The committed step in the pathway for leucine, isoleucine, and valine catabolism is catalyzed by branched-chain \( \alpha \)-ketoacid dehydrogenase (BCKD). This multienzyme complex is itself regulated through reversible subunit phosphorylation by a specific kinase (BCKD-kinase). Although BCKD is present in the mitochondria of all mammalian cells, BCKD-kinase has a tissue-specific pattern of expression. Various experimental, nutritional, and hormonal conditions have been used to alter the expression of BCKD-kinase, yet little is known regarding the regulation of basal BCKD-kinase expression under normal conditions including the mechanism of its tissue specificity in any organism. Here we use tissue-derived cultured cells to explore the mechanisms used to control BCKD-kinase expression. Whereas the amount of BCKD-kinase protein is significantly higher in mitochondria from \( \text{C}_{4}\text{C}_{12} \) myotubes than in BNL CL2 liver cells, gene transcription and stability of BCKD-kinase mRNA share similar properties in these two cell types. Our results show that the amount of protein synthesized is regulated at the level of translation of BCKD-kinase mRNA and that an upstream open reading frame in the 5′-untranslated region of this transcript controls its translation. The location and putative 19-residue peptide are conserved in the mouse, rat, chimpanzee, and human genes. Likewise, gene structure of mouse, chimpanzee, and human BCKD-kinase is conserved, whereas the rat gene has lost intron 9.

Branched-chain \( \alpha \)-ketoacid dehydrogenase kinase (BCKD-kinase)\(^1\) expression decreases catabolization of the corresponding ketoacids of leucine, isoleucine, and valine by inhibiting the function of the branched-chain \( \alpha \)-ketoacid dehydrogenase (BCKD) enzyme complex through reversible phosphorylation of the BCKD-E1\(\alpha\) subunits (1, 2). Whereas the BCKD complex is located in the mitochondria of all mammalian tissues, the BCKD-kinase protein content varies with tissue type. The concentration of BCKD-kinase in liver cells is low, whereas skeletal muscle has the highest level of BCKD-kinase expression. Other tissues, such as the brain and kidney, hold intermediate amounts between these two extremes (3, 4). This distribution inversely correlates with the highest BCKD activity state in the liver and the lowest BCKD activity state in skeletal muscle (5, 6). As a result, the general activity state of the BCKD complex varies from tissue to tissue, probable based on the ratio of phosphorylated (inactive) to dephosphorylated (active) E1\(\alpha\) subunits within the BCKD complex.

The need for regulation of BCKD activity is noted by pathological conditions that exist at the extreme activity states. Abnormally high or inappropriate BCKD complex activity has been shown to contribute to increased branched-chain amino acid catabolism and protein degradation in skeletal muscle, the hallmark of cachexia (5, 7). Treatment of this wasting condition, often seen in late stages of cancer and other illnesses, is of continuing concern in the care of chronically ill patients, because it often occurs even in the presence of otherwise adequate parenteral nutrient intake (8). In contrast, impaired BCKD complex activity results in the rare inborn error of metabolism maple syrup urine disease, a condition that can result in mental retardation, brain edema, seizures, coma, and death if not treated by life-long limitation of branched-chain amino acid intake (9–11). Numerous reports have demonstrated that insulin, glucocorticoids, and conditions such as acidosis, diabetes, hyperthyroidism, and amino acid deprivation can affect the distribution and expression of BCKD-kinase in cells and tissues with the expected change in activity state of the BCKD complex (12–15).

To date, little is known regarding the means of regulating basal BCKD-kinase expression under normal nutritional conditions including the mechanism of its tissue specificity in any organism. Similar to the subunits of the BCKD complex, the BCKD-kinase is a mitochondrial protein encoded in the nuclear genome. The gene is on human chromosome 16p11.2, mouse chromosome 7F3, and rat chromosome 1q36. The gene structure and amino acid sequence between mouse and human is highly conserved.\(^2\) Regulation of protein expression may occur at any of several steps, including transcription, post-transcriptional mRNA stability, translational efficiency, mitochondrial membrane transport, and post-translational protein stability (16–18). Previously, the binding of the transcription factor Sp1

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\(^{1}\) The abbreviations used are: BCKD-kinase, branched-chain \( \alpha \)-ketoacid dehydrogenase kinase; BCA, bicinic acid; BCKD, branched-chain \( \alpha \)-ketoacid dehydrogenase; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOPS, 3-(N-morpholino)propanesulfonic acid; ORF, open reading frame; PBS, phosphate-buffered saline; SSC, sodium chloride/sodium citrate; uORF, upstream open reading frame; UTR, untranslated region; RT, reverse transcription; TBE, Tris-buffered EDTA.

\(^{2}\) NCBI Entrez Gene: www.ncbi.nlm.nih.gov/entrez/query.fcgi?db= gene. Gene ID numbers: 29603 (rat); 12041 (mouse); and 10295 (human).
at a weak non-canonical site was shown to be both necessary and sufficient for minimal BCKD-kinase transcription in cultured rat hepatoma and kidney cells (4, 19). Whereas this site is conserved in both sequence and location among the rat, mouse, and human, the BCKD-kinase nucleic acid sequence upstream of this site shows little similarity in these species. Because the liver has the lowest levels of BCKD-kinase protein among all of the tissues studied and the skeletal muscle has the highest, a comparison of regulatory effectors in these two tissues will provide the necessary information to begin to understand how the expression of this gene is controlled.

In this study, we use cultured BNL C1.2 normal mouse liver cells and differentiated C2C12 mouse myotubes to explore the means used to control expression of BCKD-kinase. The results demonstrate that transcription of this gene and stability of the murine BCKD-kinase mRNA do not differ substantially between these two cell types. Further, unlike in the rat cells, basal transcription is not dependent upon Sp1 binding at the previously described site. Finally, we show that translation of the BCKD-kinase transcript does differ between the cell lines and involves an upstream open reading frame in the 5′-UTR that encodes a putative peptide conserved in mouse, rat, chimpanzee, and human.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—BNL C1.2 mouse liver cells and C2C12 mouse myoblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA). NIH-3T3 mouse fibroblasts and previously described Sp1−/- mouse fibroblast-like cells (20) were a gift from Dr. Stephen Warren (Emory University, Department of Human Genetics). BNL C1.2 and C2C12 cells were propagated in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter α-glucose, 2 mM l-glutamine, and 1 mM sodium pyruvate (MediaTech, Herndon, VA) and supplemented with 10% fetal bovine serum (Table 1) at room temperature over 2 h. Equality of protein loading and transfer was determined by Ponceau S staining of the membrane. Development of the immunoblot with BCKD-kinase antibody was as previously described (21). Visualization of the antibody binding was with West Pico chemiluminescent substrate detection protocol (Pierce). The chemiluminescent signal was exposed to Blue BioFilm (Denville Scientific Inc., Metuchen, NJ) for 2–5 min. In some experiments, the blot was stripped of all of the antibodies and then re-probed with a 1: 3,000 dilution of primary polyclonal rabbit anti-BCKD sera and a 1: 25,000 dilution of goat anti-rabbit-horseradish peroxidase-conjugated antibody (Bio-Rad). All of the incubations, antibody dilutions, and washes were performed at room temperature in IM2 (50 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 0.2% gelatin, 1% Tween 20).

**Whole Cell BCKD Assay**—Whole cell BCKD activity state, expressed as percent activity, was determined by measuring the pmol 14CO2 released from [1,14C]leucine per milligram of total cellular protein under basal conditions relative to that produced after a 10-min pre-incubation with 1 mM α-chloroisocaproate, an inhibitor of BCKD-kinase, as previously described (17, 22). Activity-state values represent an average ± S.D. of four experiments per cell line, each performed in triplicate.

**Luciferase Reporter Vector Construction**—A 930-bp PCR product was amplified from mouse genomic DNA using the Expand Long Template PCR system (Roche Applied Science) with the PAGE-purified 5′-phosphorylated primers mkrp3for (5′-GGAGGCCTCATAAATTTGTGTTG) and mkrp2rev (5′-ATCATTCCTGCATCTTTGTTGAA-3′). The PCR reactions were cleaned with QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. The purified PCR product was then cloned into the Klenow-treated Ncol site of pGL3Basic vector (Promega, Madison, WI). The inserted PCR fragment spanned from 79 bp 5′ of the mouse BCKD-kinase transcriptional start site up to, but not including, the ATG translational start site (Fig. 1A). The initiation site is located at the −5′-UTR of mouse BCKD-kinase to the translational start ATG of the luciferase reporter gene in this vector, creating the luciferase construct pEMLuc3mk1 (7–9).

A Lambda FIX II phage mouse genomic library (Stratagene, La Jolla, CA) was screened with various rat and mouse BCKD-kinase exon DNA sequence probes. Of the two positive clones found, clone 4 hybridized with a probe-bearing sequence from BCKD-kinase exons 1–4.
sert was cloned into pBluescript II KS+ vector (Stratagene) via the flanking NotI restriction sites. The resulting 16.2-kb plasmid, designated pmkin4, contained several kilobases of genomic DNA 5' to the mouse BCKD-kinase gene through the first 234 bp of intron 4. A 945-bp BglII/BamHI fragment of the mouse BCKD-kinase gene was cut from pmkin4 and subcloned into the BglII site of pGL3Promoter (Promega). This fragment was sequenced to −298 bp 5' to the start of transcription through most of intron 1. Vector pEMLuc3mk[−298] then was constructed by ligating the BglII/NdeI restriction fragment from this subclone into BglII/NdeI-cut pEMLuc3mk[−79]. Plasmid pEMLuc3mk[−4174] was created by sub-cloning a 4.2-kb fragment from pmkin4 into pEMLuc3mk[−79] using KpnI and NdeI restriction enzymes. Vector pEMLuc3mk[−1107] resulted from digesting pEMLuc3mk[−4174] with KpnI and EcoRI and blunting the ends with T4 DNA polymerase followed by ligation. Internal deletion of this vector with NheI and XhoI followed by blunting of the ends re-ligation yielded pEMLuc3mk[−1107/5’ORF].

Restriction endonuclease digestions and DNA sequence analysis (Emory Core Sequencing Facility) were used to verify all of the plasmid constructs. Restriction enzymes, Klenow fragment, T4 DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA).

**Transient Transfection and Luciferase Reporter Analysis**—An overview of each 10° NIH-3T3, 7.5° × 10° Sp1−/−, 1.5° × 10° C57BL/6 CL.2, or 1.5° × 10° C57BL/6 CL.2 cells, plated in 6-well tissue culture plates was transfected with a mixture containing a 3:1 volume-to-mass ratio of FuGENE 6 transfection reagent (Roche Applied Science) to total plasmid DNA in supplement-free growth medium, 3 μg of firefly luciferase reporter construct, and 120 ng of pRL-null Renilla plasmid (Promega). 24 h post-transfection, the growth medium was replaced with fresh medium switched to differentiation medium for the C2C12 cells and replaced again every 24 h thereafter. Cells were harvested 72 h post-transfection according to the passive lysis conditions in the Dual luciferase reporter system protocol (Promega). The ratio of firefly luciferase to Renilla luciferase luminescence was manually measured on a Turner Systems 20/20–1E lumimeter, setting the luminescence from untransfected cell lysates of the appropriate cell type as background. Data represent the mean ± S.E. of three independent experiments, each performed in triplicate, for each reporter construct. Average values for different vectors were compared using a one-tailed Student’s t test (Microsoft Excel, version X) (p values <0.05 were considered significant).

**Nuclear Extract Preparation**—This procedure was performed essentially as described (23) with modifications. Just prior to use, a 25°× protease inhibitor solution was made by dissolving Complete protease inhibitor mixture tablet (Roche Applied Science) in 2 ml of RNase-free ultrapure dH2O and then it was added fresh to each buffer at a 1× concentration. All of the glass tubes and pestles used for nuclear extract preparation were pre-chilled on ice.

Cells were harvested with trypsin/EDTA, washed with PBS, and resuspended in 1 ml of hypotonic buffer (20 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1× protease inhibitors) per flask. Cells were incubated on ice for 15 min and then dounced 30 times in a glass using Pestle B. Nuclei were pelleted by centrifugation at 2,000 × g for 10 min at 4 °C and washed in hypotonic buffer. The nuclear pellets were resuspended in 125 μl of high salt extraction buffer (20 mM Tris-HCl, pH 7.5, 420 mM KCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM MgCl2, 1 mM DTT, 1× protease inhibitors) per flask, mixed by vortexing, and incubated on ice for 30 min. The suspension was then centrifuged at 15,000 × g for 15 min at 4 °C. The supernatant (nuclear extract) was assessed for protein concentration by BCA protein assay (Pierce), aliquotted, flash-frozen in liquid nitrogen, and stored at −80 °C.

For mouse tissues, the protocol was modified from that described for cells (23, 24) and partially based on the instructions from the nuclear extract kit available from Active Motif (Carlsbad, CA). Freshly isolated adult male C57BL/6 mouse liver or thigh muscle was minced, weighed, and homogenized at low speed on a Polytron homogenizer in 3 ml of hypotonic buffer. The homogenates were incubated on ice for 15 min, and any undisrupted tissue was removed by centrifugation at 850 × g for 10 min at 4 °C. The supernatants (cytoplasmic fraction) were removed, and the cell pellets were resuspended in 1 ml of hypotonic buffer. After a 15-min incubation on ice, the cells were dounced in glass using Pestle B. Nuclei were pelleted by centrifugation at 2,000 × g for 10 min at 4 °C. The supernatants were removed and pooled with the earlier cytoplasmic fractions. The nuclear pellets were resuspended in 1 ml of high salt extraction buffer, vortexed, and incubated on ice for 30 min. The nuclear extract was clarified and aliquotted as in the procedure for cells. Rat liver nuclear extract was supplied by Active Motif.

**DNase I Protection**—This procedure was performed as described previously (25) with some modifications. A 442-bp substrate for DNase I digestion was produced from PCR amplification of plasmid pEMLuc3mk[−1107] with primer mkpro4for (5’-GAGGAGTGCAATGCTTGTTACG-3’) and mkpro5rev (5’-CGCCGGAGGGGTCGTG-3’) using an anneal temperature of 57 °C. For each amplification, one of the primers in each pair was end-labeled with 32P prior to PCR for visualization of a single DNA strand.

Each radiolabeled PCR product was cleaned with the QIAquick PCR purification kit (Qiagen), and aliquots of the resulting purified product were assessed by electrophoresis on 1.5% agarose and by scintillation counting. For DNase I protection experiments, 50–100 μl reactions containing 10 μg Tris-Cl, pH 7.5, 5 mM MgCl2, 50 μM EDTA, 75 mM KCl, 12% glycerol, 0.4 mM phenylmethanesulfonf fluoride, 1 mM DTT, 50 ng/μl poly(dI-dC), and ∼20,000 cpm of purified radiolabeled PCR product was incubated at room temperature for 30 min in the presence of 50 μg of crude nuclear extract or bovine serum albumin. Subsequently, 50 μl of RQ1 RNase-free DNase I (Promega), freshly diluted in digestion buffer (10 μg MgCl2, 5 mM CaCl2), was added. DNase I was used at 0.13, 0.4, or 1.2 units per reaction. Incubations proceeded for 1.5 min at room temperature. All of the reactions were stopped by adding 100 μl of a buffer containing 200 mM NaOH, 20 mM EDTA, 1% SDS, and 60 μg/ml yeast RNA (Sigma). DNA was phenol/chloroform-extracted, ethanol-precipitated, and dried under vacuum. The DNA pellet was resuspended in sequencing stop buffer (10 mM NaOH, 95% deionized formamide, 0.05% bromphenol blue, 0.05% xylene cyanol) and heated at 95 °C for 3 min. Sample aliquots were resolved on a 6% acrylamide/8 M urea gel in 1× TBE.

The fmol DNA cycle sequencing system (Promega) was used to generate dideoxyribonucleotide-terminated ladders for sequence comparison using the same primers and template as was used to generate the DNase I substrate. Size comparison was also made with a 25-bp DNA ladder (Invitrogen) that was 32P end-labeled with T4 polynucleotide kinase. Sequencing gels were dried under vacuum at 80 °C for 2 h, and autoradiography was performed on Kodak Biomax MS film at −80 °C with intensifying screens.

**Electrophoretic Mobility Shift Assay (EMSA)**—To make radiolabeled double-stranded DNA oligomers for EMSA, equimolar amounts of complementary single-stranded DNA oligomers were mixed in annealing buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0, 50 mM NaCl) and slowly cooled from 95 to 15 °C over the course of 1.5 h in a PerkinElmer Gene-Amp 8600 PCR machine. Of the resulting double-stranded products, 20 pmol were 32P end-labeled with T4 polynucleotide kinase and

![](http://www.jbc.org/)
the column was purified in G25 Sephadex spin columns (Roche Applied Science). In some experiments, 3'-biotinylated double-stranded DNA oligomers were used. To make these detection probes, single-stranded DNA oligomers were first labeled with the Biotin 3'-End DNA-labeling kit (Pierce) and then annealed at room temperature for 1.5 h. EMSA binding reactions containing 10 mM Tris-Cl, pH 7.5, 5 mM MgCl2, 50 μM EDTA, 75 mM KCl, 12% glycerol, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 50 ng/μl poly dl-dC, and 10 μg of crude nuclear extract were incubated with unlabeled double-stranded oligomer for 10 min at room temperature. Subsequently, 20 fmol of 3'-biotinylated probe or 10 fmol (20,000–30,000 cpm) of 5'-32P-labeled probe was added and incubation continued at room temperature for an additional 10 min. In antibody supershift experiments, 2 μg of Sp1, Sp3, or TFIIID antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the reactions, and incubation continued for an additional 10 min. In some reactions, oligomers bearing a consensus sequence for either Sp1 (Promega) or EBNA-1 (Pierce) transcription factor binding were used as competitors. All of the unlabeled competitor oligomers were used at 200× the molar amount of labeled probe. Resolution of free probe from protein-bound probe was performed by non-denaturing electrophoresis through 4% acrylamide/bisacrylamide (29: 1) in 0.5× TBE at room temperature. For the biotinylated probes, gels were semi-dry-electroblotted onto Hybond N+ nylon membrane (Amersham Biosciences) in 0.5× TBE for 1 h at room temperature. Band detection was performed with the LightShift chemiluminescent EMSA kit (Pierce) according to the manufacturer’s instructions. Gels using radiolabeled probes were dried and autoradiographed as described under “Experimental Procedures.”

Cytoplasmic Fractionation and Polyribosome Analysis—This procedure was performed as described previously (26) with modifications. BNL Cl.2 cells were plated, transfected, and maintained as described under “Experimental Procedures.” After 72 h, the medium in each well was replaced with fresh medium containing 100 μg/ml cycloheximide and the cells were incubated normally for 30 min. The cells were then washed with PBS and harvested with trypsin/EDTA. The cells were again washed in PBS and then lysed in 1 ml of lysis buffer (20 mM Tris, pH 7.5, 100 mM KCl, 5 mM MgCl2, 0.3% Igepal (Sigma), 1× Complete protease inhibitors). Lysis proceeded for 10 min on ice without vortexing. Lysates were centrifuged at 15,000 × g for 10 min at 4 °C to remove mitochoondria and nuclei. Each clarified supernatant was then loaded on the top of a 15–45% linear sucrose buffer gradient in 20 mM Tris, pH 7.5, 100 mM KCl, 5 mM MgCl2, and created on a Gradient Master model 106 (BioComp Instruments, Fredericton, New Brunswick, Canada) with the following settings: 4.06 min, time; 80.8° angle; and 0.8 speed. The lysate was distributed through the sucrose gradient by centrifugation at 39,000 rpm at 4 °C for 75 min in a pre-chilled Beckman SW 41Ti ultracentrifuge rotors. The polyribosomes fractions were pushed up from the bottom through a UA-6 fraction collector (ISCO Inc., Lincoln, NE) set for continuous monitoring of UV absorbance at 254 nm with 60% sucrose. Ten 1-ml fractions were collected from the top of the gradient in this manner. Each fraction was then phenol:chloroform-extracted, and total RNA was ethanol-precipitated. The resuspended RNA was transferred to a MagnaGraph neutral nylon membrane using a Minifold I dot-blot apparatus (Schleicher & Schuell, Keene, NH). Subsequent hybridization and densitometry were performed as described under “Experimental Procedures” using cDNA-based PCR-amplified probes from mouse BCKD-kinase exons 9–11 and firefly luciferase and full-length rat GAPDH cDNA.

RESULTS

Murine BCKD-kinase mRNA Level Does Not Correlate with Protein Level—To assess whether BCKD-kinase expression in BNL Cl.2 and C2C12 cell lines is representative of that found in...
liver and skeletal muscle, we determined both message and protein levels in these cells and in multiple mouse tissues. The previously observed difference in the expression of BCKD-kinase protein among mouse tissues (3, 4) is maintained in cultured cells where differentiated C2C12 cells expressed 2.5-fold more protein than did BNL Cl.2 cells (Fig. 1). The steady-state amount of BCKD-kinase mRNA did not directly correlate with the amount of protein in the tissues or cell lines, suggesting the existence of a regulatory control point between message transcription and translation (Fig. 2). As expected, the amount of

**Fig. 4. EMSA of the −80/−26-bp region from the BCKD-kinase genomic locus.** End-labeled 55-bp double-stranded DNA oligonucleotide probe was used as a substrate for protein binding by 10 μg of nuclear extract from mouse liver, mouse skeletal muscle, BNL Cl.2 mouse liver cells, or 3-day differentiated C2C12 mouse myotubes. *A,* sequences of the double-stranded DNA oligomers used for probes and competitors in EMSA. The boxed nucleotides represent the previously described non-canonical Sp1-binding site. Underlined nucleotides are the consensus Sp1-binding sequence in the oligomer provided by Promega. A 60-bp oligomer of proprietary sequence, but containing the EBNA-1 transcription factor binding site, was obtained from Pierce and used as an unrelated nonspecific competitor (data not shown). *B,* a mouse non-Sp1, a non-Sp3 nuclear protein that specifically binds between bases −80 and −54, as indicated by an arrow. Although the illustrated gel used mouse liver nuclear extract, it is representative of three different experiments for each of the four murine nuclear extracts. One or more bands were present inconsistently in nuclear extract-containing lanes from all of the cells and tissues tested in different experiments and are indicated as nonspecific (N.S.). Unbound probe was run off the bottom of the gel.
BCKD-kinase protein present in the mitochondria inversely correlated with the activity state of the BCKD complex, confirming that the BCKD-kinase protein is functional within these cells. In BNL Cl.2 liver cells, the BCKD complex was 63 ± 5.5% active (n = 4), whereas even undifferentiated C2C12 myoblasts had a lower activity state at 15 ± 4.5% (n = 4).

Tissue-specific Regulation of BCKD-kinase Expression Does Not Occur at Transcription—Examination of the nucleic acid

![Diagram of BCKD-kinase expression in different cell types.](https://example.com/diagram.png)
sequence upstream of the murine BCKD-kinase gene revealed numerous potential transcription factor binding sites within the 300 bases 5' to the start of BCKD-kinase transcription (27). To determine areas of potential tissue-specific protein binding within this region, DNase I footprinting was performed on the sequence from 307 bp (relative to the start of transcription, 1) through the first 45 bp of BCKD-kinase intron 1. Nuclear extracts from BNL Cl.2 liver cells, C2C12 myotubes, mouse liver, and mouse skeletal muscle were compared. Four identical areas on the coding strand were consistently found to be protected from DNase I digestion with each nuclear extract (Fig. 3). No protected regions were observed for the non-coding strand (data not shown).

Eleven double-stranded 55-bp biotinylated oligonucleotides, which overlapped by 27–28 bp, were used as probes in EMSA reactions to confirm protein binding at the protected sites. The entire region from 307 through 30 bp was covered in this manner. Only one probe bearing bases 80 through 26 consistently demonstrated specific protein binding and was further evaluated (data not shown). Neither a consensus sequence Sp1-binding site oligonucleotide nor the 5'-32P-labeled oligonucleotide, bearing the non-consensus putative Sp1-binding site, was achieved with nuclear extracts from mouse liver, mouse skeletal muscle, BNL Cl.2 liver cells, and C2C12 myotubes (Fig. 4 and data not shown). Neither Sp1 antibody nor antibody against Sp3, a related transcription factor that can utilize the same or similar binding sites (20, 28, 29), was able to further decrease electrophoretic mobility of the probe from that initially achieved with the nuclear protein (Fig. 4). One or more low mobility bands were observed in lanes containing nuclear protein. As they appeared inconsistently in different experiments and occurred with every extract at least once, even in the absence of antibody, these bands were considered to be the effect of nonspecific protein binding of the probe (data not shown). In contrast to the murine nuclear extracts, rat liver nuclear extract produced a specific shift of this probe that was effectively competed with a consensus Sp1 binding site but not with either half of the probe (data not shown). Together these results suggested that the observed difference in protein expression was not mediated by different tissue-specific transcription factors binding in the promoter region of the murine BCKD-kinase gene.

This conclusion was explored through the examination of firefly luciferase reporter enzyme activity maintained under the control of the murine BCKD-kinase promoter and 5'-UTR in different cell lines. Increasing the length of the upstream putative promoter sequence produced no differential effect between the cell lines for the various fragments, although overall relative enzyme activity was greater in the C2C12 cells than in the BNL Cl.2 cells (Fig. 5A). The pEMluc3mk[−79] vector produced greater luciferase activity than the control vector, pGL3Basic, in both cell lines (p < 0.042). No significant additional effects on luciferase activity were achieved by including up to 4.1 kilobases of 5'-genomic DNA in these vectors in either cell line, indicating that the minimal functional promoter was within the 79 bases proximal to the start of transcription.

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**FIG. 6.** BCKD-kinase mRNA stability. Total RNA was extracted from BNL Cl.2 liver cells or differentiated C2C12 skeletal muscle myotubes after treatment with 10 μg/ml actinomycin D for the indicated times. A, Northern blot on 10 μg of total RNA using cDNA probes for BCKD-kinase and 18 S rRNA. A representative blot of two independent experiments is shown. B, decay rate of the BCKD-kinase was plotted as a percentage of the starting steady-state amount present at time zero, and a best-fit trend line is shown on a logarithmic scale. The data points represent the mean ± S.D. of two independent experiments.
FIG. 7. Conservation of BCKD-kinase gene elements across species. A, the BCKD-kinase genes from human, chimpanzee, mouse, and rat are illustrated schematically as in Fig. 5. The rat gene has lost the small intron found between exons 9 and 10 in the other species. A conserved BamHI restriction site in intron 1 is indicated by the letter B. B, the mRNA sequence from the 3'-end of exon 1 and the 5'-end of exon 2, which bears both an uORF and the BCKD-kinase ORF, are shown from the human, chimpanzee, mouse, and rat genes. The nucleotides in boldface represent the initiating methionine codon of the uORF. The numbers in parentheses indicate the number of nucleotides omitted for space, which are followed by the underlined BCKD-kinase-initiating methionine codon. C, amino acid sequence of the peptide putatively encoded by the uORF from each organism. Amino acids in boldface are conserved or are similar among all four species. Amino acids conserved between three of the four organisms are in gray.

FIG. 8. BCKD-kinase 5'-UTR uORF disruption differentially affects relative luciferase reporter activity. The ratio of firefly luciferase to Renilla luciferase luminescence in BNL C12 liver cells and 48-h differentiated C2C12 muscle cells was determined 72 h after transfection. Luciferase constructs and the native mouse BCKD-kinase gene (mBCKDK) are represented as in the other figures. In the graph, colored bars indicate the average ratios ± S.E. of three independent experiments, each performed in triplicate, for the pEMLuc3mk[-1107/ΔuORF] construct. The values for other constructs are the same as in Fig. 5 and are included for reference.
Although we found no evidence by EMSA that Sp1 protein binds in this location in the mouse, we further tested this possibility using the reporter assay in a murine fibroblastic cell line in comparison with the NIH-3T3 mouse fibroblast cell line. Reporter expression was similar in the two cell lines for the pEMluc3mk vector, confirming that Sp1 binding within the proximal 79 bases is not necessary (Fig. 5B).

To determine whether mRNA stability was the post-transcriptional regulator of steady-state BCKD-kinase protein concentration, mouse liver and muscle cell lines were cultured in the presence of actinomycin D to stop gene transcription by RNA polymerase II. Northern blot analysis demonstrated an exponential decrease in BCKD-kinase mRNA for both cell lines, whereas the amount of 18 S rRNA remained unaffected.

**Fig. 9. Polyribosome-associated mRNA in BNL Cl.2 liver cells.** BNL Cl.2 cells were transfected with pEMluc3mk[−1107] or pEMluc3mk[−1107/ΔuORF] vector and, after 72 h, treated with cycloheximide, lysed, and fractionated on a linear sucrose gradient. Total RNA in each eluted fraction was dot-blotted and probed with endogenous mouse BCKD-kinase exones 9–11, rat GAPDH, and luciferase DNA probes. A, a representative polyribosome profile tracing of 254-nm absorbance is shown for BNL Cl.2 cells transfected with each vector. Fractions 1–4 contain ribonucleoproteins, dissociated ribosomal subunits, and monosomes. Fractions 5–10 contain polyribosomes. B, distribution of mRNA in polyribosomes from transfected BNL Cl.2 cells. Values represent the average percentage ± S.E. (n = 3–7) of total lysate probe signal that is present in each eluted fraction.
not bind Sp1 in the mouse. Conceivably, differential post-translational Sp1 modification between the mouse and rat nuclear extracts could account for these experimental results. Alternatively, as protein binding was observed in nearby DNA sequences, one or more other transcription factors could promote minimal transcription of murine BCKD-kinase. In recent years, several newly identified basic zinc-finger transcription factors have been shown to drive transcription of GC-rich genes lacking canonical promoter elements (30). We have not yet identified any of the proteins binding in this region.

The mouse BCKD-kinase mRNA has an extensive 5’-UTR, which contains a highly conserved 19 amino acid-encoding uORF, and is predicted to take on multiple stable secondary structures (31, 32). The presence of an uORF has been demonstrated to affect the translation of a variety of mRNAs from Saccharomyces cerevisiae, Neurospora crassa, Xenopus laevis, and several mammalian species including humans (33–39).

The reporter constructs used in this study all contained the firefly luciferase ORF fused to the entire mouse BCKD-kinase 5’-UTR structure at the location where the endogenous BCKD-kinase ORF occurs in the native mRNA sequence. Consequently, reporter gene expression should reflect the effects of the BCKD-kinase uORF on the downstream luciferase and, by inference, BCKD-kinase in its native context. Although reporter gene expression is usually used to model transcription of the gene from which the promoter and/or enhancer elements used to drive expression is derived, it is apparent in our studies that the firefly luciferase reporter activities modeled BCKD-kinase translation as well. Deletion of the uORF had a significant effect on luciferase reporter activity in BNL Cl.2 cells but not in C2C12 cells, indicating that it is a likely means of specifically inhibiting BCKD-kinase protein expression in mouse liver cells. To date, no naturally occurring mutations have been identified in the BCKD-kinase gene, yet experimental overexpression of the gene does significantly reduce BCKD activity. This finding suggests that a gain-of-function mutation in BCKD-kinase would cause maple syrup urine disease (40–42). Perhaps uninhibited activity of BCKD-kinase in the liver alone would result in similar sequelae.

There are different mechanisms possible for the tissue-specific translational control exerted by the presence of the BCKD-kinase uORF. Ribosome stalling at the uORF AUG during translation of BCKD-kinase transcript in both cell lines, despite its not having the same favorable A

| mRNA                | Fractions | Total |
|---------------------|-----------|-------|
|                     | 1–4       | 5–10  |
| BCKD kinase         | 78.2      | 21.8  | 100.0 |
| [−1107]-Luc         | 77.9      | 22.1  | 100.0 |
| [−1107/ΔuORF]-Luc   | 57.3      | 42.7  | 100.0 |
| GAPDH               | 64.4      | 35.6  | 100.0 |

DISCUSSION

In the mouse, as in humans, liver and muscle represent the tissues at the extremes of BCKD-kinase protein expression (Fig. 1) (3, 4). Our data demonstrate that these cell types use translational processes to control expression of BCKD-kinase protein. Although the amount of BCKD-kinase mRNA varies with tissue type, it does not accurately reflect the amount of protein in the mitochondria of that tissue. BCKD-kinase is GC-rich near the start of gene transcription and lacks both a TATA-box and an initiator element, as is commonly associated with constitutively expressed regulatory genes (4). Thus, our finding that regulation does not occur at the level of transcription is not surprising. Unexpectedly, we found the basal expression of the mouse BCKD-kinase gene to be different from that of the rat gene in that the conserved Sp1-like binding site did
post-transcription. As BCKD-kinase is a nuclear-encoded mitochondrial protein, these control points could include mitochondrial import and co- or post-translational modification of the protein as has been observed for other BCKD subunits (16–18).

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