Multiple positive and negative elements regulate human brain creatine kinase gene expression

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ABSTRACT

We characterized the developmental expression of the brain creatine kinase (BCK) gene in the C₂C₁₂ myogenic cell line with the use of isoenzyme, Western blot, and Northern blot analyses. The results show that both BCK subunit protein and mRNA are upregulated early in myogenesis, and then downregulated in fully differentiated myotubes. To characterize the transcriptional regulatory mechanisms, a chimeric construct containing 1.2 kilobase pairs of 5'-flanking DNA from the human BCK gene placed upstream of the chloramphenicol acetyltransferase gene in the promoterless plasmid pSVOCAT was transiently transfected into C₂C₁₂ cells. In myoblasts and differentiating myotubes, the time course of expression of the constructs paralleled that of endogenous BCK mRNA. Additional constructs prepared by deleting 5'-flanking DNA were also transfected into C₂C₁₂ cells. All constructs were preferentially expressed in myoblasts relative to myotubes with absolute levels of expression increasing with deletion of 5'-flanking DNA. In nonmyogenic cells expression of the plasmids also increased with deletion of 5'-flanking DNA. An element from -1150 to -388 was isolated and found to be capable of suppressing expression of the BCK promoter and of heterologous promoters independent of orientation and position and hence to function as a silencer. Thus, BCK expression is mediated by sequences contained in the 5'-flanking DNA, including negative elements active in both C₂C₁₂ cells and nonmyogenic cells and elements that mediate the developmental expression of the BCK gene in C₂C₁₂ myogenic cells.

INTRODUCTION

During myogenesis several of the muscle-specific genes encoding enzymes, contractile proteins, and surface markers characteristic of mature skeletal muscle are transcriptionally activated (1–10). The identification of cis-acting sequences and their interacting trans-acting factors has led to a partial understanding of the mechanisms regulating the developmental expression of these genes (11–17). Along with the increase in expression of muscle-specific genes, there is a decrease in the expression of genes characteristic of immature muscle or myoblasts such as β -actin

and brain creatine kinase (BCK) (5,6,8,13,18). Little is known of the mechanisms mediating this suppression, although β -actin appears to be regulated through transcriptional control mediated by cis-acting sequences located in the 3' nontranslated region of the gene (19). However, the mechanisms controlling expression of the BCK gene have not yet been determined.

The BCK gene is expressed in a variety of tissues, including brain, heart, intestine, kidney, and uterus, cell lines such as C₂C₁₂ myoblasts, and Hep G2 cells, and in cell samples from human small cell carcinoma and prostate and breast cancers (20–24). In addition to responding to peptides and hormones, BCK gene expression is developmentally regulated (25–28). For example, as heart and skeletal muscle differentiate an isoenzyme switch occurs characterized by replacement of the embryonic BB with the mature MM isoenzyme, which is mediated at the level of mRNA abundance (13,18,29–31).

The human BCK gene has been isolated and the complete sequence including 807 base pairs of 5' flanking DNA upstream from the CAP site has been reported (32,33,34). Sequence identity between the 5' upstream regions of the human and rat BCK genes and the adenovirus E1A promoter has been noted (22,32). The transcription start site has been identified in two cell lines (32,33). Sequence analysis of the human BCK gene has revealed two CAAT boxes, two TATA boxes, and three SpI binding sites located within 150 base pairs upstream of the cap site (33). The level of BCK gene expression in *in vitro* transcription reactions is determined by the specific TATA box utilized by the RNA polymerase II initiation complex (35).

Striking sequence identity within the 3' nontranslated region of the BCK cDNA has been observed across species (22,33,36). Recent results demonstrate that the 3' nontranslated region is important in posttranscriptional control of BCK expression in human U937 histiocytic cells (37,38). Thus, the regulation of BCK gene expression is mediated by cis-acting sequences located in the 5' upstream and 3' nontranslated regions of the gene.

To further define the mechanisms regulating expression of the BCK gene, we characterized BCK expression in different cell lines by CK isoenzyme, Northern blot, and Western blot analyses; cloned the human gene; and characterized the 5'-flanking region of the gene. Our results show that BCK gene expression is regulated by multiple elements located in the 5'-flanking region of the gene, including a differentiation specific element and a silencer that is capable of suppressing BCK gene expression in both myogenic and nonmyogenic cell lines.

MATERIALS AND METHODS

Isolation and cloning of the human brain creatine kinase gene

A human genomic DNA library containing size-fractionated DNA cloned into charon 4A was obtained from the ATCC (No. 37333) (39). Approximately 5×10^5 plaques were screened in duplicate at a density of 5×10^4 plaques/150 mm plate (40). The plaque lifts were hybridized with a ^{32}P -labeled probe derived from a human BCK cDNA isolated in our laboratory by standard methods. Selected plaques were purified to homogeneity. One full-length 12.7 kilobase BCK genomic clone (BI) was characterized by extensive restriction enzyme and Southern blot analysis. Results of DNA sequence analysis of specific genomic fragments were then compared with the published sequence (33,34).

Preparation of probes

Mouse CK cDNAs were isolated in our laboratory by screening a λ gt11 library constructed from RNA extracted from C_2C_{12} myotubes. The sequences of the cDNA clones were compared with the published sequences of mouse muscle (M) (41) and mouse B (42) CK cDNA. Glyceraldehyde-3-phosphate dehydrogenase cDNA (No. 57091) was obtained from the American Type Culture Collection (43). Probes from the 3' nontranslated region of mouse BCK (76 base pairs) and mouse MCK (100 base pairs) cDNAs were gel purified and isolated by electroelution after digestion with *EcoRI*/*PstI* and *SmaI*, respectively. A glyceraldehyde-3-phosphate dehydrogenase cDNA probe (600 base pairs) was gel purified and isolated by batch affinity absorption with sodium iodide glass beads (Geneclean, Bio 101, Inc.) after digestion with *XbaI*/*HindIII*. The probes were radiolabeled with [α - ^{32}P]dCTP (Amersham Corp.) to a specific activity of 1 to 2×10^9 dpm/ μg by the random primer method of Feinberg and Vogelstein (44).

DNA sequence determination

Select restriction fragments of the original genomic clone, including 5' and 3' flanking regions, were subcloned into Bluescript vectors for further restriction enzyme mapping and DNA sequencing. Double-stranded DNA sequencing was performed by dideoxynucleotide chain termination with [α - ^{35}S]dATP (Amersham Corp.) and Sequenase (United States Biochemical) (45). Oligonucleotide primers were synthesized on an Applied Biosystems 380B automated synthesizer in the Protein Chemistry Laboratory at Washington University.

Cells and cell culture

The mouse skeletal muscle cell line C_2C_{12} (No. CRL1772) (46), mouse fibroblast cell line NIH3T3 (No. CRL1651), human cervical carcinoma cell line HeLa (No. CCL2), and the human hepatoma cell line Hep G2 (No. HB8065) (47) were obtained from the ATCC. C_2C_{12} cells were maintained in an atmosphere of 8% $\text{CO}_2/92\%$ air in growth media (Dulbecco's modified Eagle's medium [DMEM] supplemented with 20% fetal calf serum, penicillin [50 $\mu\text{g}/\text{ml}$], and streptomycin [50 $\mu\text{g}/\text{ml}$]) or differentiation media (DMEM supplemented with 10% horse serum, penicillin [50 $\mu\text{g}/\text{ml}$], and streptomycin [50 $\mu\text{g}/\text{ml}$]). NIH3T3 cells were maintained under the same conditions in DMEM plus 10% calf serum. Hep G2 cells were maintained in 5% $\text{CO}_2/95\%$ air in growth media (minimum essential medium with 10% NuSerum [Collaborative Research], Earle's salts, L-glutamine [2 μM], penicillin [50 $\mu\text{g}/\text{ml}$], and streptomycin [50

$\mu\text{g}/\text{ml}$]). HeLa cells were maintained in 5% $\text{CO}_2/95\%$ air in minimum essential medium supplemented with 10% fetal calf serum, Earle's salts, L-glutamine (2 μM), penicillin (50 $\mu\text{g}/\text{ml}$), and streptomycin (50 $\mu\text{g}/\text{ml}$). C_2C_{12} myoblasts were harvested at 80% confluence and C_2C_{12} myotubes were harvested at specified times after switching to differentiation media. Hep G2 cells used for transfection experiments were fed the day of transfection with DMEM/Ham's nutrient F-12 with HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) supplemented with 10% NuSerum and refed with growth medium after transfection. All cell lines were free of mycoplasma contamination as determined by the Gen-Probe Mycoplasma Tissue Culture II Rapid Detection Kit (Fisher).

Northern blot hybridization

Total cellular RNA from C_2C_{12} myoblasts and myotubes was isolated by a single step acid guanidinium thiocyanate-phenol-chloroform extraction with RNAzol B (Cinna/Biotech) (48). RNA was quantified by absorbance at 260 nm and integrity was assessed by electrophoresis in 1.2% agarose gels after denaturation with methyl mercuric hydroxide. Ten micrograms of RNA were fractionated on 1.5% formaldehyde agarose gels and transferred to Genescreen membranes (DuPont-New England Nuclear) (49). Northern blots were prehybridized at 42°C in a solution of 50% formamide, 1M NaCl, 1% sodium dodecyl sulfate (SDS), 0.1% sodium pyrophosphate (NAPPi), 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% ficoll, 0.05 M Tris-HCl, pH 7.5, 10% dextran sulfate, and 100 $\mu\text{g}/\text{ml}$ denatured sonicated salmon sperm DNA. Hybridization with [^{32}P]-labeled probes at a concentration of 3 to 6×10^5 dpm/ml was performed at 42°C for 16 to 20 hours in the same solution. Membranes were washed twice for 5 minutes at room temperature with $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) with 1% SDS and then once in the same solution for 20 to 30 minutes at 68°C . Autoradiograms were prepared with Kodak XAR-5 film with the use of Cronex intensifying screens at -70°C . Blots were quantified by laser densitometry (Ultrascan XL, LKB) or radioisotopic scanning (Ambis scanner, Automated Microbiology Systems, Inc.). MCK and BCK signals were normalized to the glyceraldehyde-3-phosphate dehydrogenase signal to correct for unequal loading or transfer of RNA. To prepare blots for rehybridization, membranes were stripped of probe by boiling for 20 minutes in a solution of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS.

Western blot analysis

C_2C_{12} myoblasts, C_2C_{12} myotubes, Hep G2 cells, and NIH3T3 cells were washed with phosphate-buffered saline, harvested with a cell scraper, pelleted by centrifugation at 1500 rpm for 10 minutes, and stored at -80°C .

Pellets were resuspended in a solution of 50 mM Tris-HCl, pH 8.5, 5 mM 2-mercaptoethanol, 1% triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 $\mu\text{g}/\text{ml}$ antipain, and 5 $\mu\text{g}/\text{ml}$ leupeptin; incubated for 10 minutes at 4°C ; and centrifuged for 2 minutes. The protein content of the supernatant was quantified by standard Bradford microassay (50).

Western blots were performed essentially as described (51). An aliquot of supernatant containing 100 μg of protein was fractionated by 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher and Schuell). The membranes were incubated with a 1:200 dilution of a goat

antihuman BB CK antibody (Pel-Freez), which cross-reacts with mouse M and B subunits. The immune complexes were detected with rabbit anti-goat immunoglobulin G (IgG) (Pel-Freez), labeled with Na[¹²⁵I] as described (52). Relative M and B subunit mass was determined by scanning laser densitometry of autoradiograms with signal intensities shown to be in the linear range by comparison with a standard curve generated by analysis of known amounts of MB CK by Western blot.

Analysis of creatine kinase isoenzyme activity

Total CK activity in the cell supernatant fraction was determined with the use of a miniature centrifugal analyzer (Gemeni). CK isoenzymes in the supernatant fraction were separated by anion-exchange chromatography with the use of fast protein liquid chromatography (FPLC) and a high-resolution column (Mono Q HR 5/5, Pharmacia), as described previously (53). The column was equilibrated with 20 mM Tris-HCl, pH 8.4. A sample of supernatant containing CK activity of 1000 to 2000 mIU (*C₂C₁₂* cells), 200 mIU (Hep G2), or 40 mIU (NIH3T3) was applied to the column and eluted with a linear gradient of NaCl from 0 to 400 mM (10 mM/ml) in 20 mM Tris-HCl, pH 8.4, containing 5 mM 2-mercaptoethanol. CK activity in the column effluent was assessed on-line by incubating the effluent at 37°C with substrates (CK S.V.R., Behring Diagnostics, San Diego, CA) for the coupled enzyme assay of CK yielding NADPH and monitoring of absorbance at 340 nm as described previously (54). The relative proportions of activity of each individual isoenzyme were expressed as percentages of the total area under the absorbance curve.

Cell transfection and chloramphenicol acetyltransferase assays

The plasmid pMSV β -gal, which contains the β -galactosidase gene under the control of the Maloney murine sarcoma virus long terminal repeat, was generously provided by Nadia Rosenthal (Boston University). The plasmids pSVOCAT, pSV2CAT, and pRSVCAT were obtained from the ATCC (55,56). The chimeric constructs MCKCAT2620 and MCKCAT935 comprising the human MCK 5' upstream DNA were previously prepared in our laboratory (16). Unless indicated, *C₂C₁₂* cells were plated 24 hours before transfection at 1.5×10^5 cells per 60 mm dish in 3 ml of growth media. Transfections were performed by the calcium phosphate coprecipitation method (57). Precipitates contained a total of 20 μ g of DNA comprised of 15 μ g of test plasmid and 5 μ g of pMSV β gal used as an internal standard to correct for transfection efficiency and yield of cell extract. After a 4 hour incubation with the precipitate, cells were subjected to a 3 minute glycerol shock. They were then washed with Optimem (Gibco) and fed with growth media. Unless otherwise noted, half the dishes were harvested 48 hours later as myoblasts and the remainder were fed with 3 ml of differentiation media and harvested 60 hours later as fully differentiated myotubes. NIH3T3, HeLa, and Hep G2 cells were plated at a density of 3 to 5×10^5 cells per 60 mm dish, transfected as described above, and harvested 48 hours after transfection unless otherwise noted. Cell extracts were prepared and assays for β -galactosidase and chloramphenicol acetyltransferase were performed as described previously (16,57,58). The volume of extract used for chloramphenicol acetyltransferase assays was based on the results of the β -galactosidase assay used as an internal standard and varied from 6 to 50 μ l.

Construction of BCK-chloramphenicol acetyltransferase (CAT) chimeras

A 1230 base pair *BalI*/*SmaI* fragment containing 1150 base pairs of 5'-flanking DNA, the untranslated first exon, and 10 base pairs of the first intron was isolated from clone BI. *KpnI* linkers were added and the fragment was inserted into a *KpnI* site prepared by filling in the unique *HindIII* site of pSVOCAT with the large fragment (Klenow) of *Escherichia coli* DNA polymerase I (BRL) and adding *KpnI* Linkers. This construct is designated BCKCAT1150. Convenient restriction enzyme sites at -388 base pairs (*HindIII*), -362 base pairs (*SacII*), and -92 base pairs (*SacII*) relative to the cap site were used to prepare two promoter deletion constructs of BCKCAT1150 with identical 3'-ends. The *HindIII*/*SmaI* fragment was inserted into the unique *HindIII* site 5' of the CAT gene in pSVOCAT with the use of *HindIII* linkers and is referred to as BCKCAT388. A partial *SacII* digest of this construct deleted the DNA sequences from -362 to -92 base pairs relative to the cap site. The plasmid was then ligated to produce the BCKCAT92 chimeric construct. A fragment containing sequences from -1150 to -388 was inserted in both orientations into the unique *BamHI* site 3' of the CAT gene in the BCKCAT92 construct with the use of *BamHI* linkers. These constructs are referred to as BCKCAT silencer (sense) and BCKCAT silencer (antisense). The -1150 to -388 fragment was also inserted into the unique *BamHI* site 3' of the CAT gene in the MCKCAT935 chimeric construct. These two chimeras are named MCKCAT935 silencer(sense) and MCKCAT935 silencer (antisense).

The orientation of all constructs was confirmed by DNA sequence analysis across the *BamHI*, *HindIII*, or *KpnI* sites. Plasmid DNA isolated by standard methods was greater than 50% supercoiled and free of RNA or other contaminants as assessed by electrophoresis in 0.75% agarose gels and staining with ethidium bromide.

RESULTS

Isolation and characterization of the human brain creatine kinase gene

One clone (BI) containing the human BCK gene was isolated and characterized by extensive restriction enzyme mapping and Southern blot analysis (Figure 1). The first two exons, the eighth

Figure 1. Partial restriction map and sequencing strategy of the human BCK gene. A partial restriction map of the 12.7 kilobase clone BI is shown. Exons are depicted as boxes with introns and flanking regions as a thin line. Coding regions are solid; untranslated regions are cross-hatched. The sequencing strategy is presented as horizontal arrows.

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-1153      TGGCCACTCCCGCTGATGGGCGATCGGGTGTATCCAGTTATGTTGTTTTCCCTCCA      -1093
-1092      CAGATCTCTTGGTCCATTAGTGGAGAGAACTGAGACATCTTTCTAAGGGCTGCATTCT      -1033
-1032      AGAAGGGGAGATTGCTGGGTACGGAGTTCGAACATTGTTAAGCTTAATAGATTCTGCCA      -973
-972      AATTATCTTGCAAAAAGCATGCAGCAATTTAAGTTGCAGGCAGCGTGGACGCGCTGTCT      -913
-912      TCCCGCATTCCAGCTCAAAGCCAGCAATGGCTGGGGTCGATCTGGGAGTGTGGGGGTAG      -853
-852      GGCAGGTGGAGTCACTGCTTAATTTGCATGATTCTGGTTATTCGTGA      -805
          GGGCAGGTGGAGTCACTGCTTAATTTGCATGATTCTGGTTATTCGTGA
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Figure 2. Nucleotide sequence of BCK 5'-flanking DNA. DNA sequence was determined by the strategy depicted in Figure 1. The nucleotide sequence shown represents 5'-flanking DNA extending from -805 to -1150 relative to the cap site. * Oct-1 consensus sequence.

exon, and nucleotides from -1150 to +80 relative to the cap site were sequenced and compared with the published sequence (33,34). The clone spans 12.7 kilobase pairs of DNA and contains the 3.2 kilobase BCK gene, approximately 1.8 kilobase pairs of 5'-flanking DNA, and 7.7 kilobase pairs of 3'-flanking DNA. There were no differences between the sequenced fragments of our genomic clone and the previously published sequence for the human BCK gene. The 348 nucleotides of 5'-flanking DNA from -1153 to -805 relative to the CAP site and not included in the published sequence, are shown in Figure 2 because this region is part of a functionally important element (see below).

Developmental expression of brain creatine kinase in the C₂C₁₂ myoblast line

To characterize the regulatory program of the BCK gene in C₂C₁₂ cells, we performed analyses of isoenzymes of CK, M and B subunit protein, and M and B subunit mRNA from C₂C₁₂ myoblasts and myotubes undergoing differentiation.

BCK and MCK subunits combine to form the homodimeric BB, MM, and heterodimeric MB isoenzymes. To characterize the contribution of the three isoenzymes to total CK activity in C₂C₁₂ myoblasts and myotubes, CK isoenzyme analysis was performed on cell extracts from C₂C₁₂ myoblasts harvested at 80% confluence, and C₂C₁₂ myotubes harvested 24, 72, 96, and 120 hours after media switch. The results of analysis of three time course experiments showed that BB was the predominant isoenzyme (86% of total CK activity) in myoblasts (0 hour) and MM was the predominant isoenzyme (51% of total CK activity) in myotubes at 120 hours (Figure 3). The activity of both the BB and MB isoenzymes increased during the initial 96 hours of differentiation before decreasing in fully mature myotubes at 120 hours, indicating accumulation of B subunit protein in the initial stages of myogenesis. The contribution of both the BB and MB isoenzymes to total CK activity decreased during the final stages of differentiation as the MM isoenzyme was induced. In contrast, the MM isoenzyme was present in only small amounts in myoblasts and MM CK activity increased throughout myotube differentiation. CK isoenzyme analysis of cell extracts from NIH3T3 and Hep G2 cells harvested 48 hours after plating was also performed by FPLC. BB was the only cytoplasmic isoenzyme of CK detected in extracts of these cells (data not shown).

Because analysis of enzymatic activity of CK isoenzymes suggested accumulation of BCK subunit protein during the early stages of myogenesis in C₂C₁₂ cells, Western blot analysis was performed on cell extracts from C₂C₁₂ myoblasts harvested at 80% confluence and C₂C₁₂ myotubes harvested 24, 72, 96, and

Figure 3. CK isoenzyme analysis of C₂C₁₂ cells undergoing differentiation. Total CK activity in extracts of C₂C₁₂ myoblasts and C₂C₁₂ myotubes was determined with the use of a miniature centrifugal analyzer. For each time point, 1000 mIU of total CK activity was separated into isoenzyme fractions with the use of FPLC. Select stages from a representative experiment selected from three time course studies are shown. Dashed line represents NaCl concentration (mM).

120 hours after changing to differentiation media. M and B subunit protein was identified with the use of an antibody that cross-reacts with mouse M and B subunits. BCK subunit protein was present in myoblasts (0 hour) and gradually increased, peaking at 96 hours before decreasing at 120 hours (Figure 4). The MCK subunit was also present in myoblasts but increased throughout differentiation. Thus, changes in CK isoenzyme composition in differentiating C₂C₁₂ cells are mediated by accumulation of BCK and MCK subunit protein. Western blot analysis performed on extracts of NIH3T3 and Hep G2 cells detected only the B subunit, consistent with results of isoenzyme analysis (data not shown).

To determine whether the CK isoenzyme switch in C₂C₁₂ cells undergoing differentiation is regulated at the level of mRNA abundance, total cellular RNA for Northern blot analysis was isolated from C₂C₁₂ myoblasts harvested at 80% confluence and C₂C₁₂ myotubes harvested at 24, 48, 72, and 120 hours of differentiation. Membranes were probed, stripped, and reprobed with M and B subunit-specific probes derived from the 3'-non-translated regions of mouse M and BCK cDNA clones, which show no sequence identity. The glyceraldehyde-3-phosphate dehydrogenase cDNA probe was used to normalize for unequal loading of RNA and transfer to nitrocellulose membranes because expression of glyceraldehyde-3-phosphate dehydrogenase mRNA does not change during differentiation of C₂C₁₂ cells. MCK mRNA was not detected at 24 hours after media switch, (data not shown) was first seen at 48 hours and subsequently increased throughout differentiation. In contradistinction, BCK mRNA was present in myoblasts, increased five-fold 48 hours after media switch, and then gradually decreased in differentiating myotubes to a final level comparable to that seen in myoblasts (Figure 5).

Figure 4. Expression of B and M subunit protein in differentiating C_2C_{12} cells. Western blots were prepared with an aliquot of cell extract containing 100 μ g of protein obtained from C_2C_{12} myoblasts harvested at 80% confluence (0 hour) and C_2C_{12} myotubes harvested 24, 72, 96, and 120 hours after switching to differentiation media. Blots were probed with goat antihuman BB CK antibody and the immune complexes were detected with rabbit anti-goat IgG. M and B subunits were quantified by scanning laser densitometry of autoradiograms. The results of a representative time course from six Western blot experiments are shown. AU = arbitrary units derived from measurement of peak height by densitometry.

Figure 5. Expression of BCK and MCK mRNA in differentiating C_2C_{12} cells. Northern blots were prepared with 10 μ g of total cellular RNA isolated from C_2C_{12} myoblasts harvested at 80% confluence (0 hour) and C_2C_{12} myotubes harvested 48, 72, and 120 hours after switching to differentiation media. The three panels labeled B, M, and GAP are autoradiograms of the same blot hybridized, stripped, and re-probed consecutively with subunit specific probes from the 3'-untranslated region of mouse BCK and MCK cDNA and the glyceraldehyde-3-phosphate dehydrogenase cDNA probe. The blot was quantified by laser densitometry (BCK and MCK) or radioisotopic scanning (GAP). The results of a representative Northern blot are shown above the bar graphs. The bar graphs represent pooled data from five experiments, with normalization of the MCK and BCK mRNA signals to the GAP signal. AU = arbitrary (densitometric) units.

These results show that BCK and MCK subunit accumulation is regulated at the level of mRNA abundance, with subunit protein lagging mRNA expression by 24 to 48 hours. Although induction of MCK mRNA during differentiation of myogenic cell lines in

Figure 6. Time course of expression of BCKCAT and MCKCAT constructs in C_2C_{12} cells undergoing differentiation. BCKCAT1150, pSVOCAT, and MCKCAT2620 were transiently transfected into C_2C_{12} myoblasts. Cells were harvested 24 hours later or were refed with differentiation media or growth media and harvested 48, 72, 96, and 108 hours after transfection. The time course of expression of the plasmids BCKCAT1150 (B) and MCKCAT2620 (M) in growth (g) or differentiation (d) media is shown. Experimental points represent pooled data from 10 to 12 plates of cells transfected, with each construct representing five separate transfection experiments with different batches of cells. Induction in arbitrary units (AU) is relative to expression of pSVOCAT.

culture has been described (16,59), the regulatory program of BCK mRNA appears to be more complex than had previously been assumed (60). Rather than simply being downregulated with differentiation, BCK mRNA is upregulated during the first 48 hours of myogenic differentiation and then downregulated during the later stages of myogenesis.

Regulation of the brain creatine kinase gene

To determine whether 5'-upstream sequences of the BCK gene are capable of transcriptionally controlling its developmentally regulated expression, the BCKCAT1150 chimeric construct was transiently transfected into C_2C_{12} myoblasts. We characterized the time course of expression of the plasmid BCKCAT1150 in C_2C_{12} myoblasts and myotubes. The promoterless plasmid pSVOCAT was used as a background reference and pSV2CAT and pRSVCAT were positive controls. The construct MCKCAT2620 is developmentally regulated in C_2C_{12} myotubes and was therefore used as a differentiation specific control (16). The constructs were transfected into C_2C_{12} myoblasts plated at 3×10^5 cells per dish. These cells were harvested 24 hours after transfection or refed with either growth media, to inhibit differentiation, or differentiation media, to reproduce the conditions of our Northern blot experiments (see above), and harvested at 48, 72, 96, and 108 hours after transfection. The plasmid pSVOCAT exhibited minimal expression in myoblasts and differentiating myotubes and showed no change with differentiation. After transfection with pSV2CAT and pRSVCAT, CAT activity in all cell extracts increased 25- to 35-fold relative to pSVOCAT (data not shown). As shown in Figure 6, results of CAT assays performed on extracts from cells maintained in differentiation media showed that expression of the plasmid MCKCAT2620 was below background at 24 hours but well expressed 48 hours after transfection, with expression increasing throughout the time course. In cells maintained in growth media, MCKCAT2620 was not expressed until 72 hours after transfection, with expression increasing to 2.9-fold over

Figure 7. Promoter deletion analysis of the human BCK gene. A *Bal*I/*Sma*I 1.2 kilobase BCK genomic fragment was placed upstream of the CAT gene in the promoterless plasmid pSVOCAT. This chimeric construct is designated BCKCAT1150. The unique *Hind*III site located at -388 base pairs relative to the cap site of the BCK gene was used to prepare the BCKCAT388 chimera. *Sac*II sites at -362 and -92 were used to prepare the chimeric construct BCKCAT92. The plasmid MCKCAT2620 was previously prepared in our laboratory (51). C_2C_{12} myoblasts were transfected with the calcium phosphate coprecipitation method. Myoblasts were harvested 48 hours after transfection. Cells switched to differentiation media 48 hours after transfection and harvested 60 hours later as myotubes. Cell extracts were assayed for [^{14}C]chloramphenicol conversion. Results are shown in the accompanying table. The first column represents the percent [^{14}C]chloramphenicol converted to acetylated products. Induction represents the CAT activity present in extracts of cells transfected with the chimeric plasmids relative to pSVOCAT. N is the total number of dishes of cells transfected with each construct. At least eight separate transfection experiments were performed by use of at least two preparations of each plasmid and with different batches of C_2C_{12} cells. The hatched boxes represent sequences contained in the untranslated first exon. Except for the CAT gene, figures are drawn to scale.

background at 108 hours as the cells became confluent and showed evidence of biochemical differentiation (increase in total CK/ μ g cell protein, data not shown). In contrast, expression of the plasmid BCKCAT1150 in cells maintained in differentiation media was above background 24 hours after transfection, increased to peak expression 48 hours after transfection, and then gradually declined, resembling very closely the time course of BCK mRNA expression in C_2C_{12} cells undergoing differentiation. A similar pattern of expression for BCKCAT1150 (but with peak expression delayed to 72 hours) occurred in cells maintained in growth media. Therefore, the 5'-flanking DNA between -1150 and $+80$ of the BCK gene confers a regulatory pattern of expression to chimeric plasmids that closely resembles the time course of expression of the endogenous BCK gene in C_2C_{12} cells undergoing differentiation. These results also demonstrate that C_2C_{12} myoblasts maintained in growth media show evidence of biochemical differentiation when the cells become confluent, as demonstrated by expression of MCKCAT2620 and downregulation of BCKCAT1150. Accordingly, myoblasts were maintained in growth media and harvested before reaching confluence 48 hours after transfection and myotubes were harvested 108 hours after transfection to maximize expression of myoblast and myotube-specific proteins.

The 5'-flanking DNA of the BCK gene was further characterized by promoter deletion analysis (Figure 7). Transfection with BCKCAT1150 resulted in a 2.7-fold increase in CAT activity relative to pSVOCAT in C_2C_{12} myoblasts. In C_2C_{12} cells transfected with BCKCAT1150 and harvested as myotubes, CAT expression was less than pSVOCAT. Transfection with BCKCAT388 resulted in an 8.7-fold increase in CAT activity in myoblasts, whereas CAT activity was only 2.9-fold over background in myotubes. BCKCAT92 was expressed 14.6-fold over background in myoblasts and 5.8-fold in myotubes. These results show that the expression of BCKCAT chimeric constructs in myoblasts and myotubes increased with

deletion of 5'-flanking DNA, suggesting the presence of two negative elements. These data also demonstrate that all three BCKCAT chimeric constructs were preferentially expressed in myoblasts relative to myotubes. When expression of each construct in myoblasts and myotubes was compared, the magnitude of the decrease in expression with differentiation was similar for all BCKCAT chimeric constructs, with expression in myotubes approximately 30% of that seen in myoblasts. Therefore, the differentiation-specific decrease in expression of the BCKCAT constructs is mediated by sequences contained in the smallest (BCKCAT92) plasmid.

To determine whether downregulation of the BCKCAT constructs with time after transfection of C_2C_{12} cells was due to differentiation, the constructs BCKCAT1150 and BCKCAT92 were transiently transfected into HeLa cells, a nonmyogenic cell line that expresses BCK (see above). Cells were harvested at 48 and 108 hours after transfection. Transfection with BCKCAT1150 resulted in CAT activity 2.4-fold above background in cells harvested at 48 hours after transfection and four-fold above background in cells harvested at 108 hours after transfection (Table 1). The construct BCKCAT92 was expressed 14.1-fold over background at 48 hours and 20.3-fold above background at 108 hours. The increase in expression of the BCKCAT constructs with time after transfection of HeLa cells contrasts with the decrease in expression in C_2C_{12} cells undergoing differentiation after transfection. Hence, the decrease in expression of BCKCAT constructs in C_2C_{12} cells is due to differentiation and is mediated by sequences contained in the BCKCAT92 construct.

Two other nonmyogenic cell lines that express BCK, NIH3T3 and Hep G2, were also used to evaluate expression of BCKCAT constructs (Table 1). Cells were harvested 48 hours after transfection for CAT assays. Results of transient transfection of NIH3T3 and Hep G2 cells show that expression of chimeric BCKCAT constructs increased with deletion of 5'-flanking DNA,

Table 1. Transfection of BCK Promoter Constructs in HeLa, HepG2, and NIH3T3 cells.

	HeLa				HepG2 (48 hours*)		NIH3T3 (48 hours*)	
	48 hour* % Conversion	Induction	108 hour* % Conversion	Induction	% Conversion	Induction	% Conversion	Induction
pSVOCAT	0.9	—	1.1	—	2.5	—	1.3	—
BCKCAT 1150	2.2	2.4	4.3	4.0	2.6	1.1	1.5	1.2
BCKCAT 388	—	—	—	—	32	7.1	5.6	4.3
BCKCAT 92	12.7	14.1	22.3	20.3	74	29.6	8.3	6.4
BCKCATsilencer (sense)	2.1	5.3	7.6	7.6	18.2	7.3	—	—

Percent conversion [^{14}C]chloramphenicol and induction are as described in the legend to Figure 7 and represent pooled data from at least three transfection experiments.
 *Represents time of harvest (in hours) after transfection.

Figure 8. A BCK silencer studies. A fragment of 5'-flanking DNA from -1150 to -388 relative to the cap site of the BCK gene was isolated and placed in both orientations in the unique BamHI site located 3' of the CAT gene in the chimeric construct BCKCAT92. Paired dishes of C_2C_{12} myoblasts were transfected and harvested as myoblasts or myotubes as discussed in the legend to Figure 7 and in the Methods section. Induction represents expression of each plasmid relative to pSVOCAT. Inhibition represents the decrease in CAT activity in extracts prepared from cells transfected with BCKCAT silencer constructs relative to that of cells transfected with the parent construct, BCKCAT92. N represents the number of plates of cells transfected with each construct. Three to five separate transfection experiments were performed. Except for the CAT gene and silencer cartridges, figures are drawn to scale. B: MCK silencer studies. A fragment of 5'-flanking DNA from -1150 to -388 relative to the cap site of the BCK gene was placed in both orientations in the unique BamHI site located 3' of the CAT gene in the chimeric construct MCKCAT935. C_2C_{12} myoblasts were transfected and harvested as early myotubes or late myotubes as described in the text.

as seen after transfection of HeLa cells. Thus, both negative elements function in nonmyogenic cell lines as well as in myogenic cell lines.

To determine whether the negative regulatory element from -1150 to -388 could function in a position- and orientation-independent fashion, we placed this element 3' of the CAT gene in the construct BCKCAT92, which is expressed at relatively high levels in all cells lines tested. The plasmids pSVOCAT, BCKCAT92, and the two BCKCAT silencer constructs were transiently transfected into C_2C_{12} myoblasts. As shown in Figure 8, panel A, the presence of this regulatory element 3' to the CAT gene in either orientation resulted in marked inhibition of expression of BCKCAT92. Similar results were obtained in

C_2C_{12} myotubes. In transiently transfected Hep G2 and HeLa cells, BCKCAT silencer (sense) also showed reduced expression relative to BCKCAT92 (Table 1). Hence, the sequence elements from -1150 to -388 have the properties of a transcriptional silencer. To determine whether the BCK silencer was also capable of suppressing expression of a heterologous promoter, we placed the BCK silencer 3' of the CAT gene in the plasmid MCKCAT935, which is developmentally regulated in C_2C_{12} myotubes in a fashion similar to MCKCAT2620. The plasmids pSVOCAT and MCKCAT935 and the two MCKCAT935 silencer constructs were transfected into C_2C_{12} myoblasts. C_2C_{12} myoblasts were plated at 3.0×10^5 cells per plate, transfected, and cells were harvested either 24 hours (early myotubes) or 72

hours (late myotubes) after the cells were fed with differentiation media. As shown in Figure 8 panel B, the activity of the MCKCAT935 construct doubled when expression in early and late myotubes was compared. The silencer element -1150 to -388 downregulated the developmental expression of MCKCAT935 in a position- and orientation-independent fashion. Interestingly, inhibition of expression of MCKCAT constructs was more marked in late than in early myotubes at a time when expression of the MCKCAT935 construct was increasing indicating that the silencer more effectively inhibits late events in the developmentally regulated expression of MCK.

DISCUSSION

In skeletal muscle (61), heart (62) and myogenic cell lines undergoing differentiation in culture (63) the CK isoenzyme shift is characterized by gradual transition from the embryonic BB form through the hybrid MB form to the adult type MM isoenzyme indicating that both M and B subunits are probably up-regulated early in development. Recently, with the use of M and B subunit-specific cDNA probes we showed that B subunit mRNA and M subunit mRNA are coordinately up-regulated in rat hearts during early development while at late stages of development the B subunit mRNA shows marked down-regulation at a time when M subunit mRNA continues to accumulate (18). A similar pattern of M and B subunit mRNA expression is seen in developing skeletal muscle, although the timing of the isoenzyme switch is different than in heart, occurring later in development (18). Therefore, the developmental regulation of expression of the BCK gene in muscle and heart is complex. The cis-acting sequence elements that mediate this complex pattern of regulation of the BCK gene during myogenesis have not been characterized. As an initial step in determining the molecular mechanisms that regulate expression of the BCK gene we characterized expression of the CK isoenzymes, M and B subunit protein and M and B subunit mRNA in C₂C₁₂ cells undergoing differentiation in culture. Our results show that M and B subunit mRNAs are coordinately upregulated during early development resulting in accumulation of M and B subunit protein and expression of MM, MB and BB isoenzymes while at late stages of differentiation the BCK gene is selectively turned off. The CK isoenzyme transition may have evolved in sarcomeric tissues because of energy requirements met by the unique ability of MM CK to localize to M bands during myofibrillar maturation where the enzyme is thought to serve as an intramyofibrillar ATP regenerating system (64). The need, if any, for accumulation of BCK subunits during the early stages of myogenesis is unknown. Upregulation of BCK may represent a developmental program that muscle cells retain in common with cells that do not demonstrate the CK isoenzyme switch and continue to express BCK.

Our results show that 5'-flanking DNA between -1150 and +80 of the human BCK gene confers a regulated pattern of expression to chimeric plasmids that closely resembles the time course of expression of the endogenous BCK gene in C₂C₁₂ cells undergoing differentiation. Within this segment of the BCK gene there are several candidate sequence motifs that may modulate the coordinate activation of the BCK and MCK genes. Myogenic factors including MyoD and myogenin form heterodimers with E12, the ubiquitous immunoglobulin enhancer-binding factor, and bind to a consensus sequence CANNTG (E-box or MEF-1 motif). The E-box motif is present in two copies in the MCK gene

enhancer representing an essential component mediating developmental regulation of the MCK gene (65,66). Two E-box motifs are also present in the BCK gene; CACTTG at -653 to -648 (34) and CAGGTG at -849 to -844 (see Figure 2). In addition, a motif flanking -862 of the human MCK enhancer (Billadello, J., unpublished) that we have found to be essential for full expression in C₂C₁₂ myotubes (CCCGAGAC) is present at -596 to -589 in the BCK gene (34).

The rat BCK gene contains an AT-rich sequence (TATAAATA) at -60 that binds a factor called TA-rich recognition protein (TARP) that is expressed in both muscle and non-muscle cells and may be a positive acting transcription factor for the BCK gene (22,67). An identical motif is present in the human BCK gene at -66 to -59 (32,33). The trans-acting factor TARP interacts with AT-rich sequences in both the MCK gene enhancer (also referred to as the MEF-2 binding site (68)) and the BCK 5' upstream regulatory region. In addition, the TARP recognition sequence from the BCK gene and the AT-rich sequence from the MCK gene enhancer can substitute for each other functionally in transfection experiments (67). Hence, these sequences may represent common regulatory elements shared between the two genes (67). Alternatively, these two motifs may differ in their relative affinities for trans-acting factors (69), the MCK sequences binding the myotube-specific factor MEF-2 preferentially and the BCK sequences ubiquitous factors (69) that may include TARP (22,67). Perhaps competition between TARP and MEF-2 for binding to the TA-rich sequence in the BCK gene in myotubes results in down-regulation of the BCK gene while nonmuscle tissues and myoblasts that express TARP but not MEF-2 continue to express BCK. Perhaps TARP and other ubiquitous factors expressed in muscle and nonmuscle cells mediate the early increase in expression of the BCK gene during myogenesis while myotube specific factors such as MEF-2 mediate downregulation during the late stages of myogenesis.

The cis-acting elements present in BCKCAT92 are also capable of downregulating expression with myogenic differentiation, yet they produce a consistently high level of expression in nonmyogenic cell lines that normally express BCK. The effect of this cis-acting element is unique because no other well-expressed positive regulatory element reported is also capable of mediating a differentiation-specific decrease in expression in muscle. A recent report shows that a single cis-element can mediate different effects on expression of linked promoters, depending on physiologic context (70). The critical cis-acting sequences present within -92 to +80 of the BCK gene are unknown, but several sequence motifs of potential importance are present. This region is very rich in GC and contains an SpI binding site and two CAAT boxes. SpI binding sites and CAAT boxes increase transcription *in vitro* via their respective trans-acting binding factors SpI and CTF-1 (71-73). Recent evidence suggests that SpI is also capable of decreasing transcription (74). GC-rich sequences are ubiquitous and are found in many housekeeping genes and cellular oncogenes (75-78). Several factors have been reported to enhance transcription through binding to GC-rich regions (79,80). In addition, negative trans-acting factors that recognize these regions have been reported. One such factor, GCF, has recently been cloned and characterized (81). This factor recognizes the consensus sequence NNG/CCG/CG/CG/CGCN and downregulates expression of β -actin and the receptor for epidermal growth factor (EGFR) by binding to the β -actin and EGFR GCF binding sites. The BCK gene contains a sequence GCGCGGCC at -92 to -83 that

resembles both the GCF consensus sequence and the GCF binding site of EGFR. In addition, the sequence CCCGCCCGC at +47 to +57 is the complement of the GCF binding site of β -actin. We can therefore speculate that SpI, GCF, or other uncharacterized trans-acting factors expressed as myotubes differentiate mediate the developmentally regulated decrease in expression of the BCK gene through these cis-acting elements.

The negative element at -1150 to -388 suppressed the expression of a construct containing its cognate promoter BCKCAT92, and a construct containing a heterologous promoter, MCKCAT935, in an orientation and position-independent fashion and thus fits the definition of a silencer. Since the first description of silencers in yeast in 1985 (82), silencers have been described in genes encoding human β -interferon (83), rat gastrin (84), rat myosin heavy chain (85), rat α -fetoprotein (86), human ϵ -globin (87), rat and human insulin (88,89), human interleukin 3 (90), and others (91-98). Analysis of the 765 base pair BCK silencer did not reveal consensus sequences found to be important for the function of other silencers that have been described. However, sequence elements with 60% to 75% identity with silencers reported to repress yeast, collagen II, gastrin, ϵ -globin, and lysozyme gene expression are present. Cis-acting motifs that do not function as traditional silencers (99) but are capable of suppressing transcription have been identified in genes encoding rat myosin heavy chain (85), mouse immunoglobulin heavy chain (100), and rat insulin (89). The BCK silencer contains no areas of >50% identity with these previously reported negative elements. However, consensus sequences for the SpI and Oct-1 binding sites are present and are located at -422 to -417 and -831 to -824, respectively (Figure 2). As noted above, SpI can repress transcription. However, this effect has only been described with SpI binding sites more proximal to the transcription initiation complex (72). The precise mechanism by which this silencer suppresses expression has not been delineated, but is probably mediated through trans-acting factors that repress transcription through protein-protein or DNA-protein interaction (101,102), with the SpI or Oct-1 binding sites, or through other previously uncharacterized cis-acting motifs.

The negative elements of the BCK gene are active in cells normally expressing the gene and thus differ from the negative elements identified for most other genes, such as those found in the mouse immunoglobulin heavy chain enhancer (100), rat insulin I promoter (89), and rat growth hormone gene (96), which are active only in nonexpressing cells. The BCK negative elements most closely resembles the negative regulatory regions of the gastrin and α -fetoprotein (AFP) genes. Like the BCK elements, the AFP and gastrin negative elements are active in expressing cells. However, the AFP and gastrin elements are inactive in nonexpressing cells (84,103). Because BCK is expressed in many different cells we have not determined whether the silencer mediates suppression of the BCK gene in cells that do not normally express BCK. However, determination of activity of our BCKCAT constructs in fully differentiated myotubes does show that both negative elements are active in cells that downregulate BCK. AFP and gastrin gene expression, like BCK gene expression, is also developmentally regulated. The AFP gene is expressed in fetal liver and is repressed after birth. In transgenic animals, cis-acting sequences containing the negative element identified in the AFP gene are essential for the postnatal extinction of an AFP transgene in liver cells (104). The negative elements of the BCK gene differ from those of the AFP and gastrin genes in that the BCK negative elements suppress BCK

expression in each cell line tested and are not dependent on differentiation for full activity.

We have characterized BCK expression in C₂C₁₂ cells and have determined that the 5'-flanking sequences of the BCK gene are capable of transcriptionally controlling its developmentally regulated expression. Multiple positive and negative elements are present in the upstream sequence and likely act as a molecular switch controlling BCK gene expression. The negative elements of BCK are unique with respect to their ability to suppress transcription regardless of cell type or state of differentiation. The positive element is also remarkable in its ability to mediate a differentiation-specific decrease in expression. In addition, the combination of positive and negative elements of this gene and their specific regulatory properties are novel. Thus, BCK expression is determined by the interaction of different cis-acting elements and their associated trans-acting factors in a manner not previously described.

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