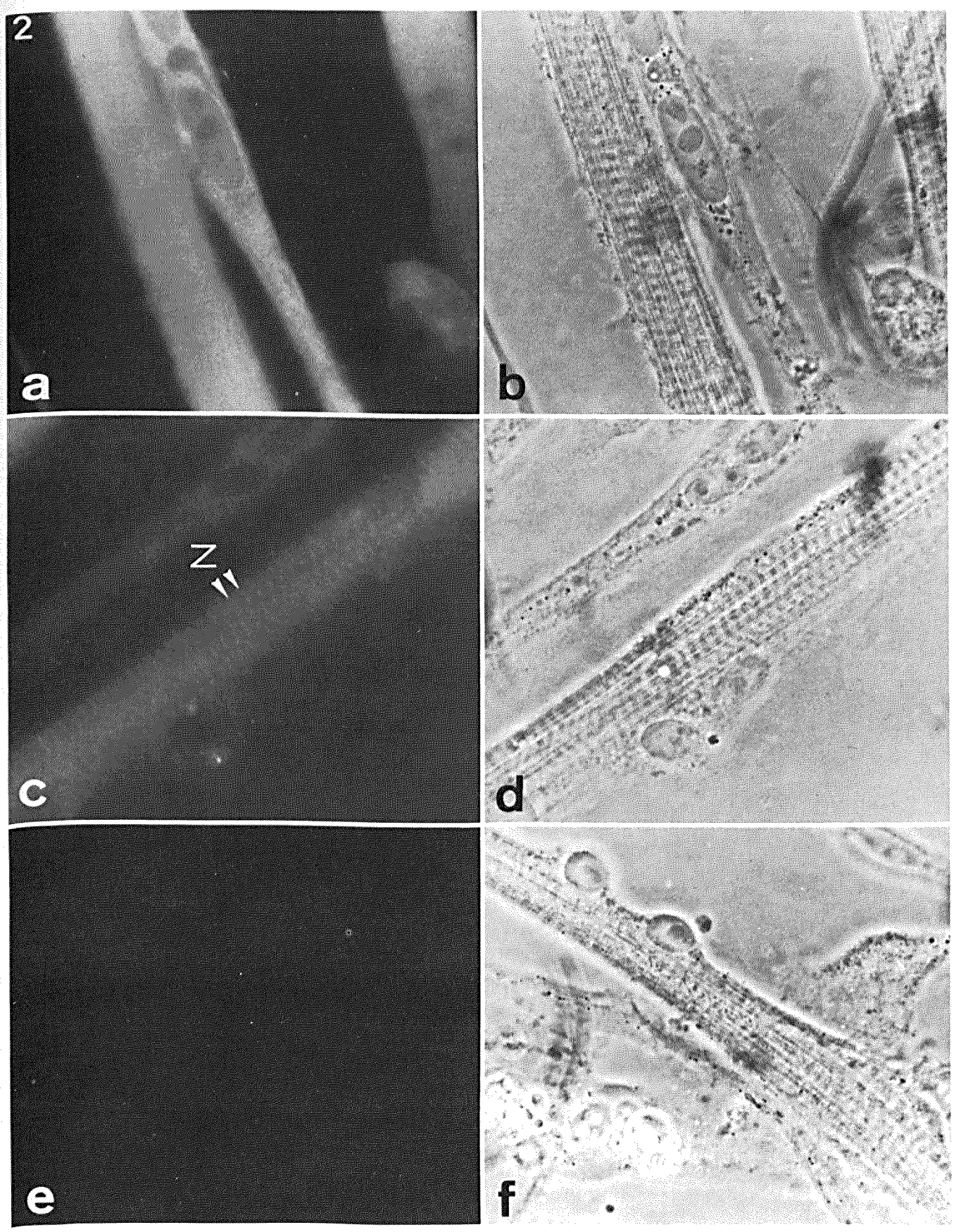
middle of the A-band (Fig. 1c). No fluorescence is found at the Z-line. Background fluorescence due to staining of soluble sarcoplasmic CK which despite permeabilization was present in these cells is substantially reduced (Fig. 1c,e, cf. Fig. 1a) and unspecific staining with preimmune antibodies is not detectable (Fig. 2e). M-Line staining is seen most clearly in large myotubes after about three days of culture under standard conditions. Occasionally a cross-striated pattern is observed in mononucleated cells presumably representing postmitotic myoblasts which are known to synthesize and accumulate muscle-specific proteins like M-CK before fusion (Fig. 1e) (Turner *et al.*, 1976a,b). Cells which have been prevented from fusion by a low calcium medium also show M-line bound MM-CK in single myofibrils extending into elongated cell processes (not shown).

*Subcellular localization of B-CK*

Myogenic cells stained with anti-B-CK antibodies following the standard fixation procedure display fluorescence which is distributed throughout the myotubes except the nuclei (Fig. 2a). Again no subcellular localization can be observed using this method. If, however, cells are permeabilized and soluble CK removed prior to staining with anti-B-CK antibody, faint, diffuse fluorescence is observed at the myofibrillar Z-band region (Fig. 2c). No specific fluorescence is found at the M-line. Although Z-bands of myofibrils often exhibit unspecific fluorescence after immunostaining, the weak signal observed with anti-B-CK at this location was above background (Fig. 2e). Similar Z-band staining with anti-B-CK has been observed in unfixed myofibrils isolated from 4–11-day-old chicken embryonic muscle tissue (Wallimann *et al.*, 1977a), from chicken heart muscle (Wallimann *et al.*, 1977b) and in unfixed, permeabilized cultured muscle cells (not shown). Therefore, Z-band staining is not a fixation artefact. These results indicate that, at early stages of skeletal muscle development when BB-CK is the predominant CK isoenzyme species, a very small amount of this isoenzyme is associated with the Z-band region and gradually disappears during the isoenzyme transition. The fluorescence staining obtained with anti-B-CK and anti-M-CK antibodies was unambiguously assigned to the Z-band region and M-line respectively, by direct observation in the fluorescence microscope switching the fluorescence epi-illumination on and off while keeping the phase contrast illumination very low and by superimposition of enlarged negatives or transparent tracings of the parallel fluorescence and phase-contrast pictures. In addition, if cells were stained simultaneously with both antibodies a double cross-striated pattern with strong M-line and weak Z-line fluorescence was seen (not shown). Since the antibodies are subunit

Fig. 2. Fluorescence (a, c, e) and phase-contrast pictures (b, d, f) of myogenic cells stained by indirect immunofluorescence using anti-B-CK antibodies (a–d) and control IgG (e, f), after complete fixation of six-day-old cultured cells (a, b), and after superficial fixation of cells of the same age followed by Triton X-100 permeabilization and subsequent removal of soluble cell constituents by washing (c–f). Z, Z-band. (For details see Materials and methods.) .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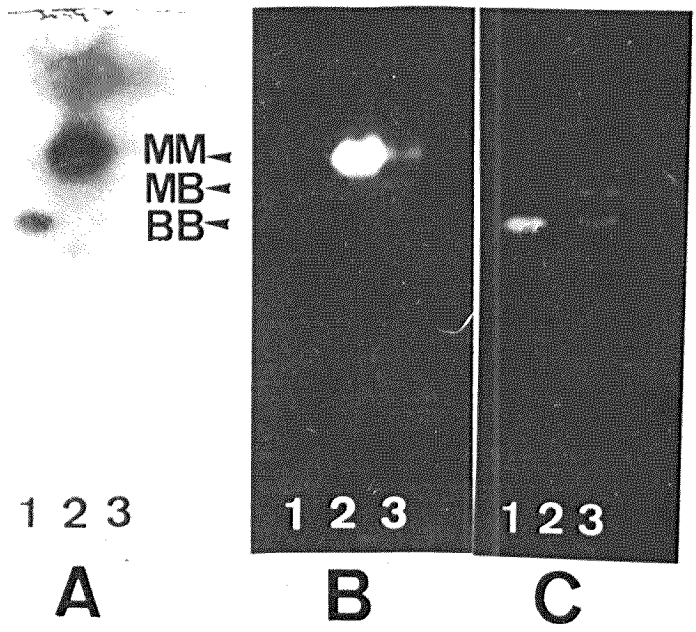


Fig. 3. Total tissue extracts from adult chicken brain (1), skeletal muscle (2), and five-day-old myogenic cell cultures (3), subjected to PAGE under native conditions whereby the three CK isoenzymes (MM, MB and BB) are resolved and shown after staining for CK activity (panel A), immunoblotting with anti-M-CK antibody (panel B) and anti-B-CK antibody (panel C). Note the subunit specificity of both antibodies showing no cross-reactivity with the heterologous dimers but both recognizing the homologous subunit of the MB hybrid.

specific, the results presented indicate that neither the BB-form nor the MB-form, but only the MM-form of CK is incorporated into the M-line. A similar isoenzyme-specific localization of CK has also been found in bovine heart myofibrils in which MM-CK and MB-CK are found simultaneously (Moser *et al.*, unpublished observation).

*Detection by immunoblots of CK isoenzymes in extracts from tissues and cultured muscle cells: subunit specificity of anti-CK antibodies*

Only one single band of total extracts from skeletal muscle and brain was stained after transfer to nitrocellulose and indirect immunofluorescence staining by anti-M-CK and anti-BB-CK antibodies, respectively (Fig. 3, panels B and C, lanes 1 and 2). The position of these bands corresponded to the position of CK activity as detected by the CK overlay-gel staining technique (Fig. 3, panel A; Turner *et al.*, 1973). Both anti-M-CK and anti-B-CK antibodies did not cross-react with the heterologous homodimers. However, the hybrid form, MB-CK, which is present in embryonic muscle and myogenic cell cultures and moves with intermediate electrophoretic mobility was stained by both antibodies (Fig. 3, panels B and C, lane 2). This demonstrates the subunit specificity of the antibodies and the isoenzyme-specific tissue distribution of CK. In addition, even

though B-type CK was found by this method in chicken fibroblast cells and extracts from embryonic skin, no evidence for the presence of M-type CK has so far been obtained in this laboratory.

## Discussion

Developmentally regulated isoenzyme transitions are frequently found in differentiating cells and are the rule rather than the exception in muscle. The advantages they afford to the developing tissue are at best hypothetically perceived. Under these circumstances information concerning the subcellular compartmentalization, developmental timing of emergence and retreat, and tissue specificity of the isoenzymic forms will undoubtedly provide useful clues to a better understanding of the physiological significance of such transitions.

Unlike isolated myofibrils cells contain all of the possible CK isoenzyme forms and are obviously the ideal subject for subcellular compartmentalization studies on CK. Even though immunofluorescence staining of fully fixed myogenic cells has been successfully applied to demonstrate the CK-isoenzyme transition from BB-CK to MM-CK (Turner *et al.*, 1976b) it did not reveal any details on the subcellular localization of the CK isoenzymes. Therefore, due to the large excess of soluble over myofibril-bound CK it has to date been impossible to determine unequivocally the cellular location of the bound forms with respect to the three isoenzymes of CK. By the prefixation–permeabilization technique described here detachment of cells is prevented by mild prefixation after which cells are permeabilized. During permeabilization unfixed soluble cell constituents are removed, thus rendering subcellular structures accessible to antibody staining without prior fixation. An artificial staining with anti-M-CK and anti-B-CK antibodies of the M-band and Z-band, respectively due to relocation or selective trapping of these antigens during permeabilization of the cells seems unlikely since even in fully fixed cells, especially in well-developed myotubes that are filled with bundles of myofibrils, hints of the underlying cross-striated fluorescence pattern are occasionally observed despite the high 'background fluorescence' of stained soluble CK [not shown here, but nicely demonstrated by Konieczny *et al.* (1982)]. Furthermore, the localization of M-CK at the M-line has been established on the electron microscope level with both unfixed and glutaraldehyde-prefixed tissue (Wallimann *et al.*, 1977a,b, 1978, 1983) or prefixed and frozen tissue ultracryotomy (Strehler *et al.*, 1983).

The permeabilization method may also be generally applicable to other cell culture systems in helping to localize on a subcellular level multicompartmented cellular constituents. Using this procedure for cultured myogenic cells the myofibril-bound MM-CK clearly resides in the M-line, the small amount of myofibril-bound BB-CK is diffusely located in the Z-region and all MB-CK appears to be soluble. This demonstrates the isoenzyme-specific localization of CK isozymes in skeletal muscle.

The developmental timing of MM-CK incorporation into the M-line of myofibrils is not yet clear. As a point of reference the appearance of MM-CK can be compared to that

of myomesin, another M-line protein (Masaki & Takaiti, 1974; Trinick & Lowey, 1977; Strehler *et al.*, 1980) which is known to be present at early stages of myofibrillogenesis (Eppenberger *et al.*, 1981), or to the relative appearance of these two proteins with respect to M-line electron density. Interpretation of the data has been hampered by presumed problems of detectability at the light and electron microscope levels and by apparent tissue-specific differences in the myofibril assembly processes in skeletal and heart muscle (Anversa *et al.*, 1981; Eppenberger *et al.*, 1981). In myogenic cells derived from skeletal muscles a clear cross-striated fluorescence pattern is more frequently observed with anti-myomesin than with anti-M-CK antibody at early stages of development (mononucleated cells and very small myotubes). However, preliminary double immunofluorescence experiments do not show the presence of one antigen without the other (Doetschman, unpublished). At the electron microscope level, anti-myomesin staining of cells reveals the presence of myomesin at the M-region of nascent myofibrils which are still lacking an electron-dense M-line (Eppenberger *et al.*, 1981) indicating, with respect to myomesin, a somewhat delayed incorporation of CK into the M-line at very early stages of skeletal muscle development. In the heart of some mammals incorporation of CK into the M-line and the concomitant appearance of an electron-dense M-line structure seems to be delayed even until some days after birth (Anversa *et al.*, 1981; Carlsson *et al.*, 1982). It has been suggested that incorporation of MM-CK into the M-line structure occurs at the same time contractile properties of the muscle improve (Hopkins *et al.*, 1973) and when a marked enhancement of the major systems involved in ATP metabolism takes place (Baldwin *et al.*, 1977). Thus, the acquisition of bound CK into the myofibrillar apparatus may represent an important step in the physiological maturation of myofibrils. This is supported by recent experiments in which we have shown that the M-line bound CK is enzymatically active and is able to contribute significantly to immediate ATP regeneration during muscle contraction (Schlösser *et al.*, 1982; Wallimann *et al.*, 1982).

In contrast to the work reported by Eckert *et al.* (1980), we have found no indication for either the presence of muscle specific MM-CK or an association of this enzyme with intermediate filaments in fibroblast cell cultures. In chicken fibroblasts no specific immunofluorescence staining has been observed with anti-M-CK antibodies and the relatively weak staining with anti-B-CK antibodies did not reveal any striking subcellular distribution (Turner *et al.*, 1976b). Very recent experiments using monoclonal anti-M-CK antibodies which stain the M-line of myofibrils do not confirm any evidence (Eckert *et al.*, 1980) for the presence of this CK-isoenzyme in Ptk-2 cells nor its association with intermediate filaments (Cerny, unpublished observations). No detectable M-CK synthesis has been noticed in fibrogenic cells using pulse-labelling techniques followed by immunoprecipitation with anti-M-CK antibodies (Caravatti *et al.*, 1979). *In vitro* translation studies with poly-A containing mRNA derived from chicken fibroblasts have not shown, in contrast to corresponding experiments using mRNA from myogenic cells, any measurable amount of muscle type M-CK being formed (Perriard *et al.*, 1978b). Furthermore, chicken skin fibroblasts have been shown

to contain only BB-CK (Lough & Bischoff, 1977) and no M-CK was found in these fibroblasts (Konieczny *et al.*, 1982). MM-CK can therefore still be considered as a tissue-specific marker for differentiated skeletal muscle and some heart muscles.

In conclusion, the isoenzyme-specific localization of CK within the myofibrillar apparatus, as well as possible tissue-specific differences in the timing of its incorporation into the M-line where it has the capacity to increase the efficiency of energy metabolism inside the myofibril, have begun to provide clues to the physiological significance of an isoprotein transition.

## Acknowledgements

We are grateful to Dr B. K. Grove for a gift of mouse anti-myomesin serum for the double immunofluorescence (performed by Dr Thom Doetschman, unpublished) and to Dr Coleman for sending a preprint of the work by Konieczny *et al.* (1982). We would like to thank M. Bähler and Drs Thom Doetschman, J. C. Perriard and E. Strehler for help and discussion and Miss E. Abächerli for typing the manuscript. This work was supported by Swiss NF-grant No. 3.707-0.80 and a grant from the Muscular Dystrophy Association of America to H. M. Eppenberger.

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