Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6589–6592, November 1982
Developmental Biology

Molecular cloning and expression during myogenesis of sequences coding for M-creatine kinase

(hybrid-selected translation/muscle gene expression)

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Communicated by Nathan O. Kaplan, August 11, 1982

ABSTRACT **Sequences complementary to muscle poly(A)$^+$RNA were cloned in the plasmid pBR322 and the resulting colonies were screened by colony hybridization with labeled cDNA derived from skeletal muscle and smooth muscle (gizzard). The skeletal muscle-specific clones were further screened by RNA blotting hybridization for a muscle mRNA having the size expected for a putative type M creatine kinase (M-CK) mRNA. The remaining clones with the expected hybridization properties were finally characterized by hybrid-selected translation, and a cloned sequence was shown to contain DNA hybridizing to mRNA that could be translated into M-CK. This plasmid, pMCK1, was further characterized by restriction mapping. Blot analysis of total cell RNA from differentiating myogenic cell cultures showed accumulation of M-CK mRNA in cultures older than 42 hr but not in young little-differentiated cultures.**

During the development of myogenic cells, mononucleated cells fuse to form myotubes which become contractile at the time when well-organized myofibrils, the characteristic organelles of cross-striated muscle, appear in the cytoplasm of fully differentiated cells. The myofibrils are composed of a set of myofibrillar contractile proteins that are produced only in terminally differentiated muscle cells. In the course of differentiation, these proteins replace the cytoplasmic contractile isoproteins, which are expressed in nonmuscle cells and in the nonterminally differentiated myogenic progenitor cells. Hence, isoprotein "switches" occur in differentiating myogenic cells and have been described for some contractile proteins (1, 2) as well as for enzymes such as creatine kinase (CK) (3–5) that have an important role in energy metabolism in muscle.

In early stages of differentiation of cultured myogenic cells as well as in embryonic muscle, BB-CK is accumulated but, as differentiation reaches its terminal phases, isoenzymes containing the muscle-specific CK subunit (M-CK) progressively appear (3–5) until, in adult muscle, the latter subunit replaces the type B CK subunit (B-CK) previously present (5). In an early transitory phase of terminal differentiation the synthesis of B-CK is enhanced and the synthesis of M-CK is initiated; finally differentiated cells that synthesize predominantly M-CK and only traces of B-CK are produced (6). The levels of CK synthesis are paralleled by the availability of translatable polysomal mRNA (7, 8). The switch to M-CK expression can be inhibited reversibly by growth of the cells in bromodeoxyuridine-containing medium. It is likely that the CK isoprotein switch is governed at the transcriptional level and involves repression of the B-CK gene(s) and activation of the M-CK gene(s).

This isoenzyme switch was chosen for a detailed analysis of the elements that regulate gene activity during myogenic development and to compare its features to those of other muscle-specific genes—e.g., the isoproteins of actin (9, 10) or myosin heavy and light chains (10, 11). These comparisons hopefully will reveal common regulatory mechanisms for the coordination of muscle-specific gene expression as have been postulated by others (12). To this end we set out to study in detail M-CK expression during myogenesis with the aid of sequence-specific nucleic acid probes obtained by the use of recombinant DNA technology. We report here the isolation of a cDNA probe containing a sequence complementary to mRNA for M-CK. This cloned sequence was shown to hybridize specifically to RNA from differentiated muscle and to RNA from differentiated cultured muscle cells but not to RNA from smooth muscle and other non-skeletal-muscle tissue in which the M-CK gene is not expressed.

EXPERIMENTAL PROCEDURES

Materials. The plasmid p1E6 was purified and characterized as cDNA containing α-actin sequences by P. Gerschwiler. Oligo(dT)-cellulose (T2) was from Collaborative Research (Waltham, MA); avian myeloblastosis virus reverse transcriptase was a gift from J. Beard (Life Sciences, St. Petersburg, FL); DNA polymerase I and terminal deoxynucleotidyltransferase were from P-L Biochemicals. Restriction enzymes were from Boehringer Mannheim, P-L Biochemicals, or Bethesda Research Laboratories. Labeled nucleotide triphosphates and nick-translation kits were from the Radiochemical Centre (Amersham, England). Nitrocellulose membranes BA-85 were from Schleicher & Schüll (Feldbach, Switzerland), and "Gene-Screen" membranes were obtained from New England Nuclear (Dreieich, Federal Republic of Germany). All other chemicals were of the highest grade available. The solutions were autoclaved and glassware was heat sterilized in order to avoid nuclease and bacterial contamination.

RNA Isolation. RNA was isolated from differentiated breast, leg, or gizzard muscle of 19- to 20-day-old chicken embryos or from adult animals by grinding the tissue under liquid nitrogen with mortar and pestle to a fine powder, which then was further processed by a hot phenol method (13). To avoid any contamination with cellular DNA, all RNA preparations were sedimented through a CsCl step gradient (7, 14), washed with 3 M Na acetate and ethanol, and redissolved in TE buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA). Poly(A)$^+$RNA was purified

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Abbreviations: CK, creatine kinase; M-CK, subunit of MM-CK; B-CK, subunit of BB-CK; BB-CK, MM-CK, enzymatically active dimers; bp, base pair(s); kb, kilobase(s).

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from these preparations by chromatography on oligo(dT)-cellulose (15). The quality of such preparations was tested by cell-free *in vitro* translation.

RNA Translation *in Vitro*. Rabbit reticulocyte lysates were pretreated with micrococcal nuclease (16) to abolish endogenous mRNA. Chicken RNA was translated and the *in vitro* translation products were analyzed as described (7). Analysis of immunoprecipitated CK peptides on two-dimensional gels was carried out as described (17).

Molecular Cloning of Muscle cDNA. Double-stranded cDNA was synthesized from muscle poly(A)$^+$RNA as described (18). After mild S1 nuclease treatment, the double-stranded cDNA was "sized" on sucrose density gradients or directly tailed by using terminal deoxynucleotidyltransferase with oligo(dC). Plasmid pBR322 was digested with *Pst* I and the resulting linear molecules were tailed with oligo(dG). Tailed cDNA and tailed plasmid were annealed and transfected into Ca^{2+}-treated *Escherichia coli* cells (χ1776). Aliquots of the transformation mixtures were plated on nutrient agar plates containing tetracycline (10 μg/ml) to select for bacterial clones containing plasmid (18). Colonies were transferred into the wells of microtiter plates containing selection medium.

Colony Hybridization. Colonies containing plasmids with muscle DNA sequences were identified by colony hybridization (19) using ^{32}P-labeled cDNA made from muscle or gizzard RNA. Colonies that hybridized to cDNA from muscle but not from gizzard were further selected.

Plasmid Preparation. Plasmid DNA was prepared with a modified method of the detergent lysate method (20); an isopropanol fractionation step was used after the RNase digestion.

Synthesis of Single-Stranded cDNA and Labeling of Plasmid DNA. Single-stranded cDNA was synthesized (21) by using poly(A)$^+$RNA from muscle or gizzard as template in the presence of [^{32}P]dCTP. Plasmid DNA was labeled with [^{32}P]dCTP by nick-translation with commercially available materials to a specific activity of 1–2 $\times$ 10^8 cpm/μg.

Restriction Endonuclease Digestions. DNA was digested with restriction enzymes as directed by the supplier, usually in volumes of 10–20 μl at average concentrations of 2 units/μg of DNA. Incubation was for 2–12 hr.

Agarose Electrophoresis of RNA and DNA. Aliquots of RNA were ethanol precipitated, denatured with deionized glyoxal (22), and electrophoresed on 1.2% agarose gels with recirculation of the 10 mM phosphate buffer (pH 7.0). DNA was separated in Tris acetate buffer (40 mM Tris/20 mM acetic acid/2 mM Na_2EDTA, pH 8.1). Chicken rRNA (28/18 S) or *E. coli* rRNA (23/16 S) and 5S RNA were used as size markers for RNA separations; *Taq* I or *Hae* III digests of pBR322 DNA were used for DNA size determination.

Transfer of Nucleic Acids onto Membranes. Glyoxal-denatured RNA was blotted as described (23). The transferred nucleic acids were hybridized to radioactive probes of either nick-translated plasmid DNA or radioactively labeled cDNA from muscle poly(A)$^+$RNA. RNA as well as DNA was hybridized at 37–42°C in the presence of formamide and dextran sulfate (24) with 5$\times$ SSPE (1$\times$ SSPE = 0.18 M NaCl/10 mM $NaPO_4$/1 mM Na_2 EDTA, pH 7.0) instead of standard saline citrate. After hybridization for 16–18 hr, the membranes were washed for 2 hr in 0.3$\times$ SSPE containing 0.2% $NaDodSO_4$ at 68°C and then for 1 hr in 0.5 M Na phosphate at pH 7.0 at the same temperature. The washing was repeated until the washes contained negligible radioactivity. The dried membranes were exposed to preflashed x-ray film at −70°C with an Ilford intensifier screen.

Hybrid-Selected Translation. Selected plasmid DNA (5–10 μg) was linearized by digestion with *Eco*RI and bound to 1-cm^2 membrane pieces (Gene-Screen) (25). The DNA was immobilized onto the membrane by baking; the membranes were cut into small pieces and transferred to microcentrifuge tubes. Muscle poly(A)$^+$RNA at 1 mg/ml was hybridized in 50 μl of hybridization buffer [50% (vol/vol) formamide/0.75 M NaCl/0.075 M Na citrate/20 mM Tricine, pH 8.0/0.2% $NaDodSO_4$ containing 200 μg of tRNA per ml] at 37°C for 16 hr. The membrane fragments were thoroughly washed, and the bound RNA was eluted by heating (boiling water bath) in 300 μl of water containing 10 μg of tRNA. The RNA in the supernatant was precipitated with ethanol in the presence of LiCl and analyzed by *in vitro* translation.

RESULTS AND DISCUSSION

The initial cDNA library containing muscle sequences consisted of 600 independent bacterial clones that had plasmids with inserted sequences. These clones were screened for plasmids yielding positive hybridization signals with radioactive cDNA from leg muscle but not with cDNA from gizzard. Gizzard consists of smooth muscle tissue and does not contain M-CK or any translatable mRNA for M-CK (not shown). This selection left a group of 40 plasmids. These were further screened with a cDNA probe made from a sample of poly(A)$^+$RNA enriched for M-CK mRNA on a density gradient (not shown). Plasmids hybridizing specifically well with these probes were again selected. Because α-actin mRNA was also one of the major mRNA species in the M-CK mRNA enriched fraction (26), this group of clones was further analyzed with cloned α-actin sequences (unpublished data).

The selected 26 clones were analyzed by dot hybridization for their content of actin sequences. Because the probe contained an almost full-length copy of α-actin cDNA, such α-actin sequences among the selected plasmids could be eventually detected with this probe. None of the 26 remaining plasmids showed a strong signal with nick-translated insert sequences of the actin clone (not shown). The actin sequences of skeletal muscle cDNA and gizzard cDNA show enough crossreactivity so that the α-actin-containing clones were already preselected from the cDNA library before by the differential colony hybridization.

Screening of the remaining clones was accomplished as follows. Muscle RNA was denatured and separated on 1.2% agarose gels; the RNA was blotted onto membranes and hybridized with the various nick-translated plasmids. From previous density gradient analysis, the approximate size of the mRNA for M-CK was known to be in the range of actin mRNA and slightly smaller than 18S RNA (not shown). Some of the selected plasmid sequences hybridized to RNA of this size class. These plasmids were further tested by hybrid-selected translation of muscle RNA, and specific immunoprecipitation methods were utilized for identification of plasmids containing M-CK DNA sequences.

In the first series of experiments, membrane-bound plasmid DNA, including the one later identified as containing M-CK sequences (named pMCK1) was hybridized to poly(A)$^+$RNA from leg muscle at high stringency. The RNA eluted from these membranes yielded an extremely weak signal or no signal at all for M-CK after translation. However, lowering the hybridization temperature by 5°C, from 42°C to 37°C, showed that the specific signal for M-CK was clearly enhanced when pMCK1 DNA was used as the selecting agent. This might indicate that the inserted DNA contains A+T-rich sequences.

The immobilized pMCK1 DNA retained specifically M-CK mRNA after hybridization (Fig. 1*a*). The RNA of lane 1 was translated and yielded a protein band with the same mobility

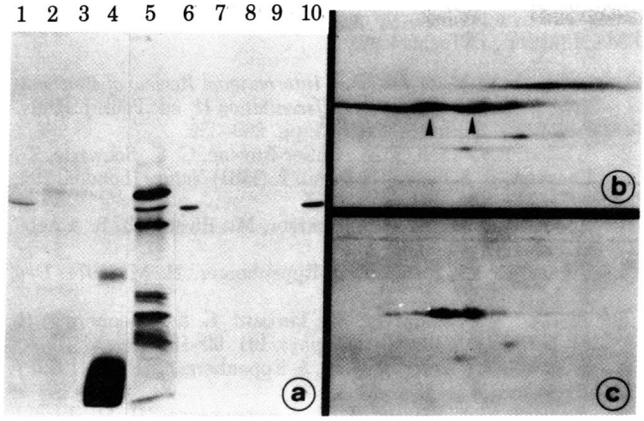

FIG. 1. Identification of cloned M-CK sequences by positive hybrid-selected translation. (*a*) Fluorograph of *in vitro* translation products separated on 14% polyacrylamide/$NaDodSO_4$ gels before (lanes 1–5) and after immunoprecipitation with anti-M-CK antibody (lanes 6–10). The RNA used in the translational assay 1 was selected by hybridization to membrane-bound plasmid DNA. Lanes: 1 and 6, RNA eluted from a membrane containing bound pMCK1 DNA after hybridization with leg muscle poly(A)$^+$RNA; 2 and 7, RNA subjected to the same selection procedure as in lane 1, but membrane-bound pBR322 DNA was used as selecting agent; 3 and 8, no RNA was added to the *in vitro* translation system; 4 and 9, 50 ng of rabbit reticulocyte RNA enriched for globin mRNA; 5 and 10, 1 μg of untreated poly(A)$^+$RNA from chicken leg. (*b* and *c*) M-CK region of a two-dimension gel (17). Isoelectric focusing (first dimension) was from left to right; $NaDodSO_4$ electrophoresis (second dimension) was from top to bottom. The sample in *b* was an aliquot from the reaction analyzed in lane 1 of *a*; the sample in *c* was the immunoprecipitate as in lane 6 of *a*. Arrowheads point to the two subspecies of the M-CK subunit as determined by coelectrophoresis of purified M-CK. The gels were exposed at −70°C for 5 days to preflashed x-ray film.

as M-CK on a $NaDodSO_4$ gel and was also precipitable with anti-M-CK antibody (lane 6). An additional band of radioactive protein migrating with slightly higher molecular weight was also labeled in the absence of added RNA (lane 3) and represented background incorporation of the cell-free system (16). Results from positive control assays with poly(A)$^+$RNA from muscle not preselected by hybridization (lanes 5 and 10) were identical with respect to M-CK synthesis to the assay with mRNA retained on the immobilized pMCK1 DNA. In other control experiments we showed that pBR322 DNA bound to solid supports did not retain any RNA translated into M-CK (lanes 2, 7). No M-CK signal was observed when the RNA was absent from the assays (lanes 3, 8) or when RNA enriched for globin mRNA was used (lanes 4, 9).

To exclude the possibility that proteins that may have comigrated and coprecipitated with M-CK could give rise to the observed bands in Fig. 1*a* (lanes 6 and 10), the translation products were further analyzed on two-dimensional gels. A major part of the radioactivity from such a translation assay comigrated with M-CK subunits (Fig. 1 *b* and *c*). The more acidic smear with a slightly higher molecular weight represents background incorporation and was also observed in the absence of added RNA. The gel in *b* was overexposed and therefore the slight smearing of the synthesis products is overrepresented on the autoradiograph. This smearing was the result of overloading the first-dimension gel with reticulocyte proteins and was decreased accordingly when M-CK was purified by immunoprecipitation as in Fig. 1*c*. When the immunoprecipitate was run on a two-dimensional gel, two species of labeled peptides were found to react with antibody against M-CK as expected (17) but not the radiolabeled contaminating species (background).

On the basis of the experiments described above we concluded that the cloned sequences of pMCK1 hybridize specifically with sequences contained in the mRNA for M-CK.

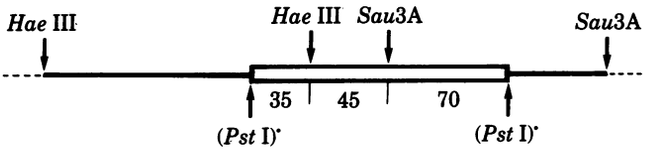

FIG. 2. Restriction map of pMCK1. DNA from pMCK1 was digested with *Hae* III, *Sau*3A, and a combination of *Hae* III and *Sau*3A. The size of the restriction fragments was estimated by comparing the gel patterns of pMCK1 with those of digested pBR322 DNA. The numbers indicate the sizes (in bp) of the fragments. The *Pst* I sites at either end of the inserted sequence were not reconstituted and could not be recut with *Pst* I. Various other enzymes were tested but did not result in cleaving the inserted sequence (see text).

The pMCK1 DNA was further characterized by restriction mapping. The *Pst* I sites, into which the cDNA had been cloned, were not reconstructed and hence the inserted segment could not be excised by *Pst* I digestion of the pMCK1 DNA. Digestion with enzymes such as *Eco*RI, *Hin*dIII, or *Bgl* II did not result in cleavage of the 150-base-pair (bp) insert. However, restriction sites for *Hae* III and *Sau*3A could be localized within the inserted sequence (Fig. 2).

After the sequence homology between pMCK1 and M-CK mRNA was established, this clone was used for further experiments concerning the expression of M-CK during myogenesis. In preliminary experiments using sucrose density gradient fractions of poly(A)$^+$RNA from differentiated cell cultures, the mRNA that stimulated the synthesis of M-CK and the one for B-CK were found to sediment close to but on the small side of the 18S peak of chicken ribosomal RNA (not shown). The size of M-CK mRNA was also estimated by blot analysis. As shown in Fig. 3 (lane 4) the M-CK mRNA derived from differentiated

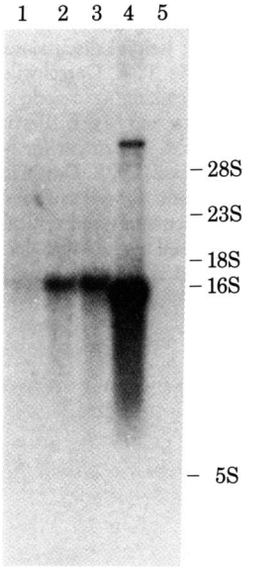

FIG. 3. Accumulation of RNA sequences specific for M-CK during myogenic differentiation and in muscle tissue. Total cellular RNA was denatured by glyoxylation, resolved on a 1.2% agarose gel, blotted onto a membrane, and hybridized with the nick-translated ^{32}P-labeled pMCK1 DNA probe. Samples analyzed in lanes 1–3 contained 50 μg of total cell RNA from myogenic cultures extracted 24, 42, and 72 hr after inoculation. Lane 4 contained 0.5 μg of poly(A)$^+$RNA from differentiated chicken leg muscle, and lane 5 contained 2 μg of poly(A)$^+$RNA from chicken gizzard smooth muscle. Markers for S values were the ribosomal RNAs from *E. coli* and chicken. The hybridized blot was exposed at −70°C for 10 days.

muscle was found to be slightly smaller than the 18S ribosomal RNA from chicken. The relation of the hybridizing high molecular weight bands to M-CK mRNA is not clear. A sample of poly(A)$^+$RNA isolated from gizzard did not hybridize to the pMCK1 probe (lane 5). This observation is in accord with earlier findings that gizzard muscle does not contain MM-CK or translatable mRNA that codes for M-CK (unpublished data). In lanes 1–3, samples of 50 μg each of total cell RNA from differentiating myogenic cell cultures were applied to the gel, blotted onto a membrane, and hybridized with radioactive pMCK1. In the sample derived from 24-hr cells (lane 1) only a trace of or no hybridizing RNA can be seen in the autoradiograph. In the sample with the identical amount of RNA extracted from 42-hr cells or 72-hr cells, the mRNA for M-CK can easily be detected. The discrepancy in migration of the hybridizing band in the total cell RNA preparation and the major hybridizing band of the poly(A)$^+$RNA from leg was attributed to an artifact due to different loading of the gel.

The present results corroborate our earlier findings that newly synthesized mRNA for M-CK accumulates in differentiating myogenic cells, as had already been shown by *in vitro* translation methods (8). The present findings substantiate the hypothesis that differential transcription likely governs the CK transition.

The cloned sequences described above will be useful for study of the expression and the gene structure of the muscle-specific form of CK. M-CK DNA derived from plamid 85C6 containing M-CK sequences (obtained from C. Ordahl) did not cross-hybridize with pMCK1 sequences (unpublished data). Rescreening of the cDNA library with this probe revealed the plasmid pMCK2 with an insert of 640 bp; it hybridized to the pMCK1 sequences as well as to the plasmid p85C6. This result indicates that different parts of the same sequence have been obtained in the two laboratories.

We are grateful to Dr. C. Ordahl (Department of Anatomy, Temple University, Philadelphia) for helpful discussions and exchange of materials (clone 85C6) and to Ms. P. E. Gerschwiler for help in the characterization of the plasmid containing α-actin sequences. Thanks are also due to Mr. H. Thurnherr and Ms. E. Perriard for part-time technical assistance and to Ms. M. Leuzinger for help in the preparation of the manuscript. We also thank Dr. R. Dottin for discussions and communicating unpublished results as well as Drs. W. Schaffner, M. Caravatti, and T. Koller for discussions and critical reading of the manuscript. The work was supported by the Muscular Dystrophy Association of America, by a grant to H.M.E. from the Swiss National Science Foundation, and by a predoctoral training grant to U.B.R. from the Swiss Federal Institute of Technology.

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