Identification of recombinant phages containing sequences from different rat myosin heavy chain genes

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ABSTRACT

The construction and identification of a recombinant plasmid containing a cDNA insert which hybridizes specifically to myosin heavy chain mRNA is described. The plasmid was used as a probe to screen a rat genomic library for recombinant phages containing myosin heavy chain sequences. Six clones with approximately 15 k bp inserts each were isolated. Digestion with several restriction enzymes and hybridization of the fractionated DNA with the plasmid probe showed that the clones contained 3 different DNA inserts. Electron microscopy of a heteroduplex made by hybridization of DNA from two clones confirmed that the inserts originated in different genes. Hybridization of size-fractionated EcoRI digested rat spleen DNA with the cloned probe suggested the existence of at least 5 myosin heavy chain genes.

INTRODUCTION

Myosin is a central protein in the contractile apparatus. Its rate of synthesis rises dramatically during the fusion of myoblasts to form myotubes and it has been used extensively as a marker to study the control of gene expression during myogenesis (for review see ref. 1). Furthermore, the myosin and other contractile proteins exist in multiple forms, which are expressed in other tissues and whose synthesis is independently controlled (2-5). Comparison of the structure and organization of the genes coding for these proteins may help understand how gene expression is controlled.

The active myosin molecule consists of two large subunits, each with a molecular weight of 200 k daltons and three to four smaller peptides with molecular weights ranging from 15 to 30 k daltons (6). We previously reported the cloning of cDNA sequences complementary to myosin light chain 2 and actin mRNA (7). Here we describe the construction of a bacterial plasmid containing sequences of myosin heavy chain mRNA, as well as the isolation from a rat genomic library of recombinant phages containing myosin heavy chain DNA sequences of three different restriction patterns. The results indicate the existence of multiple myosin genes.

MATERIALS AND METHODS

Protein extractions. Myosin heavy chain was prepared according to John (8), actin according to Spudich and Watt (9).

Preparation of mRNA and cell-free translation. RNA extraction from leg muscle of one-month old Wistar rats was carried out by the phenol/m-cresol method described by Kirby (10). Poly(A)-containing RNA was isolated by affinity chromatography to oligo(dT) cellulose (11) and ethanol-precipitated.

mRNA preparations were translated in nuclease-treated rabbit reticulocyte lysates, as described by Pelham and Jackson (12).

Partial proteolysis. Individual bands cut from SDS-polyacrylamide gels were partially digested with *Staphylococcus aureus* V8 protease (Miles) according to Cleveland et al. (13). When an entire track was digested it was laid on top of a 15% SDS-polyacrylamide gel and overlaid with the same enzyme imbedded in 1% low-temperature gelling agarose (Sigma).

Synthesis of double-stranded DNA. Single-stranded cDNA was synthesized essentially as described (14, 15) in a 100 μ l reaction mixture containing 50 mM Tris-Cl pH 8.2, 10 mM KCl, 10 mM $MgCl_2$, 30 mM beta-mercaptoethanol, 10 μ g oligo (dT)₁₂₋₁₈ (Collaborative Research), 500 μ M each dCTP, dGTP, TTP, dATP (dATP ³²P radioactively labelled at a specific activity of 0.4 Ci/mM), 15 μ g poly(A)⁺ RNA, and 45 units reverse transcriptase (kindly supplied by Dr. J. Beard). The reaction was stopped by the addition of EDTA to 10 mM and 30 μ g of yeast tRNA were added as carrier. The mixture was then phenol and ether extracted, passed over a G-50 medium Sephadex column in 10 mM Tris-Cl pH 7.4, 2 mM EDTA, 10 mM NaCl (TEN) and ethanol-precipitated overnight.

RNA hydrolysis was done by dissolving the pellet in 200 μ l TEN + 19 μ l 1N NaOH and incubating for 25 min at 70°C. The hydrolysis was stopped by addition of 25 μ l 1 M Tris pH 7.5 + 19 μ l 1 N HCl.

After ethanol precipitation, the single-stranded cDNA was made double-stranded in 100 μ l reaction mixture containing 50 mM Tris-Cl pH 7, 20 mM KCl, 7 mM $MgCl_2$, 10 mM beta-mercaptoethanol, 0.1 mM EDTA and 200 μ M dATP, dGTP, dCTP, TTP each (0.3 Ci/mM (³²P-dCTP)), the single-stranded cDNA and 10 U DNA polymerase I (gift of Prof. H. Schaller). The reaction was held at 42° for 10 min, after which EDTA was added to 10 mM. Phenol extraction and gel filtration were carried out as above, except that the Sephadex column buffer contained only 0.1 mM EDTA.

Preparative restriction enzyme digestion of cDNA. 200 ng double-stranded cDNA was digested with 0.2 U Pst 1 at 37° for 1 h. The mixture was heated to 70° for 10 min to inactivate the enzyme, and then spun in a micro-

fuge for 5 min to precipitate it.

Ligation of cDNA to plasmid. 30 ng of Pst 1 cut pBR 322 plasmid were added to restricted cDNA and ethanol-precipitated. The ligation was carried out for 15 h at 4° in a 3 µl reaction mixture containing 60 mM Tris-Cl pH 7.6, 10 mM MgCl₂, 10 mM beta-mercaptoethanol, 1 mM ATP and 0.12 U T4 ligase (gift of Dr. H. Lehrach).

Transformation of χ 1776 E. coli was done as described (16). All experiments were performed under P₃EK2 containment and in accordance with the NIH guidelines for recombinant DNA work.

Selection of clones containing inserts complementary to muscle poly(A)⁺ RNA. Tetracycline-resistant ampicillin-sensitive colonies of bacteria were transferred to nitrocellulose filters on which their DNA was denatured and baked (17). These filters were then hybridized to a cDNA probe made on a template of poly(A)⁺ RNA extracted from muscle. Plasmid DNA from clones giving good hybridization was prepared as described (7).

Hybridization of muscle mRNA to plasmid DNA covalently linked to DBM-cellulose and hybridization of plasmid DNA to agarose-fractionated RNA covalently linked to DBM paper were done as described (18).

Screening of rat library for myosin clones. Approximately 400,000 plaques from a rat library (a gift from Drs. T. Sargent and J. Bonner (19)) were screened as described by Benton and Davis (20). Duplicate nitrocellulose filters were pulled sequentially from each petri dish, denatured, neutralized, and baked. Hybridization with nick-translated plasmid 82 as a probe was done as described by Maniatis et al. (21). Plaques from the regions of the plates that gave positive hybridization on the two filters were picked and replated several times until all the plaques in the plate gave positive signals after autoradiography.

Growing of recombinant phages and preparation of DNA. Phages were grown as described by Blattner et al. (22), prepared as described by Yamamoto and Bruce (23) and purified by two successive cesium chloride density centrifugations. DNA was extracted by several successive phenol and chloroform extractions.

Gel electrophoresis and Southern blots. Restricted DNA was electrophoresed on 0.7% agarose gels. Transfers of DNA to nitrocellulose sheets, denaturation and neutralization, baking and hybridization were done as described by Maniatis et al. (21).

Hybridization of plasmid DNA to agarose-fractionated RNA covalently linked to DBM-paper. RNA samples were separated by electrophoresis on hori-

zontal slab gels containing 1% agarose and 7.5 mM methyl mercury hydroxide (Alfa) according to Bailey and Davidson (24). The fractionated RNA was transferred to DBM-paper according to Alwine et al. (25) except that the buffer used for the transfer was sodium acetate pH 5. Following transfer, the DBM paper was hybridized to nick translated (26) ^{32}P -labelled plasmid DNA by the method of Alwine et al. (25).

Nick-translation of DNA. DNA was labelled by nick-translation, as described by Maniatis et al. (26). The specific activity of the nick-translated DNA used was $1-3 \times 10^8$ cpm/ μg . Either the whole plasmid 82 DNA or the 225 bp insert was used as probe.

Preparation of spleen DNA. Rat spleen DNA was prepared from Wistar rats, as described by Gross-Bellard et al. (27).

Heteroduplex analysis. Heteroduplexes were prepared and mounted for electron microscopy by the formamide procedure of Davis et al. (28). Samples were examined in a Philips 300 electron microscope.

Myogenic cell cultures. The myogenic cells of line L8, of rat origin (29), were used to prepare cultures containing only proliferating mononucleated cells and differentiated cultures containing multinucleated muscle fibers. Cultivation of the cells and extraction of RNA have been described (7).

RESULTS

Construction and Selection of cDNA Clones Containing Myosin mRNA Sequences

Since the mRNA coding for myosin heavy chain is abundant in muscle mRNA and is at least 6 kb long, it was expected that double-stranded cDNA made from rat muscle poly(A)⁺ RNA might contain fragments of myosin cDNA with two restriction sites which would be useful for ligation into pBR 322.

Double-stranded cDNA synthesized on muscle poly(A)⁺ RNA template was restricted with Pst 1 and ligated to Pst 1 cut pBR 322. The DNA was used to transform *E. coli* χ 1776 bacteria. Colonies of bacteria resistant to tetracyclin but sensitive to ampicillin were screened for those carrying plasmids containing muscle-specific sequences, by hybridization to labelled cDNA made on a muscle poly(A)⁺ RNA template and autoradiography. Thirteen of 47 tet⁺ amp⁻ colonies were positive in this test.

Plasmid DNA was isolated from each of the colonies by CsCl equilibrium density centrifugation and was covalently bound to diazotized cellulose (DBM cellulose). Poly(A)⁺ RNA extracted from rat skeletal muscle was hybridized to the bound DNA. After elution of the bound RNA it was translated in a

reticulocyte cell-free system (CFS) and the products were run on an SDS-polyacrylamide gel. The gels were autoradiographed. Translation products of cell-free system directed by RNA eluted from one plasmid, designated clone 8, contained many bands, the largest one co-migrating with myosin heavy chain marker. This clone was picked for further characterization.

Subcloning of Plasmid 8

When the plasmid DNA isolated from clone 8 was cut with Pst 1, run on a 1.5% neutral agarose gel and ethidium bromide stained, three inserts were observed (1300, 700, 225 bp). In order to isolate plasmids containing only the desired insert, plasmid 8 DNA was digested to completion with Pst 1 and the DNA fragments were re-ligated. After transformation, 3 clones, each containing one of the three inserts, were picked for further characterization: Poly(A)-containing RNA from rat muscle was fractionated on methylmercury hydroxide agarose gels and transferred to DBM cellulose paper. DNA of each plasmid was nick-translated and hybridized to the DBM-cellulose paper. DNA of one of the plasmids, designated 82, which contained the 225 bp insert, hybridized strongly to a single band of RNA having an estimated size of 7000 nucleotides, which is the expected size of myosin heavy chain mRNA (Fig. 1).

Identification of mRNA which Hybridizes to the Recombinant Plasmid DNA

Plasmid 82 DNA was isolated, bound to DBM cellulose, and hybridized to muscle mRNA, as described above. After elution, the hybridized RNA was translated in an RNA-dependent reticulocyte cell-free system. When the products were run on an SDS-polyacrylamide gel and autoradiographed, a pattern very similar to that of the products of RNA eluted from clone 8 was observed (Fig. 2). To test the identity of the largest band, co-migrating with myosin heavy chain, rat skeletal muscle myosin was co-electrophoresed with CFS products. The myosin heavy chain marker, together with the largest radioactive band, was cut out and subjected to partial proteolysis with V8 protease and electrophoresed. As can be seen in Fig. 3, tracks 3 and 4, a very similar pattern was formed by the marker digestion products and the digested labelled cell-free system products. Digestion of labelled myosin isolated from differentiated cultures of the myogenic line L8 resulted in a peptide pattern which differed somewhat from the pattern of the products of rat skeletal muscle myosin (Fig. 3, track 2). This is in agreement with earlier observations indicating a difference between embryonic (culture) and adult muscle myosin (30).

Fig. 1. Hybridization of nick-translated plasmid 82 DNA with size-fractionated RNA. RNA from the following sources was fractionated on a 1% methylmercury hydroxide agarose gel and transferred to DBM paper: (1) differentiated L8 cultures (50 μ g); (2) proliferating L8 myoblast cultures (50 μ g); (3) rat skeletal muscle (25 μ g). 0, 28S and 18S indicate the positions of the origin of the gel, 28S and 18S rRNA, respectively.

Digestion products of CFS directed by muscle RNA, which co-migrated with actin marker, produced a completely different pattern (Fig. 3, track 1).

To test whether the CFS products smaller than myosin heavy chain (Fig. 2, track 2) were derivatives of myosin heavy chain, the entire track containing the undigested CFS products was cut out of the SDS gel and laid horizontally on another SDS-polyacrylamide gel and digested with V8 protease. As can be seen in Fig. 3, track 5, the major digestion products in the gel were of identical size and therefore appeared as horizontal stripes across the gel. In a control digestion experiment a polyacrylamide-SDS track containing products of a CFS directed by total muscle poly(A)⁺ RNA was cut and treated as the products of the selected RNA (Fig. 3, track 6). The absence of stripes is obvious. The results thus show that all major products of the CFS directed by RNA eluted from plasmid 82 DNA were fragments of a single protein. This indicated that the multiple bands were produced by early quitting of the nascent polypeptides during synthesis of myosin heavy chain in the cell-free system.

Fig. 2. SDS-polyacrylamide gel electrophoresis of translation products of RNA selected by hybridization to plasmid DNA. RNA preparations, selected by hybridization of muscle poly(A)-containing RNA to plasmid DNA linked to cellulose, were translated in the retic cell-free system. The products were analyzed by 10-20% polyacrylamide-SDS gel electrophoresis. The RNA was selected by hybridization to (1) plasmid 749, containing actin mRNA sequences (ref. 7); (2) plasmid 82; (3) plasmid 103, containing myosin light chain 2 mRNA sequences (ref. 7). Track 4 contains cell-free translation products of unfractionated rat skeletal muscle poly(A)⁺ RNA. M.A. LC₂ indicate the positions of myosin heavy chain, actin and myosin light chain 2 markers, respectively.

Hybridization of Plasmid 82 Insert DNA with RNA Extracted from Myogenic Cultures

Poly(A)⁺ RNA from proliferating myogenic cells (L8) and differentiated L8 cultures containing multinucleated fibers were fractionated on methyl-mercury hydroxide agarose gels and transferred to DMB-cellulose paper. As can be seen in Fig. 1, the labelled plasmid hybridized to RNA from differentiated cells in the myosin heavy chain mRNA region. After over-exposing the DNA blot, a very faint band could also be detected in the track containing RNA from proliferating myoblasts (not shown). Myosin isoforms are present in small amounts in nonmuscle tissues (31-34). These results could, therefore, reflect cross hybridization between different kinds of myosin. However, they could also be attributed to the existence of minute amounts of muscle-specific myosin RNA in myoblast cultures.

Hybridization of Plasmid 82 with EcoRI Digested Genomic Rat DNA

DNA was extracted from rat spleen, digested to completion with EcoRI and

Fig. 3. SDS-polyacrylamide gel electrophoresis of products of partial proteolysis. The following preparations were excised from SDS-polyacrylamide gels and digested with *Staphylococcus aureus* V8 protease, as described in Materials and Methods, and electrophoresed on a 15% SDS-polyacrylamide gel: (1) CFS translation products of skeletal muscle RNA co-migrating with actin marker; (2) ^{35}S -methionine-labelled myosin heavy chain isolated from differentiated L8 cultures; (3) myosin heavy chain isolated from rat skeletal muscle (coomassie stained); (4) the largest radioactive band (co-migrating with myosin marker) cut out of SDS gel containing products of CFS directed by muscle RNA which hybridized to immobilized plasmid 82; (5) the entire track of CFS products of RNA hybridizing to immobilized recombinant plasmid 82; (6) entire track of CFS products of unfractionated poly(A)⁺ skeletal muscle RNA (see text).

run on a 0.8% agarose gel. After transfer of the DNA to a nitrocellulose filter by the Southern blotting technique, the filter was hybridized to nick-translated plasmid 82 insert DNA probe and autoradiographed. Five different bands (estimated sizes 8.9; 8.0; 4.6; 4.4; 2.6 kbp) could be detected (Fig. 4, track 1).

Isolation from a Library and Characterization of Genomic Clones Containing Myosin Heavy Chain Sequences

A library of rat genomic DNA in λ Charon 4A was screened for clones containing sequences which hybridized with labelled plasmid 82 DNA. After screening 4×10^5 plaques (approximately 2 copies of the rat genome), 6 plaques hybridizing with the nick-translated 82 probe were isolated and cloned (de-

Fig. 4. Hybridization of nick-translated plasmid 82 insert with DNA digested with EcoRI electrophoresed on 0.8% agarose gel and transferred to nitrocellulose paper. Track 1: genomic DNA extracted from rat spleen. Track 2: mixture of clones CMH1, CMH2, CMH3.

signated here CMH1-CMH6).

DNA of one of these clones (CMH1) was bound to DBM cellulose and hybridized to total muscle RNA. The RNA was eluted and translated in a CFS and the products were analyzed by SDS acrylamide electrophoresis and autoradiography. The polypeptide pattern was identical to that of products of CFS directed by RNA selected by hybridization to plasmid 82, shown in Fig. 2.

DNA of each clone was digested with EcoRI, Hind III, Bam HI restriction enzymes and subjected to 0.7% agarose gel electrophoresis. The DNA was transferred to nitrocellulose filter and hybridized to nick-translated plasmid 82 DNA. Three different restriction and hybridization patterns were obtained with all three enzymes (Fig. 5): three of the genomic clones (CMH1, CMH3, CMH6) had the same pattern: two others were identical (CMH2, CMH5), and one was unique. Neither fragments which hybridized to plasmid 82 nor any other fragment was shared between the different groups. The size of all inserts was approximately 15 kb. Comparison of the labelled bands formed by hybridization of EcoRI digested rat spleen DNA with nick-translated plasmid 82 DNA and the

Fig. 5. Restriction pattern of DNA from 3 recombinant genomic clones restricted with EcoR1, Bam HI or Hind III and electrophoresed on a 0.7% agarose gel. (A) Ethidium bromide staining of digestion products of genomic clones: 1) CMH1; 2) CMH2; 3) CMH3. (B) Hybridization of nick translated plasmid 82 with fractionated DNA, transferred to nitrocellulose paper. Clones CMH4 and CMH6 gave restriction patterns identical to clone CMH1; clone CMH2 was identical to clone CMH5.

radioactive EcoR1 fragments of the genomic clones (Fig. 4, track 2) shows that 3 out of the 5 spleen DNA-labelled bands were identical in size to the 3 bands formed by hybridization of the 3 different genomic clones with the same probe (8.9; 8.0; 4.6 k bp).

Heteroduplex Analysis

To confirm that the different genomic DNA clones originated from different genes, heteroduplex analysis was performed between DNA from clones CMH1 and CMH3 and visualized by electron microscopy. Figure 6 shows the existence of several homologous regions interrupted by regions of nonhomology. The nonhomologous regions might indicate the existence of coding regions which do not cross hybridize or intervening sequences. The differences in size of the two strands of the loops suggest the latter.

DISCUSSION

We have described the cloning and identification of a recombinant bacter-

Fig. 6. Electron micrograph of heteroduplex between the recombinant phages CMH1 and CMH3.

ial plasmid containing sequences of myosin heavy chain mRNA. The identification of the inserted sequences was done by cell-free translation of the mRNA that hybridized to the recombinant plasmid. The cell-free products, when analyzed by acrylamide-SDS gel electrophoresis, contained a major band comigrating with myosin heavy chain, and many smaller bands. Partial proteol-

ysis has shown that (a) the plasmid 82 binds myosin heavy chain mRNA; (b) the smaller bands in the CFS products are incomplete myosin heavy chain molecules. These smaller bands are probably formed because of early termination of the polypeptide chains during the cell-free translation (for review see ref. 1). Hybridization of the labelled plasmid DNA with size-fractionated muscle poly(A)⁺ RNA showed that the probe hybridizes to RNA which fits the size of an mRNA coding for myosin heavy chain. Since the sequence of amino acids in myosin heavy chain is not known, sequencing of the DNA insert could not be used as a means of identification.

Investigation at the protein level has shown the existence of multiple forms of myosin heavy chain. Three different forms of skeletal muscle myosin have been reported (30, 35-37) and additional forms were described for nonmuscle cells (31-34). The present study shows that rat spleen DNA restricted by EcoRI contains five different fragments which hybridize to the myosin DNA plasmid probe. From a rat genomic DNA library, we isolated clones containing three out of the five EcoRI fragments. Since our myosin cDNA plasmid probe is short (225 bp) and does not contain an EcoRI site, and since for each of our genomic clones we found the corresponding EcoRI fragment in the total DNA, it is most likely that each of the five fragments in the genomic DNA represents a different myosin gene. Electron microscopy examination of heteroduplexes formed by hybridization of two of the recombinant phages confirmed that they originated from different genes. The number of myosin heavy chain genes indicated in these experiments is minimal, since the short segment in our probe may not be represented in good homology in all the myosin genes. It should be noted that the plasmid 82 probe hybridized to various extents to the five EcoRI genomic fragments.

The availability of recombinant plasmids and phages containing myosin heavy and light chain DNA sequences, as well as plasmids which hybridize specifically to the muscle α actin mRNA (7) now enables the investigation of the expression and structure of these genes.

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