Volume 331, number 1,2, 193–197 FEBS 13039 September 1993

PCR-based assignment of two myosin heavy chain cDNA clones to biochemically and histochemically defined single type IIB and IID fibers of rabbit muscle

Andreas Uber, Dirk Pette*

Fakultät für Biologie, Universität Konstanz, Postfach 5560 M641, D-78434 Konstanz, Germany

Received 22 July 1993; revised version received 17 August 1993

The present study assigns two as yet unidentified fast myosin cDNA clones to specific myosin heavy chain (MHC) isoforms and their mRNAs in different fiber types of rabbit skeletal muscle. Specific oligonucleotide primers were used for reverse transcription and polymerase chain reaction (PCR) to yield products of defined lengths. The method was sensitive enough to detect specific mRNA sequences in total RNA extracts from microdissected, freeze-dried, single-fiber fragments down to 16 ng dry weight. The fibers were typed histochemically and biochemically by their electrophoretically assessed MHC complement. The following results were obtained: clone pMHC20-40 was assigned to type IIB fibers and clone pMHC24-79 to type IID fibers.

Fast myosin heavy chain isoform; Muscle fiber type; Myosin heavy chain mRNA isoform; Reverse transcriptase/polymerase chain reaction; Single fiber study

1. INTRODUCTION

The assignment of cDNA clones to myosin heavy chain (MHC) isoforms is currently based on hybridization assays using RNA preparations from muscles with a predominance of different fiber types [1–4]. In the case of fast MHC cDNA clones, this approach has left questions unanswered. Using a cRNA probe transcribed from the pMHC24-79 cDNA of Maeda et al. [3], Dix and Eisenberg observed in situ hybridization in fast fibers of rabbit gastrocnemius muscle, tentatively designated as type IID/IIX fibers. However, cross-hybridization with other fast MHC mRNA isoforms could not be excluded [5].

The aim of the present study was to elaborate a better method for assigning MHC cDNA clones to specific fiber types. For this purpose, total RNA was extracted from single muscle fiber fragments. The MHC mRNA was reverse-transcribed with the use of specific oligonucleotide primers, followed by amplification in the polymerase chain reaction (PCR), yielding DNA fragments of defined length. Fiber typing was performed by electrophoretic identification of the MHC isoform and histochemical stainings. Three different cDNA clones were investigated: the pMHCβ174 [6], the pMHC20-40 [3], and the pMHC24-79 (Maeda and Wittinghofer, unpublished). The pMHCβ174 was previously identified as specific to the β-cardiac MHC isoform [6], whereas the exact fiber type assignment of the fast pMHC20-40 and pMHC24-79 cDNA clones has not as yet been possible.

*Corresponding author. Fax: (49) (7531) 883 940.

Abbreviations: nt, nucleotides.

2. MATERIALS AND METHODS

2.1. *Oligonucleotide primers*

20mer oligonucleotides were used. The primers specific to pMHCβ174 were derived from the hypervariable sequence between head and rod [6]. The non-coding oligonucleotide (CTT GCA TTG AGG GCA TTC AG) is specific to the sequence between positions 154 and 173 of pMHCβ174 (nomenclature according to the authors [6]). The upstream coding oligonucleotide (GGA TCC CTG GAG CAG GAG AA), corresponds to the sequence extending between positions 1 and 20 of pMHCβ174. An additional oligonucleotide (AAG CAG CAG CTG GAT GAG CG) specific to the sequence between positions 112 and 131 was labeled with digoxigenin 11-dUTP at the 3'-end and served as a diagnostic probe in Southern blot hybridizations [7], using an antibody-linked detection assay (Boehringer-Mannheim).

The primers used for the two fast rabbit skeletal MHC clones were derived from the published sequence of pMHC20-40 [3] and the unpublished sequence of pMHC24-79 (K. Maeda and A. Wittinghofer, personal communication). The non-coding primers (pMHC20-40, ACT TGA TGC ACA AGG TAG TG; pMHC24-79, TTA TCT CCC AGA ATC ATA AG) were from the hypervariable untranslated 3'-region. The coding primers (pMHC20-40, AGA GGC TGA GGA ACA ATC CA; pMHC24-79, ACT GCA AGC CAA GGT GAA AT) were derived from the translated C-terminal region. The same diagnostic oligonucleotide (CAG CAC GAG CTG GAG GAG GC) was used for both sequences.

2.2. *Whole muscle RNA extraction and preparation of cDNA*

Total RNA was extracted using a modification [8] of the method

of Chomczynski and Sacchi [9] RNA concentration was assessed spectrophotometrically. Aliquots were used for cDNA synthesis by reverse transcription [10].

2.3. *Dissection and identification of single fibers*

Fiber bundles from rabbit adductor magnus (ADM) and gastrocnemius (GAS) muscles were frozen in melting isopentane (−159°C). For analyses of unidentified single fibers, 1–3 mm-long fibers were dissected under a stereomicroscope from freeze-dried muscle fiber bundles. The method for dissecting fiber fragments from freeze-dried cross-sections was used for analysing histochemically identified fibers [11]. Serial cross sections (four 12 μm thick sections for histochemistry and two 50–100 μm thick sections for dissection of fiber fragments) were cut at −25°C on a microtome in a cryostat One thin section was stained for NADH-tetrazolium reductase [12]. Myofibrillar actomyosin ATPase (mATPase) was stained in the following three sections after preincubations at pH 4.30 or pH 4 55, and after formaldehyde fixation and preincubation at pH 9.60 [11,13]. Fragments of the identified fibers were isolated from a consecutive thick section and analysed electrophoretically for their MHC composition [14,15].

2.4. *RNA extraction from single fibers and reverse transcription*

Total RNA was extracted from single-fiber fragments using the oil well technique [16]. Fiber fragments in the range of 50–200 ng dry mass were introduced into 0 26 μl of a high-salt extraction solution [17] under mineral oil 50 mM Tris-HCl, pH 9.0, 250 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 U/μl human placenta RNase inhibitor (Boehringer-Mannheim). After 1 h incubation at +4°C, the assay was diluted to yield optimum conditions for reverse transcription [10] by adding 0 86 μl of the following solution: 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 10 mM DTT, 1.3 mM dNTP, 1 μM oligonucleotides, 0.65 U/μl AMV reverse transcriptase (Boehringer-Mannheim) After 30 min incubation at 42°C, the mixture was heated for 10 min at 65°C in order to inactivate possible DNase contamination.

2.5. *Polymerase chain reaction*

Reactions were performed in 0.5 ml polyethylene tubes, immersed into three temperature-controlled glycerol baths (Robotherm PCR machine; Bühler, Bodelshausen, Germany). The assay mixture (25 μl) contained 67 mM Tris-HCl (pH 8.8 at 20°C), 10% DMSO, 160 μg/ml BSA, 2 mM $MgCl_2$, 50 mM NaCl, 7 mM 2-mercaptoethanol, 17 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.2 mM dNTP, 0.15 μM of the primer pair, 1 μl of the cDNA, 0.8 U *Taq* DNA polymerase (Boehringer-Mannheim). The first cycle was started by 5 min denaturation at 92°C. In the following 24 cycles, the denaturation step lasted for only 1 min. Primer annealing (1 min at 54°C) was followed by the synthesis step (30 s at 74°C) In order to obtain only double-stranded DNA, the last cycle was terminated by a 5 min synthesis step PCR was performed in separate assays for each sequence.

2.5.1. PCR for single-fiber analysis

The reverse transcription assay was completely transferred into a 0.5 ml polyethylene tube containing 60 μl of the following solution: 83.75 mM Tris-HCl (pH 8.65 at 20°C), 62.5 mM NaCl, 2.5 mM $MgCl_2$, 12.5% DMSO, 200 μg/ml BSA, 8.75 mM 2-mercaptoethanol, 20.75 mM $(NH_4)_2SO_4$, 0.125% Triton X-100, 0.25 mM dNTP. For separate amplifications of the different sequences, 20 μl portions of this mixture were transferred into new tubes. After addition of 5 μl starting reagent (0 75 μM of the two primers, 0 8 U *Taq* DNA polymerase), PCR was performed as above.

2.6. *Electrophoretic product analysis*

5 μl of the amplification assays were analysed electrophoretically on a 6% polyacrylamide gel in Tris-borate buffer (100 mM Tris-HCl, pH 8.3, 83 mM boric acid, 1 mM EDTA). The amplification products were visualized by ethidium bromide or by hybridization with digoxigenin-labeled diagnostic oligonucleotides [7].

3. RESULTS

3.1. *Three specific PCR products from whole muscle RNA*

In order to check the specificity of the three primer pairs, total RNA extracted from 5 mg GAS was incubated for reverse transcription in 25 μl assay mixture containing the three non-coding primers. The cDNAs were amplified in three separate assays with 1 μl cDNA. Three products of expected lengths were obtained: 173 nt for pMHCβ174, 236 nt for pMHC20-40, and 289 nt for pMHC24-79. Control reactions without reverse transcriptase yielded no products (Fig. 1).

Because the three amplified sequences should contain *Alu*I-specific restriction sites, the PCR products were digested with this endonuclease. Digestion yielded fragments of the expected lengths for each of the three sequences (data not shown). Southern blot hybridizations of the products with diagnostic oligonucleotides unambiguously proved the specificity of the three sequences (Fig. 2).

3.2. *Detection of three MHC mRNA isoforms in whole muscle RNA preparations*

10 ng total RNA from different muscles were reverse-transcribed, followed by separate PCR for each sequence. For electrophoretic analysis, the products were combined. Cardiac ventricle displayed only the 173 nt product specificto pMHCβ174, whereas skeletal muscles yielded signals for all three sequences (Fig 3). Signal intensities did not provide information on relative

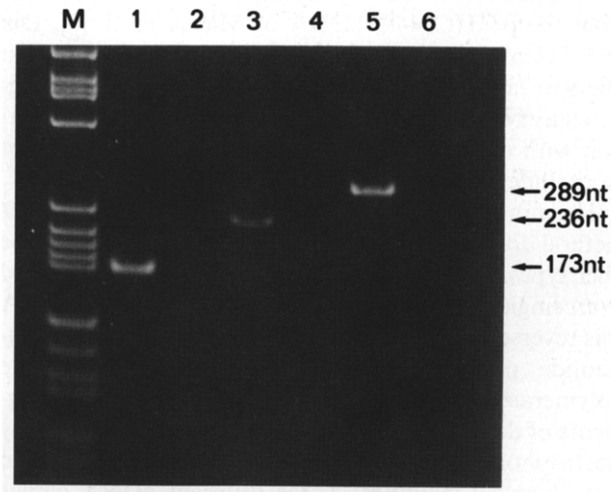

Fig. 1. Electrophoretically separated reaction products from reverse transcription-polymerase chain reactions with primers specific to three MHC cDNAs. Total RNA extracted from 5 mg rabbit gastrocnemius muscle was incubated in separate assays for reverse transcription. Subsequently, the cDNAs were separately amplified using specific primer pairs Lane 1, 173 nt product of the pMHCβ174 sequence; lane 3, 236 nt product of the pMHC20-40 sequence, lane 5, 289 nt product of the pMHC24-79 sequence; lanes 2, 4, and 6 are control reactions without reverse transcriptase. M, marker (DNA molecular weight marker V, Boehringer-Mannheim).

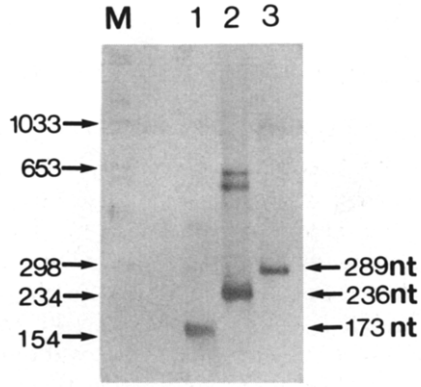

Fig. 2. Southern blot of PCR products with 3'-digoxigenin-labeled diagnostic oligonucleotides. Lanes 1–3, reaction products of pMHCβ174, pMHC20-40, and pMHC24-79, respectively. M, marker (digoxigenin-labeled molecular weight marker VI; Boehringer-Mannheim). The signals in the range of > 600 nt are non-specific, most likely resulting from excessive cycling [20].

amounts of the three mRNA isoforms in a given muscle because PCR was run to saturation. However, varying intensities of a given signal in different muscles indicated different amounts of the respective mRNA. ADM, GAS, and vastus lateralis muscles showed highest intensities of the pMHC20-40 signal. The band, specific to pMHC24-79, was dominant in GAS and plantaris muscles (Fig. 3).

3.3. *PCR studies on single fibers*

Analysis of single-fiber fragments precluded isolation of total RNA. However, incubation of the fragments in extraction medium proved to be sufficient for the assessment of specific mRNA isoforms. Fig. 4 documents the reliability of RNA extraction from a freeze-dried fiber bundle (lanes 1,4,7) and three different fibers (lanes 2,5,8). Because the fiber bundle contained different fiber types, signals specific to all three cDNAs were obtained. This experiment also showed that signals were not observed in the absence of reverse transcriptase (Fig. 4, lanes 3,6,9), thus proving that only mRNA was a template for PCR and that genomic DNA was not amplified with the primers used.

Table I

Reproducibility of results from multiple analyses performed on single-fiber fragments

Muscle	cDNA	Number of fiber pieces examined	Number of fiber pieces yielding signals	Positive (%)
Soleus	pMHCβ174	41	39	95
Adductor magnus	pMHC20-40	76	74	97
Gastrocnemius	pMHC24-79	95	93	97

Single fibers of up to 3 mm in length were dissected from fiber bundles of various muscles. Small pieces were cut from each fiber and subjected to separate analyses for sequences specific to three MHC mRNA isoforms. Sample weights measured on a quartz fiber balance were in the range of 16–300 ng dry weight.

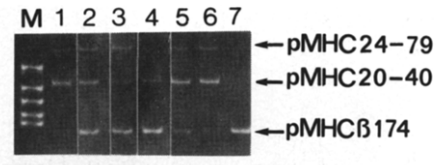

Fig. 3. Analyses for three MHC mRNA isoforms in various rabbit muscles. Equal amounts of total RNA (10 ng) extracted from 50 mg muscle were reverse-transcribed in the presence of the three MHC cDNA-derived primers. Aliquots of these incubations were subjected in separate assays to PCR in the presence of specific primer pairs. The products of the three reactions were combined and electrophoretically separated. Lane 1, adductor magnus; lane 2, gastrocnemius; lane 3, plantaris; lane 4, soleus; lane 5, vastus lateralis (deep portion); lane 6, vastus lateralis (superficial portion); lane 7, cardiac ventricle. M, marker (see Fig. 1).

3.4. *MHC mRNA isoforms in single fibers*

Because of its dominance in ADM (Fig. 3), we examined the mRNA specific to pMHC20-40 in single fibers of this muscle. Rabbit ADM muscle is composed mainly of type IIB and type IID fibers [13,18]. Analyses revealed the presence of the pMHC20-40 isoform in fibers identified as type IIB (Fig. 5). Type IID fibers and the few type IIA fibers examined did not yield the 236 nt

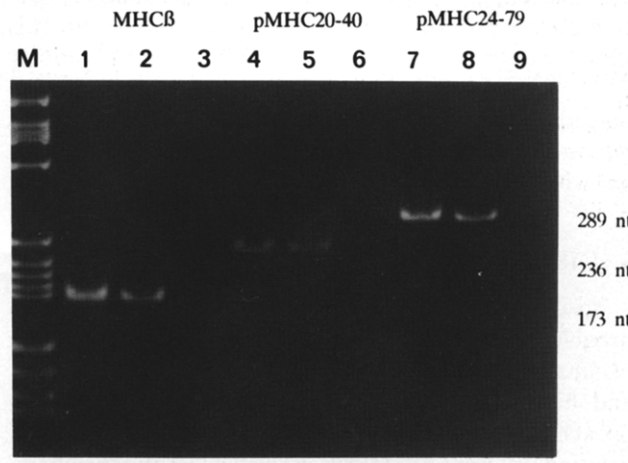

Fig. 4. PCR after reverse transcription of unpurified RNA from freeze-dried muscle fibers. Total RNA was extracted from a gastrocnemius fiber bundle (lanes 1,4,7) weighing approximately 100 μg and three different single fibers (lanes 2,5,8) by incubation in high-salt extraction solution. Control reactions with RNA from the fiber bundle in the absence of reverse transcriptase are shown in lanes 3,6,9. Lanes 1 and 2 show the 173 nt product specific to pMHCβ174, lanes 4 and 5 the 236 nt product specific to pMHC20-40, and lanes 7 and 8 the 289 nt product for pMHC24-79. M, marker (see Fig. 1).

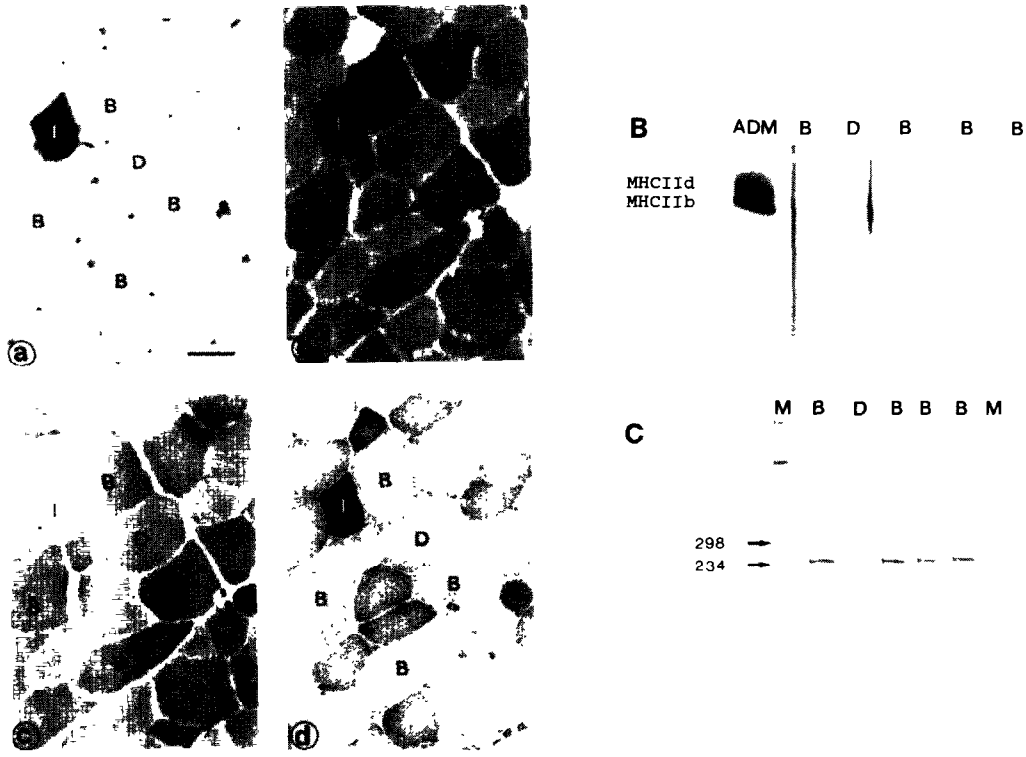

Fig 5. Representative single-fiber analyses for specific MHC mRNA isoforms in rabbit adductor magnus muscle. (A) Histochemical classification of fiber types in serial cross sections by mATPase staining after preincubations at pH 4.30 (a), pH 4.55 (b), pH 9.60 (c), and by staining for NADH-tetrazolium reductase (d). Bar, 50 μm (B) Electrophoretic analyses for MHC isoforms in the fibers labeled in A. (C) mRNA analyses of the same fibers. Products of reverse transcription and PCR were visualized by hybridization with a digoxigenin-labeled diagnostic oligonucleotide. The 236 nt signal specific to pMHC20-40 is seen only in type IIB fibers, but not in type IID fiber. Arrows mark two fragments in lane M of 234 nt and 298 nt length. ADM, whole muscle extract from adductor magnus; B,D, fiber types IIB and IID; I, fiber type I, M, digoxigenin-labeled DNA molecular weight marker VI; MHCIIb,MHCIId, myosin heavy chain isoforms.

signal specific to pMHC20-40. Type IID fibers, predominant in GAS [13,18], yielded the 289 nt signal specific to the pMHC24-79 clone (Fig. 6). However, this signal was not found in some fibers unambiguously defined as type IID. The investigated type I fibers displayed the signal specific to pMHCβ174. None of the oligonucleotide pairs under study yielded a positive signal with type IIA fibers.

4. DISCUSSION

To our knowledge, the present study is the first approach for characterizing cDNA clones by the analysis of single fibers identified by their MHC complement and histochemical profile. The reliability of this approach depends on the specificity of the methods for defining different fiber types, as well as on the specificity and sensitivity of the method for specific detection of mRNA isoforms.

Three fast fiber types (IIA, IIB and IID), defined histochemically and by their MHC complement, exist in rabbit skeletal muscles [13,18]. Consequently, both methods of fiber typing were used in the present study. The specificity and sensitivity of the method for monitoring MHC mRNA isoforms in single-fiber fragments result from the use of sequence-derived, specific primers in reverse transcription and PCRs. Restriction analysis and Southern blot hybridization with diagnostic oligonucleotides [7] independently prove the identity of the amplified products.

Our results demonstrate the possibility of reverse-transcribing and amplifying mRNA extracted from fiber fragments down to sample weights of 16 ng. The method is highly reproducible (Table I). Its sensitivity could be further improved by chemiluminescence for PCR product detection [19].

The main result of our study is the assignment of three MHC mRNA isoforms to three fiber types. mRNA specific to the pMHCβ174 clone is present in type I fibers. This sequence, previously assigned to the β-cardiac MHC [6], served as a model for ascertaining the feasibility and specificity of our method. The pMHC20-40 sequence can be assigned to a MHC mRNA present in type IIB fibers. We suggest that this clone is specific to MHCIIb. The pMHC24-79 sequence is specific to a MHC mRNA isoform present in type IID fibers. However, it is possible that this mRNA isoform is not expressed in all type IID fibers.

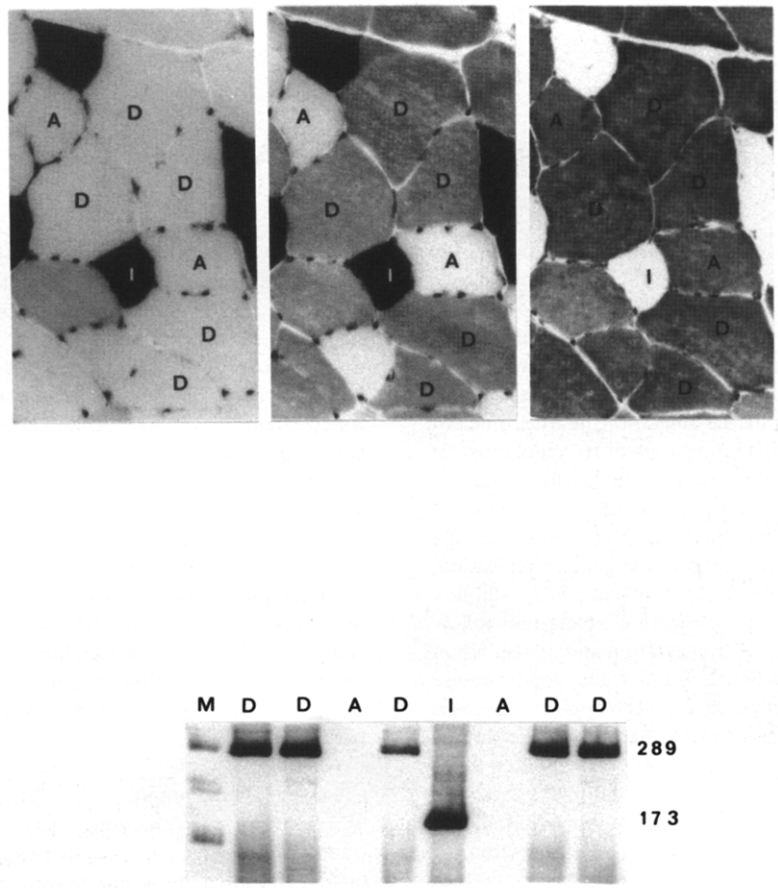

Fig. 6. Representative single fiber analyses for specific MHC mRNA isoforms in rabbit gastrocnemius. (Upper panel) Histochemical classification of fiber types in serial cross-sections by mATPase staining after preincubations at pH 4.30 (left), pH 4.55 (middle), pH 9.60 (right). (Lower panel) MHC mRNA analyses performed on fragments of the same fibers shown in the upper panel. Reaction products were visualized by hybridization with digoxigenin-labeled diagnostic oligonucleotides. The 289 nt signal specific to pMHC24-79 is seen in type IID fibers, but not in type IIA fibers. The type I fiber displays the 173 nt signal specific to pMHCβ174. A,D, fiber types IIA and IID, respectively; I, fiber type I; M, digoxigenin-labeled DNA molecular weight VI marker (Boehringer-Mannheim).

Acknowledgements: This study was supported by the Deutsche Forschungsgemeinschaft, SFB 156. We thank Dr. H.-P. Vosberg for support, as well as Drs. K. Maeda and A. Wittinghofer for supplying the pMHC24-79 sequence. We acknowledge the technical assistance of Mrs. Bärbel Gohlsch, and thank Heidemarie Peuker for helpful discussions.

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