# Intracellular Targeting of Isoproteins in Muscle Cytoarchitecture

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*Abstract.* Part of the muscle creatine kinase (MM-CK) in skeletal muscle of chicken is localized in the M-band of myofibrils, while chicken heart cells containing myofibrils and BB-CK, but not expressing MM-CK, do not show this association. The specificity of the MM-CK interaction was tested using cultured chicken heart cells as "living test tubes" by microinjection of in vitro generated MM-CK and hybrid M-CK/B-CK mRNA with SP6 RNA polymerase. The resulting translation products were detected in injected cells with isoprotein-specific antibodies. M-CK molecules and translation products of chimeric cDNA molecules containing the head half of the B-CK and the tail half of the M-CK coding regions were localized in the M-band of the myofibrils. The tail, but not the head portion of M-CK is essential for the association of M-CK with the M-band of myofibrils. We conclude that gross biochemical properties do not always coincide with a molecule's specific functions like the participation in cell cytoarchitecture which may depend on molecular targeting even within the same cellular compartment.

In most cells of higher organisms isoforms, representing closely related proteins with conserved amino acid sequences and often similar functional properties are the products of multigene families. Distinct isoforms may exist as related proteins in different tissues, be developmentally regulated within a single tissue or even coexist within a single cytoplasmic compartment. In part divergence in protein sequence among isoforms may serve to target a given isoprotein to its appropriate compartment within the cell. Specific sequences, often located at the amino terminus, have been found to be responsible for the extracellular export of many secretory proteins (5) or the intracellular targeting of proteins destined for intracellular compartments like mitochondria, chloroplasts or the nucleus (11, 17, 26).

Here we examine the molecular basis for the localization of muscle creatine kinase (MM-CK)[1] within the M-band of the muscle myofibril (50, 54, 55, 56). Creatine kinase is the major enzyme ensuring energy production in the muscle cell. The enzyme is a dimer of two subunits which exist as a nonskeletal muscle form, brain creatine kinase (BB-CK), in embryonic muscle and in nonmuscle cells like brain. Upon muscle differentiation the synthesis of the M-CK subunit is induced and BB-CK is gradually replaced by the skeletal muscle-specific enzyme MM-CK (8, 14, 40, 41, 51). Although myofibers that differentiate in culture have been shown to contain all three isoenzymes, MM-CK, MB-CK and BB-CK (40, 51) only MM-CK appears to be localized and specifically bound to the M-band of the myofibril (53). This result can be explained in two ways. Either MM-CK as the major CK species in differentiated cells is interacting with the M-band region because it is more abundant or the MM-CK molecules possess unique properties not shared by MB-CK or BB-CK that are responsible for its skeletal muscle typical association with the M-band.

Unlike heart cells from mammalian species chicken heart cells do not express any M-CK, contain relatively high levels of BB-CK (42), and are devoid of the electron dense material typical of the M-band region in which M-CK is usually localized (52). The well structured myofibrils display the high molecular weight M-band components myomesin and M-protein (21, 22). Consequently chicken heart cells provide a test system to examine the basis for the specificity of MM-CK isoenzyme localization within the cell. Homogeneous mRNA was produced "in vitro" from full length cDNA of B-CK and M-CK or from chimeric genes and was then microinjected into living heart cells. We conclude that localization of M-CK within muscle cytoarchitecture is isoprotein and peptide sequence specific.

## Materials and Methods

### Cells and Microinjection

Cultures of chicken heart cells were derived from 8–10 d-old chicken embryos. Standard techniques used for the isolation of chicken skeletal and heart muscle cells (6, 51) were adapted: after preparation of heart tissue dissociation was carried out using 0.2% trypsin (Difco Laboratories, Inc., Detroit, MI) in calcium- and magnesium-free salt solution (CMF) during 45 min at 37°C. The tissue, homogenized in culture medium by trituration, was then filtered through a nylon filter to remove uncompletely dissociated tissue. Finally, the cells were plated at a density of $\sim 3.5 \times 10^5$ cells per gelatin coated culture dish (35-mm diameter) in medium containing 85 parts of minimal essential medium with Earle's salts (Amimed AG, Basel, Swit-

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1. *Abbreviations used in this paper*: B-CK, creatine kinase subunit; BB-CK, brain creatine kinase; CMF, calcium- and magnesium-free; MB-CK heterodimeric creatine kinase; M-CK, creatine kinase subunit; MM-CK, muscle creatine kinase.

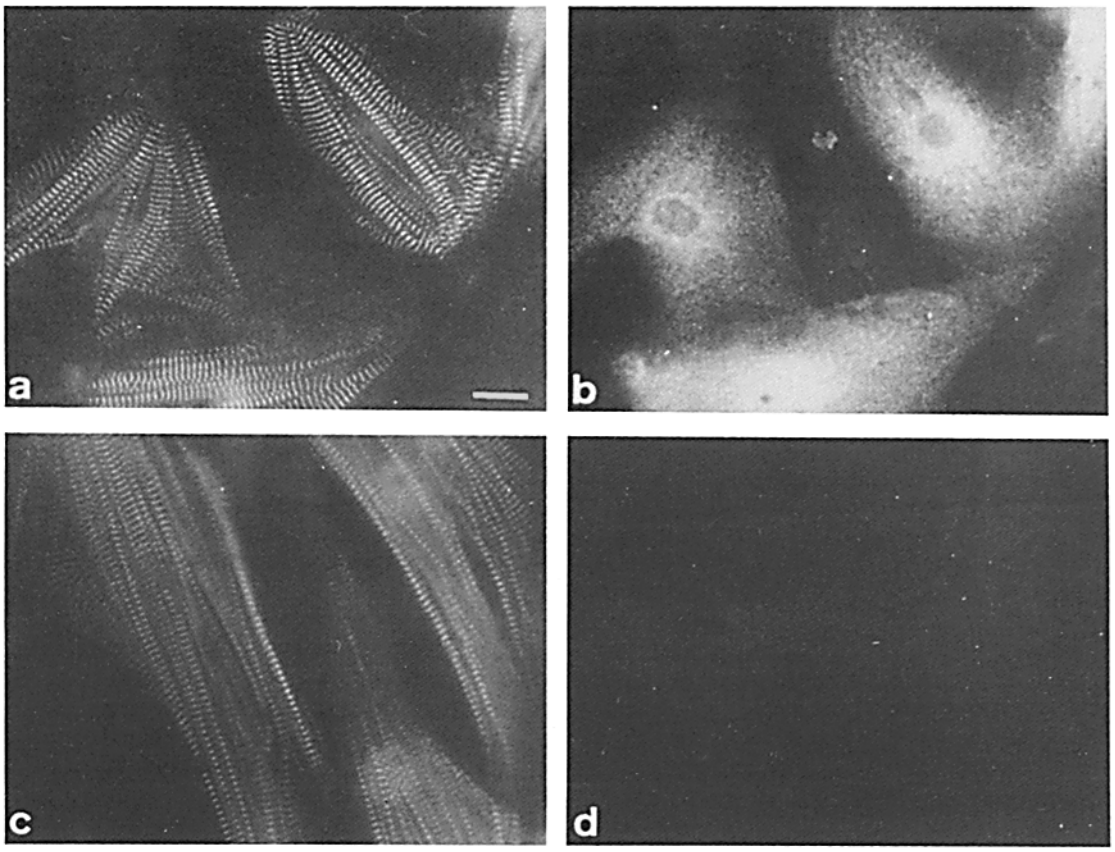


*Figure 1.* Creatine kinase isoprotein expression in cultured chicken heart cells. Heart cells were prepared from 8-d old chicken embryos as described in Materials and Methods. After 3 d in culture the cells were fixed and double-stained with the following antibodies: (*a* and *c*) monoclonal antibody B4 against the M-band protein myomesin, second antibody FITC conjugated; (*b*) polyclonal anti BB-CK antibody and polyclonal anti-MM-CK antibody; (*d*) and rhodamine-labeled second antibody. All heart cells contain myomesin, staining only the M-band region of these cells, and B-CK while none of the cells express M-CK. Note that the B-CK was found in a diffuse distribution showing no interaction with the myofibrils. Bar, 10 μm.

zerland), 10 parts of selected horse serum (Gibco, Grand Island, NY), 3.5 parts of embyro extract and 1.5 parts of antibiotica (penicillin/streptomycin, Gibco). To reduce overgrowth of fibroblast no glutamine was added to the culture medium (10).

Microinjection experiments were performed as described (20) using injection buffers of either 10 mM Tris-HCl, 0.5 mM EDTA pH 7.2 or 140 mM KCl for the injection of synthetic RNA. The maintenance of constant injection pressures was achieved using the microinjector apparatus of Eppendorf (Eppendorf Gerätebau, Hamburg, FRG).

## *In Vitro Expression and Gel Analysis*

For the transcription experiments the pSP65 constructs were linearized and transcribed with SP6-RNA polymerase (Boehringer Mannheim Diagnostics, Inc., Houston, TX) as described (33) using 0.5 mM ribonucleotides (A, U, and C) and 0.05 mM GTP. The transcripts were capped by the inclusion of 0.5 mM methylated cap analogue (m7GpppG; Pharmacia Fine Chemicals, Uppsala, Sweden) in the transcription reaction. Transcripts were purified by centrifugation through a linear 5–25% sucrose gradient in SET (10 mM Tris-HCl, 5 mM EDTA, 1% SDS, pH 7.4; SW60 55,000 *g*, 15 h). The RNA containing fractions were precipitated with 2.5 vol EtOH, 1:0.1 vol 3 M NaAc, pH 5.5, and dissolved in injection buffer.

Cell-free translation was carried out in a rabbit reticulocyte lysate (Promega Biotec, Madison, WI) containing [$^{35}$S]methionine (Amersham International plc, Amersham, U. K.) as described by the manufacturer. Aliquots from translation assays were analyzed either on a 10% polyarcylamide gel with the buffer system described by Laemmli (28) or on a two-dimensional gel according to O'Farrel (36). After electrophoresis gels were prepared for autoradiography using PPO/DMSO (New England Nuclear, Boston, MA) and exposed to x-ray film (Fuji RX). Immunoprecipitations were performed as described (41) and immune complexes were collected with *Staphylococcus aureus* protein A (Pansorbin; Calbiochem AG, Lucerne, Switzerland).

## *Immunolocalization*

Double-label immunofluorescence microscopy was performed using a modification of methods described by Wallimann et al. (53) and Goldenthal et al. (19) (*a*) Cells were rinsed three times with the relaxation buffer solution A (0.1 M KCl, 5 mM EDTA, 1 mM EGTA, 1 mM BME, pH 7.0) (*b*) Cells were prefixed with 3% paraformaldehyde in PBS for 1 min, then shortly permeabilized using 0.1% saponin in PBS for 1 min and then carefully washed three times with PBS to extract most of the soluble proteins. (*c*) The final fixation was done with 3% paraformaldehyde in PBS for 15 min. (*d*) After three washes with PBS the cells were further permeabilized with 0.1% saponin in PBS for 20 min. (*e*) Incubation with the first antibodies diluted in 0.1% saponin-PBS solution for 20 min at room temperature. Monoclonal antibody B4 was used as undiluted hybridoma culture supernatant, polyclonal antibodies against CK isoproteins were diluted 1:100. (*f*) After three washes with 0.1% saponin in PBS (5 min each) the incubation with second antibodies was done as described in step *e*. The dilution 1:200 for both goat anti-rabbit RITC-conjugated and sheep anti-mouse FITC-conjugated antibodies. (*g*) After three washes with 0.1% saponin in PBS (5 min each) and with PBS (2 times) the cells were embedded in 0.1 M glycin, 50% glycerol, pH 9.0.

## *Cloning Procedures*

Restriction endonucleases, Klenow fragment of DNA polymerase and T4 DNA ligase were obtained from Boehringer and Pharmacia and were used according to the manufacturer's directions. DNA fragments were ligated directly in low melting agarose (FMC, Sea Plaque Corporation, Rockland,

ME) after electrophoresis. Plasmid minipreps and maxipreps were performed using the alkaline lysis method (4). pSP65 constructs were cloned in *Escherichia coli* HB101.

## Results

### *Chicken Heart Cells do not Express MM-CK*

Heart muscle cells provide an unusual opportunity to examine the molecular specificity of M-CK interaction with the M-band, because they contain all known components of the M-band except M-CK. Heart cells derived from 8–10-d-old chicken embryos were cultured for 48–96 hours and subjected to double-immunofluorescence staining using monoclonal antibody B4 against the M-band protein myomesin (21, 22) and polyclonal antibody against chicken nonmuscle specific BB-CK or the skeletal muscle-specific MM-CK (40). The results are shown in Fig. 1 and indicate that the heart cells contain myomesin in a pattern of sharp bands coinciding with the M-bands of myofibrils (Fig. 1, *a* and *c*). The other known M-band component M-protein was also found (not shown, 21). B-CK, however was found in all heart cells in a diffuse distribution (Fig. 1 *b*) and showed no specific interaction with the myofibrils. None of the cells contained M-CK (Fig. 1 *d*). These results corroborate earlier observations on embryonic and adult heart tissue (42, 52) and make cultured heart cells an ideal model system to probe for the molecular specificity of M-CK interaction with the myofibrils, since all known elements of the M-band including myomesin and M-protein (21) are present except M-CK. By introduction of M-CK into these cells it should, in principle, be possible to create a cytoarchitecture of the M-band similar to the one observed in skeletal muscle. Therefore MM-CK or its mRNA was microinjected into chicken heart cells and the distribution of M-CK was studied by double-immunofluorescence as described above.

In preliminary experiments we determined that the heart cells proved to be suitable living "test tubes" to study the interaction since they can easily be microinjected. Heart cells were injected with purified MM-CK or poly $A^+$ RNA from skeletal muscle which had been enriched for M-CK mRNA by size fractionation. The purified protein showed a fuzzy pattern of interaction in the A-band region while the M-CK produced after injection of poly $A^+$ RNA displayed an improved pattern (47). However the injection of both protein or poly $A^+$ RNA are not completely satisfactory for several reasons. The protein may be degraded or partially denatured during the purification procedure and therefore may not be able to interact properly. On the other hand poly $A^+$RNA contains additional messengers for proteins that may also be involved in M-band interaction. Moreover, with these components there are no possibilities to construct mutant proteins that would allow an investigation of the specificity at the molecular level. To overcome these problems we generated homogeneous RNAs from full length cDNA clones with SP6 RNA polymerase, tested their biological activity in a cell-free protein synthesis system and used them in injection experiments as described below.

### *Cell-free Translation Products of Synthetic CK mRNAs Comigrate with the Purified Proteins*

Full length cDNA coding for M-CK was constructed from the clones described (37, 44) and incorporated in the correct orientation in the vector pSP65. The B-CK cDNA was directly subcloned from the full length clone H4 isolated in our laboratory (25). Generation of homogeneous transcripts was carried out as described in Materials and Methods and aliquots were translated in a cell-free protein synthesizing system from rabbit reticulocytes. As shown in Fig. 2 both the B-CK (lanes *5* and *6*) and the M-CK (lanes *9* and *10*) derived from the synthetic RNA have the same electrophoretic mobility as the respective isolated protein (lanes *1* and *2*) or the translation product from gizzard (lane *3* and *4*) or leg muscle RNA (lane *7* and *8*). They could also be immunoprecipitated with anti-BB-CK antibody (lane *6*) or anti MM-CK antibody (lane *10*) in a manner similar to that observed for the authentic proteins (lanes *4* and *8*). Therefore the translation products of the synthetic mRNAs have identical behavior on gels as compared with the authentic proteins. It appears that the synthetic B-CK message was translated up to 10 times more efficiently than the synthetic M-CK mRNA (compare lanes *5* and *9*, Fig. 2). The basis of this effect is unclear but could be due to different length of the 5′ untranslated region (which in both clones is not quite complete) or to an intrinsic difference in translatability within the cell-free protein synthesis system. Both synthetic mRNAs give rise to distinct subbands of apparent molecular mass of 36,300 kD, 34,800 kD for M-CK and a B-CK subband with almost identical mobility to that of the complete B-CK subunit. Upon extended exposure, similar bands were observed in analyses of immunoprecipitated proteins derived from translation of poly $A^+$ RNA (not visible in Fig. 2). These subbands most likely represent initiation of protein synthesis at internal methionines, as was observed for other synthetic mRNAs (48).

Analysis of the translation products from synthetic mRNAs on two-dimensional gels showed comigration of the M-CK product with both spots of the isolated protein as described previously. However, the synthetic B-CK product comigrated only with the more basic spot of the two spots formed by the isolated B-CK protein (43). The subbands discussed above focused into distinct spots with isoelectric points shifted in the direction expected from the charges of the amino acids lost due to internal initiation (not shown). We conclude that the in vitro generated mRNA makes protein of expected molecular weight and charge corresponding to M-CK and B-CK.

### *Only the M-CK Product of Microinjected Synthetic mRNA Associates in an Isoprotein-Specific Manner with M-bands of Myofibrils*

To examine the localization we injected these RNAs into cultured chicken heart cells which were cultured for additional periods of time and then subjected to double immunofluorescence staining with antibodies to BB-CK and to myomesin. All heart cells showed the expected cross-striated pattern with the anti-myomesin antibody (not shown) showing intact M-bands. However, even after extended mild detergent extraction (see Materials and Methods) to remove soluble antigen no myofibril-bound B-CK remained visible. From these experiments it is evident that even after injection of relatively high amounts of additional mRNA (up to 1,000 molecules per cell) no interaction of B-CK with the M-band was found. Thus B-CK appears to be unable to interact with the myofibrillar M-band and increased concentration of the protein

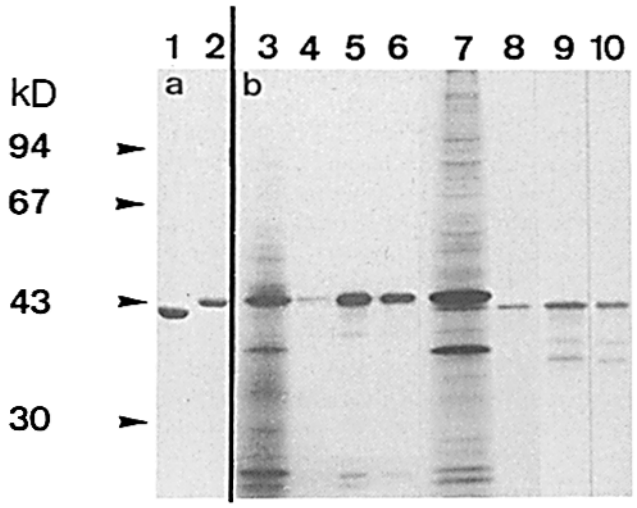


*Figure 2.* In vitro translation of synthetic mRNA coding for B-CK and M-CK Full length cDNAs coding for B-CK and M-CK have been cloned into the multiple-cloning site of pSP65. The expression plasmids were linearized with Hind III, and M-CK- or B-CK-specific transcripts were obtained by in vitro transcription with SP6 RNA polymerase. The transcripts were capped by the addition of methylated cap analogue (m7GpppG) to the transcription reaction. Next, synthetic RNAs and isolated RNAs were translated in a rabbit reticulocyte translation system in the presence of [$^{35}$S]methionine. Aliquots were analyzed directly or after immunoprecipitation with the corresponding antisera on a denaturing 10% polyacrylamide gel and then autoradiographed. (*A*) Gel stained for protein: (lane *1*) purified M-CK; (lane *2*) purified B-CK. (*B*) Autoradiography of the translation products of lane *3* poly A$^+$ RNA from chicken gizzard (0.3 μg); (lane *4*) same as in lane *3* but after immunoprecipitation with anti-BB-CK antibody; (lane *5*) synthetic RNA coding for B-CK (20 ng); (lane *6*) same as lane *5* after immunoprecipitation with anti-BB-CK antibody; (lane *7*) poly A$^+$ RNA from chicken leg muscle (0.15 μg); (lane *8*) same as lane *7* after immunoprecipitation with anti-MM-CK antibody; (lane *9*) synthetic RNA coding for M-CK (30 ng) and lane *10* same as lane *9* after immunoprecipitation with anti MM-CK antibody. Molecular masses are in kD. No background of the rabbit reticulocyte translation system was observed during the exposure times used which were 16 h for lanes *3–6* and 64 h for lanes *7–10*. The minor bands observed in lanes *4–6* and *8–10* are most probably due to initiation of translation at internal methionines.

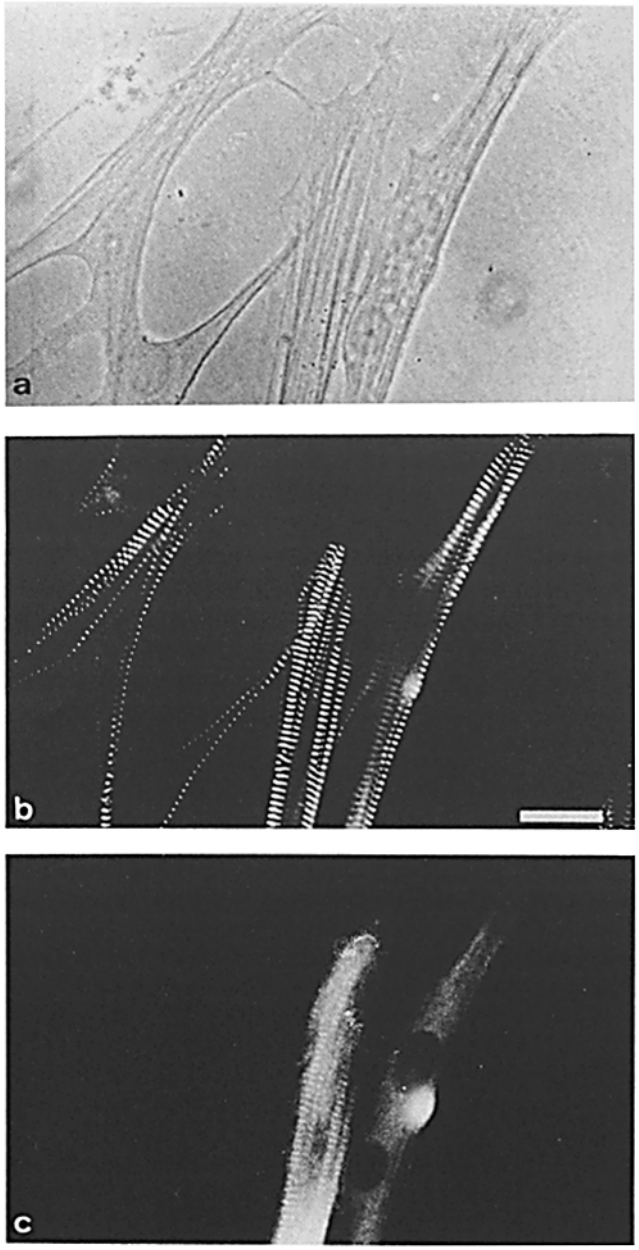


*Figure 3.* Microinjection of synthetic M-CK mRNA into chicken heart cells. Synthetic M-CK mRNA was transcribed from the corresponding cDNA clone linearized with Hind III. The RNA was microinjected in a concentration of 0.1 μg/μl into heart cells derived from 9-d-old embryos. After an additional period of 14 h the cells were processed for double-immunofluorescence staining using monoclonal anti-myomesin antibody B4 (*b*) and polyclonal anti-MM-CK antibody (*c*). Only the injected cell in the middle shows a clear M-CK staining in a cross-striated pattern while all cells stain with the anti-myomesin antibody. The very weakly stained cell at right most probably died during the injection procedure as indicated by its mottled appearance in the phase contrast picture (*a*). Bar, 20 μm.

resulting from microinjection does not lead to an artifactual localization.

Localization was observed when in vitro generated M-CK mRNA was microinjected into heart cells. As before cells were cultured for 4–16 h, and subjected to double-immunofluorescence staining using anti-MM-CK and anti-myomesin antibodies. As shown in Fig. 3, one microinjected cell shows fluorescence staining for M-CK, in addition to myomesin. The appearance of M-CK in this cell indicates that synthetic M-CK mRNA can be translated by the heart cells although they do not normally express the corresponding M-CK gene. After gentle detergent extraction most of the M-CK specific fluorescence was localized in a sharp cross-striated pattern (Fig. 3 *c*) which corresponded with the control staining with anti-myomesin in the same cells. Not all of the soluble M-CK could be completely extracted and gave rise to a relatively uniform background staining of the whole cytoplasm. As expected all heart cells including noninjected cells show the typical M-band staining with the antibody against myomesin (Fig. 3 *b*). If the incubation time after injection was reduced to less than 1 h no signal was observed while a clear M-CK signal could be obtained with postinjection incubation periods of more than 1.5 h. Prolonged incu-

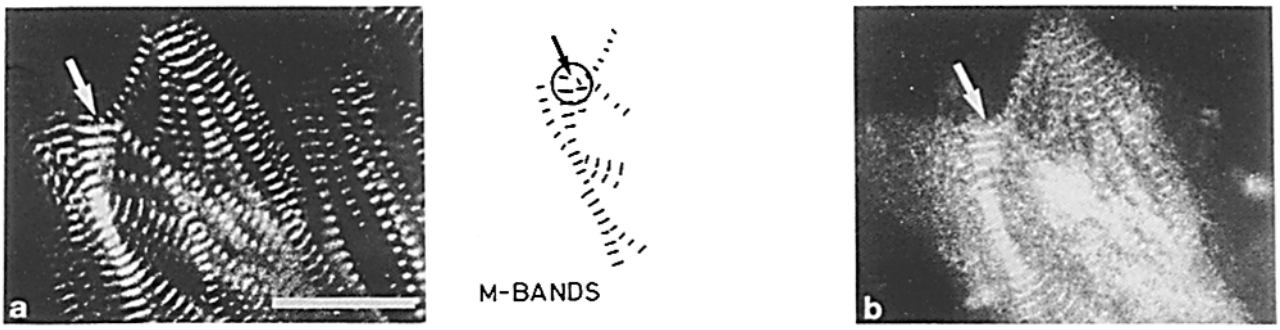


*Figure 4.* Interaction of the newly synthesized MM-CK occurs at the M-band of chicken heart myofibrils. Synthetic M-CK mRNA was microinjected into heart cells derived from 9-d-old chicken embryos at a concentration of 0.05 μg/μl. Double immunofluorescence staining was performed 16 h after injection with the monoclonal anti-myomesin antibody (*a*) and polyclonal anti MM-CK antibody (*b*). Increased magnification of part of an injected cell reveals that the staining patterns of myomesin and M-CK are identical, as can be seen clearly in the circled area. The sketch in the middle is taken from the myomesin staining (*a*) and superimposes exactly with the M-CK staining pattern (*b*). Bar, 20 μm.

bation before M-CK specific staining did not result in a different behavior of the newly synthesized M-CK. Using concentrations below 10 ng/μl no clear M-CK signals were observed and usually a concentration of 100 ng/μl for all subsequent experiments was used. In all cases the RNA was synthesized in the presence of the CAP nucleotide, if this substance it was deleted from the transcription mixture the RNA was not efficiently translated and no M-CK staining was observed.

If the injection of M-CK mRNA indeed results in the creation of a M-band cytoarchitecture identical to the one observed in skeletal muscle the newly synthesized M-CK should colocalize at the light microscope level exactly with the known M-band protein myomesin. In Fig. 4 *a* detailed analysis of part of another injected cell is shown in higher magnification. The encircled areas show the same myofibrils stained with myomesin in Fig. 4 *a* and with M-CK in Fig. 4 *b*. The sketch in the middle corresponds to a schematic representation of the M-bands as taken from the myomesin staining and superimposes perfectly with one designed on the basis of M-CK staining. This is especially evident at the cross point of two myofibrils in the circle. It has been shown that anti-myomesin stains invariably the M-band of isolated myofibrils and of myofibrils in cultured cells as well (21, 22). The coincidence of myomesin and M-CK staining patterns establishes unequivocally an M-CK interaction in the M-band region. From these experiments it is clear, at least on the level of light microscopy, that the microinjected synthetic RNA is translated by the living cells and that the protein product can associate in an isoprotein-specific manner to create the skeletal muscle like cytoarchitecture typical of the M-band region.

### *Isoprotein Typical Electrophoretic Behavior of Chimeric CK is Determined by the $NH_2$-Terminal Half of the Molecule*

Amino acid sequence comparisons of M-CK to B-CK sequences have shown an extensive homology among all CKs analyzed to date. In 34 positions the amino acids are conserved in an isoprotein- specific fashion and allow distinction of M-CK from B-CK sequences. Almost two thirds of these residues are located in the COOH-terminal half of M-CK (2). To investigate the possible involvement of these residues in determining the isoprotein-specific localization, chimeric molecules were constructed. The cDNA of either M-CK or B-CK was bisected by digestion at a conserved Pvu II site at amino acid 186 and the head portion ($NH_2$-terminal half) of M-CK was fused to the tail portion (COOH-terminal half) of B-CK and vice versa (Fig. 5 *A*). From both chimeric cDNAs, transcripts were produced and translated in vitro, immunoprecipitated with anti-MM-CK antibody and analyzed on two-dimensional gels (Fig. 5 *B*). Surprisingly the resulting isoprotein chimeras did not behave in an intermediate manner. The B-CK head/M-CK tail isoprotein chimera migrated as one spot only with an apparent isoelectric point slightly more acidic than the basic spot of the B-CK doublet and an apparent molecular weight somewhat higher than purified B-CK (Fig. 5 *B*, *a* and *b*). The M-CK head/B-CK tail isoprotein chimera, however, migrated as a double spot with apparent isoelectric points slightly more basic than the M-CK doublet with an apparent molecular weight somewhat lower than purified M-CK. In both analyses the minor spots observed in Fig. 5 *B*, *b* and *d* are very likely derived from initiation of protein synthesis at internal methionines as discussed above. These experiments indicate that electrophoretic properties of both isoprotein chimeras are determined by the head portions of their respective isoproteins.

### *Isoprotein Typical Interaction with the Muscle Cytoarchitecture is Influenced by the COOH-Terminal Half of the Molecule*

However, depending on the nature of the injected RNA the interaction of resulting translation products with the myofibrils was dramatically different. In the next series of experiments the in vitro generated RNA transcripts coding for the isoprotein chimeras M/B-CK and B/M-CK were injected into cultured heart cells and their distribution within cells analyzed by double-immunofluorescence. As shown in Fig. 5, *B* and *C* both chimeric proteins reacted with anti-MM-CK antibody. Fig. 6 shows a series of three individual microinjected cells. The left panels show the internal control staining with anti myomesin antibody to localize M-bands and the right panels show the staining with anti-MM-CK antibody. In the control experiment (Fig. 6, *a* and *b*) RNA from the

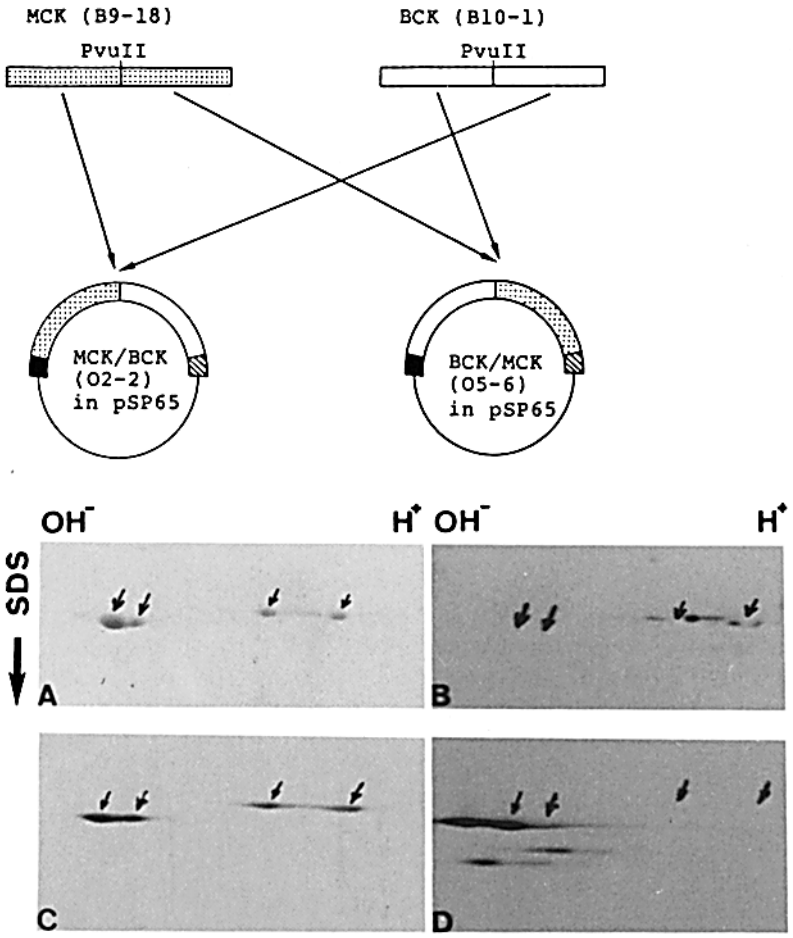


*Figure 5.* Construction and in vitro expression of CK-isoprotein chimeras. (*A*) cDNA sequences of M-CK (*hatched bar*) and of B-CK (*open bar*) were cut at the conserved Pvu II restriction site at amino acid 186. The vector containing the head portion (amino-terminal sequences) of B-CK resp. M-CK was ligated to the isolated tail portion (carboxy-terminal sequences) of the other isoprotein. The resulting chimeras maintained the correct reading frame. (*B*) Transcripts were produced from both chimeric cDNAs and translated in a rabbit reticulocyte translation system. After immunoprecipitation with anti-MM-CK antibody the translation products were analyzed by two-dimensional gel electrophoresis together with purified M-CK and B-CK. The gels were stained for protein and the position of the isolated enzymes marked by an arrow of [$^{35}$S]methionine containing ink before exposure. (*a* and *c*) protein staining of M-CK (more basic) and B-CK (3 μg each); (*B*) translation product of the B-CK head/M-CK tail chimera (*d*) translation product of the M-CK head/B-CK tail chimera. The head portion of the chimeras determines the migration properties during electrophoresis. The subbands seen in *B* and *D* are most probably due to initiation of translation at internal methionines (also see Fig. 2).

full length M-CK cDNA clone was used and the interaction at the M-band is again clearly visible as already shown before (Figs. 3 and 4). In contrast the protein derived from the M-CK head/B-CK tail construction did not show any interaction with the myofibrils (Fig. 6, *c* and *d*), and even if the cells were extracted more thoroughly no signs of M-band staining could be observed. On the other hand the translation product from the inverse construction, B-CK head/M-CK tail, could be localized in a cross-striated pattern as shown in Fig. 6, *e* and *f*. Although the M-band is stained the fluorescence extends into other segments of the sarcomeres indicating additional sites of interaction with the myofibril. We have observed the fluorescence pattern does superimpose with the A segments of the phase contrast pattern if the two patterns were compared directly at very low visible light intensity in the microscope. Therefore, it is unlikely that the interaction occurs randomly like in the region of the I-Z-I-bands. It is probable that application of computerized image reconstruction methods would lead to a more detailed assignment where in the myofibril this chimeric protein interacts. These experiments indicate that the COOH-terminal half of the isoprotein chimeras influences isoprotein-specifically their interaction with the myofibrils and that a protein containing additional M-CK specific features is required for the creation of a skeletal muscle like M-band cytoarchitecture (e.g., complete M-CK). In contrast the $NH_2$-terminal halves that largely determine the electrophoretic behavior of the isoprotein chimeras do not seem to direct the specificity of interaction with the myofibrils.

## Discussion

In most cases little is known about the functional significance of most isoproteins. On one hand the different genes coding for the isoproteins may have evolved to allow differential gene control mechanism to operate during the course of differentiation and in the various tissues of the adult stage. The isoproteins encoded by the various genes of such a gene family would be indistinguishable as in the case of some of the actin gene products of *Dictyostelium discoideum* (32). In these cases regulatory sequestration might have been responsible for the evolution of genetically distinct but functionally indistinguishable isoprotein sequences. On the other hand the multiple forms of a given protein may provide the cell with functionally different polypeptides which may be better adapted for a cell type-specific or organelle-specific task than the original ancestral protein. Such ideas have been discussed for the tubulin isoproteins (16, 24) but the same questions can be asked for other isoprotein families (29).

Our experiments show that a skeletal muscle-like M-band cytoarchitecture could be produced in heart muscle cells and thus provide evidence for the importance of intracellular targeting of tissue-specific isoprotein to their proper sites also within the cytoskeletal organization. The use of RNA tran-

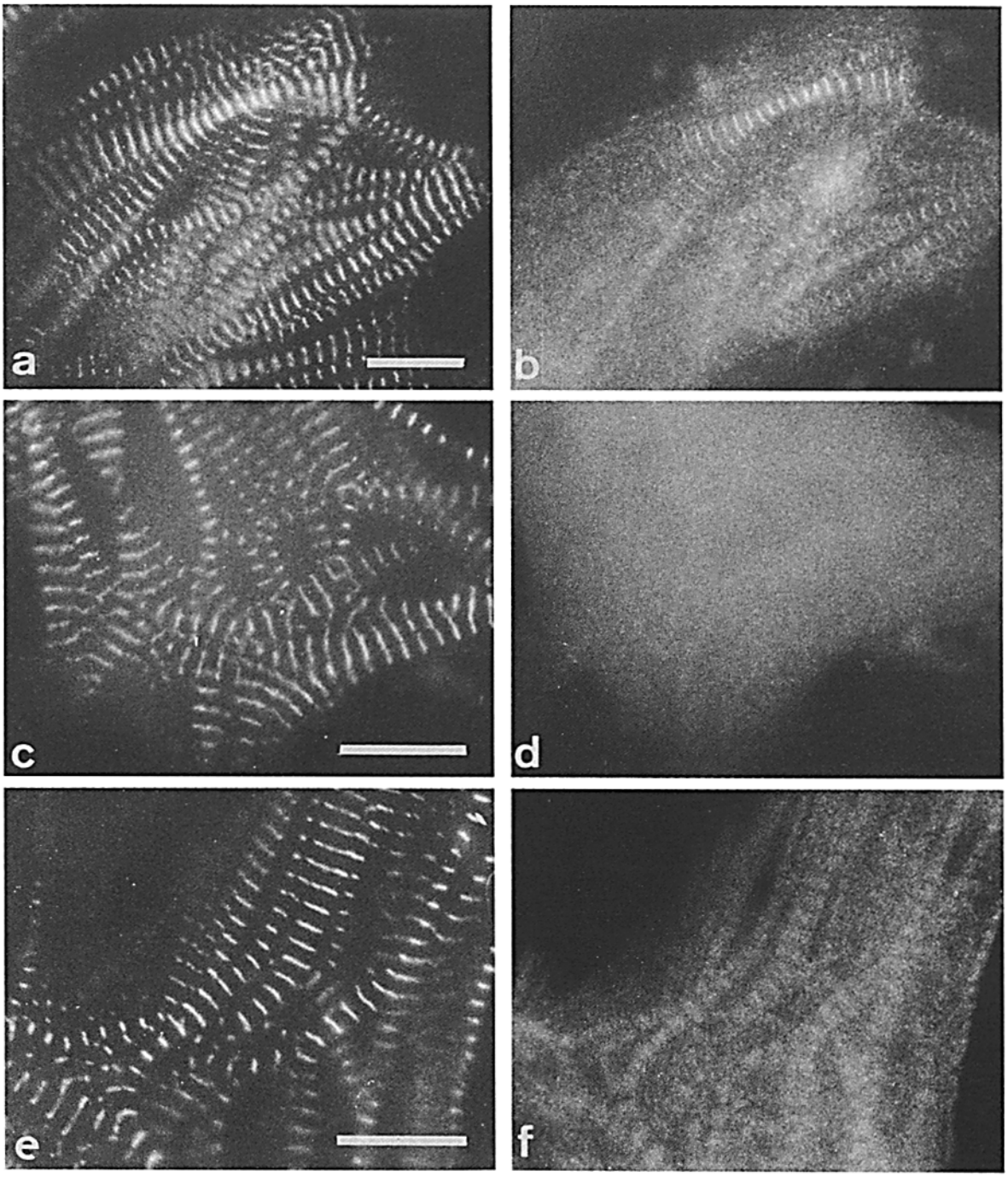


*Figure 6.* Immunofluorescence localization of CK proteins after microinjection of synthetic mRNA coding for CK isoprotein chimeras into chicken heart cells B-CK head/M-CK tail and M-CK head/B-CK tail synthetic mRNA (at 0.1 μg/μl) was microinjected into chicken heart cells derived from 9-d-old embryos. The cells were further incubated for 16 h and double immunofluorescence staining was performed with anti myomesin antibody B4 (*a*, *c*, and *e*) and anti-MM-CK antibody (*b*, *d*, and *f*). (*a* and *b*) Control injection of synthetic mRNA coding for entire M-CK. (*c* and *d*) Injection of M-CK head/B-CK tail mRNA. (*e* and *f*) Injection of B-CK head/M-CK tail mRNA. Note that the M-CK head/B-CK tail chimera is not able to interact with the myofibrils indicating that the M-CK tail portion is involved in this interaction. Bars, 10 μm.

scribed by SP-6 RNA polymerase from cloned templates for cell-free translations and microinjections into living cells has allowed the efficient analysis of the biochemical nature and the behavior of such translation products within a cell. Furthermore, this approach represents a convenient way to construct and test chimeric or otherwise mutant proteins. In contrast to transfection, injection of RNA also avoids problems that might occur like transcriptional activation of the vector promoter etc.

By injection of homogeneous RNA we have successfully avoided the problems of impurity, partial degradation, or denaturation observed in experiments with biochemically

purified M-CK (47). The generation of RNA from cloned templates also avoids problems arising from the heterogeneity of even the most highly purified cellular RNA preparations. In addition, nascent polypeptides clearly represent the ideal form of any protein molecule that is destined to participate in the formation of cellular structures, not only because such polypeptides can undergo folding as required by the nascent structure but also because posttranslational modification (proteolytical or chemical) possibly affecting the proper interaction may depend on the cellular environment.

M-CK and B-CK molecules show nearly identical biochemical activities and catalyze the same reaction. Therefore, one should anticipate an extensive homology (2) in the regions of the molecule participating in the catalytic site. Other homologous regions might guarantee the folding and dimerization of CK. However, the two isoenzymes are rather distinct in other characteristics such as their apparent $M_r$ on SDS gels or their isoelectric points on 2D-gels. Although the molecular weights calculated from the sequence data yield 43,340 for M-CK and 42,875 for B-CK, the apparent $M_r$ on SDS gels differs by as much as 3,000 and quite surprisingly it is M-CK that appears to be smaller than B-CK (8). On two-dimensional gels both CK species form characteristic double spots with apparent isoelectric points more basic for the M-CK spots than for the B-CK spot. As shown in the results section the M-CK isoprotein can specifically interact with the M-band region of the myofibrils from skeletal and heart muscle (see Fig. 4). It is quite clear that the different primary structures of the CK must somehow account for these isoprotein typical characteristics.

Most interesting is the fact that the part coding for the $NH_2$-terminal region of M-CK mRNA appears to be responsible for double spot formation of M-CK while the full length B-CK mRNA and constructions with B-CK mRNA $NH_2$-terminal halfs gave rise to only one major spot (Fig. 5). Since RNA isolated from B-CK-containing tissues can be translated into B-CK comigrating with both subunit types from isolated purified B-CK (43) we assume that there may be additional B-CK mRNA species in chicken tissues (25). It is unlikely that the M-CK doublet arises by proteolytic processing as was suggested for human plasma M-CK at the COOH-terminal Lys (3). We have shown that the double spot formation is dependent on the $NH_2$-terminal sequence, and it is unlikely that a protease specific for M-CK processing is present in the cell free translation system from rabbit reticulocytes. Furthermore no sequence heterogeneity has been detected in the $NH_2$ terminus of rabbit, monkey, and human M-CKs (9) confirming our finding that the M-CK doublet arises from one sequence only.

M-CK sequences contain in 34 positions amino acids that are conserved isoprotein typically among all known M-CK. The pattern at the same positions is also conserved in a different configuration typical for all B-CKs (2). It is possible that part or all of the M-CK specific properties are associated with these amino acids conserved in the M-CK sequence. Our experiments indicate that the M-CK is capable of specific interaction with the M-band of the myofibril and already the presence of the COOH-terminal half in a hybrid molecule mediates this process at least in part. In this portion of the molecule, 23 of the 34 above mentioned isoprotein specific amino acids are localized. Although as shown before they do not contribute to gross differences in electrophoretic migration, most of them are conservative exchanges, they seem to be involved in the determination of the pattern of interaction.

The study with chimeric CK molecules has indicated that the COOH-terminal half of M-CK is responsible, at least to a large extent, to promote the interaction with the myofibril as shown in Fig. 6 *f*. A similar pattern was found with a mutant M-CK protein carrying a stop codon near the COOH-terminus (replacing amino acid Val 297) and producing a M-CK that is ~100 amino acids shorter than the wild-type molecule. Again this molecule showed a binding pattern similar to the one obtained with the B-CK/M-CK into chimera extending into other segments of the sarcomere of the heart myofibrils of the injected cells (Schäfer, B., unpublished observations).

All conditions known so far that impair the integrity of the M-CK protein like the constructions of chimeric CKs, the mutant short protein and also the purified M-CK which might have been partially denatured and/or degraded result in a localization that is far from perfect, the region decorated by such a "M-CK" protein extends over a wider range of sarcomeric structures. There are obviously regional differences within the M-CK molecule important for the interaction but as expected the integral homogeneous nascent protein has the best chances to interact properly with the cellular architecture.

Since the complete M-CK molecule gives the best reconstruction of a skeletal type M-band cytoarchitecture in heart cells it can be assumed that the whole folded molecules with the complete set of isoprotein specifically conserved amino acids of M-CK are needed to optimally interact with the myofibril. It is therefore unrealistic to hope that replacement of single amino acids by "in vitro" mutagenesis will help to point out a small number of amino acid residues responsible for proper interaction.

Many localization experiments using isoprotein-specific antibody probes have shown that organelle- or tissue-specific isoproteins coexpressed in a cell do often localize in different locations within the cytoarchitecture. It is likely that under such circumstances the specificity of the isoprotein building blocks guarantees the organelle-specific morphogenesis. Differential distribution has been observed e.g. for the isoproteins of alpha-actinin (13) actin (38), myosin heavy chain (15, 35), tubulin (16). In some cases, mutations in tissue-specific isoprotein genes led to severely damaged phenotypes (27, 34) indicating the functional implications of defective isoproteins. These observations also are consistent with studies carried out on myogenic cells undergoing cytodifferentiation, in which stress fiber-like filaments (SFLS) have been observed consisting of nonmuscle contractile isoproteins that were the precursor structures for the myofibrils made up of muscle-specific contractile proteins (1, 12).

There are, however, also a number of experiments reported in which no specificity of isoprotein assembly was observed. Chemically modified alpha-actinins or actins (18, 30, 31, 45, 46) of either nonmuscle or skeletal muscle type interacted equally well with myofibrils of microinjected cultured heart cells. Likewise muscle actin sequences transfected into fibroblastic cells were expressed as protein and interacted with the nonmuscle cytoskeleton (23). A chimeric tubulin construct made of chicken and yeast tubulin sequences was introduced into mouse cells and the protein expression prod-

uct was found in the microtubules (7). It is possible that isoprotein specificity in these cases is not stringent and/or the assays used are not sensitive enough to reveal the differences.

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