n-6 PUFAs downregulate expression of the tricarboxylate carrier in rat liver by transcriptional and posttranscriptional mechanisms

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Abstract **The tricarboxylate (citrate) carrier (TCC), a protein of the mitochondrial inner membrane, is an obligatory component of the shuttle system by which mitochondrial acetyl-CoA is transported into the cytosol, where lipogenesis occurs. The aim of this study was to investigate the molecular basis for the regulation of TCC gene expression by a high-fat, n-6 PUFA-enriched diet. Rats received for up to 4 weeks a diet enriched with 15% safflower oil (SO), which is high in linoleic acid (70.4%). We found a gradual decrease of TCC activity and a parallel decline in the abundance of TCC mRNA, the maximum effect occurring after 4 weeks of treatment. At this time, the estimated half-life of TCC mRNA was the same in the hepatocytes from rats on both diets, whereas the transcriptional rate of TCC mRNA, tested by nuclear run-on assay, was reduced by ~38% in the rats on the SO-enriched diet. The RNase protection assay showed that the ratio of mature to precursor RNA, measured in the nuclei, decreased with the change to the n-6 PUFA diet. These results suggest that administration of n-6 PUFAs to rats leads to changes not only in the transcriptional rate of the TCC gene but also in the processing of the nuclear precursor for TCC RNA.**—Siculella, L., F. Damiano, S. Sabetta, and G. V. Gnoni. **n-6 PUFAs downregulate expression of the tricarboxylate carrier in rat liver by transcriptional and posttranscriptional mechanisms.** ***J. Lipid Res.*** **2004.** 45: **1333–1340.**

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PUFAs are potent regulators of cellular metabolism. PUFAs, as components of dietary lipids, not only influence hormonal signaling events by modifying membrane lipid composition but also have a direct effect on the molecular events that govern gene expression (1). PUFA ingestion leads to a noticeable inhibition of the activity of many proteins/enzymes involved in both carbohydrate metabolism and lipogenesis (2). In this regard, PUFAs have long been known to decrease the capacity of liver cells to synthesize fatty acids de novo (3, 4). This pathway is most active in liver and adipose tissue and involves a set of enzymes referred to as lipogenic enzymes (5). These include FAS, acetyl-CoA carboxylase (ACC), malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PD), and 6-phosphogluconate dehydrogenase. Consistent with their role in energy metabolism, the activities of these enzymes are induced when animals are fed a high-carbohydrate diet and decreased during starvation or by a high-fat, PUFA-enriched diet [for review, see ref. (5)]. Similarly, the activities of key enzymes involved in regulating the flux of glucose to fatty acids, such as glucokinase, pyruvate kinase (L-PK), and pyruvate dehydrogenase, are influenced by a high-PUFA diet (6). It has been shown that PUFAs inhibit rodent hepatic lipogenesis by suppressing the mRNA encoding for several proteins, including ACC, FAS, L-PK, G6PD, ME, and the S14 protein (2). Different mechanisms are responsible for the regulation of the lipogenic enzymes at the molecular level. The PUFA-mediated inhibition of ACC, FAS, L-PK, stearoyl-CoA desaturase (SCD-1), and, in part, S14 protein occurs at the transcriptional level (6–11), whereas PUFA suppression of G6PD and ME is thought to occur through a posttranscriptional mechanism (1, 5, 12).

Lipogenesis requires cooperation between mitochondrial and cytoplasmic enzymes and involves fluxes of metabolites across the mitochondrial membranes (13). The tricarboxylate (citrate) carrier (TCC), an integral protein of the mitochondrial inner membrane, plays a fundamental role in intermediary metabolism because it represents a link between carbohydrate catabolism and fatty acid syn-

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Abbreviations: ACC, acetyl-CoA carboxylase; G6PD, glucose-6-phosphate dehydrogenase; ME, malic enzyme; L-PK, pyruvate kinase; SCD-1, stearoyl-CoA desaturase; SO, safflower oil; TCC, tricarboxylate carrier.

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thesis. Indeed, definitive evidence was obtained indicating that citrate is an obligatory component of the shuttle system by which mitochondrial acetyl-CoA, mainly derived from a sugar source, is transported to the cytosolic compartment, where it becomes a substrate for ACC (14). In addition, this shuttle supplies NAD^+ and NADPH to support glycolysis and lipid biosynthesis, respectively (15). TCC has been extensively characterized in liver mitochondria from mammals (16–18) and fish (19, 20). The TCC cDNA sequences of rat (21), yeast (22), cow (23), and human (24) are known. The nucleotide sequence of the human TCC gene has been determined (25). A coordinated reduction of lipogenic enzyme and of TCC activities has been described in starved rats (26). The simultaneous decrease of TCC mRNA level, observed in the mitochondria of these animals, was ascribed to a posttranscriptional mechanism (27). Moreover, a recent study from our laboratory showed that lipogenic enzyme and TCC activities were reduced in parallel by a diet supplemented with 15% safflower oil (SO), which is rich in n-6 PUFAs (28). A lower content in the liver of both immunoreactive TCC protein and its mRNA was demonstrated to be responsible for the observed reduction (28).

The aim of this study was to investigate the molecular basis for the regulation of TCC gene expression as mediated by a high-fat, n-6 PUFA-enriched diet. We showed that in hepatic cells, the effect of a SO-supplemented diet on TCC gene expression is modulated by both transcriptional and posttranscriptional mechanisms.

EXPERIMENTAL PROCEDURES

Materials

[α-^{32}P]dATP (3,000 Ci/mmol) and [α-^{32}P]UTP (3,000 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Milano, Italy). [^{14}C]citrate (specific activity, 100 mCi/mmol) and Hybond N^+ nylon filters were purchased from Amersham Biosciences (Milano, Italy). Restriction enzymes were obtained from Promega (Milano, Italy). RNase-free DNase I, α-amanitin, actinomycin D, and 1,2,3-benzenetricarboxylic acid were purchased from Sigma-Aldrich Co.; the Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories (Milano, Italy). T3 RNA polymerase, RPAIII kit, RNase inhibitor, and β-actin antisense control template were obtained from Celbio (Milano, Italy). All other reagents were of analytical grade.

Animals

Male Wistar rats (200–250 g), purchased from Harlan, were housed individually at 22 ± 1°C with a 12 h light/12 h dark cycle. Animal treatment was the same as reported by Zara et al. (28). Briefly, control rats were fed ad libitum with a standard rodent diet (25% protein, 4.3% lipid, 59.7% carbohydrate, of which 7.1% was cellulose, and a salt and vitamin mixture), whereas a second group of rats was fed a high-fat diet [the standard diet supplemented with 15% (w/w) SO/kg diet] for the indicated periods of time. The SO diet was freshly prepared each week and stored at −20°C until required. The fatty acid composition of dietary lipids, determined by gas-liquid chromatographic analysis of their respective fatty acid methyl esters, is reported in **Table 1**. The animals had free access to food and water. Body weight and food intake of the animals were recorded throughout the experiment, and no significant differences were found between the two dietary groups. All of the experiments were performed in accordance with local and national guidelines regarding animal experiments.

TABLE 1. Fatty acid composition of control and SO-supplemented diets

Fatty Acid	Control Diet	SO Diet
$C_{14:0}$	0.97 ± 0.13	0.32 ± 0.09
$C_{16:0}$	16.56 ± 0.50	8.66 ± 0.13
$C_{16:1}$ (n-7)	1.16 ± 0.09	0.38 ± 0.06
$C_{18:0}$	2.18 ± 0.19	1.54 ± 0.13
$C_{18:1}$ (n-9)	28.78 ± 1.51	16.84 ± 0.56
$C_{18:2}$ (n-6)	49.46 ± 1.28	70.40 ± 1.98
$C_{20:5}$ (n-3)	1.40 ± 0.15	0.19 ± 0.06
$C_{22:6}$ (n-3)	1.14 ± 0.12	0.30 ± 0.05
Σ saturated	19.71 ± 0.45	10.52 ± 0.38
Σ unsaturated	81.94 ± 0.87	88.11 ± 0.92
Σ saturated/Σ unsaturated	0.24 ± 0.02	0.12 ± 0.02

Values are percentages of total fatty acids. Results are expressed as means ± SE of five determinations. Interbatch variation in composition did not exceed 0.5% in each case. SO diet indicates a control diet supplemented with 15% (w/w) safflower oil. Fatty acids were extracted from the two different diets and analyzed by gas-liquid chromatography as fatty acid methyl esters. Σ saturated, sum of saturated fatty acids; Σ unsaturated, sum of unsaturated fatty acids.

Citrate transport in rat liver mitochondria

The citrate transport assay was carried out in freshly isolated rat liver mitochondria essentially as described by Palmieri et al. (29). Briefly, mitochondria (~50 mg of protein) were resuspended in 100 mM KCl, 20 mM Hepes, 1 mM EGTA, and 2 μg/ml rotenone (pH 7.0) in a final volume of 10 ml and loaded with 0.75 mM malate as described by Zara and Gnoni (26). Transport was initiated by the addition of 0.5 mM [^{14}C]citrate (specific activity, 100 mCi/mmol) to malate-loaded mitochondria and terminated by adding 12.5 mM 1,2,3-benzenetricarboxylic acid.

Isolation of RNA and Northern blot analysis

Total RNA from liver was isolated as described by Chomczynski and Sacchi (30). Total RNA (15 and 30 μg) was separated, blotted, and hybridized as reported by Siculella et al. (27). The RNA blots were hybridized with [α-^{32}P]cDNA probe, labeled by random primer technique (31), corresponding to nucleotides 459–1,421 of the rat TCC cDNA (21). For the normalization of the hybridization signals, a probe encoding part of the human β-actin was used. After autoradiography, the intensity of the bands was determined by densitometry.

Isolation of nuclei and nuclear run-on assay

Nuclei were isolated from hepatocytes obtained by liver perfusion and collagenase digestion as reported by Gnoni et al. (32). After collecting and washing twice with cold PBS, hepatocytes were lysed in 1 ml of buffer 1 [0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Hepes (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, and 0.5% (v/v) Nonidet P40] by homogenization with a Dounce homogenizer. Nuclei were purified from hepatocytes by centrifugation through a 2.0 M sucrose cushion as described by Siculella et al. (27). The nuclei were stored at −80°C in 210 μl of storage buffer [50% (v/v) glycerol, 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.1 mM EDTA, 10 mM DTT, and 0.25 mM phenylmethylsulfonyl fluoride] before use in a nuclear run-on assay, which was carried out as described by Liu, Sun, and Jost (33). Total RNA was extracted as indicated above. Denatured DNA was applied to

Hybond N^+ nylon membranes by a dot-blot apparatus. The filter-bound DNA was hybridized as reported by Siculella et al. (27). Hybridization signals were quantified as described above.

mRNA turnover assay

Hepatocytes from control and SO-fed rats were maintained on plastic Petri dishes (60 mm) until monolayer formation (32) and were further incubated in Ham's F12 medium in the presence of 4 μg/ml actinomycin D. At different times, 10 plates ($\sim 4 \times 10^6$ cells) from each group were washed with cold PBS and total RNA was extracted as above described. For each time point, 10 μg of RNA was separated on 1% agarose gel with formaldehyde and Northern blot hybridization was carried out as indicated above, using TCC cDNA as a probe. The same filter was stripped by washing twice in a boiling solution of 0.1% SDS. The membrane was rehybridized with β-actin cDNA. The autoradiogram was quantified by densitometric scanning.

Isolation of nuclear RNA

The isolated nuclei were lysed by adding 4 ml of denaturing solution [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 100 mM β-mercaptoethanol, and 0.5% *N*-lauroylsarcosine]. All remaining RNA isolation steps were as described by Chomczynski and Sacchi (30). Final RNA pellets were resuspended in 100 μl of diethyl pyrocarbonate-treated water and stored at −80°C.

Probe design for RNase protection assay

Two probes were designed for use in the RNase protection assay and were obtained by PCR amplification using genomic clone p5B8 containing the TCC gene as a template (data not shown). The first probe, designated intron2-exon3 (I2-E3), was obtained by PCR amplification using the following primers: rp1, 5′-GAATTCTGCTGCAGGAACGACCAGGA-3′, and rp2, 5′-AAGCTTCACGGTCTCCATGGG-3′. The second probe, designated exon7-intron7 (E7-I7), was obtained by PCR amplification using the following primers: rp3, 5′-GAATTCGGCCTGGAGGCACACAAATAC-3′, and rp4, 5′-AAGCTTCTGGGTAGAGCAGAGAGCC-3′. For subcloning purposes, an *Eco*RI site was added at the 5′-end of primers rp1 and rp3, whereas a *Hin*dIII site was added at the 5′-end of primers rp2 and rp4 (underlined). After amplification by PCR, the amplified fragments were subcloned into the *Eco*RI and *Hin*dIII sites of pBluescript II vector. The subclones were linearized with *Eco*RI (Promega) and used in the in vitro transcription reactions. The transcribed RNA was larger than the protected fragments, so that incompletely digested probe could be differentiated from the target signal in the RNase protection assay.

RNase protection assay

RNA probes complementary to TCC RNA were synthesized by an in vitro transcription reaction using 10 units of T3 RNA polymerase in a buffer containing 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 500 μM NTPs (A, G, C), 50 μCi of [α-^{32}P]UTP (specific activity, 800 Ci/mmol), and 5 units of RNase inhibitor. For each reaction, 0.3 μg of plasmid DNA was used as a template. The reaction mixture was incubated for 60 min at 37°C and stopped by adding 1 unit of RNase-free DNase I for 15 min at 37°C. The integrity of the synthesized RNA probes was checked by 6% denaturing polyacrylamide gel electrophoresis. The RNase protection assay was performed using the RPAIII kit, following the instructions given by the supplier. Nuclear RNA (10 μg) was hybridized with 2×10^5 cpm of ^{32}P-labeled RNA probe in 20 μl of hybridization solution at 50°C for 16 h. In each hybridization reaction, a β-actin antisense ^{32}P-labeled RNA probe was added for normalization purposes. As a control for testing the RNase activity, probes were also hybridized with 10 μg of yeast RNA. The hybrids protected from digestion with RNase were resolved on a 6% denaturing polyacrylamide gel. Gels were dried and exposed for autoradiography, and the intensity of the bands was evaluated by densitometry with Molecular Analyst Software.

Statistical analysis

All data are presented as means ± SE for the number of experiments indicated in each case. Statistical analysis was performed by Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Time-dependent effect of SO on TCC activity

Previous data from our laboratory (28) have shown that feeding rats a high-fat diet (15% SO) for 4 weeks resulted in a noticeable decrease in TCC activity. To search for an earlier effect, experiments were carried out for up to 4 weeks using the same SO treatment. Equal amounts of liver mitochondrial proteins from the two groups of animals were used. **Figure 1** gives the citrate exchange by malate-loaded liver mitochondria, isolated from control animals and from rats fed the SO-enriched diet for the indicated periods of time. Compared with control animals, a gradual decrease of the carrier activity was observed, reaching ∼50% inhibition after 4 weeks of SO diet administration (15.6 ± 1.1 vs. 30.3 ± 1.3 nmol/min × mg protein for the control). The reduction at 4 weeks is in agreement with our previous finding (28). No significant changes in the carrier activity were detected with longer treatment times (data not shown).

Effect of the SO-enriched diet on TCC mRNA accumulation in rat hepatocytes

To investigate the molecular basis of the regulation of TCC activity by dietary n-6 fat, Northern blot analysis was carried out. Total RNA from livers of control and SO-fed rats was separated onto a denaturing agarose gel, trans-

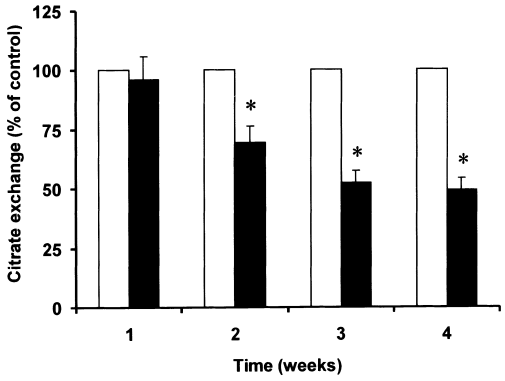

Fig. 1. Dependence of the citrate/malate exchange on the time of safflower oil (SO) treatment. Liver mitochondria freshly isolated from rats fed the standard diet (white bars) or the SO-supplemented diet (black bars) for the indicated times were used. Data shown are means ± SE of six independent experiments. *$P < 0.05$.

ferred to Hybond N^+ membranes, and hybridized with the TCC [α-^{32}P]cDNA probe and normalized with the β-actin [α-^{32}P]cDNA probe. The latter was used for the normalization, as described previously (28), in agreement with other findings (11, 34) that the hepatic abundance of its mRNA is not affected by PUFA feeding. Densitometric analysis showed a time-dependent reduction of mRNA abundance, reaching an inhibition of ~35% at 4 weeks of SO treatment with respect to control animals (**Fig. 2**). Like the TCC activity, a prolonged treatment of rats with SO-added diet did not further decrease the TCC mRNA level (data not shown). Dietary treatment of 4 weeks was then used in all subsequent experiments.

mRNA turnover assay

Previous studies have shown that mRNAs encoding for several different proteins change in stability after dietary treatment (35–37). To investigate the half-life of TCC mRNA, isolated hepatocytes from control and SO-fed rats were cultured in the presence of actinomycin D (4 μg/ml). At the indicated times (**Fig. 3**), total RNA was extracted from hepatocytes and analyzed as reported in Experimental Procedures. For each time point, 10 μg of RNA was separated onto a 1% agarose gel containing formaldehyde and Northern blot analysis was performed.

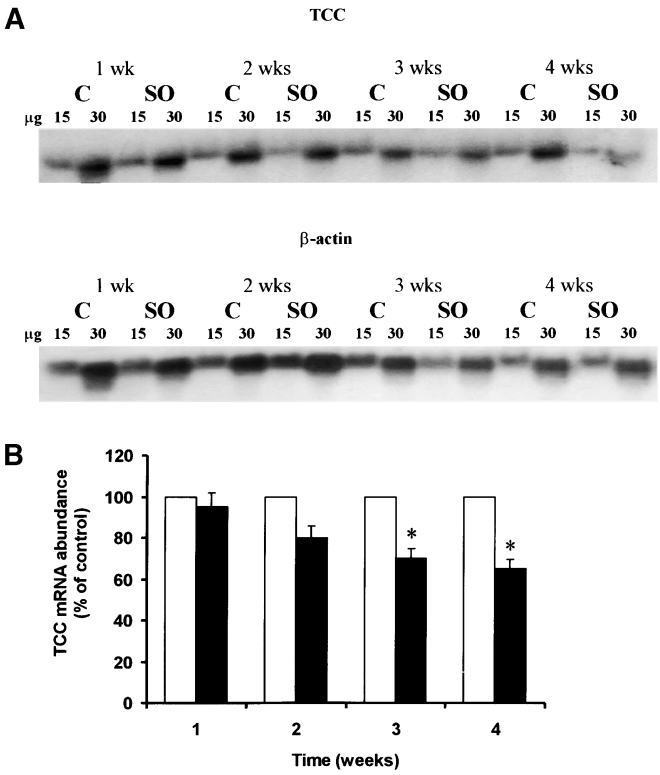

Fig. 2. A: Effect of the SO-enriched diet on the tricarboxylate carrier (TCC) mRNA accumulation in rat liver as a function of weeks of treatment. Approximately 15 and 30 μg of RNA from two pools (two animals each) were applied to the filter. C, control rats; SO, SO-fed rats. B: Quantification of Northern blot data. The bars represent an optical scan of the autoradiogram. Data shown are means ± SE of five independent experiments. White bars, control rats; black bars, SO-fed rats. $*P < 0.05$.

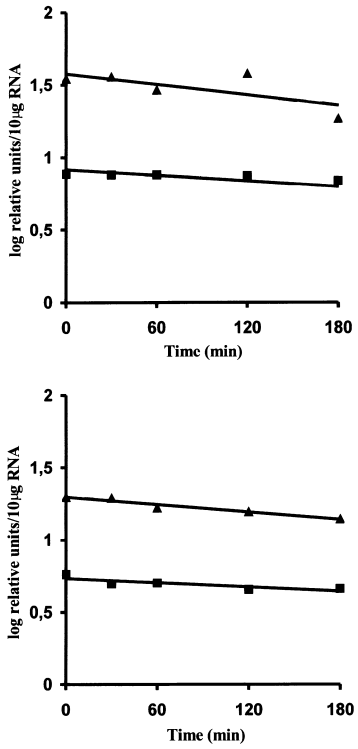

Fig. 3. Turnover of TCC mRNA in cultured hepatocytes from control and SO-fed rats. The semilog plots in the top and bottom panels represent the decay curves of TCC mRNA and β-actin mRNA, respectively. Triangles, control rats; squares, SO-fed rats. Each point represents the average of two measurements. Data shown are from a representative experiment. Similar results were obtained in five independent experiments.

Autoradiograms were quantified by densitometric scanning. The log of TCC and β-actin mRNA abundance was reported as a function of time. The semilog plot represents the decay curve of TCC mRNA (Fig. 3, top) and β-actin mRNA (Fig. 3, bottom) from both control and SO-fed rats. Results from five independent experiments showed that the apparent half-life of the TCC mRNA in cultured hepatocytes from both groups of rats was very similar (10.9 ± 0.8 h in SO-fed rats vs. 10.7 ± 0.7 h in control rats). Moreover, in the same RNA preparation, the relative rate of degradation of β-actin mRNA also remained constant.

Nuclear run-on assay

To determine whether the reduction in the steady-state level of the mRNA observed after SO treatment (Fig. 2) was attributable to a decrease in gene transcription, nuclear run-on assays were performed. To this end, nuclei isolated from control and SO-treated rat hepatocyte suspensions were allowed to incorporate [α-^{32}P]UTP. Then, [α-^{32}P]RNA was extracted and hybridized to dots (5 μg) of TCC cDNA, β-actin cDNA, pUC19, and FAS cDNA applied to the filters. Nonrecombinant plasmid pUC19 was used as a negative control, whereas β-actin cDNA represented a control for the normalization and selectivity of the response. FAS cDNA was used as a positive control, as

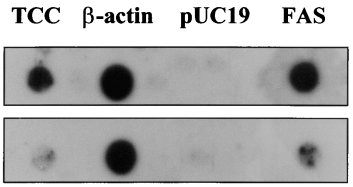

Fig. 4. Dot-blot hybridization of α-^{32}P-labeled nuclear RNA to an excess of TCC cDNA and β-actin cDNA. Nuclei, isolated from hepatocytes from control (C) and SO-fed (SO) rats, were allowed to incorporate [α-^{32}P]UTP; labeled RNA was extracted and hybridized to dots (5 μg) of the indicated cDNAs applied to the filters. Nonrecombinant plasmid pUC19 was used to determine background hybridization. FAS cDNA was used as a positive control.

it has been reported that n-6 PUFA-supplemented diet modulates this gene at the transcriptional level (8). Transcription was assessed by measuring the elongation of preexisting TCC mRNA in isolated nuclei, a procedure that should measure primarily the rate at which the initiation of transcription was occurring in vivo at the time the animals were killed (33). Incorporation of [α-^{32}P]UTP into specific TCC, β-actin, and FAS transcripts was inhibited by ~95% in the presence of α-amanitin (4 μg/ml) (data not shown), confirming that these RNAs were transcribed by RNA polymerase II. The results of the nuclear run-on assays (**Fig. 4**) clearly indicated a consistent difference of ~60% in the transcriptional rate of TCC mRNA between control and SO-fed rats. In both cases, the β-actin transcription rate remained constant. The FAS transcription rate decreased by ~70% in SO-fed rats, in agreement with Blake and Clarke (8).

Processing of TCC precursor RNA

To investigate whether dietary SO inhibits the efficiency of splicing of the TCC transcript, we compared the amount of unspliced and spliced TCC mRNA in the nuclei of hepatocytes from SO-fed versus control rats.

Two probes (I2-E3 and E7-I7), separated by ~1.1 kb, were used to detect TCC RNA (**Fig. 5**). Each probe hybridized across an exon/intron junction and thus measured TCC RNA containing that intron and RNA from which the same intron had been spliced. The protected fragments are referred to as unspliced and spliced RNA, respectively, even though both protected fragments represent a mix of RNA containing one or more of all the TCC introns. Using the first probe, we found in the control nuclei that the amount of spliced RNA (E3 protected fragment) was 39% greater than the amount of unspliced RNA. On the other hand, in the nuclei from SO-fed rats, the amount of spliced RNA was smaller by ~20% than that of unspliced RNA, thus determining an inversion in the ratio of the amount of spliced to unspliced RNA (**Fig. 6**). Furthermore, the reduction in the amount of spliced RNA in the nuclei of SO-fed rats versus control rats was similar (~35%) to the decrease in the accumulation of the mature TCC RNA measured in the cytoplasm (Fig. 2). Unexpectedly, the amount of unspliced RNA was similar in both SO-fed and control rats, despite the 60% reduction in the transcriptional rate observed in nuclei of SO-fed rats (Fig. 4). Therefore, these data are consistent with a regulation in RNA processing. A decrease in the ratio of the amount of spliced to unspliced RNA in rats fed the SO diet, compared with control rats, indicated that the splicing reaction itself is inhibited by dietary SO. In contrast, we found not only the same amount of unspliced RNA but also a similar ratio of spliced to unspliced RNA in both SO-fed and control rats using the second probe (E7-I7) (Fig. 6).

DISCUSSION

Nutrients govern the tissue content and activities of different proteins by functioning as regulators of gene transcription, nuclear RNA processing, mRNA degradation, and mRNA translation as well as acting as posttranslational modifiers of proteins (1). Fat is a dietary constituent with a strong influence on cell differentiation, growth, and metabolism. The fatty acid composition of dietary lipid has a noticeable and distinct influence on the molecular events that govern gene expression (1). In particular, it has been demonstrated that a n-6 PUFA diet suppresses the transcription of the hepatic genes encoding for FAS, S14 protein, SCD-1, and L-PK [for review, see ref. (2)]. Examples of genes that are regulated by posttranscriptional mechanisms include ME (35) and partly S14 (38, 39). In fact, the putative lipogenic protein S14 gene undergoes

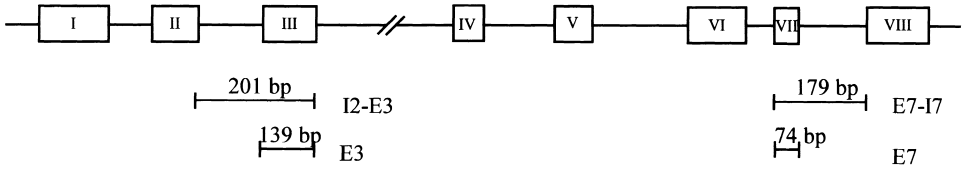

Fig. 5. Probes and predicted fragments for the RNase protection assay. The top line represents schematically a diagram of the TCC primary transcript; the boxes and lines depict exons and introns, respectively. The lower lines represent TCC-specific RNA fragments that are detected in the RNase protection assay by specific TCC probes. Two probes were designed to hybridize across intron-exon boundaries of TCC mRNA such that pre-mRNA with and without introns could be detected in the assay. The first probe (I2-E3) protects a 201 nt fragment of pre-mRNA deleting intron 2 and in addition recognizes processed transcripts (139 nt fragment) that have undergone the splicing of intron 2. The second probe (E7-I7) protects a 179 nt fragment containing intron 7 and also spliced transcript without the 74 nt intron.

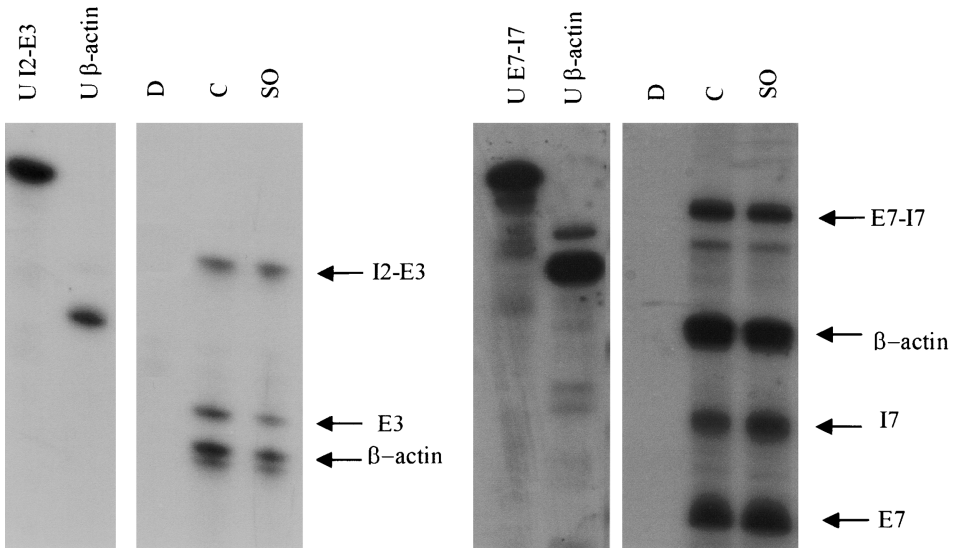

Fig. 6. Effect of SO-enriched diet on the processing of TCC precursor RNA. Nuclear RNA (10 μg) was analyzed using an RNase protection assay and two TCC-specific probes. U, undigested probes (hybridization of probes to 10 μg of yeast RNA without subsequent RNase digestion); D, digested probes (hybridization to 10 μg of yeast RNA followed by RNase digestion); C, hybridization of probes to 10 μg of nuclear RNA isolated from livers of control rats, followed by RNase digestion; SO, hybridization of probes to 10 μg of nuclear RNA isolated from livers of SO-fed rats, followed by RNase digestion.

large changes in transcriptional activity in response to dietary fat (2); however, it has been shown that, after high-carbohydrate diet or insulin administration to rats, the S14 gene is regulated by both transcriptional and post-transcriptional mechanisms (38, 39). The latter mechanism involves enhanced splicing of its pre-mRNA. The increase in ME activity in response to a high-carbohydrate diet is accounted for by alterations in the stability of the cytoplasmic mRNA and not by changes in the transcriptional activity of this gene (35). PUFA suppression of G6PD involves a posttranscriptional mechanism that is quite different in that it occurs in the nucleus early after transcription and could be ascribed to a differential partitioning of pre-mRNA into various nuclear compartments (12, 40, 41).

Our previous finding indicated that the activity of TCC, a protein of the mitochondrial inner membrane strictly correlated with lipogenesis, is specifically reduced by a 15% SO-enriched diet administered to rats for 4 weeks. This reduction has been ascribed to a lower content of the immunoreactive carrier protein (28). In the present work, to search for possible earlier regulation, we tested the effect on TCC activity of the same diet administered to rats for up to 4 weeks. We found a gradual decrease of TCC activity during this period, with the strongest inhibition occurring after 4 weeks of treatment. At this time, in agreement with our previous data (28), a decrease of TCC activity of ~50% was observed. Interestingly, Northern blot analysis with total RNA from liver showed a decline of the abundance of TCC mRNA, which paralleled that of TCC activity. A 4 week treatment, therefore, was chosen in the subsequent experiments. To determine the step(s) involved in the inhibition in rat liver of TCC expression by SO feeding, transcriptional activity, mRNA stability, and pre-mRNA processing were investigated. We first asked whether changes in the TCC mRNA turnover rates could account for the reduced mRNA accumulation in SO-treated rats. To address this question, the apparent half-life of TCC mRNA was estimated. The data in Fig. 3 show that the decay of the TCC mRNA occurred at the same rate in the hepatocytes from treated and control animals. In fact, the estimated half-life was ~11 h in both cases. Therefore, the absence of a consistent variation in TCC mRNA turnover suggests that other mechanisms for the carrier gene regulation must be involved. To determine whether changes in the transcription activities were responsible for the SO-induced reduction in the abundance of TCC mRNA, a nuclear run-on assay was performed. As shown in Fig. 4, the transcriptional rate of TCC mRNA, tested in nuclei isolated from treated rat hepatocytes, was reduced by ~60%. This finding indicated that the inhibition of transcription of the TCC gene parallels the observed decline in TCC mRNA abundance. It should be noted that, in agreement with a previous observation (8), FAS gene transcription activity, used as a positive control, was markedly reduced by the dietary treatment. The fact that under the same conditions the transcription rate for β-actin mRNA was unchanged by SO treatment indicated that the n-6 PUFA action was gene specific. Although the reduced transcription activity we observed could account for the reduced TCC mRNA level, further experiments were performed to investigate whether any posttranscriptional process occurs in rat liver nuclei after SO treatment. Based on the fact that dietary fat did not interfere with the stability of the mature TCC mRNA, as the turnover assay demonstrated (Fig. 3), we chose another exper-

imental approach to evaluate the posttranscriptional regulation of TCC mRNA. To this end, we measured the content of both the precursor and the spliced RNA in the nuclei at steady state. If only transcriptional events were responsible for the changes in the mRNA amount, the same ratio of spliced RNA to precursor should be found in both control and treated rats. Nevertheless, using the probe I2-E3, our data showed that the ratio of mature to precursor RNA decreased with the change to a high-fat, SO diet. This does not appear to be a generalized effect of SO treatment on splicing because we did not observe any variation using the probe E7-I7. Based on these results, we hypothesize that a *cis*-acting element maps to exon 3. Therefore, neither transcriptional rate changes nor precursor stability alone can account for our observations. Taken together, these results indicate that administration of a high-fat, n-6 PUFA-enriched diet to rats leads to changes not only in the transcriptional rate of the TCC gene but also in the processing of the nuclear precursor for TCC RNA. Experiments are in progress in our laboratory to further elucidate this pathway using different probes representing other exon-intron junctions. The reduction of spliced mRNA in the nuclei of SO-fed versus control rats was similar to that observed in cytoplasmic TCC mRNA accumulation, ruling out nuclear-cytoplasmic transport as a potential regulatory mechanism. Therefore, modulation of TCC gene expression by both transcriptional and posttranscriptional mechanisms would result in a coordinated response to nutritional changes, consistent with the carrier role in intermediary metabolism. To our knowledge, among genes in the lipogenic pathway, alterations in RNA processing attributable to dietary fat have been reported only for G6PD expression (40, 41). The present study presents the first information on the molecular mechanism by which TCC gene expression is modulated by a nutritional factor.

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