

The carboxyl terminus of myosin binding protein C (MyBP-C, C-protein) specifies incorporation into the A-band of striated muscle

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SUMMARY

Myosin binding protein-C (MyBP-C), also known as C-protein, is a major constituent of the thick filaments of vertebrate striated muscles. The protein, ~130 kDa, consists of a series of 10 globular motifs (numbered I to X) each of ~90-100 amino acids, bearing resemblance to the C2-set of immunoglobins (Ig C2) and to the fibronectin type III (FnIII) motifs. Using pure preparations of myosin and MyBP-C, it has been demonstrated that the major myosin binding domain of MyBP-C resides within the C-terminal Ig C2 motif (motif X). However, in the context of the *in vivo* thick filament, it is uncertain if the latter domain is sufficient to target MyBP-C correctly to the A-band or if other regions of the molecule are required for this process. To answer this question, cultures of skeletal muscle myoblasts were transfected with expression plasmids encoding seven truncation mutants of MyBP-C, and their targeting to the A-band investigated by immunofluorescence microscopy. To distinguish the recombinant proteins from endogenous MyBP-C, a myc epitope was inserted at each amino terminus. Recombinant MyBP-C exhibited an identical dis-

tribution in the sarcomere to that of native MyBP-C; i.e. it was found exclusively in the C-zone of the A-band. A mutant encoding the C-terminal 372 amino acids, but lacking motifs I-VI (termed $\Delta 1-6$), also targeted correctly to the A-band. This fragment, which is composed of two Ig C2 and two FnIII motifs, was the minimal protein fragment required for correct A-band incorporation. Larger amino-terminal deletions or deletion of motif X, the myosin binding domain, abolished all localization to the A-band. One construct ($\Delta 10$) lacking only motif X strongly inhibited myofibril assembly. We conclude that the myosin binding domain of MyBP-C, although essential, is not sufficient for correct incorporation into the A-band and that motifs VII to IX are required for this process. The data suggest a topological model in which MyBP-C is associated with the thick filament through its C terminus.

Key words: Myosin, MyBP-C, Muscle protein, C-protein, Development, Myofibril assembly, A-band, Thick filament, Myogenesis, Sarcomere

INTRODUCTION

The myofibrils of the vertebrate striated muscles are complex, semi-crystalline structures consisting of interdigitating thin and thick filaments predominantly composed of actin and myosin, respectively, and of a battery of myofibril associated proteins having structural and/or regulatory functions (reviewed by Epstein and Fischman, 1991; Small et al., 1992). Besides myosin, at least eight different proteins have been found in association with vertebrate thick filaments: (1) titin (Maruyama et al., 1977; Wang et al., 1979; reviewed by Trinick, 1992); (2) skelemin (Price et al., 1993); (3) M-protein (Masaki and Takaiti, 1974; Trinick and Lowey, 1977; Noguchi et al., 1992); (4) myomesin (Grove et al., 1985); (5) MM-creatine kinase (Wallimann et al., 1983); (6) AMP-deaminase (Cooper and Trinick, 1984); (7) MyBP-H (H-protein) (Starr and Offer, 1983; Bahler et al., 1985; Bennett et al., 1986) and MyBP-C (C-protein) (Offer et al., 1973).

MyBP-C is a relatively large protein (~130 kDa) that represents about 2% of myofibrillar protein (Offer et al., 1973). It is located in the A-bands of the thick filaments of all vertebrate

cross-striated muscles, where it is restricted to a series of 7-9 transverse stripes, 43 nm apart, in the cross-bridge bearing region (Craig and Offer, 1976; Dennis et al., 1984; Bennett et al., 1986). Several isoforms of MyBP-C that are fiber-type specific or expressed at selected stages of development have been identified. In the chicken, for example, at least six different isoforms have been characterized (Takano-Ohmuro et al., 1989). We and others have cloned and sequenced several isoforms of MyBP-C from various organisms (Einheber and Fischman, 1990; Fürst et al., 1992; Weber et al., 1993; Okagaki et al., 1993; Gautel et al., 1995). From the deduced primary structure, MyBP-C has been predicted to contain a series of globular domains, based on repetitive motifs, each 90-100 amino acids in length, which bear resemblance to the C2-set of the immunoglobulin superfamily (Ig C2) and the fibronectin type III motif (FnIII). In the case of MyBP-C, seven Ig C2 and three FnIII motifs are present (Fig. 1). One of the best characterized biochemical properties of MyBP-C is its relatively strong affinity for myosin (Offer et al., 1973; Moos et al., 1975). Using an *in vitro* binding assay, we have shown that the principle myosin binding domain of MyBP-C resides within

the C-terminal Ig C2 motif, a region of the MyBPs which is highly conserved among the C- and H-protein families (Okagaki et al., 1993). MyBP-C also binds to titin and weakly to actin (Moos et al., 1978; Yamamoto, 1986; Fürst et al., 1992; Koretz et al., 1993). Although MyBP-H binds to myosin it does not appear to bind titin (Soteriou et al., 1993).

The precise function of MyBP-C is uncertain but evidence exists for both structural and regulatory roles. When compared with other myofibrillar proteins, MyBP-C appears relatively late during myofibril assembly in both cultured skeletal (Kawashima et al., 1986; Lin et al., 1994) and cardiac muscle (Schultheiss et al., 1990; Rhee et al., 1994). It is first detected by immunofluorescence in A-bands as non-striated myofibrils (NSMFs) (Lin et al., 1994) or nascent myofibrils (Rhee et al., 1994) transform into mature myofibrils. Since the expression of MyBP-C correlates with the emergence of cross-striations, the protein may be a regulatory component in the assembly reaction, potentially as a rate-limiting molecule. When purified myosin is polymerized in vitro, at physiological stoichiometries with MyBP-C, the resulting thick filaments are slightly longer and more regular than in the absence of MyBP-C (Koretz, 1979; Davis, 1988). The presence of MyBP-C reduces the critical concentration required for myosin polymerization, suggesting that it may regulate thick filament assembly (Davis, 1988). This is consistent with the observation that co-expression of MyBP-C or MyBP-H with myosin heavy chain in COS cells promotes the formation of long cable-like co-polymers of both proteins (Seiler et al., 1996). Deletion of the C-terminal Ig C2 motif from MyBP-C or MyBP-H prevents such cable formation.

The observation that MyBP-C inhibits actin-activated skeletal muscle myosin ATPase but stimulates actin-activated cardiac muscle myosin ATPase (Offer et al., 1973; Moos and Feng, 1980; Yamamoto and Moos, 1983; Hartzell, 1985) suggests a potential role in the regulation of contraction. The cardiac isoform of MyBP-C is phosphorylated by a catecholamine-sensitive pathway and this phosphorylation correlates with the rate of twitch relaxation (Hartzell and Titus, 1982; Hartzell, 1984). The reactive serine residues have been identified but the precise kinase awaits characterization (Gautel et al., 1995). Furthermore, partial extraction of MyBP-C from myofibrils enhances tension generation at submaximal concentrations of Ca^{2+} and accelerates the contractile velocity at low levels of Ca^{2+} activation (Hofmann et al., 1991a,b). Both changes are reversible upon the reinsertion of MyBP-C.

It is clear that better knowledge of the various biochemical properties of MyBP-C and their precise mapping on the protein is crucial to understand the function as well as the mechanism of action of MyBP-C. As described above, the major myosin binding domain of MyBP-C has been mapped to the C-terminal Ig C2 motif of the protein (motif X). However, in the context of the in vivo thick filament, it is uncertain if the latter domain is sufficient to target the protein to the A-band. It is likely, because of the complexity of the thick filament, that other regions of MyBP-C are important for correct localization to the A-band. To address this question, we have analyzed the expression and distribution of seven truncation mutants of MyBP-C in cultured skeletal myoblasts. We show here that a mutant encoding the last 372 amino acids at the C terminus (33% of the full length protein) is efficiently and correctly incorporated into the A-band. This fragment, consisting of two Ig C2 and two FnIII motifs (motifs VII-X), is the minimal

length required for correct targeting to the C-zone of the A-band. Larger deletions at the amino terminus or deletion of motif X, the myosin binding domain, abolished localization to the A-band. We conclude that the myosin binding domain of MyBP-C, although essential, is not sufficient for correct incorporation into the A-band and that motifs VII to IX are required for this process. The data suggest a topological model in which MyBP-C is associated with the thick filament through its C terminus; sequences nearer the N terminus may subserve properties associated with thin filament interactions.

MATERIALS AND METHODS

Vector constructs

Recombinant DNA procedures followed standard methods (Sambrook et al., 1989). The mutations and the junctions of the spliced regions were confirmed by double-stranded DNA sequencing, using the protocols and the reagents provided by the Sequenase sequencing kit (United States Biochemical). Unless otherwise specified, the primers and enzymes were purchased from New England Biolabs. The cDNA encoding the full length MyBP-C (Einheber and Fischman, 1990; Okagaki et al., 1993) was cloned into the *EcoRI* site of pBluescript II SK+/- (Stratagene) and the polyadenylation signal removed by digestion with exonuclease III. The cDNA was ligated to *XbaI* linkers, and cloned into the *XbaI* site of the expression vector pJDp- (Mikawa et al., 1992). This plasmid was called pJDC. To construct a cDNA encoding a myc epitope at the N terminus of MyBP-C (MyBP-C^{myc}), the three bp at the 5' end of the initiation codon were mutated to create a *NdeI* restriction site. The cDNA was digested with *NdeI* and the ends were filled in by reaction with the large fragment of DNA polymerase I (Klenow). The cDNA was then excised by digestion with *NotI* and inserted into the *SmaI* and *NotI* sites of pBSmyc. Plasmid pBSmyc was generated by inserting the oligonucleotide 5'-AAGCTTGGGCGACCTCAC-CATGGAGCAAAAAGCTCATTCTGAAGAGGACTTGAATTC-3', encoding the myc epitope (Munro and Pelham, 1986), into the *HindIII* and *EcoRI* sites of pBluescript II KS+/- (Stratagene). The plasmid encoding MyBP-C^{myc} was digested with *XhoI* and filled in with Klenow. The cDNA was excised by digestion with *NotI* and cloned into the *SmaI* and *NotI* sites of pJDC. This plasmid was called pMC. To construct $\Delta 1-2$, pMC was digested with *XmnI* and filled in by reaction with Klenow. The 3' portion of MyBP-C was excised by digestion with *XbaI* and inserted into the *EcoRI* and *XbaI* sites of pBSmyc. Before ligation, the *EcoRI* ends were filled in with Klenow. The plasmid encoding $\Delta 1-2$ was digested with *HindIII* and filled in with Klenow. The insert was removed by digestion with *XbaI* and cloned into the *SmaI* and *XbaI* sites of pJDp-. To construct $\Delta 1-6$, a *NheI* linker (5'-CTAGCTAGCTAG-3') was inserted into the *AccI* site at position 2,303 of pMC. The 3' portion of MyBP-C was isolated by digestion with *NheI* and *XbaI*, and cloned into the *EcoRI* and *XbaI* sites of pBSmyc. Before ligation, the *NheI* and the *EcoRI* ends were filled in with Klenow. The plasmid encoding $\Delta 1-6$ was digested with *HindIII* and filled in with Klenow. The insert was removed by digestion with *XbaI* and cloned into the *SmaI* and *XbaI* sites of pJDp-. To construct $\Delta 1-8$, pMC was digested with *NotI* and filled in with Klenow. The 3' portion of MyBP-C was excised by digestion with *XbaI* and cloned into the *EcoRI* and *XbaI* sites of pMC. Before ligation, the *EcoRI* ends were filled in with Klenow. The plasmid encoding $\Delta 1-8$ was digested with *HindIII* and filled in with Klenow. The insert was removed by digestion with *XbaI* and cloned into the *SmaI* and *XbaI* sites of pJDp-. To construct mutant $\Delta 1-9$, pET-C2, a plasmid encoding the 14 kDa peptide (Okagaki et al., 1993) was digested with *NcoI* and filled in with Klenow. The insert was excised by digestion with *BamHI* and cloned into the *SmaI* and *BamHI* sites

of pBSmyc. The plasmid encoding $\Delta 1-9$ was digested with *HindIII* and filled in with Klenow. The insert was removed by digestion with *XbaI* and cloned into the *SmaI* and *XbaI* sites of pJDp-. Mutants $\Delta 10$, $\Delta 9-10$ and $\Delta 7-10$ were constructed by inserting a *NheI* linker (5'-CTAGCTAGCTAG-3') encoding a termination codon in any of the three reading frames. To construct $\Delta 10$ and $\Delta 7-10$, the linker was inserted into the *AccI* sites at position 3,014 and 2,303 of pMC, respectively. In the case of $\Delta 7-10$, the 3' end of the cDNA was removed by digestion with *NheI* and *XbaI*. Mutant $\Delta 9-10$ was constructed by inserting the linker in the unique *NotI* site of pMC.

Western blots

Canine fibroblasts (D17, available from ATCC) were grown at 37°C under an atmosphere of 2% CO₂ in Dulbecco's modified Eagle's medium (DME) supplemented with 7% FBS, glutamine and antibiotics. D17 and primary cultures of myoblasts (see below) were transfected with a mixture of LipofectAMINE (Gibco BRL) and DNA according to the manufacturer's recommendations. The next day (D17) or three days later (myoblasts), the cells were washed with PBS and lysed with hot (80°C) 2× sample buffer (Laemmli, 1970) containing 2 mM of PMSF and 2 µg/ml of aprotinin. The chromatin was sheared by several passages through a 20G1 needle. The samples were then boiled for 3 minutes and separated by electrophoresis using the Laemmli system (Laemmli, 1970) in either 7, 12, or 15% SDS-PAGE gels. The proteins were transferred to nitrocellulose membranes overnight at 4°C in 25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3 (Harlow and Lane, 1988). In some experiments, to improve the transfer of positively charged proteins, such as $\Delta 1-9$, the proteins were transferred to nitrocellulose membranes according to the method of Szewczyk and Kozloff (1985). The membranes were then incubated with mAbs specific for the chicken fast isoform of MyBP-C (MF1; Reinach et al., 1982) or for the myc epitope (9E10; Evan et al., 1985), and then with horseradish peroxidase-labeled affinity-purified goat anti-mouse antibodies (Sigma). The antibody complex was visualized by chemiluminescence (Dupont).

Myoblast cultures and immunofluorescence microscopy

Myoblasts were isolated from the pectoralis muscles (PM) of chicken (embryonic day (E) 11) and grown as described (Nawrotzki et al., 1995). After isolation, they were plated in collagen-coated 4 cm² Chamber-Slides (Nunc, Inc.) at a density of 4.0×10⁵ cells/chamber. The next day the myoblasts were transfected with a mixture of LipofectAMINE (Gibco BRL) and DNA according to the manufacturer's recommendations. One to three days later, they were fixed with 2% paraformaldehyde for 10 minutes and permeabilized with 1% Triton X-100 for 10 minutes at room temperature. For immunofluorescence, the cells were incubated with selected primary antibodies for 30 minutes at room temperature. The following primary antibodies were used: MF1, a mAb against the chicken fast isoform of MyBP-C, isotype IgG 1 (Reinach et al., 1982); 9E10, a mAb against the myc epitope, isotype IgG 1 (Evan et al., 1985); T11, a mAb against the chicken titin, isotype IgG 2b (Sigma; Fürst et al., 1988); F59, a mAb against the S1 fragment of chicken skeletal myosin heavy chain, isotype IgG 1 (Miller et al., 1989). To study the distribution of the recombinant proteins and myosin, the cells were incubated with MF1 or 9E10, followed by Texas Red-conjugated affinity-purified rabbit anti-mouse IgG 1 antibodies (Hyclone). They were then incubated with F59 conjugated to biotin (Harlow and Lane, 1988), followed by FITC-conjugated avidin (Cappel). The cells were fixed with 2% paraformaldehyde for 5 minutes and mounted in 50% glycerol in PBS containing 100 mg/ml of 1,4-diazabicyclo-[2.2.2]octane (Sigma). To study the distribution of the recombinant proteins and titin, the cells were incubated with MF1 or 9E10 followed by Texas Red-conjugated affinity-purified rabbit anti-mouse IgG 1 antibodies (Hyclone). They were then incubated with T11, followed by FITC-conjugated affinity-purified rabbit anti-mouse IgG 2b antibodies (Hyclone), fixed and mounted as described above. Specimens were observed with a Nikon

epifluorescence microscope using 60× and 100× objectives. The following filters were used: a filter block for FITC (UV-1A, Nikon), a filter block for Texas Red (G-1B, Nikon), and a dual filter block allowing visualization of Texas Red and FITC simultaneously (XF53, Omega Optic). Photographs were taken using Kodak TMAX 400 or Ektachrome 400 films.

Statistical analysis

To study the effects of the truncation mutants on myofibril assembly, myoblasts were transfected and processed for immunofluorescence 3 days later as described above. The percentage of cells expressing the truncation mutants and having cross-striated myofibrils as observed after staining for myosin or titin was determined for each chamber. The data obtained from 3 to 5 independent transfections were analyzed. For each construct, 6 to 7 chambers were examined and between 286 and 482 transfected cells were scored. The data were analyzed by an analysis of variance followed by the test of Scheffé (Woolson, 1987).

RESULTS

Description of the MyBP-C truncation mutants

Our first step was to generate and characterize several truncation mutants of MyBP-C. The mutants, depicted in Fig. 1, were constructed by deleting portions of the 5' and 3' coding region of the cDNA encoding the chicken fast isoform of MyBP-C (Einheber and Fischman, 1990; Okagaki et al., 1993). High expression of the recombinant proteins was achieved in tissue culture by subcloning the various cDNAs into the plasmid pJDp-. This plasmid encodes the promoter and the 5' untranslated sequence of the spleen necrosis virus which produces strong, constitutive expression of cDNAs in most eukaryotic cells (Mikawa et al., 1991, 1992). During differentiation in culture, the mononucleated myoblasts fuse and begin to express a battery of myofibril associated proteins including three different variants of MyBP-C: cardiac, slow and fast isoforms (Kawashima et al., 1986). To distinguish the recombinant proteins from the endogenous isoforms of MyBP-C, a short linker encoding a myc epitope of 12 amino acids was inserted at the 5' end of each expressed cDNA (Fig. 1). Hereafter, the recombinant full length MyBP-C containing the myc epitope is referred to as MyBP-C^{myc}.

Mutant $\Delta 1-2$ was constructed by deleting Ig C2 motifs I and II, except for 15 amino acids of the latter motif. Mutant $\Delta 1-6$ lacks motifs I to VI and 29 amino acids of motif VII. To create mutant $\Delta 1-8$, motifs I to VII were deleted as well as 54 amino acids of motif VIII. Mutant $\Delta 1-9$ contains only motif X, which contains the major myosin binding domain of MyBP-C (Okagaki et al., 1993). Except for the presence of the myc epitope, mutant $\Delta 1-9$ is identical to the recombinant 14 kDa fragment previously described. Mutant $\Delta 10$ was constructed by deleting motif X plus 15 amino acids of motif IX. To create mutant $\Delta 9-10$, motifs IX and X were deleted along with 27 amino acids of motif VIII. Mutant $\Delta 7-10$ was generated by deleting motifs VII to X except for 29 amino acids of motif VII.

Before studying the distribution of MyBP-C^{myc} and its truncation mutants in cultured skeletal myoblasts, we examined the size and antigenicity of the expressed proteins. Fibroblasts were transfected with plasmids encoding MyBP-C, MyBP-C^{myc} and the truncation mutants. At selected time points the cells were then lysed and analyzed by western blotting. As a control, extracts of pectoralis muscle (PM) of chicken embryos

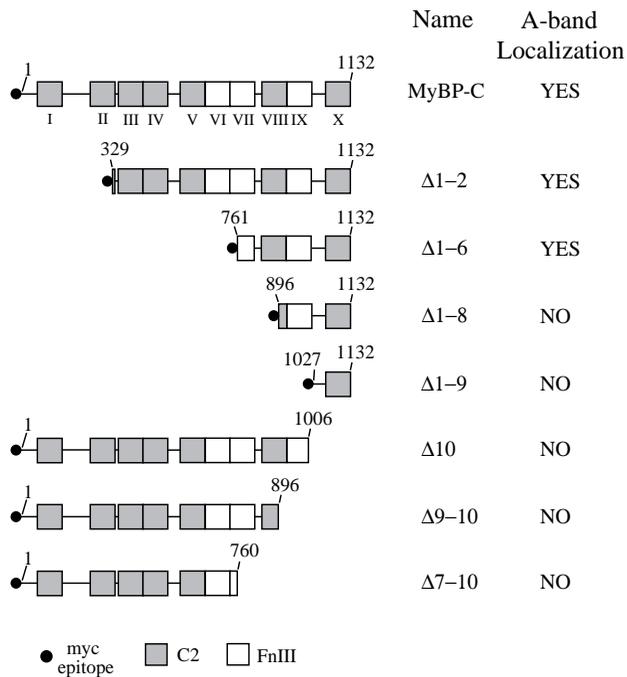


Fig. 1. Expression constructs used in the present study. The numbers above each protein correspond to the first and last amino acids, starting from the N-terminus (left). The numbers below MyBP-C indicate the position of each Ig C2 (C2) and fibronectin type III (FnIII) motif. The position of the myc epitope at the N terminus is shown. The distribution of each protein was investigated in well differentiated myotubes and their presence in the A-band is indicated.

(E20) were analyzed simultaneously. When the blots were incubated with MF1, a mAb specific for MyBP-C, a single product of 130 kDa was detected in cells transfected with MyBP-C or with MyBP-C^{myc} (Fig. 2A). This product migrated with the same mobility as endogenous MyBP-C in the PM. Its size and reactivity with MF1 clearly indicated that it corresponded to MyBP-C. No MyBP-C antigen was observed when mock transfected cells or cells transfected with pJDP- were used. As expected, a band of 130 kDa was detected when lysates of cells expressing MyBP-C^{myc} were analyzed using the mAb against the myc epitope. This band was not observed when extracts of cells expressing MyBP-C were analyzed, thus demonstrating the specificity of the anti-myc antibody.

When cells expressing the truncation mutants were analyzed with western blots, the seven constructs generated proteins migrating on SDS-PAGE with the predicted mobilities (Fig. 2A). Mutants Δ1-2, Δ10, Δ9-10 and Δ7-10 were recognized by MF1 and by the anti-myc antibody, whereas mutants Δ1-6, Δ1-8, and Δ1-9 were recognized solely by the anti-myc antibody. The latter observation indicates that the MF1 epitope lies between repeats II and VII (between amino acids 329 and 761). Except for Δ7-10 and Δ1-9, the mutants appeared as single, sharp and intense bands on each blot. When cells expressing Δ7-10 were analyzed, more than one band was observed; the larger and more intense of these bands corresponded in size with the expected product of Δ7-10. The other bands may represent partial degradation products, suggesting that Δ7-10 is slightly less stable than the other truncated proteins. In the case of Δ1-9, the band corresponding to the expected product was relatively faint and often difficult to detect. The theoretic

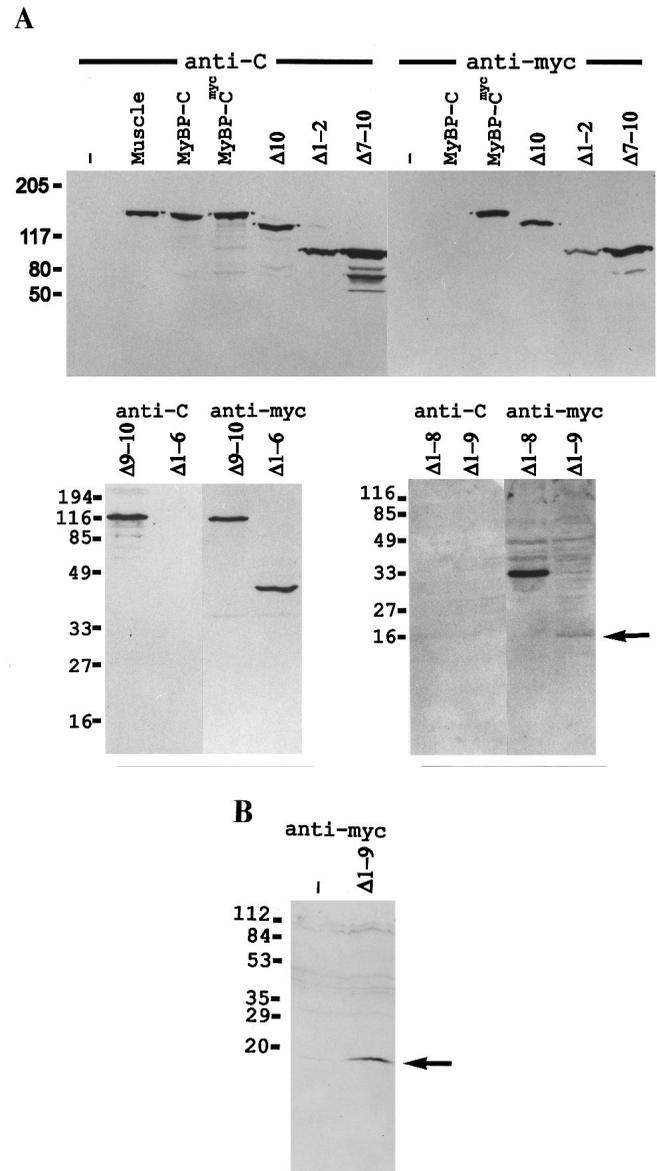


Fig. 2. Evidence for the expression of the recombinant proteins in cultured cells. (A) Fibroblasts transfected with plasmids without any insert (-), coding for MyBP-C (MyBP-C), coding for MyBP-C containing the myc epitope (MyBP-C^{myc}), or coding for the mutants described in Fig. 1, were analyzed by SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane and subsequently reacted with mAbs specific for MyBP-C (anti-C) or for the myc epitope (anti-myc). Muscle: lane containing a lysate of the PM of chicken embryos (E 20); Arrow: position of the product encoded by Δ1-9. (B) Mock transfected myoblasts (-) or myoblasts transfected with Δ1-9 were analyzed by SDS-PAGE three days after transfection. The expressed peptide was identified with mAbs specific for the myc epitope (anti-myc, arrow). Numbers on the left of each gel indicate the relative mobility of molecular mass markers (kDa).

cal isoelectric point of Δ1-9 (9.8) could have rendered the protein difficult to transfer.

However, when the pH of the transfer buffer was increased from 8.8 to 11, the signal intensity was only slightly improved, suggesting that other factors, e.g. low level of expression or protein degradation, could be responsible for poor detectability

of this fragment. To see if $\Delta 1-9$ would be expressed in differentiated myotubes, cultures of myoblasts were transfected with this truncation mutant. Three days later the cells were lysed and analyzed by western blots. A protein band migrating with an approximative molecular mass of 14 kDa was detected on the membrane indicating that $\Delta 1-9$ was correctly expressed (Fig. 2B).

The intracellular distribution of recombinant MyBP-C

To test if recombinant MyBP-C would incorporate into the A-bands of the cross-striated myofibrils, myoblasts were isolated from chicken embryos (E11) and transfected with a plasmid encoding MyBP-C^{myc}. Two to three days after transfection, the time required for the myoblasts to fuse and to form myotubes with robust cross-striated myofibrils, the cells were fixed and

processed for immunofluorescence. The distribution of MyBP-C^{myc} was assessed by staining the cells with a mAb against the myc epitope, and the distribution of MyBP-C compared with myosin and titin, by double labeling the cells with mAbs specific for these latter proteins. Since the conclusions reached with mAbs against myosin or titin were similar, only the photomicrographs obtained after labeling with anti-myosin are presented below.

In the few myoblasts that did not differentiate or in the scattered fibroblasts within the muscle cultures, MyBP-C^{myc} was homogeneously distributed in the cytoplasm, not associated with any obvious cellular structures. As the myoblasts differentiated MyBP-C^{myc} became associated with non-striated myofibrils (NSMFs), also known as nascent myofibrils (Fig. 3A,B). The NSMFs are precursors of cross-striated myofibrils

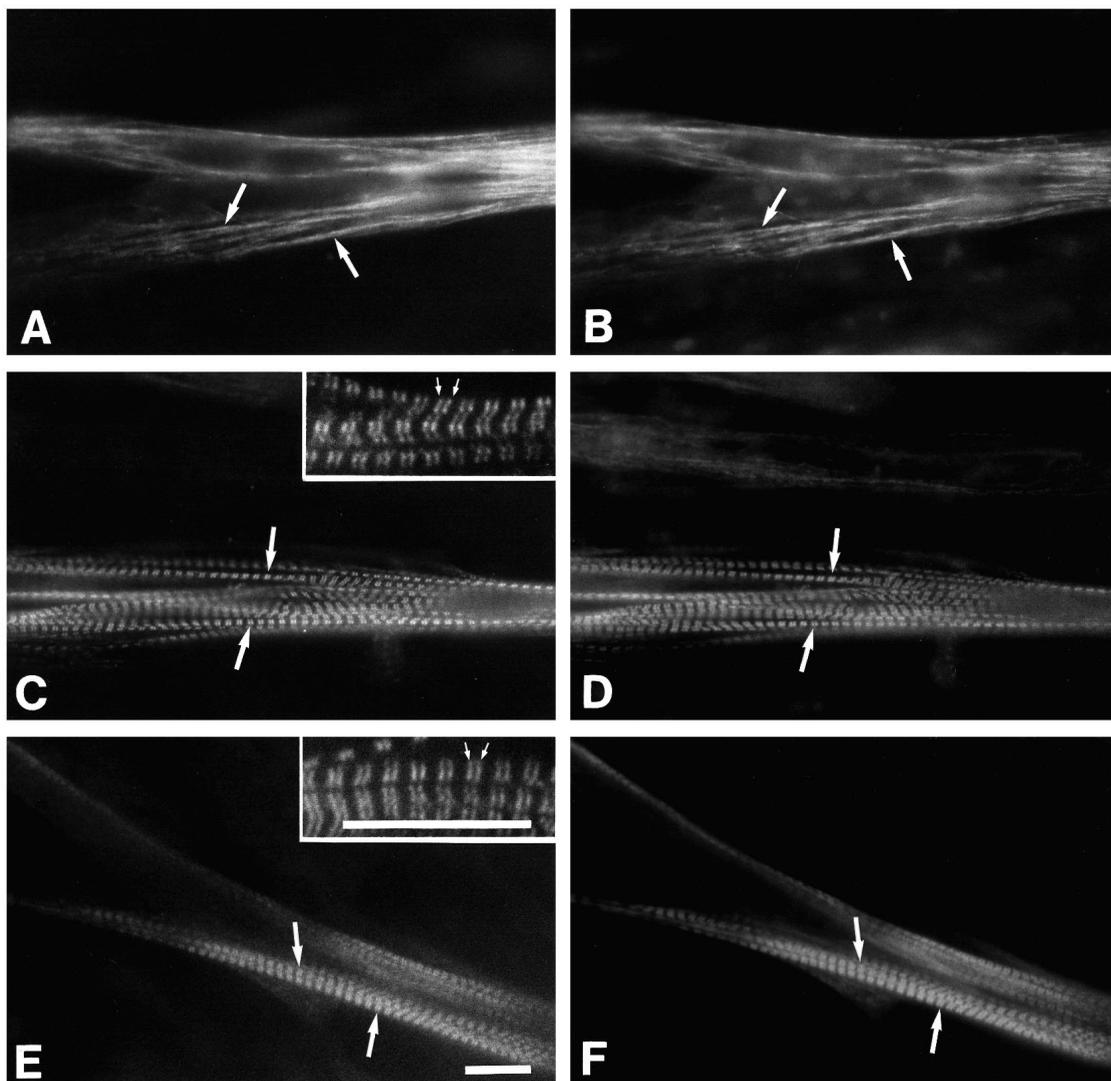


Fig. 3. Evidence for the incorporation of recombinant MyBP-C into A-bands. Myoblasts transfected with MyBP-C^{myc} (A-D), or not transfected (E,F), were processed for immunofluorescence. The cells were double immunostained with mAbs specific for the myc epitope and sarcomeric myosin (A-D), or for MyBP-C and myosin (E,F). The left row of figures indicates the distribution of the recombinant (A,C) and the endogenous MyBP-C (E) using the Texas Red channel. The right row shows the distribution of myosin in the same cells within the FITC channel. In young myotubes the recombinant MyBP-C is localized to the NSMFs which are positive for myosin (arrows, A,B). In more mature myotubes, the recombinant and endogenous MyBP-C are localized to the A-bands of the cross-striated myofibrils (arrows, C-F). Insets in C and E: higher magnification illustrating that both the recombinant and the endogenous MyBP-C are distributed as doublets within each A-band (arrows). Bars, 20 μ m.

and contain several myofibrillar proteins including myosin, titin, actin, α -actinin, tropomyosin and the troponin complex (Einheber and Fischman, 1991; Rhee et al., 1994; Lin et al., 1994). In differentiated myotubes MyBP-C^{myc} staining was

confined to the A-bands of myofibrils, since these same regions of the sarcomere co-stained with mAbs against myosin (Fig. 3C,D). At the resolution of the light microscope, the distribution of MyBP-C^{myc} was identical to that of endogenous

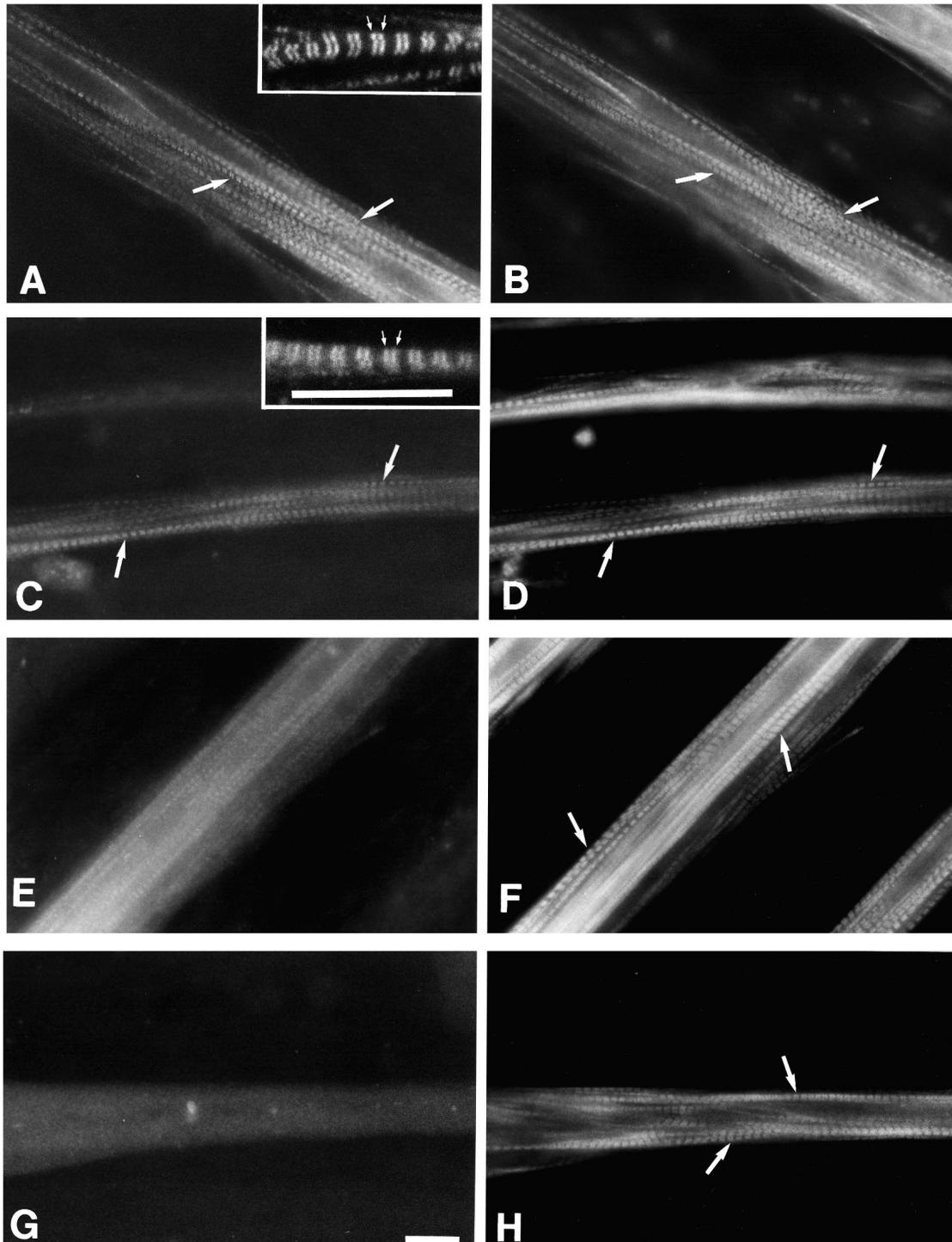


Fig. 4. Distribution of mutants with deletions at the N terminus. Myoblasts transfected with mutants $\Delta 1-2$ (A,B), $\Delta 1-6$ (C,D), $\Delta 1-8$ (E,F) and $\Delta 1-9$ (G,H) were processed for immunofluorescence three days after transfection. The cells were double immunostained with mAbs specific for the myc epitope and sarcomeric myosin. The left row shows the distribution of the recombinant proteins (Texas Red channel), whereas the right row indicates the distribution of myosin in the same cells (FITC channel). Mutants $\Delta 1-2$ and $\Delta 1-6$ are localized to the A-bands of cross-striated myofibrils (arrows, A-D), whereas most of the product of $\Delta 1-8$ remains diffuse in the cytoplasm (E) and was not incorporated into the A-band (arrows, F). Mutant $\Delta 1-9$ distributes as a diffuse pattern in the cytoplasm (G), not localized to the A-band (arrows, H). Insets in A and C: higher magnifications illustrating that $\Delta 1-2$ and $\Delta 1-6$ are distributed as A-band doublets (arrows). Bars, 20 μ m.

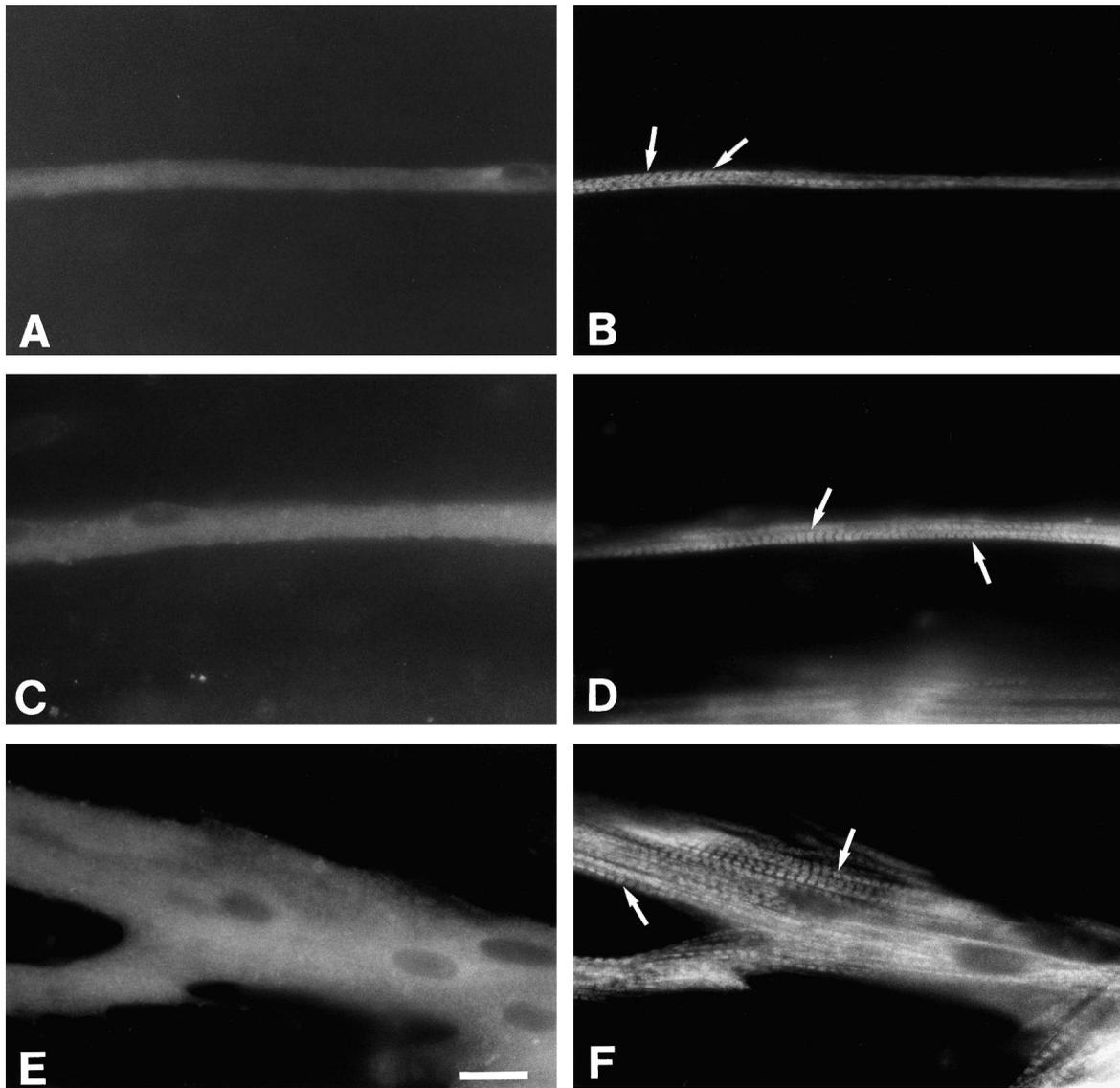


Fig. 5. Distribution of mutants with deletions at the C terminus. Myoblasts transfected with mutants $\Delta 10$ (A,B), $\Delta 9-10$ (C,D) and $\Delta 7-10$ (E,F) were processed for immunofluorescence three days after transfection. The cells were double immunostained with mAbs specific for the myc epitope and sarcomeric myosin. The left row of figures show the distribution of recombinant proteins (Texas Red channel), while the right row shows the distribution of myosin within the same cells (FITC channel). The mutants are distributed diffusely in the cytoplasm and not localized to the A-band (arrows, B,D,F). Bar, 20 μ m.

MyBP-C (compare Fig. 3C,E). Both proteins stained as doublets, one fluorescent stripe in each half A-band (insets Fig. 3C,E). In the transfected myotubes, when cross-striated myofibrils were clearly apparent, MyBP-C^{myc} was always detected within the A-bands of cross-striated myofibrils. No example of transfected cells with mature cross-striated myofibrils lacking MyBP-C^{myc} in the A-bands was ever observed. These data suggest that MyBP-C^{myc} was as effectively incorporated into the A-bands of the cross-striated myofibrils as endogenous MyBP-C, presumably as these structures were assembled.

Cytoplasmic distribution of the truncation mutants

To identify those domains of MyBP-C containing the information required for incorporation of the protein into A-bands,

myoblasts were transfected with a series of MyBP-C truncation mutants. In well differentiated myotubes, $\Delta 1-2$ and $\Delta 1-6$ were identified as fluorescent doublets (Fig. 4A,D), one stripe in each half A-band (insets, Fig. 4A,C). In the light microscope the distribution of these two mutants was indistinguishable from that of MyBP-C^{myc}. Thus, the six N-terminal motifs of MyBP-C are not required for A-band targeting of the complete molecule. Presumably, the C-terminal peptide of 372 amino acids, consisting of motifs VII to X, contains sufficient information to specify correct incorporation of MyBP-C into the A-band. When myotubes were stained using the anti-myc antibody, the intensity of the cross-striations in cells expressing $\Delta 1-2$ was similar to that observed in cells expressing MyBP-C^{myc}. However, the fluorescent signal was slightly weaker in cells expressing $\Delta 1-6$, suggesting that either less

protein was incorporated into these A-bands, or that the availability of the myc epitope was obscured in the $\Delta 1-6$ peptide after A-band incorporation.

Mutant $\Delta 1-8$ did not target to the A-bands of the cross-striated myofibrils (Fig. 4E,F). Most of the product of $\Delta 1-8$ remains diffuse in the cytoplasm, although a weak accumulation of protein was observed at the level of the I-band. This weak signal in the I-band was demonstrated by observing the cells with a filter permitting dual visualization of the FITC and the Texas Red signals simultaneously (data not shown). Mutant $\Delta 1-8$ was the only protein found in detectable amounts within the I-band and the reason for this is not understood. In well differentiated myotubes, $\Delta 1-9$ was diffusely distributed in the cytoplasm and not detected in either the A- or I-bands (Fig. 4G,H). In comparison with the other truncation mutants, the signal intensity in cells expressing $\Delta 1-9$ was quite weak, consistent with western blot data suggesting low levels of expression and/or an unstable protein product. The fact that neither $\Delta 1-8$ nor $\Delta 1-9$ was localized to the A-band was unexpected, since these two mutants contain the C-terminal Ig C2 motif encoding the myosin binding domain of MyBP-C. Similarly, the mutants lacking one or more motifs at the C terminus ($\Delta 10$, $\Delta 9-10$ and $\Delta 7-10$) were not localized to the A-band. Instead, they remained diffusely distributed in the myotube sarcoplasm (Fig. 5A,F). These observations suggest that although the myosin binding domain (motif X) is required for proper incorporation of MyBP-C into the A-band, this domain alone is insufficient to properly target the protein in muscle cells containing the wild-type MyBP-C.

Cells expressing mutant $\Delta 10$ did not assemble robust cross-striated myofibrils. Rather, the myotubes expressing significant levels of $\Delta 10$ contained fewer nuclei and were smaller in length and diameter than comparably aged control myotubes. Cross-striated myofibrils were observed in only 3% of the cells

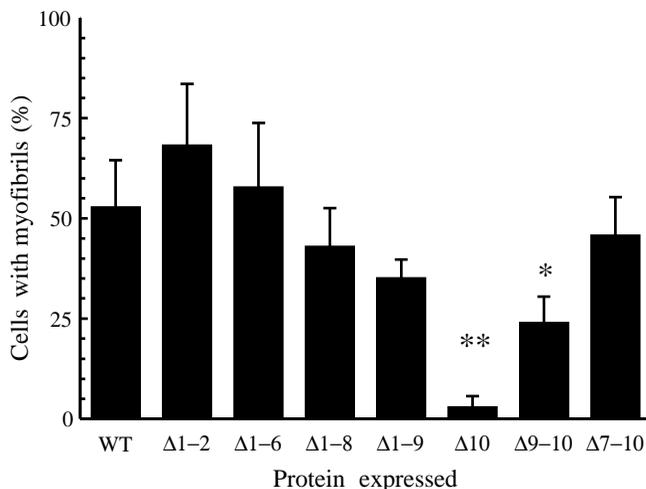


Fig. 6. Effects of the truncation mutants on myofibril assembly. Myoblasts were isolated and transfected with plasmids encoding MyBP-C^{myc} (WT) and the truncation mutants. Three days later, the cells were processed for immunofluorescence and the percentage (%) of cells expressing the recombinant proteins and having cross-striated myofibrils was determined. Data represent average value; bars represent one standard deviation; **average different from WT ($P < 0.005$); *average different from WT ($P < 0.01$).

expressing $\Delta 10$, in comparison with a figure of 53% in myotubes expressing MyBP-C^{myc} (Fig. 6). When cross-striated myofibrils were present in the cells expressing $\Delta 10$, these were shorter and contained fewer sarcomeres than the myofibrils in adjacent, untransfected cells (Fig. 5A,B). Expression of mutant $\Delta 9-10$ also had a negative effect on the myoblast differentiation program or the assembly of the myofibrils, but this effect was less dramatic than that of mutant $\Delta 10$ (Fig. 6).

DISCUSSION

This study demonstrates that recombinant MyBP-C can be efficiently incorporated into the A-bands of the myofibrils after transient expression in cultured embryonic skeletal muscle. As judged by light microscopy, the distribution of the recombinant protein is identical to that of endogenous MyBP-C. The experiments prove that ~60% of the N-terminal portion of MyBP-C (motifs I-VI) is unnecessary for A-band incorporation. A large deletion at the N terminus generating a C-terminal peptide of 372 amino acids, encoding part of motif VII and all of motifs VIII to X (mutant $\Delta 1-6$), permits faithful A-band insertion. This peptide is the minimal requirement for A-band insertion, since mutations with larger deletions from the N terminus, or mutants lacking the C terminus, do not localize to the A-band. These observations indicate that motifs VII to X are both necessary and sufficient for incorporation of MyBP-C into the C zone of the A-band. Based on these data we propose a model in which MyBP-C makes contact with, and is positioned on, specific thick filament sites by repeats VII to X (Fig. 7). We further suggest that the N-terminal section of MyBP-C does not interact with the myosin backbone of the thick filament, or does so very weakly, since this portion of the molecule does not distribute to the A-band if it lacks the C

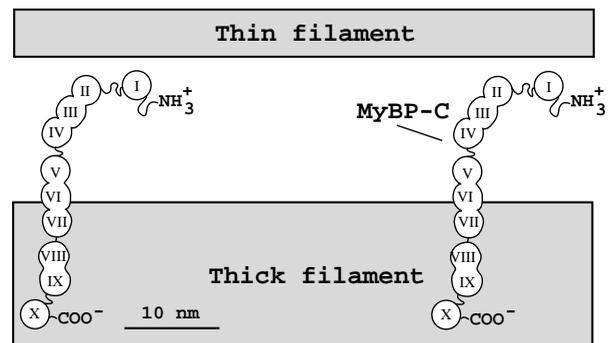


Fig. 7. Hypothetical model indicating the positioning of MyBP-C on the thick filament. The preceding data suggest that MyBP-C is associated with the thick filament through motifs VII to X at the C terminus of each MyBP-C. In this model, the N-terminal moiety does not bind to the thick or to the thin filament in resting muscle. The sites on the thick filament where the molecules of MyBP-C are localized are separated from each other by 43 nm (Dennis et al., 1984; Bennett et al., 1986). Although rough estimates suggest the presence of two to three molecules of MyBP-C at each of these sites (Dennis et al., 1984; Hartzell and Sale, 1985), for diagrammatic purposes only one molecule is indicated. In this model the C terminus of MyBP-C is depicted as laying on the surface of the thick filament but none of our data preclude embedment of motif X in the thick filament. Roman numerals indicate the position of the Ig C2 and FnIII motifs of MyBP-C (see Fig. 1).

terminus (mutants $\Delta 9-10$ and $\Delta 7-10$). It should be recognized, however, that the A-band signal of $\Delta 1-6$ is weaker than that of full length MyBP-C or of $\Delta 1-2$, suggesting that $\Delta 1-6$ is incorporated less efficiently into the A-band than the longer peptides or its myc epitope is less accessible to its antibody probe. Another possibility is that the myc epitope itself interferes with peptide incorporation into the thick filament, for as seen in the model presented in Fig. 7, the epitope is close to the filament backbone in $\Delta 1-6$ and might alter the binding reaction between the expressed peptide and the myosin rod.

Although the function of MyBP-C *in vivo* is uncertain, evidence exists for roles in both the regulation of contraction and in myofibril assembly. These two functions are not mutually exclusive; separate domains of the molecule may subserve different physiological properties. MyBP-C binds relatively weakly to filamentous actin; i.e. with a K_d in the micromolar range (Moos et al., 1978; Yamamoto, 1986). Also, MyBP-C alters positively or negatively the actomyosin ATPase activity of cardiac and skeletal muscles, respectively. MyBP-C might modify the interactions between myosin and actin, or directly affect thin filament functions (Offer et al., 1973; Moos and Feng, 1980; Yamamoto and Moos, 1983; Hartzell, 1985). When purified fractions of MyBP-C are observed by transmission electron microscopy, the molecules appear as flexible rods with dimensions of 3 nm \times 32 nm (Hartzell and Sale, 1985; Swan and Fischman, 1986), or 4 nm \times 50 nm (Fürst et al., 1992). Since the distance between the surface of the thin and the thick filaments ranges between 9 and 16 nm (Julian et al., 1978), the protein could span the inter-filament gap, even if 30% of MyBP-C is bound to the thick filament. Thus, the model proposed in Fig. 7 would permit direct interactions between MyBP-C and both thick and thin filaments, and be consistent with contractile regulation. The most highly conserved segment of MyBP-C is an 18 amino acid stretch between motifs I and II (Weber et al., 1993). The high degree of conservation of this portion of the molecule and its proposed topology near the thin filament (Fig. 7), suggests that it could be involved in the regulation of contraction. In support of this hypothesis, the cardiac isoform of MyBP-C is phosphorylated by a catecholamine-sensitive pathway and this phosphorylation correlates with the rate of twitch relaxation (Hartzell and Titus, 1982; Hartzell, 1984). The phosphorylated serines have been mapped to the N terminus between motifs I and II (Gautel et al., 1995).

When purified myosin is polymerized *in vitro* with physiological concentrations of MyBP-C, the resulting thick filaments are slightly longer and more regular than filaments assembled in the absence of MyBP-C (Koretz, 1979; Davis, 1988). Furthermore, when COS cells are co-transfected with two cDNAs, one encoding MyBP-C and the other the myosin heavy chain (MyHC), MyBP-C promotes the formation of long myosin-containing, cytoplasmic cables (Seiler et al., 1996). In view of the fact that MyBP-C appears relatively late in myofibrillogenesis, and that its expression coincides with the first appearance of cross-striations (Lin et al., 1994; Schultheiss et al., 1990), we suggest that MyBP-C might function as a rate-limiting component of A-band assembly. Since MyBP-C interacts with the thick filament through motifs VII to X at its C terminus, the latter domain is likely to be directly involved in the assembly reaction.

Mutants $\Delta 1-9$ and $\Delta 1-8$ do not localize to the A-band. This

was a surprising observation since both proteins encode the myosin binding domain of MyBP-C. Detection of $\Delta 1-9$ by western blots was difficult and the signal observed by immunofluorescence microscopy was relatively weak, suggesting low expression or protein instability. However, $\Delta 1-8$ was easily detected by both procedures indicating that low expression or instability was not the reason for its absence in the A-band. Although we cannot rule out the possibility that $\Delta 1-8$ and $\Delta 1-9$ do not fold properly once expressed, it is more likely that they fail to be incorporated into the A-band because of lower affinities for myosin binding sites than endogenous MyBP-C. This agrees with *in vitro* binding studies, in which it has been observed that a peptide encoding the myosin binding domain of MyBP-C does not displace MyBP-C from reconstituted thick filaments, nor does it compete with MyBP-C for the myosin binding sites in a mixture of both proteins (Okagaki et al., 1993). Conceivably, motifs VII and VIII encode subtle myosin binding activities which are required for correct incorporation into myofibrils, but these were not detected earlier because they function in concert with motif X. This would explain why none of the mutants lacking motif X were incorporated into the A-band.

MyBP-C also binds to titin (Fürst et al., 1992; Koretz et al., 1993; Soteriou et al., 1993), the huge filamentous molecule thought to be associated with the surface of the thick filament (Maruyama et al., 1977; Wang et al., 1979; reviewed by Trinick, 1992; Small et al., 1992). Conceivably, both the titin and myosin binding domains of MyBP-C are required for the incorporation of MyBP-C into the thick filament. According to this hypothesis, mutant $\Delta 1-6$ would be correctly incorporated into the A-band because it possesses both the titin and myosin binding domains, whereas mutants $\Delta 1-8$ and $\Delta 1-9$ do not, because they encode only the latter domain. The titin binding domain on MyBP-C has not been mapped but it should now be feasible to assess its localization within repeats VII and VIII, as the previous hypothesis implies.

We have also observed that myotubes expressing $\Delta 10$ rarely assemble cross-striated myofibrils and when myofibrils were observed, these were shorter and contained fewer striations than adjacent, untransfected myotubes. Mutant $\Delta 9-10$ also possesses a negative effect on either the myoblast differentiation program or myofibril assembly, however, this effect is less dramatic than that of $\Delta 10$. The common characteristic of these two mutants is the absence of motif X which contains the major myosin binding domain of MyBP-C. The mechanism for this negative effect is not understood. One possibility is that $\Delta 10$ and $\Delta 9-10$ compete with endogenous MyBP-C, or another myofibrillar protein, for an essential binding site on titin or myosin needed for myofibril assembly.

In normal development, MyBP-C is expressed relatively late in myofibrillogenesis (Kawashima et al., 1986; Schultheiss et al., 1990; Lin et al., 1994; Rhee et al., 1994). When MyBP-C is detected, it is always localized in a typical doublet in the A-bands. This observation is compatible with its involvement in late stages of myofibril assembly, possibly as a rate limiting factor. In concordance with this, when cross-striations were apparent in cells expressing recombinant MyBP-C, the latter protein was always limited to the C-zone of the A-band. Recombinant MyBP-C was also detected in NSMFs, also termed nascent myofibrils, which do not contain typical A-bands and which are the likely precursors of cross-striated

myofibrils (Lin et al., 1994; Rhee et al., 1994). To our knowledge, this is the first report showing that MyBP-C can be incorporated into NSMFs. There are several possibilities to explain the presence of recombinant MyBP-C in the NSMFs: (a) the concentration of recombinant protein may have been insufficient to trigger full assembly of the A-bands in the NSMFs; (b) the cells may have been caught in the midst of A-band assembly; (c) the precocious expression of MyBP-C alone may not be sufficient to trigger A-band assembly. Other myosin binding proteins, e.g. MyBP-H, myomesin and M-protein may act cooperatively with MyBP-C in late stages of myofibril assembly. Thus, the latter protein may be one of the late appearing proteins needed for the transition of NSMFs to mature cross-striated myofibrils. Further work is needed to discriminate between these possibilities.

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