Regulation of Creatine Kinase Induction in Differentiating Mouse Myoblasts

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The regulation of creatine kinase (CK) induction during muscle differentiation was analyzed with MM14 mouse myoblasts. These cells withdraw from the cell cycle and commit to terminal differentiation when fed with mitogen-depleted medium. Myoblasts contained trace amounts of an isozyme of brain CK (designated BB-CK), but differentiation was accompanied by the induction of two other isozymes of muscle and brain CKs (designated MM-CK and MB-CK). Increased CK activity was detectable within 6 h of mitogen removal, 3 h after the first cells committed to differentiation and 6 h before fusion began. By 48 h, MM-CK activity increased more than 400-fold, MB-CK activity increased more than 150-fold, and BB-CK activity increased more than 10-fold. Antibodies prepared against purified mouse MM-CK cross-reacted with muscle and brain CKs (designated M-CK and B-CK, respectively) from a variety of species and were used to demonstrate that the increase in enzymatic activity was paralleled by an increase in the protein itself. CK antibodies were also used to aid in identifying cDNA clones to M-CK. cDNA sequences which corresponded to protein-coding regions cross-hybridized with B-CK mRNA; however, a subclone containing the 3'-nontranslated region was unique and was used to quantitate M-CK mRNA levels during myoblast differentiation. M-CK mRNA was not detectable in myoblasts, but within 5 to 6 h of mitogen withdrawal (6 to 7 h before fusion begins) it accumulated to about 30 molecules per cell. By 24 h, myotubes contained approximately 1,100 molecules per nucleus of M-CK mRNA.

The induction of muscle-specific protein synthesis can be studied in myogenic cell lines by appropriate manipulation of the culture conditions. Myoblasts, the muscle precursor cells, can be triggered to terminally differentiate by feeding with mitogen-depleted medium (20, 31, 35, 49, 68). Differentiating myoblasts are characterized by the rapid accumulation of muscle-specific proteins, the cessation of proliferation, and the ability to fuse into multinucleated myotubes, or muscle fibers (52). Numerous proteins are induced during myogenesis, including components of the contractile apparatus, metabolic enzymes, and membrane proteins (7). The mechanism by which a wide range of different proteins is induced is largely unknown; those studied in the most detail appear to be regulated at the level of mRNA accumulation, which has frequently been assumed to imply transcriptional control (2, 26, 43, 56, 65). No specific inducing factors have been identified, although several groups have presented evidence suggesting that differentiated muscle cells contain diffusible activators of muscle-specific protein synthesis (4, 67). Induction of the contractile proteins occurs in a coordinate manner, but several other classes of proteins appear with unique kinetics (13, 15, 16). These observations suggest the need to study a variety of proteins to obtain a complete understanding of gene regulation during muscle development.

Our approach has been to study the induction of creatine kinase (CK), a metabolically critical enzyme in muscle (3). There are three known isotypes of CK, designated M-CK (muscle), B-CK (brain), and Mt-CK (mitochondrial). CK exists as a dimer, and the cytoplasmic, nonmitochondrial isotypes M-CK and B-CK randomly associate to form three isozymes: MM-CK, MB-CK, and BB-CK (22). Mt-CK does not dimerize with M-CK or B-CK and has other properties which readily distinguish it from the cytoplasmic CKs (5,

24). The differences between M-CK and B-CK in terms of structure, function, and mode of regulation are poorly understood. M-CK cDNAs have been prepared from chicken RNA (55, 57), and their sequences have recently been determined (32). B-CK has not been sequenced, but based upon differences in amino acid composition, and, in most cases, a lack of immunological cross-reactivity, M-CK and B-CK are almost certainly the products of different genes (21, 64). In developing embryonic muscle and in myogenic cultures, CK undergoes an isozymic transition; the predominant form switches from BB-CK to MM-CK while the total enzymatic activity increases greatly (50, 53-55). Further analysis of CK represents an attractive complement to the study of contractile protein induction due to its unique metabolic role, its isozymic transition, and because it is a major soluble protein in muscle (64).

We have analyzed CK synthesis with MM14 mouse myoblasts, a permanent cell line whose differentiation is under the stringent control of the mitogen fibroblast growth factor (37, 38). These myoblasts proliferate indefinitely in mitogen-rich medium, but when shifted to mitogen-depleted medium, G₁ cells withdraw from the cell cycle and irreversibly commit to terminal differentiation (36). Mitogen deprivation therefore converts >99% of a culture of proliferating myoblasts into postmitotic myocytes (committed cells which have not yet fused) within the time span of one cell cycle (12.5 h). By 24 h, virtually all of these myocytes fuse into multinucleated myotubes. MM14 cells thus represent a homogeneous and rapidly differentiating system with which to study the induction of muscle-specific protein synthesis after myoblast commitment.

In this paper, we characterize the timing and magnitude of M-CK and B-CK induction in relationship to the commitment kinetics of MM14 myoblasts. Both subunit types were induced, although M-CK increased to a much greater extent. We also report the isolation and characterization of cDNA

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clones to M-CK and their use in quantitating M-CK mRNA levels. These experiments lay the groundwork for a detailed study of the mechanism of CK induction during mouse muscle development.

MATERIALS AND METHODS

DNase I (Worthington Diagnostics, DPFF) was further purified free of RNase as described before (6). S1 nuclease was purified from crude α -amylase powder (Sigma Chemical Co.) as described before (63) through the DEAE step. Reverse transcriptase was from J. Beard (Life Sciences, Inc.). *Escherichia coli* DNA polymerase I was a gift of L. Loeb, University of Washington, Seattle. Restriction enzymes were from New England Biolabs or Bethesda Research Laboratories. *Hind*III and *Eco*RI linkers were from Collaborative Research. Antiserum against bovine serum albumin was purchased from Miles/Yeda.

Isolation of CKs. MM-CK was purified from adult Swiss HA/ICR mouse skeletal muscle by a slight modification of a procedure for rabbit MM-CK (30). That procedure was followed through the magnesium acetate extraction step, and then the extract was dialyzed into 0.1 M Tris-hydrochloride–0.1 mM EDTA (pH 8.0), concentrated by precipitation with 70% $(\text{NH}_4)_2\text{SO}_4$, and chromatographed on DEAE-cellulose. In the latter step, the enzyme was loaded on a column (1.2 by 12 cm) equilibrated with 10 mM Tris-hydrochloride–0.1 mM EDTA (pH 8.0) and eluted with a linear gradient composed of 100 ml each of 10 and 60 mM Tris-hydrochloride–0.1 mM EDTA (pH 8.0). Peak fractions were pooled, concentrated by precipitation with 70% $(\text{NH}_4)_2\text{SO}_4$, and assayed for activity (28). Purified mouse MM-CK has a specific activity of 300 mU/mg of protein at 25°C.

Human MM-CK was purified, and human BB-CK and mouse BB-CK were partially purified as described previously (30). However, the BB-CK isozymes were only taken through the first $(\text{NH}_4)_2\text{SO}_4$ fractionations. Rabbit MM-CK and BB-CK were purchased from Worthington Diagnostics and Sigma Chemical Co., respectively. Chicken MM-CK and BB-CK were a generous gift from M. Robinson and M. Nameroff, University of Washington, Seattle.

Preparation of antibodies to mouse M-CK. New Zealand white rabbits were initially injected (subcutaneously and intramuscularly) with a 1:1 (vol/vol) emulsion of mouse MM-CK (2.5 mg) in phosphate-buffered saline (PBS) and Freund complete adjuvant. After 2 weeks, the rabbits were boosted with 1 mg of CK in the same manner. This procedure was repeated 2 weeks later and then at monthly intervals. After 3 months, one rabbit exhibited a low titer and was boosted every 2 to 3 months with 3 mg of CK prepared as follows. One-third of the CK was native, one-third was denatured with 1% sodium dodecyl sulfate (SDS) and embedded in a 10% polyacrylamide gel, and one-third was cross-linked with glutaraldehyde as described by Bulinski et al. (8). After homogenization, this mixture was emulsified with an equal volume of Freund complete adjuvant and injected subcutaneously along the back and neck. Antibodies specific to CK were affinity purified from the rabbit serum (54) on a column of Sepharose 4B-mouse MM-CK containing 2 mg of antigen per ml (41).

Immunological assays. Antibody titer, cross-reactivity with other CK isozymes, and specificity of binding was demonstrated by using an immunoblot technique (61) modified as described before (8). Tissue extracts were made for the immunoblot assay by homogenizing adult skeletal muscle or brain directly into SDS gel sample buffer (prepared as

described by Laemmli [34]) at a concentration of 4 ml/g of tissue, boiling for 15 min, and centrifuging at $16,000 \times g$ for 10 min. Cell cultures were prepared for immunoperoxidase staining by rinsing three times with Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-hydrochloride [pH 7.4]), fixing for 45 s in 60% ethanol–3% formaldehyde–4% acetic acid, and then again rinsing three times with TBS. Fixed cultures were incubated overnight with an appropriate dilution of antibodies in TBS containing 0.5% normal goat serum, and immunoperoxidase staining was performed with a Vectastain ABC biotin-avidin-peroxidase reagent kit (Vector Laboratories) as described by the manufacturer.

Growth of cells and extraction of CKs. Mouse MM14 myoblasts were grown and induced to differentiate as previously described (36) except that Ham's F-10 medium containing 1.1 mM CaCl_2 (F-10C), 1 μM insulin, 100 nM dexamethasone, and 5% horse serum was used in place of conditioned medium. Commitment of cells to terminal differentiation was measured as previously described (35). CKs were extracted from cultures containing 2×10^5 to 4×10^5 cells per 100-mm dish as follows. The cultures were rinsed extensively in warm F-10C medium and then in ice-cold PBS. Cells were then serially scraped into 5 ml of ice-cold PBS and centrifuged for 5 min at $700 \times g$. The supernatant was removed, and pellets were lysed by addition of 0.1 ml of 5 mM Tris-hydrochloride–3 mM EDTA (pH 8.0) followed by rapid freeze-thawing. Cell debris was removed by centrifugation at $16,000 \times g$ for 1 min, and the supernatant was assayed as described below. The cell number was monitored with a Coulter Counter (Coulter Electronics, Inc.) or, when measuring RNA levels, by a fluorimetric assay for DNA (33).

Measurement of CK activity and isozymes. CK extracted from cultures was assayed for total activity by the procedure of Horder et al. (28); background activity in the absence of creatine phosphate was always subtracted. Isozymes of CK were electrophoretically separated on cellulose-acetate and visualized with a fluorescent activity stain, using a Super CPK Isoenzyme Kit (Helena Laboratories) as described by the manufacturer. Relative levels of each isozyme were determined by scanning the stained proteins with a Helena Quick Scan Flur-Vis densitometer equipped with a Quick Quant II digital automatic integrator.

Isolation of nucleic acids. Total nucleic acids were isolated from myogenic cultures by scraping the cells into $1 \times \text{SET}$ (1% SDS, 10 mM Tris-hydrochloride [pH 7.8], 5 mM EDTA) containing 100 μg of proteinase K per ml, digesting for 1 h at 45°C, extracting with phenol and chloroform, and then precipitating with ethanol. ^3H mRNA was obtained by oligodeoxythymidylate-cellulose chromatography (1) of DNase I-treated nucleic acids isolated from myotubes which had been labeled for 12 h in F-10C containing 10% horse serum and 100 μCi of ^3H uridine per ml (New England Nuclear Corp.). Muscle and brain polyadenylated RNAs were purified from 10-day-old Swiss HA/ICR mice as follows. The animals were sacrificed by cervical dislocation, and limb skeletal muscle and brain tissue were rapidly excised and frozen in liquid N_2 . Frozen tissue was then thoroughly homogenized into a 1:1 (vol/vol) mixture of solution A (0.5% SDS, 25 mM EDTA, 75 mM NaCl [pH 8.2]) and redistilled phenol saturated with solution A (15 ml/g of tissue) (56). After centrifugation, the aqueous layer was removed, reextracted with phenol and chloroform, and then precipitated with ethanol. Total nucleic acids were suspended in 20 mM sodium acetate (pH 5.0), and high-molecular-weight RNA was precipitated with 2 M LiCl. Polyad-

enylated RNA was isolated by chromatography on oligodeoxythymidylate-cellulose (1).

Isolation of cDNA clones. Preparation of rabbit reticulocyte lysate, in vitro translation, size selection of RNA, synthesis of double-stranded cDNA, and cloning into pBR322 were all performed essentially as described before (18). An M-CK cDNA (pMCK-m3') was identified by hybrid selection with CK-enriched mRNA (12). Cloning of pMCK-m2 in the yeast expression vector pMAC561 was performed as described before (42). The internal *Hind*III fragment of pMCK-m2 was used to isolate an almost full-length M-CK cDNA designated pMCK-m4. A complete description of this latter cDNA will be provided elsewhere (J. Jaynes, J. Chamberlain, J. Buskin, and S. Hauschka, manuscript in preparation). Restriction map analysis of the cDNA clones was performed by standard methods (40), as were RNA dot blots and Northern blots (60).

Cloning into M13 and measurement of M-CK mRNA levels. The insert from plasmid pMCK-m3' was excised with *Eco*RI and *Hind*III, isolated by sedimentation on 5 to 20% (wt/vol) sucrose gradients in 20 mM Tris [pH 7.5]–2 mM EDTA–0.2 M NaCl–0.1% Sarcosyl for 15 h at $280,000 \times g$, and cloned into *Eco*RI- and *Hind*III-cut phage vectors M13mp10 and M13mp11 (46). The subclone designated M13mp11MCK-m3' contained viral strand DNA of the same sense as mRNA (mDNA strand). Viral strand DNA was purified from M13 phage vectors as described before (45). Nick translation of the pMCK-m3' insert, isolation of 32 P-labeled single-stranded cDNA probes with M13mp11MCK-m3', and measurement of mRNA levels via solution hybridization were performed as described before (17). Briefly, the nick-trans-

TABLE 1. CK isozyme levels after mitogen withdrawal from myoblasts

Time (h)	% CK isozyme			Sp act ^a			
	MM	MB	BB	Total	CK isozyme		
					MM	MB	BB
0	~20	0	~80	<.5	<.5	<.5	<.5
8	35	37	28	3.8	1.3	1.4	.8
12	45	38	17	16.7	7.5	6.3	2.8
18	51	42	7	122	62	51	8.5
24	65	32	3	234	152	75	7.0
36	68	30	2	270	184	81	5.4
48	72	26	2	294	212	76	5.8
60	74	24	2	291	215	70	5.8
72	79	20	1	236	186	47	2.4

^a Milliunits of CK activity per 10^6 nuclei.

lated pMCK-m3' insert was denatured and hybridized to viral-strand DNA from the mp11MCK-m3' subclone and then the hybrid was separated from single-stranded mDNA by chromatography on Bio-Gel A-50 M (Bio-Rad Laboratories). The cDNA strand was separated from M13 DNA by a second passage over the Bio-Gel column, this time in the presence of 0.1 N NaOH and 0.25 mM EDTA. The single-stranded cDNA probe was subsequently hybridized to nucleic acids under high stringency conditions (40% formamide, 0.6 M NaCl [68°C]) and then treated with S1 nuclease to digest any nonhybridized probe. S1-resistant counts per minute were converted to the concentration of M-CK mRNA in a sample by comparison with a standard curve obtained by hybridization with known amounts of M-CK mRNA. M-CK mRNA concentrations in the standard were determined as described before (17).

RESULTS

Induction of CK activity in differentiating MM14 myoblasts.

The accumulation of CK enzymatic activity in MM14 mouse myoblasts after a switch to low-mitogen medium is shown in Fig. 1. No CK was detected in proliferating cells; however, the maximum sensitivity of this assay (minimum detectable activity divided by the number of cells assayed) was 0.5 mU/ 10^6 cells. Enzymatic activity remained undetectable until 6 h after mitogen withdrawal (3 h after the first cells committed to terminal differentiation), when it was present at 0.65 mU/ 10^6 nuclei, and then increased to about 300 mU/ 10^6 nuclei at 48 to 60 h before declining in conjunction with myofiber degeneration.

The relative distribution of CK isozymes was determined in parallel with the above experiment (Table 1). Although the assay used detected minute levels of CK in proliferating cells, background fluorescence coupled with low activity precluded accurate densitometry. However, visual examination of the separated isozymes revealed that about 80% of the activity in proliferating myoblasts was BB-CK and 20% was MM-CK; virtually no MB-CK was seen. By 8 h, accurate densitometry was possible, and all three isozymes had accumulated to similar levels; by 12 h, MM-CK became the predominant isozyme, and by 72 h, MM-CK accounted for 80% of the CK, whereas BB-CK accounted for only 1%.

The relative contribution of MM-CK, MB-CK, and BB-CK to the increase in CK activity can be approximated by multiplying the total activity at each time point by the fraction of each isozyme. This calculation revealed that MM-CK, MB-CK, and BB-CK were all induced during

FIG. 1. Induction of CK activity after mitogen withdrawal from myoblasts. Cultures fed with mitogen-depleted medium at time zero were assayed for CK at the indicated intervals. Extraction of CK and determination of enzymatic activity were as described in the text. The insert shows an enlargement of the first 12-h period.

FIG. 2. Cross-reactivity of rabbit anti-mouse MM-CK with CK isozymes from various species. Affinity-purified antibodies were checked for specificity by an immunoblot assay as described in the text. Shown are the immunoperoxidase-stained reacting proteins. M, Purified MM-CK; B, BB-CK (rabbit and chicken BB-CKs were purified, whereas mouse and human BB-CKs were partially purified); Bx, mouse brain extract. The amounts of protein loaded were adjusted to give similar staining and were approximately: mouse, rabbit, and human MM-CKs, 150 ng; chicken MM-CK, 1.2 μ g; rabbit BB-CK, 1 μ g; chicken BB-CK, 5 μ g; human and mouse B-CKs and Bx were not pure but contained approximately 1 μ g of BB-CK as judged by Coomassie blue staining of the acrylamide gels. The mouse brain extract contained small quantities of MM-CK (as judged by the isozyme assay, which probably arose from blood contained in the tissue sample).

mouse myoblast differentiation but that most of the increase was contributed by MM-CK and MB-CK (Table 1).

Preparation, purification, and characterization of antibodies to mouse MM-CK. To assay CK independently of its enzymatic activity and to aid in identifying cDNA clones (see below), antibodies were prepared against purified mouse MM-CK. Initial attempts to raise rabbit antibodies by using standard procedures (23) produced very low-titer serum. An

alternate protocol involving immunization with denatured, cross-linked, and polyacrylamide gel-embedded CKs was therefore utilized. The serum obtained immunoprecipitated purified MM-CK but precipitated small amounts of other muscle proteins as well. Antibodies specific for CK were therefore obtained by chromatography on an MM-CK-Sepharose 4B affinity column. The specificity of the affinity-purified antiserum and its cross-reactivity with other CK types was demonstrated with an immunoblot assay. Figure 2 demonstrates that the antibodies reacted (although to different extents) with M-CK and B-CK subunits from humans, rabbits, chickens, and mice and that no other proteins were recognized. The affinity-purified antiserum was used in all subsequent experiments.

CK accumulation in differentiating MM14 cultures. As a verification that the increase in CK enzymatic activity parallels the increase in the protein itself, an immunoblot was performed against culture extracts obtained at various intervals after mitogen withdrawal (Fig. 3). The acrylamide gels used in these experiments resolved the M-CK and B-CK subunits (Fig. 2 and 3B) as long as the enzyme concentrations were not too great. Trace amounts of CK, primarily of the B type, were barely detectable in proliferating myoblasts (time zero). Induction of the protein over basal levels was first detected 6 h after mitogen withdrawal and was mostly of the M type. By 12 h, the intensity of staining resulted in a loss of resolution between the two subunits, but the total intensity continued to increase until 48 to 60 h. Thus, the increase in CK detected with a specific antibody was similar to the increase in enzymatic activity (Fig. 1).

Accumulation of CKs in individual cells after mitogen deprivation. Withdrawal of mitogens from MM14 myoblasts does not trigger a simultaneous commitment of all cells to terminal differentiation. Rather, since myoblasts commit only when traversing the G_1 phase of the cell cycle, a period equivalent to one full cell cycle is required for commitment of the entire population (35). Thus, although the data shown in Fig. 1 and 3 demonstrate a general increase in CK levels, the changing population of committed cells during differentiation of the cultures precludes extrapolation of that data to CK accumulation in individual cells. The fraction of cells making CK at any given time can, however, be determined

FIG. 3. Induction of immunoreactive CK after mitogen withdrawal from myoblasts. CK extracted from differentiating myoblast cultures was assayed via an immunoblot with affinity-purified anti-mouse MM-CK antibodies. (A) total proteins transferred to nitrocellulose; (B) immunoperoxidase-stained proteins. The time points after removal of mitogens are shown in hours and are the same as those in Fig. 1.

by immunoperoxidase staining of cultures with antibodies. Proliferating cultures contained a background of approximately 1% CK-positive cells (Fig. 4). An increase in the number of CK-positive cells was first detected 6 to 7 h after mitogen withdrawal (3 to 4 h after the first cells commit to terminal differentiation). The percentage of CK-positive cells continued to increase over the next 12 to 14 h until nearly 100% of the cells contained detectable CK. Parallel cultures stained with a monoclonal antibody to myosin heavy chain (MF-20, courtesy of D. Fischman) exhibited identical behavior (data not shown), and the increase in cells making both proteins lagged behind the increase in committed cells by 3 to 4 h (Fig. 4). This experiment could not distinguish whether the CK-positive cells in proliferating cultures contained M-CK or B-CK since the antibody reacted with both. However, since the same low frequency of cells was also myosin positive, it seems probable that these cells represent a background of myocytes that have already accumulated high levels of muscle-specific proteins. Such cells would thus be responsible for the trace amounts of MM-CK in extracts of proliferating myoblasts (Table 1).

Isolation and characterization of cDNA clones to mouse M-CK. Polyadenylated RNA size selected for CK sequences was prepared and used to make a cDNA library in pBR322. The cloning strategy involved ligating blunt-ended double-stranded cDNA to a 1:1 mixture of *Hind*III and *Eco*RI linkers and then inserting the DNA into *Eco*RI- and *Hind*III-cut pBR322. A clone containing an M-CK cDNA was then identified by using the technique of hybrid selection. Here, plasmid DNA from various clones was isolated, bound to nitrocellulose, and hybridized to CK-enriched RNA. Bound RNA was then identified by in vitro translation followed by polyacrylamide gel electrophoresis. Figure 5 shows a fluorogram of the electrophoresed translation products obtained after hybridization to four different plasmids. Comparison of the labeled proteins with purified standards tentatively identified plasmids 2 and 3 as actin (lanes 5 and 6) and plasmid 4 as CK (lane 7); plasmid 1 did not hybridize to any CK-enriched RNA (lane 4). Furthermore, the 41,000-

FIG. 4. Increase in number of CK-positive cells after mitogen withdrawal from myoblasts. Myoblast cultures fed with mitogen-depleted medium at time zero were assayed for the percentage of the cells irreversibly committed to terminal differentiation (Δ) or staining positively for CK with affinity-purified anti-MM-CK antibodies ($\bullet$). Also shown is the increase in total CK activity ($\square$) (Fig. 1). Committed and CK-positive cells were determined as described in the text.

FIG. 5. Identification of a CK cDNA clone via hybrid selection. Plasmid DNA from various clones (4 μ g) was applied to nitrocellulose and hybridized to 1 μ g of CK-enriched mRNA. Bound RNA was eluted and translated in vitro in the presence of [35 S]methionine. Labeled products were separated on 10% polyacrylamide gels either before or after immunoprecipitation and visualized by autoradiography. (A) Total translation product obtained from the following sources of RNA: lane 1, 100 ng of CK-enriched mRNA; lane 2, 10 ng of CK-enriched mRNA; lane 3, no added RNA; lane 4, plasmid 1-selected RNA; lane 5, plasmid 2-selected RNA; lane 6, plasmid 3-selected RNA; lane 7, plasmid 4-selected RNA. The products in lane 5 and 6 comigrated with actin, and that in lane 7 comigrated with M-CK. The 45,000 molecular-weight band was endogenous to the reticulocyte lysate (lane 3). (B) Immunoprecipitated translation products from RNA selected by the following plasmids: lane 1, plasmid 2 (anti-CK); lane 2, plasmid 2 (anti-bovine serum albumin); lane 3, plasmid 4 (anti-CK); lane 4, plasmid 4 (anti-bovine serum albumin). The results demonstrated that plasmid 4 contained a CK cDNA insert. MW, Molecular weight of size standards in thousands.

dalton protein in lane 7 (plasmid 4) was immunoprecipitated with affinity-purified antibodies to MM-CK (Fig. 5B, lane 3), whereas no immunoprecipitation was detected with translation products from RNA selected by the actin clone (Fig. 5B, lane 1). As an additional specificity control, antisera to bovine serum albumin precipitated neither translation product. Plasmid 4 thus contained a CK cDNA insert.

Proof that this 316-base-pair plasmid, hereafter referred to as pMCK-m3' (for mouse M-CK), is a clone of M-CK and not B-CK required further experimentation. Rescreening the cDNA library with a nick-translated pMCK-m3' insert produced many identical, but no larger, clones. Therefore, a new library was constructed in the yeast expression vector pMAC561. Screening of this library identified a plasmid containing a 1,200-base-pair CK insert designated pMCK-m2. Restriction maps of both cDNAs, various subclones, and a third cDNA (pMCK-m4) are presented in Fig. 6. As can be seen, the pMCK-m3' cDNA insert is wholly contained within pMCK-m2. In addition, sequence determination has shown that the pMCK-m3' insert ends in a stretch of polyadenylate and thus corresponds to the 3' end of the mRNA (the complete sequence will be reported elsewhere).

Accumulation of M-CK mRNA after mitogen withdrawal from myoblasts. To directly measure M-CK mRNA levels, it was necessary to obtain a probe which did not cross-hybridize with B-CK mRNA. The 1,200-base-pair cDNA pMCK-m2 was therefore cleaved into three pieces with *Hind*III (Fig. 6), and each piece was hybridized to muscle, brain, and chicken oviduct RNA. Figure 7 shows that the 5' and central portions of the cDNA hybridized to brain RNA at moderate but not high stringency. However, the 3' probe (equivalent to pMCK-m3') did not hybridize with brain RNA even at low stringency. None of the probes hybridized with oviduct

FIG. 6. Partial restriction map of pMCK-m2 and pMCK-m4 and their relation to M-CK mRNA and three subclones. The size of the mRNA was deduced from Northern blot analysis of muscle polyadenylated RNA (Fig. 8). A, *Ava*I; H, *Hind*III; P, *Pst*I; B, *Bgl*II; A_n, polyadenylate tail. Shown at the bottom is the scale in base pairs.

RNA, and all three hybridized with muscle RNA even at high stringency. As a further characterization, a Northern blot of muscle and brain polyadenylated RNA was hybridized to the central *Hind*III fragment of pMCK-m2. Under moderate stringency, a single band of approximately 1,700 bases was detected in both the muscle and brain samples (Fig. 8). Hybridization to brain RNA was much less than that to muscle RNA and was abolished at high stringency (data not shown). Together these results confirm that the cDNA is to M-CK and not B-CK and that the 3' probe does not cross-hybridize with B-CK mRNA.

For measurement of M-CK mRNA levels, the 3' probe was subcloned into the single-stranded phage vector M13mp11. Viral-strand DNA from this subclone contains the mDNA strand of pMCK-m3' insert and could therefore be used to separate single-stranded cDNA probes from nick-translated pMCK-m3' insert (17). Before the single-stranded cDNA probe was used for mRNA measurements, its optimal hybridization stringency was determined by hybridization to muscle and brain polyadenylated RNA over a range of temperatures. The probe hybridized to muscle but not brain RNA at up to 68°C in 40% formamide, but hybridization was abolished at 72°C (data not shown). The 68°C-40% formamide stringency was therefore used for all measurements.

FIG. 7. Cross-hybridization of CK subclones with muscle, brain, and chicken oviduct polyadenylated RNAs. CK cDNA subclones (Fig. 6) were nick translated and hybridized to the indicated amounts of the various RNAs. Stringency refers to the posthybridization washing temperature in 0.5% SDS-5 mM Tris-hydrochloride (pH 7.8)-2.5 mM EDTA. The CK cDNAs were complementary to M-CK but not to B-CK; however, the 5' and H0.4 subclones cross-hybridized with B-CK mRNA at moderate stringency.

The M-CK single-stranded cDNA probe was then used to determine the level of M-CK mRNA in MM14 myoblasts at various intervals after mitogen withdrawal. Proliferating myoblasts contained no detectable M-CK mRNA, i.e., less than 10 molecules per cell (Fig. 9). Induction of the M-CK message was apparent at 5 to 6 h after mitogen withdrawal (at which time the cultures contained 30 molecules of M-CK mRNA per cell). However, since only about 25% of the total population had become committed to terminal differentiation after 5 to 6 h of mitogen deprivation (Fig. 4), the committed cells that contained M-CK probably had at least four times more molecules per cell than was calculated for the entire population. By 12 to 14 h, all of the cells had become committed to terminal differentiation, and by 24 h, M-CK mRNA levels attained a maximum of about 1,100 molecules per nucleus. By 72 h, M-CK mRNA levels decreased by about 50%, but observation of the cultures suggested that this was at least partially due to myotube degeneration. These results indicated that the induction of M-CK was controlled at the level of mRNA accumulation. Further experimentation is required to show whether the increase in M-CK mRNA is accompanied by an increased rate of transcription from the M-CK gene.

DISCUSSION

The accumulation of CK has been characterized in differentiating MM14 mouse myoblasts. The increase in CK

FIG. 8. Northern blot of muscle and brain polyadenylated RNAs (M and B, respectively) versus an M-CK cDNA. Polyadenylated RNAs were isolated, separated by agarose gel electrophoresis, transferred to nitrocellulose paper, and hybridized to a nick-translated pMCKH0.4 insert. After hybridization, the filters were washed at 45°C in 0.5% SDS-5 mM Tris-hydrochloride (pH 7.8)-2.5 mM EDTA and then exposed to X-ray film. Amounts of RNA in lanes M and B were 1 and 10 µg, respectively. Molecular sizes are in kilobases.

isozymes agrees qualitatively with earlier studies of CK accumulation during chicken muscle development (22), as well as with studies of rat (19), quail (59), and chicken (54) myoblasts and of the musclelike mouse cell line BC₃H1 (50). However, the use of different assays and methods of data reporting preclude direct comparisons of relative enzyme levels. Our data were normalized to the number of cells (or nuclei) used in the assays since parameters such as the amount of DNA, RNA, and protein per nucleus as well as the number of cells per culture dish vary considerably during myoblast differentiation (10; unpublished data). For example, proliferating myoblasts contain unequal amounts of DNA per cell, depending on which phase of the cell cycle they are in, whereas myotube nuclei all exhibit a G₁ DNA content.

The magnitude and timing of CK induction reported by different investigators varies considerably, reflecting intrinsic differences between the myogenic cells of various species. Chicken and rat myoblasts require several days and numerous cell cycles for complete differentiation *in vitro* (49, 62), and induction of CK is thus spread out over an extended period. However, MM14 myoblasts differentiate in response to mitogen deprivation within the 12.5 h period of a single cell cycle (35). Furthermore, primary cultures are invariably contaminated by nonmuscle cells which do not induce CK, thus lowering the overall magnitude of induction; in contrast, MM14 mouse myoblasts are a homogeneous, clonally derived cell line (27). Thus, increased levels of M-CK mRNA and protein were detectable in MM14 myoblasts 5 to 6 h after exposure to mitogen-depleted medium (only 2 to 3 h after the first cells commit to terminal differentiation) and well before fusion had begun (Fig. 1 and 9 and Table 1). The peak of CK activity at 48 h after mitogen withdrawal was at a level nearly 600-fold higher than that in proliferating myoblasts (Fig. 1). However, at that time, 26% of the activity was contributed by MB-CK, which is composed of one subunit each of M-CK and B-CK, and 2% is contributed by BB-CK (Table 1). Therefore, both M-CK and B-CK were induced during MM14 myoblast differentiation, although M-CK was induced to a greater degree. Similar inductions of B-CK during myogenesis have been reported for rat and chicken cultures (19, 53, 54), although this fact has received little attention because of the far greater induction of M-CK. The significance of B-CK induction in cultures is not clear, however, since skeletal muscle from adult chickens (54) and mice (unpublished data) contains virtually no B-CK.

Similar differences between the isoform composition of adult versus cultured muscle cells have been reported for several other proteins, including the myosin heavy and light chains (9, 26, 58, 66). A "coactivation-selective-repression" model of gene regulation during muscle development has been proposed to explain these differences (29). In that model, early muscle formation would be accompanied by a general induction of many isoforms, followed by selective deinduction appropriate to the developmental fate of each type of muscle. Such a mechanism is certainly compatible with our data.

The necessity for multiple CK isozymes in different cell types or even in the same cell is not clear. Presumably, slight differences in the roles of each isozyme are reflected in their structures; yet little is known about these differences because only small portions of the B-CK sequences have been determined. Most antibodies raised against MM-CK or BB-CK have not cross-reacted with the other isozyme; it is therefore of interest that the antiserum described here showed

FIG. 9. M-CK mRNA levels after mitogen withdrawal from myoblasts. Total nucleic acids were extracted from differentiating cultures at the indicated intervals and assayed for M-CK mRNA as described in the text. This assay has a limit of detectability (minimum amount of RNA detectable divided by maximum number of cells assayed) of 10 molecules per nucleus.

such a broad specificity (Fig. 2) (21, 47, 50). Based upon quantities of the various CKs required to obtain equivalent staining intensities, the relative affinity of the purified anti-mouse MM-CK antiserum was about 10 times greater for mouse, rabbit, and human MM-CK than for either chicken MM-CK or for mouse, rabbit, and human BB-CK, and the affinity was about 30 times greater than for chicken BB. Because we prepared antiserum against denatured and cross-linked MM-CK rather than against the native enzyme, one could argue that internal parts of the protein are more highly conserved than are external parts. Sequence data from a small peptide surrounding a cysteine residue required for enzymatic activity in rabbit MM-CK and BB-CK show virtual identity (64). Furthermore, since heterodimers of CK isozymes can be formed between M-CK and B-CK from widely different species, the structural features required for dimerization must be conserved as well (14). The cross-hybridization of M-CK cDNA clones with B-CK mRNA (Fig. 6 and 7) also demonstrates considerable homology between coding regions of the two isotypes, although the 3' nontranslated regions are apparently unique. Although parts of the M-CK cDNA cross-hybridized with B-CK, no other RNA species were detected on Northern blots (Fig. 8; unpublished data). This is in contrast to results with chicken M-CK, in which the 3' nontranslated region cross-hybridizes with at least one higher-molecular-weight species (32).

Since pMCK-m3' did not cross-hybridize with B-CK mRNA, it could be used to measure M-CK mRNA levels during myoblast differentiation (Fig. 9). The results agree with those of Rosenberg et al. (55) that the increase in M-CK during myogenesis is regulated by an increase in M-CK mRNA. However, it has now been possible to directly quantitate the mRNA increase as well as to demonstrate that it begins well before fusion. Our results are also similar to those reported for several other muscle-specific proteins. For example, α -actin (43, 56), myosin heavy chain (2, 65), troponin C and I (26), and α -tropomyosin (26) mRNAs are all induced during myogenesis.

The maximal M-CK mRNA level (1,100 molecules per nucleus) was within the range of values reported for the intermediate abundance class of muscle messages (51), yet it is considerably less than the peak levels of 36,000 and 50,000 mRNA molecules per nucleus reported for the major muscle proteins α -actin and myosin heavy chain (2, 56). As with M-CK, the mRNA levels for these latter two proteins decline after reaching their peaks. The observation that M-CK protein does not peak until after the decline in M-CK mRNA (compare Fig. 1 and 9) suggests a much longer half-life for the protein than for the mRNA. It is possible that a burst of mRNA synthesis serves to quickly produce required levels of muscle proteins which, once achieved, can be maintained without continual high levels of mRNA. Although an increase in mRNA levels is commonly interpreted as demonstrating transcriptional control, formal proof of such a mechanism must still be provided. In this regard, it has recently been demonstrated that deinduction of the cell cycle-specific protein thymidine kinase during mouse muscle differentiation is regulated by changes in thymidine kinase mRNA levels which occur at a posttranscriptional level (25, 44).

The high specificity of pMCK-m3' has recently been exploited to isolate the entire mouse M-CK gene (Jaynes et al., in preparation). This gene spans 11 kilobases and contains seven introns. A comparison of the cDNA and genomic clone sequences with known CK protein and cDNA sequences (5, 24, 32, 64) confirms the identity of these clones as M-CK. Isolation of the M-CK gene will greatly facilitate future studies of the mechanism of CK induction during myogenesis.

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