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Influence of Subunit Transcript and Protein Levels on Formation of a Mitochondrial Multienzyme Complex

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Abstract Constitutive expression of nuclear genes encoding mitochondrial proteins raises the question of whether these proteins are present in similar amounts in mitochondria of different tissues. We report that amounts of a single multienzyme complex can vary on a per mitochondrion basis depending on the number of mitochondria per cell. Human branched–chain α-keto acid dehydrogenase (BCKD) expression is used as a paradigm in these studies. Expression is compared and contrasted in HepG2 and DG75 cells in which mitochondrial content is twofold higher in the hepatocarcinoma line than in the lymphoblastoid line. Per cell, BCKD activity is equal in the two cell types, but BCKD protein concentration per mitochondrion is twofold higher in DG75 cells. Steady-state mRNA levels do not appear to be directly related to amounts of protein in the two cell lines. To test whether one subunit is limiting in formation of complex, overexpression of each BCKD subunit was elicited by plasmid transfection of the DG75 cells. Only overexpression of the β-subunit of the decarboxylase component induced more BCKD activity without apparent increase in mRNA for the other endogenously expressed subunits. This implies that free BCKD subunits exist in a cell and can be recruited into an active complex when the limiting subunit becomes available. © 1996 Wiley-Liss, Inc.

Key words: mitochondria, multienzyme complex formation, subunit transcript, protein levels, post-translational events

Mitochondria number and protein content vary with cell type based on functional and energy requirements of the tissue in which they exist [Hood et al., 1994]. However, most components of mitochondria are present in all tissues. Among these constant components are multienzyme complexes involved in the biosynthesis and catalysis of various cellular metabolites. It is not known whether the amount of these complexes varies between mitochondria from different tissues. If variations occur, points of regulation could include transcription rate, stability of mRNA, efficiency of translation, rate of protein import into mitochondria, stability of the protein subunits, and association of subunits with one another to form the complex. Variation in amounts of these complexes could also be controlled by expression of a single component of the complex, if production of the subunits is not tightly coordinated. To examine regulatory mechanisms one must first characterize the events leading to the formation of the mitochondrial multienzyme complex and then determine if expression of any one component influences expression of the other subunits.

In the present study, human branched-chain α-ketoacid dehydrogenase (BCKD) is used as a model to examine formation of ubiquitous mitochondrial multienzyme complexes. BCKD catalyzes the committed step in the catabolism of the branched-chain α-keto acids derived from leucine, isoleucine and valine. All subunits of BCKD are encoded by nuclear genes. The three of interest here are the E1α and E1β components of the decarboxylase subunit, and the E2, dihydrolipoyl acyltransferase. An interpretive model for the structure of BCKD is a symmetrical core of 24 E2 subunits surrounded by 12 $E1\alpha_2\beta_2$ tetramers [Reed and Hackert, 1990]. This structure results in a ratio for E1α:E1β:E2 of 1:1:1, as previously suggested [Yeaman, 1986]. It has been well established that activity of BCKD is regulated by phosphorylation of serine residues in the E1α component through the action of a specific kinase and phosphatase [Harris and Paxton, 1985; Reed et al., 1985]. However, recent studies have demonstrated that mRNA and protein for BCKD can increase by

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physical stress and/or chemical stimuli [England et al., 1995; Fujii et al., 1995; Honda et al., 1991; Zhao et al., 1994]. Upon induction, both coordinate and noncoordinate changes in transcript levels have been reported [Chicco et al., 1994; Chinsky et al., 1994; England et al., 1995; Zhao et al., 1994]. From these studies, it appears that changes in mRNA levels are not reflected in concomitant changes in protein levels; thus, post-transcriptional events may be important to the formation of a functional BCKD complex.

To examine potential regulatory events, BCKD expression was compared in two different cell types; HepG2, a hepatocarcinoma cell line that grows in monolayer, and DG75, a lymphoblastoid cell line that grows in suspension culture. HepG2 cells contain twice as many mitochondria as DG75 cells, and both cell lines express BCKD activity. Results indicate that although transcript levels for the subunits vary between the two cell lines, amounts of BCKD complex are equal on a per cell basis. These observations support the idea that post-transcriptional mechanisms regulate complex levels. Within a single cell type, we examined whether overexpression of a single subunit could influence BCKD complex formation. Our data shows that the amount of E1β protein is the limiting component in formation of the BCKD complex.

MATERIALS AND METHODS

Culture of HepG2 and DG75 Cell Lines

HepG2 cells were grown in Eagle's minimal essential medium [EMEM]; DG75 cells were grown in RPMI 1640 medium. Both media were supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin/fungizone for growth of cells at 37°C in humidified air maintained at 5% CO_2.

Whole Cell BCKD Activity Assay

BCKD activity was determined by measuring the release of $^{14}CO_2$ from [1-^{14}C]leucine essentially as described by Litwer et al. [1989]. Assays were done in triplicate using 2×10^6 cells assay^{-1}. Total activity was estimated in the presence of 1 mM α-chloroisocaproate and basal activity in the absence of this substrate analog. α-Chloroisocaproate specifically inhibits the BCKD kinase, allowing conversion of the complex to the unphosphorylated, active form [Litwer et al., 1989]. Decarboxylation rate of [1-^{14}C]leucine was expressed as pmol CO_2 mg cellular protein^{-1} h^{-1}. Cellular protein content was determined using the BCA Protein Assay (Pierce, Rockford, IL) following the manufacturer's instructions.

Determination of Steady-State mRNA Levels

Total RNA was isolated from actively growing HepG2 and DG75 cells using TRI reagent LS or TRI reagent, respectively, per the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). mRNA levels of specific transcripts were quantified using RNase protection analysis essentially as described by Hod [1992]. BCKD mRNA levels were normalized to γ-actin mRNA in each cell preparation. Antisense RNA probes for E1α, E1β, E2, or γ-actin were produced by incorporation of [α-^{32}P]-UTP (Amersham, Arlington Heights, IL 800 Ci/mmol, 20 mCi/ml) as the only source of UTP. Probes used corresponded to bp 180–435 for E1α [McKean et al., 1992], bp 190–585 for E1β [Nobukuni et al., 1990], bp 611–806 for E2 [Lau et al., 1992] and a 144-bp fragment for γ-actin [Gunning et al., 1983]. Total cellular RNA (15 μg) was hybridized with 5×10^5 cpm of each probe at 45°C for 16–20 h. Single-stranded RNA was digested by the addition of 10-fold volume of digestion buffer (10 mM Tris–HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA) with 50 units RNase T (Boehringer Mannheim, Indianapolis, IN) ml^{-1} and 10 μg RNase A (Sigma, St. Louis, MO) ml^{-1} for 1 h at 30°C. RNase digestion was stopped with an equal volume of guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 0.5% sodium N-lauoylsarcosine, 25 mM sodium citrate, 0.1 M β-mercaptoethanol). Protected duplex RNA was precipitated with an equal volume of isopropanol and centrifuged at 12,000*g* for 15 min at 4°C. Duplexes were denatured and separated on an 8 M urea, 5% polyacrylamide gel by electrophoresis. Intensities of the protected fragments were quantified using a Molecular Dynamics model 400 Series Phosphorimager.

mRNA Half-life Determination

HepG2 cells were grown in 60-mm petri dishes to 50% confluence. Transcription was inhibited using media containing 500 nM actinomycin D. To inhibit transcription in DG75 cells at a density of 5×10^5 cells ml^{-1}, media containing 100 nM actinomycin D was used. Concentrations of actinomycin D were experimentally determined to be minimal amounts needed to stop [^{3}H]uridine (43 Ci mmol^{-1}, Amersham) incorporation into RNA, while maintaining cell viability. RNA was isolated from the cells at various time points

up to 30 h following actinomycin D treatment. RNase protection fragments were again quantified with the PhosphorImager. Amounts of RNA used were shown to be proportional to intensity. Half-lives were calculated as described by Harrold et al. [1991], using data derived from at least three separate experiments.

Nuclear Run-on Assay

Nuclei were isolated from 5×10^7 HepG2 or DG75 cells basically as described [Keller et al., 1984]. Cells were disrupted using 10 strokes of a Dounce homogenizer with a B (loose-fitting) pestle, layered over a 0.876 M sucrose cushion and centrifuged at 1,300*g* for 10 min at 4°C. *Supernatant* was removed, and pelleted nuclei were resuspended in glycerol storage buffer (50 mM Tris–HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). Nuclei were frozen in liquid nitrogen and stored at −80°C until needed.

Transcription from isolated nuclei was carried out as described [Ausubel et al., 1989]. Briefly, nuclei were placed in a transcription buffer containing ATP, CTP, GTP, and [α^{32}P]-UTP. Transcription proceeded for 30 min at 30°C and was stopped by addition of 150 U DNase I (RNase free) for 30 min at 30°C. Then 200-μg proteinase K and ⅙ vol of SDS/Tris buffer (5% SDS, 500 mM Tris–HCl, pH 7.4, 125 mM EDTA) was added for a 30-min digestion at 42°C. Radiolabeled RNA was isolated by TCA precipitation onto Millipore glass fiber filters. Filters were treated with 200 units DNase I for 30 min at 37°C, RNA was eluted from the filters and treated with 90 μg proteinase K for 30 min at 37°C. RNA was precipitated with 0.3 M sodium acetate plus 2.5 vol of ethanol. Isolated radiolabeled RNAs were hybridized for 48 h at 55°C to 1.5 μg of synthesized antisense RNA probes for E1α, E1β, E2, and γ-actin loaded on dot-blot membranes. Following hybridization, filters were washed as follows: 2× SSPE for 15 min at 55°C, 2× SSPE + 0.25 μg RNase A ml^{-1} for 15 min at room temperature, and 1× SSPE for 15 min at 55°C. Wash conditions were optimized to ensure specificity of hybridization. Radioactive mRNAs trapped on the membranes were quantitated by phosphorimaging and the results normalized to γ-actin.

Western Blot Protein Analysis

Mitochondrial protein was isolated from approximately 5×10^7 HepG2 or DG75 cells as described by Litwer and Danner [1988] and resuspended in isolated buffer (50 mM Tris–HCl, pH 7.5, 0.9% NaCl, 1 mM EDTA, 0.4% sodium lauryl sulfate, and 0.5% sodium deoxycholate) for quantification using the BCA protein assay. Increasing quantities (1–9 μg) of mitochondrial protein from each cell line were loaded onto a 10% SDS–polyacrylamide gel and separated by electrophoresis. Proteins were electrophoretically transferred onto Hybond-ECL membrane (Amersham), and the membranes were incubated with IM2 buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.25% gelatin, 1% Tween 20] overnight at 4°C to minimize nonspecific binding. Subunits of the BCKD complex were detected with polyclonal BCKD antisera [Heffelfinger et al., 1983], using the Amersham ECL Western blotting system. Multiple exposures were quantitated using a Molecular Dynamics Densitometer. Quantities of BCKD subunits were linear in the range analyzed.

Determination of Mitochondrial Content

A pellet from 1×10^6 cells was prepared by centrifugation at 640*g* for 5 min and resuspended in 1 ml of the respective culture medium containing 15% fetal bovine serum and 10 mg Rhodamine 123 ml^{-1}. Cells were incubated for 10 min at 37°C, pelleted, and resuspended in 500 μl fresh medium. Measurements of mitochondrial fluorescence were made for 10,000 events/test with the FACS-STAR flow cytometer. Mitochondrial content of cells was compared as the mean intensities of absorption at 500 nm.

Plasmid Construction

The expression vector pREP4 (Invitrogen), harboring the hygromycin resistance gene, was engineered to contain inserts of cDNAs encoding E1α from pFP12, E1β from pTSEβ, and E2 from pTSE2A1 [Sitler et al., 1995], using the *Xho*I and *Bam*HI cloning sites. These constructed plasmids were used for transfection of DG75 cells. Plasmids are maintained as episomes due to the presence of the EBNA-1 gene and Ori-P from the Epstein-Barr virus. These plasmids contain the entire open reading frames and 3′ UTRs for E1α and E1β but only the coding region for E2. Transcription was controlled by the Rous sarcoma virus long terminal repeat promoter.

Transfection of DG75 Cells

Transfections were done by electroporation essentially as described by Anderson et al. [1991].

Actively dividing DG75 cells at a density of 5 × 10^6 cells ml^{-1} were collected by centrifugation at 640*g* for 5 min and washed once in RPMI media. Cells were resuspended to a concentration of 1.67 × 10^7 cells ml^{-1} RPMI in 0.4-cm BioRad electroporation cuvettes containing 30 μg of plasmid DNA for exposure to a 250-V pulse by a BioRad Gene Pulser. Cells were allowed to rest for 5 min before diluting in 10 ml RPMI media supplemented with 15% FBS and prewarmed to 37°C. Cells were grown for 24 h prior to selection in medium containing 300 μg hygromycin B ml^{-1}. Cells that survived for 4 weeks in this medium were used for analysis.

DNA Analysis

Low-molecular-weight DNA was isolated from 5 × 10^7 cells by Hirt extraction [Hirt, 1967]. DNA was analyzed by Southern blot hybridization, using a hygromycin B probe that was random-primer labeled with [α^{32}P]-dCTP using the Megaprime DNA labeling system (Amersham). Hybridization was performed in RapidHyb buffer (Amersham) at 65°C for 2 h. Washes were done in 2× SSC, 0.1% SDS at room temperature for 20 min, 1× SSC, 0.1% SDS at 65°C for 15 min, and 0.1× SSC, 0.1% SDS at 65°C for 15 min.

RESULTS

Relative Protein Levels and BCKD Activity in HepG2 and DG75 Cells

Concentrations of cellular components for the two cell lines are compared in Table I. Total cell protein and mRNA amounts per cell are similar. BCKD protein concentrations from mitochondrial extracts were compared in the two cell lines. Estimated amounts of protein for all BCKD subunits were at least twofold higher in DG75 cells versus HepG2 cells (Fig. 1).

Considering that these differences could be due to variation in number of mitochondria per cell, mitochondrial content was compared by fluorescent staining and flow cytometry. HepG2 cells, while having lower amounts of BCKD complex per μg of mitochondrial protein, contained twofold more mitochondria than DG75 cells. BCKD activity assays substantiated these results, showing equal levels of total BCKD activity per mg protein in DG75 and HepG2 cells (Table II). Comparison of subunit protein concentrations from whole cell extracts by quantitative Western blot analysis also showed no significant difference in subunit levels (data not shown).

TABLE I. Comparison of Cellular Components

	Hep G2	DG75
Total mg protein × 10^7 $cells^{-1}$	1.14	1.10
Total μg RNA × 10^7 $cells^{-1}$	90	58
% mRNA of total RNA	2.2	3.8
Relative number of mitochondria × $cell^{-1}$	2	1

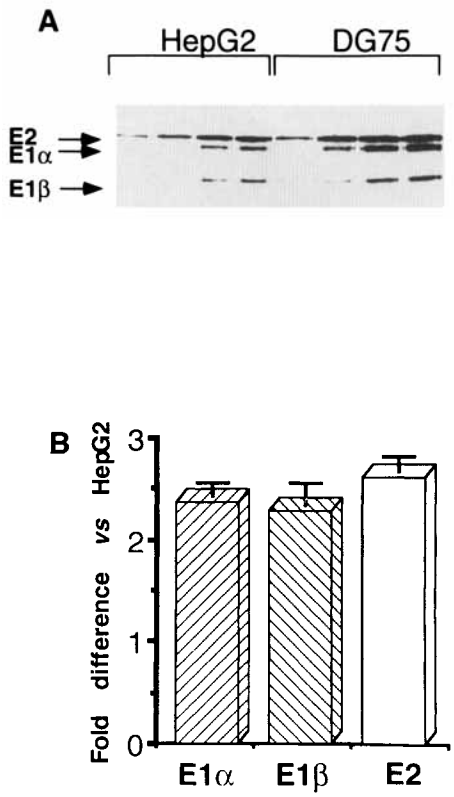

Fig. 1. Comparison of relative protein levels of BCKD subunits in mitochondria from DG75 and HepG2 cells. **A:** Western blot of 1, 3, 6, and 9 μg of mitochondrial protein from indicated cell line. **B:** Mean values ±SE of four separate experiments for DG75 relative to HepG2 for the indicated subunits.

TABLE II. Branched-Chain α-Keto Acid Dehydrogenase Activity in DG75 and HepG2 Cells*

	pmol $^{14}CO_2$-released mg $protein^{-1}$ h^{-1}		
Cell line	Basal	Total	% Active state
HepG2	784 ± 247	1604 ± 207	49
DG75	1156 ± 125	1639 ± 175	71

*Values are the average ±SE of four independent experiments from triplicate determinations in each experiment. Active state = basal activity × $total\ activity^{-1}$ × 100. Further details are in Materials and Methods.

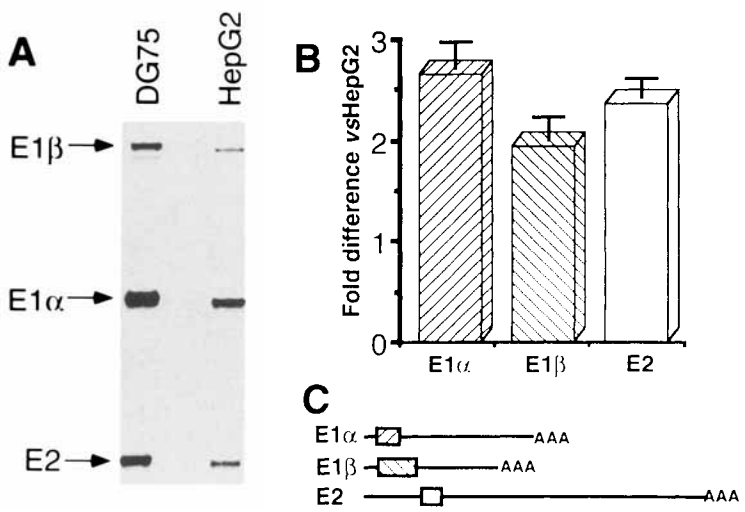

Fig. 2. Steady-state mRNA values for BCKD subunits in DG75 and HepG2 cells. **A:** Autoradiograph of RNase protected transcripts with 15 μg of total RNA from indicated cell line. **B:** Relative intensities of protected fragments determined by phosphorimaging analysis normalized to γ-actin. Values are the mean ±SE of three separate experiments comparing DG75 transcripts to those in HepG2 cells. **C:** Diagrammatic representation of the nucleotide fragments used as probes. All fragments are within the coding region of the mature subunit.

Comparison of Steady-State BCKD Transcript Levels

To determine the relationship between protein levels and mRNA levels in a cell type, relative steady-state mRNA abundances for BCKD components in HepG2 and DG75 cells were determined by RNase protection analysis. As shown in Figure 2, all three transcripts for the BCKD subunits were maintained in higher number in DG75 cells than in HepG2 cells (E1α, 2.5-fold; E1β, 1.8-fold; E2, 2.2-fold). Results were normalized to γ-actin, which reflects the percent mRNA in each cell line. Fold differences in mRNA were reproducible by Northern blot (data not shown). Molar ratios of E1α:E1β:E2 in DG75 cells were 6:1:2, whereas the ratios in HepG2 cells were 4.3:1:2. Hybridization conditions were optimized for maximal specific annealing for each probe; thus the analysis of molar ratios assumes that hybridization efficiencies were equal.

mRNA Half-Lives and Transcription Rates for the BCKD Components

Steady-state amounts of mRNA within a cell are influenced by synthesis and turnover. Half-lives of transcripts for BCKD components were determined for both cell types and varied from 11 to 21 h (Table III). Only the half-life of E2 mRNA differed between the two cell lines. Relative rates of transcription for the BCKD messages between the two cell lines were estimated by nuclear run-on assays and found to be similar in the two cell types (data not shown).

TABLE III. Half-Lives for the BCKD Transcripts in DG75 and HepG2 Cells*

	$t_{1/2}$ (h)	
Subunit	DG75	HepG2
E1α	13 ± 2	11 ± 3
E1β	17 ± 3	21 ± 2
E2	12 ± 2	21 ± 1

*Details are provided in Materials and Methods. Values are the mean ±SE from at least three separate experiments.

Increased Expression of Transcripts for Subunits of the BCKD Complex in DG75 Cells by Transfection

Hygromycin B-selected cells transfected with pREP4 (Invitrogen) plasmids bearing cDNA for one of the BCKD subunits showed approximately equal amounts of intact extrachromosomal plasmids (data not shown). Expression of mRNAs following transfection was quantitated by RNase protection analysis and reflected higher levels of transcripts: sixfold for E1α in cells transfected with pBMαR4, fourfold for E1β in cells containing pBMβR4, and twofold for E2 in cells with pBME2R4 (Fig. 3). Concentrations of endogenously produced transcripts for the other BCKD subunits remained unaffected by higher levels of the exogenously expressed transcript. Increased expression of the E1β transcript re-

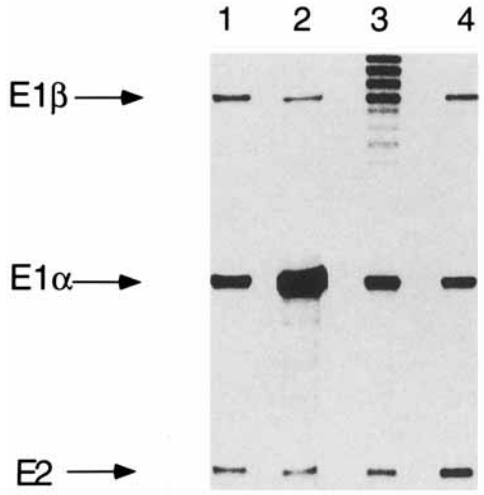

Fig. 3. RNase protection analysis of BCKD transcripts in transfected DG75 cells. Total RNA was isolated from DG75 cells transfected as follows: *lane 1,* pREP4; *lane 2,* pBMαR4; *lane 3,* pBMβR4; *lane 4,* pBME2R4.

sulted in a laddering of protected bands. Laddering could be explained by "breathing" of the duplex RNA near the ends of the E1β probe resulting from regions of repeated adenosines and uridines found within the probe [Herring et al., 1991]. The slowest moving band corresponded to a full-length protected probe. Endogenous production of E1β transcript favored protection of a single 365-nt species. Total E1β transcript was estimated as the sum of all bands in the ladder.

BCKD protein subunit concentrations were compared by quantitative Western blot analysis between cells transfected with the BCKD expression vectors and those transfected with pREP4 without insert. Overexpression of E1α mRNA resulted in twofold more E1α protein, and excess E2 mRNA produced a slight increase in E2 protein. Increased expression of either E1α or E2 did not change the amounts of the other BCKD subunit proteins present in mitochondria, as shown by statistical analysis. By contrast, increased expression of the E1β transcript resulted in twofold higher levels of E1β protein as well as increased levels in E1α and E2 proteins (Fig. 4). Differences were statistically significant at a 95% confidence level by Dunnett's test. Changes in protein levels when overexpressing E1β also resulted in increased total BCKD activity (Table IV, Fig. 4).

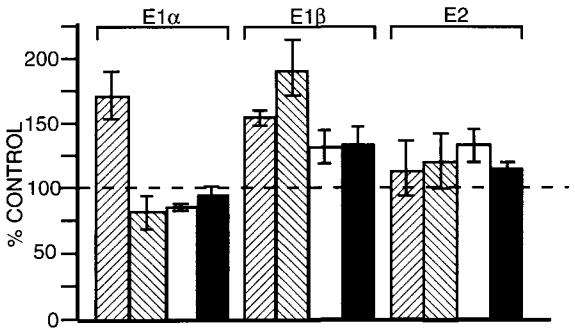

Fig. 4. Relative concentrations of BCKD protein and total activity in DG75 cells transfected as described in text. Each group represents values for all BCKD subunits and total activity as indicated by the header. *Left hatched,* E1α; *right hatched,* E1β; *stippled,* E2; *solid,* total BCKD activity. Results are expressed as a percentage of values for cells transfected with pREP4 without a cDNA insert.

TABLE IV. Influence of Overexpression of Individual Subunits of the BCKD Complex on BCKD Enzymatic Activity*

	pmol $^{14}CO_2$-releasedmg protein^{-1} h^{-1}		
Overexpressed subunit	Basal	Total	% Active state
None	1557 ± 98	2102 ± 56	74
E1α	1477 ± 111	2046 ± 33	72
E1β	2052 ± 34	2724 ± 170	75
E2	1819 ± 51	2369 ± 28	77

*Values represent the mean ±SE of triplicate assays from two independent experiments. Further details are in Materials and Methods and Table III.

DISCUSSION

Variation in mitochondria among tissues is controlled by the functional requirements of the tissue [Hood et al., 1994]. Even within a single tissue there may exist a heterogeneous population of mitochondria, again differing with function and state of development for the organelle [Cogswell et al., 1993; Goglia et al., 1988; Hood et al., 1994; Lopez-Mediavilla et al., 1989]. For ubiquitous proteins like those of the BCKD complex, it is not known whether the amount present in mitochondria will vary with tissue type. BCKD activity is regulated in different tissues by phosphorylation of the E1α subunit [Paxton et al., 1986; Wagenmakers et al., 1984]. Activity state of BCKD in muscle can change with exercise, hormones and diet but total activity remains constant [Kasperek and Snider, 1987; Patston et al., 1986; Wagenmakers et al., 1989]. Recent reports have challenged this idea suggest-

ing that increases in total BCKD activity can occur [Chinsky et al., 1994; England et al., 1995; Fujii et al., 1995; Honda et al., 1991]. Experiments were designed to define and contrast conditions within the cell that might regulate expression of BCKD.

DG75 cells double in 20 h, while HepG2 cells have a doubling time of 31 h. The number of mitochondria per cell is twofold higher in HepG2 than DG75 cells. Both have an active BCKD complex, but activity state of the complex is consistently higher in DG75 cells than in HepG2 cells. Given these obvious differences, it was unexpected to find that based on whole cell assays, BCKD activity was equal in the two cell *lines* (Table II). This implies that BCKD content per mitochondrion is two-fold higher in DG75 cells, demonstrating that ubiquitous proteins are not present in the same concentration in mitochondria from different tissues. Relative ratios of subunits to one another are the same for the different cell lines. However, absolute values for ratios of the subunits could not be determined by western blots of mitochondrial protein since polyclonal antisera may not have equal affinity for the various antigens. In addition, proteins detected can be free subunits or from assembled complexes; therefore, ratios for an active complex cannot be estimated.

Since DG75 cells divide more rapidly than HepG2 cells, it is reasonable to assume that more mRNA would be needed to maintain BCKD in the mitochondria of the DG75 cells. Steady-state mRNA amounts of all subunits were at least twofold higher in DG75 than HepG2 cells. However, half-lives of the BCKD transcripts did not appear to play a role in maintaining variation of steady-state transcript levels since half-lives were similar in the two cell types. Furthermore, run-on experiments showed that transcription rates were comparable for the BCKD mRNAs in the two cell types; thus no obvious mechanism explains the difference in steady-state levels. These experiments represent the first determination of mRNA half-lives for BCKD subunits and revealed values of 11–21 h, which are within the range of those reported for eukaryotic transcripts [Lewin, 1994]. The only difference between cell types was seen for E2-mRNA with a half-life of 12 h in DG75 and of 21 h in HepG2. Stability of the E2 transcript may be differentially affected in various tissues by the presence of an extensive 3′ untranslated region [Lau et al., 1992; McCarthy and Kollmus, 1995; Wang et al., 1995]. Further experiments are needed to examine this probable source of variation.

Despite differences seen in steady-state transcript levels for the BCKD components in the two cell types, BCKD protein abundance and total enzyme activity were strikingly similar. These findings were not surprising in light of other recent studies that showed significant induction of transcript levels for the BCKD subunits (10- to 20-fold) resulted in only twofold increases in protein levels and/or total enzyme activity [Chicco et al., 1994; England et al., 1995]. Lack of a direct stoichiometric relationship between transcript levels and protein levels has been seen for other mitochondrial complex components as well. Overexpression of the yeast 11-kd subunit of the ubiquinol-cytochrome c reductase complex resulted in a 15- to 30-fold increase in subunit mRNA levels and only a 5- to 10-fold increase in subunit protein levels [van-Loon et al., 1983]. Both translational controls and elevated turnover of excess protein were thought to be involved in inhibiting significant changes in subunit protein levels. Our findings on steady-state mRNA amounts, transcriptional rates, and amount of total BCKD complex did not address these contentions that cells resist major changes in the amount of these mitochondrial complexes. However, the overexpression studies provided data to support this idea. For example, sixfold overexpression of E1α mRNA results in only a twofold increase in protein. Similar discrepancies were observed with the other subunits.

In addressing the question of whether one of the BCKD subunits is limiting in assembly of the complex, molar ratios of the BCKD transcripts in both cell types suggest that E1β protein is the least abundant subunit. This interpretation assumes that translational efficiencies of the subunits are similar. Supporting the idea that E1β protein is limiting is the finding that E1β protein is imported into the mitochondria more slowly than the other two subunits [Sitler et al., 1995]. Results from experiments with overexpression of E1β further substantiate this hypothesis. When E1β is overexpressed, total activity is increased (Fig. 4). These results support findings from an earlier report by Fujii et al. [1995], which showed that increased muscle BCKD activity in response to physical training

was also associated with increases in E1β mRNA. In another report, Zhao et al. [1994] found that liver E1β mRNA and BCKD activity increased when animals were changed from a 0% protein diet to a 50% protein diet. Collectively, these findings point to E1β as the limiting subunit in complex formation. Since higher levels of E1β transcript and protein are not impacting transcript levels for the other subunits, these results indicate that E1β could be affecting either the import efficiency of E1α and E2 or their stability after being imported into the mitochondria.

In conclusion, our data demonstrate that ubiquitous mitochondrial multienzyme complexes can be regulated by post-transcriptional events in their formation. For these two cell lines, the amount of BCKD complex per mitochondrion varies with mitochondrial number towards a unified cellular activity level. The regulator appears to be the availability of E1β subunits.

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