

Dietary Calorie Restriction in Mice Induces Carbamyl Phosphate Synthetase I Gene Transcription Tissue Specifically*

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Dietary calorie restriction (CR) delays age-related physiologic changes, increases maximum life span, and reduces cancer incidence. Here, we present the novel finding that chronic reduction of dietary calories by 50% without changing the intake of dietary protein induced the activity of mouse hepatic carbamyl phosphate synthetase I (CpsI) 5-fold. In liver, CpsI protein, mRNA, and gene transcription were each stimulated by ~3-fold. Thus, CR increased both the rate of gene transcription and the specific activity of the enzyme. Short-term feeding studies demonstrated that higher *cpsI* expression was due to CR and not consumption of more dietary protein. Intestinal CpsI activity was stimulated 2-fold, while its mRNA level did not change, suggesting enzyme activity or translation efficiency was stimulated. CpsI catalyzes the conversion of metabolic ammonia to carbamyl phosphate, the rate-limiting step in urea biosynthesis. *cpsI* induction suggests there is a shift in the metabolism of calorie-restricted animals toward protein catabolism. CpsI induction likely facilitates metabolic detoxification of ammonia, a strong neurotoxin. Enhanced protein turnover and metabolic detoxification may extend life span. Physiologic similarities between calorie-restricted and hibernating animals suggest the effects of CR may be part of a spectrum of adaptive responses that include hibernation.

CpsI¹ is specifically expressed in hepatocytes and epithelial cells of the intestinal mucosa. It is localized in mitochondria, where it catalyzes the condensation of metabolic ammonia and HCO_3^- to carbamyl phosphate, the first step in the urea cycle in the liver (1, 2). CpsI is an abundant protein, comprising approximately 4% of liver protein (3, 4). CpsI levels are approximately 10 times lower in the small intestine (2).

The enzyme is coded for by a single copy nuclear gene (5, 6). The gene is regulated cell-type specifically, developmentally, nutritionally, and hormonally. In the liver, the enzyme and its mRNA vary with the level of dietary protein (7, 8). In rats, *cpsI* precursor RNA and mRNA are induced 3-fold by isocaloric diets containing 20% versus 4% protein (9). Increased plasma glucagon concentrations (increased intracellular cAMP) have been

shown to directly induce the level of *cpsI* mRNA (10–13). Glucocorticoids also stimulate *cpsI* mRNA in the liver (13, 14). This glucocorticoid response is reduced about 50% by insulin in hepatoma cells in culture (15). Epinephrine reduces the rate of CpsI synthesis in isolated rat hepatocytes (16). An attractive aspect of *cpsI* for studying the effects of nutrition on life-span is that enzyme activity and protein content do not vary with age in rodents, simplifying the analysis (17). Intestinal expression of the gene is not nutritionally or hormonally regulated, making possible cell type-specific studies of its regulation (11).

The transcription factors and cis elements mediating the cell-specific, hormonal, and nutritional regulation of *cpsI* expression are not well characterized. Six sequence elements proximal to the transcription initiation site are specifically bound by liver nuclear factors and by bacterially expressed C/EBP α , and one of these sites is required for activation of the promoter by overexpressed C/EBP α in transfected cells (18). Three of the six sites appear to be bound by liver-specific factors (19). More recently, four sites were shown to play roles in the expression of the gene. A direct repeat adjacent to the TATA homology activates transcription, while two other sites repress this activation. A fourth site appears to obviate the effects of the two negative sites (20).

CR delays most age-related physiologic changes and increases both mean and maximum life span in every model system tested (21). It is the only method known for extending life span in homeothermic vertebrates and the most effective means known for reducing cancer incidence and increasing the mean age of onset of age-related diseases and tumors (21, 22). CR reduces sustained plasma glucose concentrations, and this leads to reduced intracellular glucose concentrations in hepatocytes (23–26). Liver glucose transport is mediated mostly by the GLUT2 glucose transporter (27). It is present in hepatocytes, pancreatic β cells, and specialized regions of the plasma membrane of a few other cell types. Because transport through GLUT2 is symmetric, the flux of glucose is directly proportional to extracellular and intracellular glucose concentrations (28, 29). The decrease in intracellular glucose concentration is likely to affect the expression of some hepatic genes. We have found that CR results in the liver-specific, negative, post-transcriptional regulation of the gene for glucose-regulated protein 78 (26, 63).²

Although calorie-restricted mice (CR mice) have lowered plasma glucose concentrations, they are not stressed. They are healthier than mice fed *ad libitum* (21). CR mice are not starving. The diet is formulated so that the animals are not limited for any nutrient (26). Glucose transporter 1 mRNA is induced

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¹ The abbreviations used are: CpsI, carbamyl phosphate synthetase I; AL mice, *ad libitum*-fed mice; CR, dietary calorie restriction; CR mice, calorie-restricted mice; kb, kilobase; T₃, triiodothyronine.

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~3-fold in rat hepatocytes by starvation (30). We have found that this mRNA is not regulated by CR in the liver, adipose, brain, heart, kidney, lung, muscle, or intestine of mice.²

In this report, we present the novel finding that reduction of dietary calories by ~50%, without changing the intake of dietary protein, induced the level of hepatic CpsI activity, protein, mRNA, and gene transcription by 3–5-fold while having no detectable effect on the stability of the mRNA. Our results suggest that CR increases protein catabolism, probably for gluconeogenesis. The change in *cpsI* mRNA also occurred with a short-term shift from CR to *ad libitum* feeding, indicating that the change in metabolism responsible for gene induction is relatively rapid. Ammonia is a toxic end product of protein catabolism, and CpsI is the rate-limiting enzyme for the metabolic detoxification of ammonia. Whether enhanced protein catabolism and ammonia detoxification are related to the many beneficial effects of CR is unclear at present. However, enhanced protein turnover and enhanced metabolic detoxification have been postulated to have roles in life span extension (31, 32). There are also physiologic similarities between CR and hibernating animals which suggest that the effects of CR are part of the adaptive responses that include hibernation.

EXPERIMENTAL PROCEDURES

Animals—Females of the long-lived F₁ hybrid strain C3B10RF₁ have been used by us previously for studies of CR (e.g. Ref. 26). The mice are bred from C57BL10.RIII and C3H.Sw/Sn lines obtained from Jackson Laboratories. Mice were weaned and started on the diets at 28 days of age. They were housed individually and subjected to one of the two diet regimens described below. Mice were maintained at 20–24 °C and 50–60% humidity with lights on from 0600 to 1800 hours. Animals had free access to water. Sentinel mice were kept in the same room as the experimental mice, and serum samples were screened every 6 months for titers against 11 common pathogens. No positive titers were found during these studies. Three cohorts of mice were utilized. A cohort consisting of eight 21-month-old mice in each dietary group was utilized for hepatic transcription run-on assays, mRNA analysis, and determining CpsI protein levels. Another cohort, consisting of ten 6-month-old CR mice, was used for a study of short-term dietary effects. Five CR mice were fed *ad libitum* for 1 week while five remained calorie restricted. A third cohort, consisting of twelve 24-month-old mice in each dietary group, was utilized for studying *cpsI* mRNA half-life using actinomycin D. Mice were killed by cervical dislocation, and their livers and small intestines were removed. The intestines were gently flushed with phosphate-buffered saline before use (Flow Laboratories, McLean, VA).

Diets—The composition of the diets was as described (26). The mice were fed and maintained as described (33). Both dietary groups ate a purified diet containing all protein, fat, vitamins, and minerals. The *ad libitum*-fed mice (AL mice) consumed between 100 and 110 kcal per week, and the 50% CR mice consumed 49 kcal per week.

Determination of CpsI Levels—Approximately 25 mg of liver tissue was sonicated for 8 s at a setting of 3 (Branson model 350 sonifier cell disrupter) in 300 µl of 50 mM Tris (pH 6.8), 5% β-mercaptoethanol, 2% SDS, and 10% glycerol. Debris was removed by centrifugation in a Beckman microfuge at 16,000 × *g*, and protein concentrations were determined (34). Each sample (15 µg) was subjected to SDS-polyacrylamide gel electrophoresis on a 5% gel (35). The level of the ~160,000 molecular weight CpsI band was quantified by scanning the dried gel with a light densitometer (E-C Apparatus Corp., St. Petersburg, FL).

Measurement of CpsI Activity—Approximately 20 mg of liver tissue was sonicated three times for 1 s each with cooling in an ice bath, at a setting of 3 (Branson model 350 sonifier cell disrupter) in 380 µl of ice-cold water. Debris was removed by centrifugation in a Beckman microfuge at 16,000 × *g* for 10 min at 4 °C. CpsI activity assays were performed and units were calculated as described (36). The assay was linear with respect to input liver homogenate over the range of activities measured.

RNA Isolation and Visualization—Approximately 0.2 g of frozen liver tissue was homogenized for 30 s in 4 ml of TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) using a Tekmar Tissuemizer (Tekmar Co., Cincinnati, OH) at a setting of 55. RNA was isolated using TRI reagent as described by the supplier. RNA was resuspended in

FORMAzol (Molecular Research Center, Inc.). Northern and dot blots were performed as described using 20 and 10 µg of RNA, respectively (37, 38). To quantify specific mRNA, blots were serially probed. cDNA probes were labeled with [α -³²P]ATP to a specific activity of 1 × 10⁸ cpm/µg by using a multiprime labeling kit (Pharmacia Biotech Inc.). The *cpsI* probe was a 1.2 kb *Pst*I and *Eco*RI fragment excised from pHN3491 (ATCC/National Institutes of Health Repository), a plasmid vector containing the rat *cpsI* cDNA (5). Mouse transcription factor S-II cDNA was purified from the vector in a similar manner (39). Blots were also probed with a synthetic oligonucleotide complementary to mouse 18 S rRNA (40) and with oligo(dT) (Pharmacia Biotech Inc.). 5'-end labeled with [γ -³²P]ATP and T₄ polynucleotide kinase (New England Biolabs, Beverly, MA) to a specific activity of 4 × 10⁵ cpm/pmol. Blots were subjected to autoradiography using two intensifying screens, and hybridization was quantified using a phosphorimager (Molecular Dynamics).

Nuclear Run-on Assays—Frozen liver tissue (0.25–0.3 g) from three or four different animals fed *ad libitum* was pooled, and an equivalent amount of liver tissue from four different 50% CR animals was pooled separately. Samples were homogenized in 10 ml of ice-cold NA buffer (10 mM Tris (pH 8.0), 2.5 mM magnesium acetate, 0.3 M sucrose, and 0.25% Triton X-100) with five strokes at 2000 rpm using a motor-driven Potter-Elvehjem tissue grinder (Wheaton, Millville, NJ). 10 ml of NB buffer (10 mM Tris (pH 8.0), 2.5 mM magnesium acetate, 2.4 M sucrose, and 0.1% Triton X-100) was added, and the homogenate was layered onto 15 ml of NB buffer. The homogenate was centrifuged at 113,000 × *g* for 1 h at 4 °C in a Beckman SW28 rotor. The supernatant was discarded, and the nuclei were washed in 0.75 ml of 50 mM HEPES (pH 7.5), 0.1 mM EDTA, 5.0 mM dithiothreitol, and 10% glycerol. Nuclear transcription reactions were performed as described (41). After transcription, a volume of TRI reagent equal to 10 times the volume of the transcription reaction was added, and RNA was isolated as described by the manufacturer. Unincorporated nucleotides were removed by gel filtration. The total ³²P-labeled RNA was diluted to 100 µl in a final concentration of 50 mM HEPES (pH 7.0), 500 mM NaCl, 8 mM EDTA, 0.4% SDS, 35% formamide, and 1500 cpm of ³H-labeled *cpsI* cRNA. The radiolabeled RNAs were hybridized at 42 °C for 17 h to denatured DNA fixed to 38 mm² nitrocellulose filters. The filters had 5 µg of pBR322 DNA or 5 µg of rat *cpsI* cDNA (pHN3491; Ref. 5) bound to them. Filters were washed, and the amount of hybrid was determined as described (41).

In Vitro Transcription of ³H-Labeled *cpsI* cRNA—To synthesize *cpsI* cRNA for use as an internal hybridization standard for nuclear run-on assays, plasmid SP6-*cpsI* was constructed. SP6-*cpsI* contains the 1.2-kb *Pst*I to *Eco*RI fragment of the *cpsI* cDNA present in pHN3491 linked to the SP6 promoter in pLUC (42). SP6-*cpsI* was linearized with *Eco*RI and used as template in a transcription reaction with SP6 RNA polymerase and 0.5 mM GTP, UTP, and ATP and 13 µM [³H]CTP (21 Ci/mmol). The cRNA was labeled to a specific activity of 3000 cpm/fmol.

Actinomycin D Treatment of Mice—Animals were given intraperitoneal injections of 4 mg/kg, body weight, actinomycin D (Sigma) in phosphate-buffered saline. At the indicated times, livers were removed, and RNA was prepared as described above. *cpsI* mRNA levels determined using dot and Northern blots were normalized to the level of 18 S rRNA, since rRNA is much more stable than mRNA, and its level does not change with diet (26).

Statistical Analysis—Statistical significance was determined using Student's unpaired *t* test. A 95% level of confidence was considered significant.

RESULTS

CpsI Levels Were Induced by CR—We found that a protein with an apparent molecular weight of ~160,000 on an SDS-polyacrylamide gel was induced about 3-fold in the liver of CR mice (Fig. 1). This band was identified as CpsI by 4 criteria: molecular weight, tissue distribution, abundance, and N-terminal amino acid sequence. The molecular weight of the mature form of rat CpsI is ~160,000 (5). The band was well separated from other proteins and appeared to be composed of a single protein, representing approximately 4% of the total protein present. The band was absent from all other tissues examined (brain, heart, kidney, fat, muscle) with the exception of intestine, where it was present at less than 10% of the level in liver. This matches the tissue distribution and abundance reported for CpsI (2). Microsequencing of the N-terminal 10 amino acids

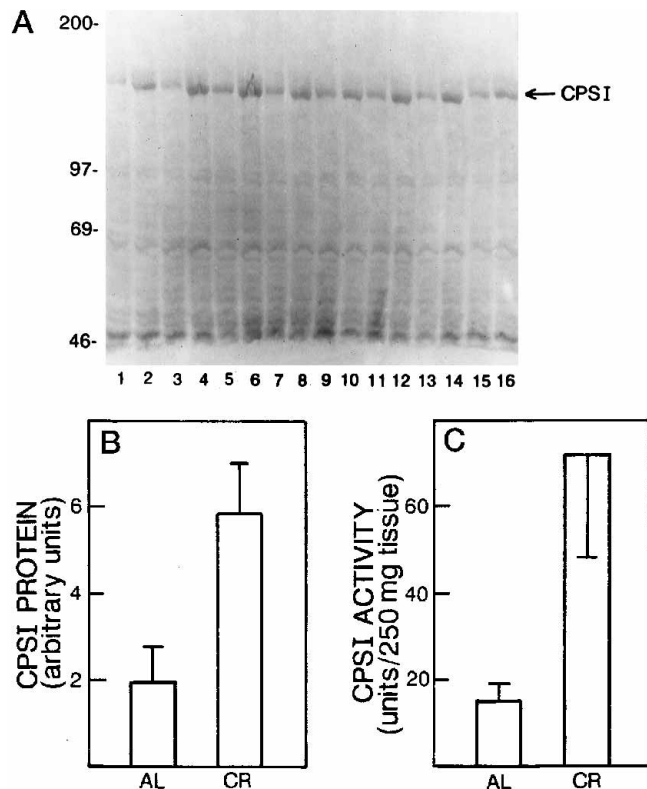


FIG. 1. CR induced CpsI protein levels and enzyme activity in liver. Panel A, total SDS soluble mouse liver protein resolved by SDS-polyacrylamide gel electrophoresis. Odd-numbered lanes represent proteins from AL mice. Even-numbered lanes represent protein from CR mice. The arrow labeled CPSI indicates the position of the protein, and the numbers to the left of the figure indicate the positions of standards in kilodaltons. Panel B, quantitation of the relative level of CpsI protein present. The means and standard deviations are shown for samples from four AL and four CR mice. Panel C, CpsI activity present in livers from each dietary group. The means and standard deviations are shown for four AL and four CR mice.

of the 160,000 molecular weight protein by the Biotechnology Instrumentation Facility (University of California, Riverside) yielded a sequence that matched 7 out of 10 of the N-terminal amino acids of the mature form of rat CpsI, as predicted from its cDNA sequence (5, 43). The sequence of the mouse cDNA is not known yet.

Hepatic CpsI levels in AL and CR mice (odd- and even-numbered lanes, respectively) are shown in Fig. 1A. Because CpsI is a highly abundant protein and of an unusually large size, it can be visualized directly by dye binding. The differences in the level of CpsI cannot be accounted for by differences in the amount of protein loaded in each lane. When the level of CpsI was quantified and corrected for the total amount of protein present in each lane, CpsI was induced approximately 3-fold ($p < 0.01$; Fig. 1B). The staining of CpsI was linear with respect to protein over the range of concentrations present in this study (data not shown).

The data shown in Fig. 1A also illustrate that the effect of CR on CpsI levels is highly specific. Of the ~20 proteins clearly visible in the stained gel, CpsI is the only protein consistently altered by diet.

Hepatic CpsI Enzyme Activity Was Induced by CR—Studies were conducted to determine whether the induction of CpsI was accompanied by an increase in the activity of the enzyme. Analyzing liver homogenates from animals in each dietary group revealed a 5-fold induction of CpsI activity in CR mice ($p < 0.01$; Fig. 1C). Therefore, the 3-fold induction of CpsI was

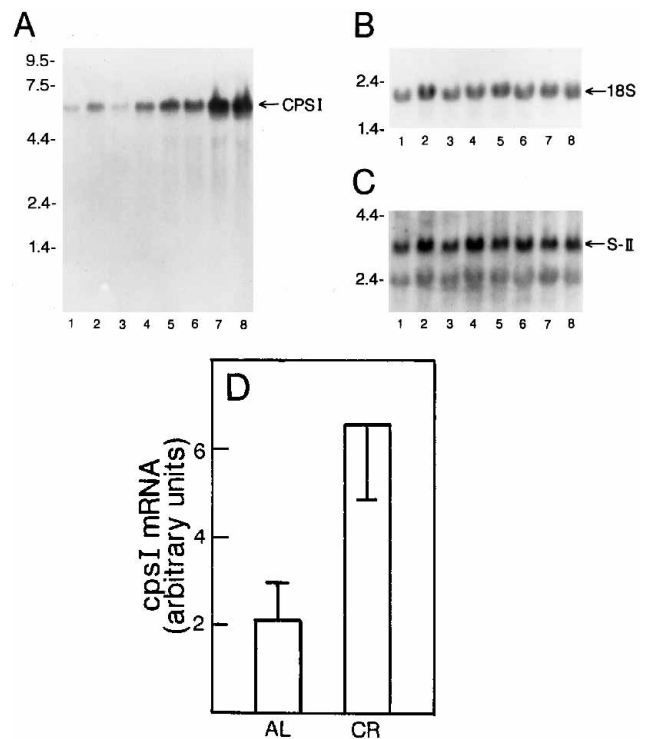


FIG. 2. CR induced hepatic *cpsI* mRNA. Panel A, hepatic *cpsI* mRNA is induced by CR. Total RNA isolated from AL (lanes 1–4) and CR (lanes 5–8) mice was subjected to Northern blot analysis. The results of probing the blot with radiolabeled *cpsI* cDNA sequences are shown. The arrow labeled CPSI indicates the position of the 6.2-kb mRNA, and the numbers to the left of the figure indicate the positions of RNA molecular weight standards in kilobases. Panel B, the level of 18 S rRNA present in each lane of the blot shown in panel A. Panel C, the levels of transcription factor S-II mRNA present in each lane of the blot in panel A. Panel D, the means and standard deviations of the *cpsI* mRNA levels in livers of four mice from each dietary group are illustrated. To control for RNA loading and transfer, *cpsI* mRNA was normalized to the level of 18 S rRNA.

accompanied by a roughly 5-fold increase in the activity of the enzyme. These results suggest that in addition to an increase in the amount of enzyme in CR mice, the specific activity of the enzyme itself may increase.

The Induction of CpsI Was Accompanied by an Increase in *cpsI* mRNA—The level of hepatic *cpsI* mRNA was determined using livers from the studies analyzing CpsI protein and enzyme activity (Fig. 2A). A single species of mRNA was detected, with an apparent size of 6.2 kb. After correction for loading and transfer of total RNA using the level of 18 S rRNA present in each lane (Fig. 2B) or the level of transcription factor S-II mRNA present (Fig. 2C), hepatic *cpsI* mRNA levels were induced just over 3-fold in CR mice (Fig. 2D). This increase was statistically significant ($p < 0.01$). We have shown that S-II mRNA does not change with respect to polyadenylated RNA or rRNA in CR or AL mice (44). The increase in *cpsI* mRNA was specific. We have examined the expression of many other mRNA, including S-II mRNA, and none of these changed with CR (Ref. 26 and data not shown). Thus, hepatic *cpsI* mRNA is specifically induced by CR, and this induction closely parallels the increase in hepatic CpsI protein and enzyme activity. These results indicate that CR acts at an early step in gene expression and does not influence the translation or stability of CpsI protein.

***cpsI* mRNA Stability Is Not Altered by CR**—Because it is possible that CR might affect the stability of *cpsI* mRNA, a study of its stability was conducted (Fig. 3). In this study, the

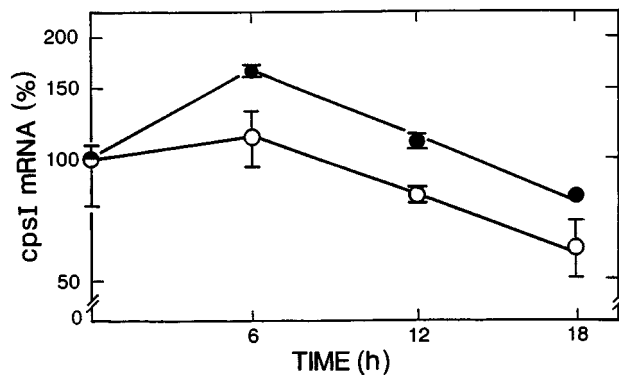


FIG. 3. CR did not change the stability of hepatic *cpsI* mRNA. Shown is the level of hepatic *cpsI* mRNA present at various times after treating animals with actinomycin D. The results obtained using CR (open symbols) and AL (closed symbols) mice are displayed. *cpsI* mRNA levels were determined by dot blot analysis and normalized to the level of 18 S rRNA present. Each point and error bar represents the mean and standard deviation obtained using 3 animals. For the zero time point, the error bar pointing up is for AL mice, and the bar pointing down for CR mice.

level of expression in the control mice, injected with the vehicle, was 0.5 ± 0.07 and 1.1 ± 0.23 for AL and CR mice, respectively (Fig. 3, zero time point; $p < 0.02$). During the first 6 h of actinomycin D treatment, there was a transient superinduction of *cpsI* mRNA in both AL and CR mice. Superinduction of other genes has been reported after actinomycin D treatment (45). This increase was greater in AL mice, suggesting that the transcription of the gene may be repressed differentially in AL mice by a relatively unstable RNA or protein. In these same animals we found that glucose-regulated protein 78 precursor RNA levels decreased by 6 h to about 10% the level found before actinomycin D treatment, suggesting that transcription is strongly inhibited by that time (data not shown). The decay rates of *cpsI* mRNA between 6 and 18 h after drug treatment indicate that the half-life of *cpsI* mRNA was ~ 12 h in both dietary groups. Thus, no change in stability of hepatic *cpsI* mRNA was detected.

CR Induced the Transcription of *cpsI*—To determine whether CR directly induced the transcription of *cpsI*, two independent transcription run-on studies were performed (Table I). In these experiments, the rate of *cpsI* transcription was enhanced an average of ~ 3 -fold in CR mice. These results are shown in Table I. The rate of transcription of the glucose-regulated protein 78 gene also was determined in these hybridization reactions. It does not change with CR (63). The magnitude of the increase in *cpsI* transcription can account for the increase in *cpsI* mRNA and protein levels.

CR Increased CpsI Activity but Not mRNA in the Small Intestine—Because *cpsI* expression in the small intestine is not altered by glucocorticoids or glucagon (cAMP), we investigated the effects of CR on intestinal CpsI activity (Fig. 4). Consistent with other reports, the level of CpsI activity was approximately ten times lower in intestine than in liver (2). Surprisingly, the activity of intestinal CpsI responded to CR. CpsI activity almost doubled in the small intestine of CR mice (Fig. 4A), while it was enhanced approximately 6-fold in liver (Fig. 4B). The increases were statistically significant in both intestine ($p < 0.05$) and liver ($p < 0.03$).

To determine whether the increase in CpsI activity in the intestine is accompanied by a change in *cpsI* mRNA, dot blots were used to quantify intestinal and liver *cpsI* mRNA in CR and AL mice (Fig. 4, C and D). *cpsI* mRNA levels were 3- and 6-fold lower in intestine than in liver of CR and AL mice, respectively. There was no statistically significant change in

TABLE I
Transcriptional regulation of *cpsI* gene expression by CR

Experiment ^a	Diet	Nuclear RNA input to hybridization	Standard RNA hybridized ^b	Nuclear RNA hybridized ^c	Transcription rate ^d
		cpm	%	cpm	ppm
1	AL	39×10^6	10	350	9
	CR	22×10^6	9	555	25
2	AL	24×10^6	8	112	5
	CR	38×10^6	7	557	15

^a Approximately equal amounts of frozen liver tissue from three or four different AL mice and an equivalent amount of liver tissue from four different CR mice were separately pooled. The experiments utilized different animals in the 21-month-old cohort. Each experiment represents a single determination.

^b Hybridization efficiency was determined from the amount of specific ³H-labeled *cpsI* cRNA hybridized to filters containing *cpsI* cDNA after subtracting the amount adsorbed to pBR322 DNA, divided by the amount of total [³H]cRNA in the reaction (1,500 cpm). The background bound to pBR322 containing filters averaged 20 cpm.

^c Nuclear RNA hybridized was calculated by subtracting background hybridization of the ³²P-labeled nuclear transcripts to pBR322 DNA from hybridization to *cpsI* cDNA. These values were corrected for the amount of standard RNA hybridized. Background hybridization to pBR322 DNA averaged 2 ppm.

^d Specific gene transcription rates in parts per million (ppm) were calculated by dividing the amount of ³²P-labeled nuclear RNA specifically bound to filters containing *cpsI* by the total amount of ³²P-labeled nuclear RNA in the hybridization reaction. The average rates of transcription were 7 and 20 in AL and CR mice, respectively.

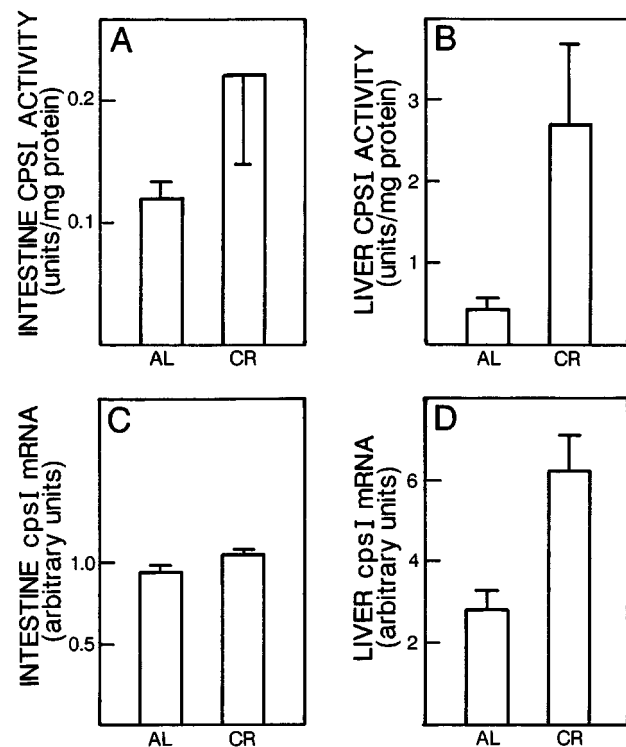


FIG. 4. Intestinal CpsI activity but not mRNA responded to CR. CpsI activity and mRNA were measured in the livers and small intestines of AL and CR mice. Each panel represents the mean and standard deviation of determinations from three animals in each dietary group. Panel A, intestinal CpsI activity levels are shown. Panel B, hepatic CpsI activity levels are shown. Panel C, intestinal *cpsI* mRNA levels are shown. Panel D, liver *cpsI* mRNA levels are shown.

the level of intestinal *cpsI* mRNA, while the level of *cpsI* mRNA in the liver was stimulated approximately 2.5-fold in these mice (Fig. 4D). These results suggest that the enzyme is translationally regulated or post-translationally modified to a more

TABLE II
Calorie restriction but not dietary protein induced *cpsI* mRNA

	CR	AL
Caloric intake/week ^a		
Total	49.0	105.0
Protein	19.8	23.4
Carbohydrate	22.6	73.6
Fat	6.7	7.9
20 month feeding ^b		
Body weight (g)	27.3 ± 2.8	45.6 ± 9.4
Protein intake (caloric intake/week/g body weight)	0.73	0.51
<i>cpsI</i> mRNA (arbitrary units)	1.58 ± 0.49	0.54 ± 0.17
1 week feeding ^c		
Body weight (g)	22.4 ± 1.53	23.3 ± 1.93
Protein intake (caloric intake/week/g body weight)	0.88	1.0
<i>cpsI</i> mRNA (arbitrary units)	1.41 ± 0.22	0.74 ± 0.09

^a Caloric intake in kcal is shown for both CR and AL mice. The diets are formulated to be nearly isocaloric for all components with the exception of calories derived from carbohydrate.

^b Mice 21 months of age were used in these studies. The diets were begun at the time of weaning and continued until mice were sacrificed. Weights were determined at the time of sacrifice. Means and standard deviations were determined for the eight animals in each dietary group.

^c Mice 6 months of age were used in these studies. Five CR mice were fed *ad libitum* for 1 week while five remained calorie restricted.

active form in the small intestine of CR mice.

cpsI mRNA Is Induced by the Reduction in Calories, Not by the Increase in Protein Consumption—*cpsI* gene expression is induced by high protein consumption. For example, rat hepatic *cpsI* mRNA increased 3-fold when the composition of isocaloric diets was shifted from 4 to 20% protein content, a 500% increase (9). For these reasons, we investigated the possibility that *cpsI* was induced in CR mice by higher protein consumption. Total protein intake is similar in both AL and CR mice (Table II). However, long term calorie restriction resulted in a 40% difference in body weight (Table II). Therefore, CR mice consumed 40% more calories from protein per gram (body weight). The increase in the protein consumed by CR mice is much smaller than the 500% increase used in studies such as those cited above.

To investigate the possibility that the 40% difference in protein consumption induced the gene, another study was performed. Two groups of mice of approximately equal weights were fed *ad libitum* and CR. After only 1 week, *cpsI* mRNA levels were twice as high in the CR mice ($p < 0.001$), even though protein consumption per gram (body weight) was 10% lower in the CR group (Table II). These results are consistent with those of the long term diet studies, suggesting that *cpsI* gene expression is induced by reduction of dietary calories and not by changes in the amount of protein consumed. Thus, protein metabolism and *cpsI* gene expression adjust rapidly to shifts in the amount of calories consumed.

DISCUSSION

In the studies reported here, we present the novel finding that chronic 50% reduction in dietary calories, without a change in dietary protein, led to a specific, statistically significant 3-fold induction of a 160,000 molecular weight hepatic protein we have identified as CpsI. To better understand the basis for nutritional regulation of gene expression, we investigated the effects of CR on key steps in the expression of the *cpsI* gene. The increased level of CpsI was accompanied by a statistically significant 5-fold induction of the enzyme activity. Hepatic *cpsI* mRNA levels and transcription were both induced by ~3-fold. Thus, CR increases both the rate of gene transcription

and the specific activity of the enzyme. Short-term feeding studies demonstrated that higher *cpsI* expression in CR mice was due to reduced consumption of dietary calories and not to consumption of more dietary protein. The change in *cpsI* mRNA occurred with a short-term shift from CR to *ad libitum* feeding, indicating that the change in metabolism responsible for gene induction is relatively rapid. In intestine, CR led to a roughly 2-fold induction in intestinal CpsI activity, without a change in the level of *cpsI* mRNA. These results suggest that CR increases the specific activity or rate of translation of CpsI in intestine. Together, our results suggest that CR increases protein catabolism, probably for gluconeogenesis.

The mechanism by which CR regulates *cpsI* transcription is not known yet. *cpsI* mRNA levels are induced by glucagon and glucocorticoids, suggesting that CR-induced changes in the levels of one or both of these hormones might be responsible. However, serum glucagon concentrations are not altered by CR in rats or mice (21, 46). Thus, glucagon regulation of intracellular cAMP levels is not a likely source of the change in the rate of *cpsI* transcription.

The effects of CR on glucocorticoid levels are more complex (47). The mean 24-h plasma total corticosterone concentrations of AL and CR rats are similar in younger animals. As the animals age, there is a modest rise in the mean 24-h plasma total corticosterone concentrations in AL mice. However, there is also a decline with age in the level of corticosterone binding globulin in CR animals, resulting in a gradual increase with age in mean 24-h free corticosterone concentrations and in the daily circadian peaks of free corticosterone. The possible effects of free, total, mean, and circadian peak concentrations of glucocorticoids on *cpsI* gene transcription are unclear. However, it is difficult to see how small changes such as a 25% increase in mean 24-h plasma corticosterone concentrations in AL mice could result in 3-fold inhibition of *cpsI* transcription.

Growth hormone suppresses CpsI activity *in vivo*, and serum growth hormone concentrations are reduced ~50% by CR in rats (48, 49). However, growth hormone decreases CpsI activity by decreasing the intracellular level of *N*-acetyl-L-glutamate, an allosteric activator of CpsI. The activator is present in excess in our *in vitro* assays and therefore cannot be responsible for the differences in activity reported here.

Insulin and epinephrine both suppress CpsI synthesis in primary cultured hepatocytes and Reuber hepatoma H-35 cells, and their effects are additive (16). Blood epinephrine levels do not change with CR in rats (50). Insulin may act by suppressing glucocorticoid stimulation of *cpsI* mRNA by 50% (15). CR does decrease serum insulin levels (46, 51, 52). However, it is not clear whether this decrease in insulin could produce the 3-fold induction of *cpsI* transcription found in the studies reported here. Thus, at this time we are unable to suggest a known regulatory signal that is likely to be responsible for the change in the rate of *cpsI* transcription found in CR mice.

Isocaloric diets containing 20% versus 4% protein increase hepatic *cpsI* precursor RNA and mRNA by ~3-fold in rats (9). In the study reported here, hepatic *cpsI* mRNA and gene transcription were induced ~3-fold by a 50% reduction of dietary calories without any change in dietary protein. In both kinds of studies, *cpsI* mRNA and gene transcription rates are high when calories derived from carbohydrates are low. Since it is not clear whether glucagon, insulin, or glucocorticoids are responsible for *cpsI* regulation in CR animals, it is possible that the gene responds directly to blood glucose concentrations. CR decreases blood glucose levels by 43% under the conditions used in this study (26). Because the transcription factors and cis elements mediating the hormonal and nutritional regulation of *cpsI* expression are poorly characterized, it is possible

that the gene contains carbohydrate response elements or other genetic elements mediating responsiveness to ammonia. A cis element has been described for the rat S14 gene, which appears to mediate responsiveness to carbohydrate concentrations (53). The six specific binding sites for bacterially expressed C/EBP α located proximal to the *cpsI* transcription initiation site could be involved in carbohydrate regulation of the gene. Gadd153, a CCAAT/enhancer-binding protein (C/EBP) which lacks a DNA binding domain and heterodimerizes with other C/EBPs, is induced by glucose deprivation in at least two cultured cell lines (40). Thus, it is possible that regulation of the level or activity of this or another C/EBP by dietary calories could influence the expression of *cpsI*.

The relative levels of *cpsI* mRNA in liver and intestine were similar to those reported by others (2). Also in agreement with others, we found no regulation of *cpsI* mRNA levels in the small intestine (11). However, we did find induction of CpsI activity in the small intestine of CR mice. This result is novel, and it suggests that the CpsI is translationally regulated or the specific activity of the enzyme is enhanced post-translationally. We consistently find that *cpsI* mRNA and protein are increased 3-fold by CR in liver, while the activity of the enzyme increases 5–6-fold (Figs. 1 and 4). Thus, hepatic CpsI appears to be post-translationally modified and we believe that CpsI is regulated similarly in the small intestine. The increase may be a response to higher ammonia production by the luminal bacteria of the intestine. These bacteria may catabolize more protein due to the lower carbohydrate intake of CR mice.

The total protein synthetic activity of liver and other tissues decreases with age in organisms as diverse as insects and humans (reviewed in Ref. 31), but the rate of synthesis of some proteins remains unchanged while the synthesis of others even increases. The effects of aging on protein degradation are not as well described. However, since protein synthetic activity decreases with age while the total protein content of cells and tissues remains constant, the rate of protein degradation is thought to decline with age. The ability of cells to degrade structurally aberrant proteins appears to decrease with age (31). Dietary restriction reduces this age-related decline, increasing the rate of protein turnover (54–57). Enhanced protein turnover leads to increased levels of metabolic nitrogen. Thus, the increase in hepatic CpsI activity in CR animals is likely to result from both increased catabolism of dietary protein for the generation of metabolic energy and enhanced turnover of cellular proteins.

The ammonia produced by protein catabolism is highly neurotoxic, playing a role in pathologies such as hepatic encephalopathy and perhaps Alzheimer's disease (58, 59). The 5-fold induction in CpsI activity during CR is likely to reduce the level of free ammonia in blood and therefore to decrease the level of brain ammonia. However, it is presently unclear how the increased metabolic capacity for ammonia detoxification compares to the increase in ammonia production from protein catabolism.

The increase in CpsI found in CR animals suggests an increase in urea production and the ability to handle ammonia and HCO_3^- as "end products" of metabolism. To our knowledge, CpsI has not been measured in hibernating animals. However, a major problem during hibernation is the accumulation of ammonia and HCO_3^- (60). Other similarities exist between CR and hibernating animals. These include reduced body temperature, lower serum T_3 , lower blood glucose, and a substantial increase in protein synthesis and turnover (21, 31, 60). It is well established that a mild reduction in environmental temperature may greatly extend the life span of poikilothermic vertebrates (61) and that in homeotherms, hibernation affects "bio-

logical time" (60). The reduction in body temperature during hibernation need not be severe. The body temperature of bears, for example, falls by only about 2 degrees during hibernation, and hibernation extends their life span. A comparable reduction in body temperature has been observed in CR mice (62). We suggest on the basis of these various parallels that the calorie restriction paradigm may be part of a broad spectrum of adaptive responses that include hibernation. This "hibernation hypothesis" suggests lines of inquiry for future studies into the mechanisms by which CR affects life span and metabolism.

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