Hypothyroidism Reduces Tricarboxylate Carrier Activity and Expression in Rat Liver Mitochondria by Reducing Nuclear Transcription Rate and Splicing Efficiency*

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Luisa Siculella, Simona Sabetta, Anna M. Giudetti, and Gabriele V. Gnoni 1

From the Laboratory of Biochemistry and Molecular Biology, Department of Biological and Environmental Science and Technologies, University of Lecce, Via Provinciale Lecce-Monteroni, I-73100 Lecce, Italy

The tricarboxylate carrier (TCC), also known as citrate carrier, is an integral protein of the mitochondrial inner membrane. It is an essential component of the shuttle system by which mitochondrial acetyl-CoA, primer for both fatty acid and cholesterol synthesis, is transported into the cytosol, where lipogenesis occurs. The effect of hypothyroidism on the activity and expression of the hepatic mitochondrial TCC was investigated in this study. TCC activity was significantly decreased in hypothyroid rats as compared with euthyroid animals. This hormone deficiency effect was due to a reduction in the amount of carrier protein, which resulted from a proportionate decrease of the specific mRNA. Hypothyroidism did not influence TCC mRNA stability. On the other hand, nuclear run-on assay revealed that the transcriptional rate of TCC mRNA decreased by ~40% in the nuclei from hypothyroid versus euthyroid rats. In addition, the ribonuclease protection assay showed that, in the nuclei of hypothyroid rats, the ratio of mature to precursor RNA decreased, indicating that the splicing of TCC RNA is affected. Furthermore, we found that the ratio of polyadenylated/unpolyadenylated TCC RNA as well as the length of the TCC RNA poly(A) tail were similar in both euthyroid and hypothyroid rats. Thus, the rate of formation of the TCC 3'-end is not altered in hypothyroidism. These results suggest that hypothyroidism affects TCC expression at both the transcriptional and post-transcriptional levels.

Moreover, thyroid hormones dramatically affect the extent to which liver contributes to total lipogenesis in rat. This contribution ranges from 5% in the hypothyroid animals to 34% in the hyperthyroid animals (6). Fatty acid synthesis in rat liver is extremely responsive to the thyroid status of the organism. Hypothyroidism in developing or adult rats depresses hepatic fatty acid synthase (FAS) activity by 50% (7). Cultures of hepatocytes obtained from hypothyroid rats synthesize fatty acids from acetate half as fast as euthyroid hepatocytes (8). The enzymatic activities of de novo fatty acid synthesis, i.e. acetyl-CoA carboxylase (ACC) and FAS, are greatly increased in hyperthyroid rats (9, 10) and, conversely, strongly reduced in propylthiouracil-induced hypothyroid rats (10). Furthermore, rat thyroidectomy decreases the activities of FAS and other lipogenic enzymes such as malic enzyme and glucose-6-phosphate dehydrogenase (11). It has been shown that tri-iodothyronine (T3) regulates fatty acid synthesis by altering the expression of the genes coding for the lipogenic enzymes (12). Different molecular mechanisms have been reported for this regulation. FAS in avian liver (13) and in chick embryo hepatocytes (14), as well as the putative lipogenic enzyme S14 protein in primary cultures of rat hepatocytes (15) and in rat liver (16), are transcriptionally regulated by T3, whereas it has been proposed that, in rats, T3 is involved not only in ACC transcription (17) but also in ACC mRNA stability (18). Transcriptional and post-transcriptional mechanisms have been suggested for T3 regulation of the malic enzyme gene (19, 20). T3 increases the ATP-citrate lyase content by changes in the rate of enzyme synthesis, which in turn correlate with the abundance of the corresponding mRNA (11).

Lipogenesis requires cooperation between mitochondrial and cytoplasmic enzymes and involves fluxes of metabolites across the mitochondrial membranes (21). The inner membrane of rat liver mitochondria contains a specific carrier, i.e. tricarboxylate carrier, for the outward transport of citrate. The activity of TCC is strictly correlated with the rate of lipogenesis and represents a link between carbohydrate catabolism and fatty acid synthesis. In fact, it exports from mitochondria to the cytosol (in the form of citrate) acetyl-CoA mainly derived from sugar sources, thus providing the carbon units for fatty acid and cholesterol biosyntheses. In addition, it supplies NAD+ and NADPH that support cytosolic glycolysis and lipid biosynthe-

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1 To whom correspondence should be addressed: Laboratorio di Biochimica e Biologia Molecolare, Dipartimento di Scienze e Tecnologie Ambientali, Università di Lecce, Via Provinciale Lecce-Monteroni, I-73100 Lecce, Italy. Tel: 39-0832298678; Fax: 39-0832298678; E-mail: gabriele.gnoni@unile.it.

2 The abbreviations used are: FAS, fatty acid synthase; TCC, tricarboxylate carrier; ACC, acetyl-CoA carboxylase; T3, tri-iodothyronine; pre-mRNA, precursor mRNA; UTR, untranslated region; nt, nucleotide(s).
sis, respectively (22). TCC may have important physiological functions in gluconeogenesis as well (23). The characteristics of this transporting system have been extensively investigated (for review, see Ref. 24), but little is known concerning its regulation. It has been reported that TCC activity is altered in type I diabetes (23), can be regulated by exogenous insulin (25), and is enhanced during hyperthyroidism (26). Recent reports from our laboratory showed that, in parallel with lipogenic enzyme activities, TCC activity is controlled by various nutritional states (27–29).

To our knowledge, there has been no report assessing mitochondrial TCC activity relative to the hypothyroid state, and no study so far has gone deep into the hormonal regulation at the molecular level of any gene for a mitochondrial carrier protein. Therefore, the aim of this study was to investigate whether hypothyroidism affects TCC activity and expression and, if so, to characterize the molecular step(s) for this hormonal TCC gene modulation. We showed that the reduced TCC activity observed in the hypothyroid state can be ascribed to either a transcriptional and a post-transcriptional TCC gene regulation.

**MATERIALS AND METHODS**

**Chemicals**—[α-32P]dATP (3000 Ci/mmol) and [α-32P]UTP (3000 Ci/mmol) were purchased from PerkinElmer Life Sciences (Milan, Italy). [14C]Citrate (specific activity, 100 mCi/mmol) and nylon filters Hybond N+ were purchased from Amersham Biosciences. Restriction enzymes were obtained from Promega (Milan, Italy). RNase-free DNase I, α-amanitin, actinomycin D, and 1,2,3-benzenetricarboxylic acid were purchased from Sigma. The protein assay kit was from Bio-Rad. The T3 RNA polymerase, RPAlIII kit, RNase inhibitor, and β-actin antisense control template were obtained from Celbio (Milan, Italy). ANT2 antibody (D. B. A. Italia, Milan, Italy) was commercially available. All other reagents were of analytical grade.

**Animal Treatments**—Male Wistar rats (150–200 g) were used throughout this study. They were housed in cages in a temperature (22 ± 1 °C)- and light (light on 8:00 a.m. to 8:00 p.m.)-controlled room and randomly assigned to one of two different groups. The first one is the control group and will be further referred to as euthyroid. The second group was made chronically hypothyroid by continuous administration of 6-n-propyl-2-thiouracil (0.1% w/v, in drinking tap water) for 3–4 weeks (30). Both groups had free access to food that was a commercial mash (Morini Spa, Milan, Italy). The experimental design was in accordance with local and national guidelines covering animal experiments.

**Tricarboxylate Translocase Activity Assay**—Rat liver mitochondria were prepared by differential centrifugation of the liver homogenates essentially as described previously (31). Mitochondrial integrity was monitored by measuring the respiratory control ratio. TCC activity was assayed in freshly isolated rat liver mitochondria as reported in Ref. 31. The malate/citrate exchange reaction was started by the addition to malate-loaded mitochondria (1–1.5 mg of protein) of 0.5 mM [14C]citrate and terminated by adding the inhibitor 1,2,3-benzenetricarboxylic acid at 12.5 mM (31). Immediately after the addition of the inhibitor, the tubes were rapidly centrifuged at 18,000 × g for 5 min at 2 °C, washed, and extracted with 20% HClO4. The mixture was then centrifuged, and the radioactivity in the supernatant was counted.

**Isolation of RNA and Northern Blot Analysis**—Approximately 15 μg of total RNA, extracted from the livers of hypothyroid and euthyroid rats, according to Chomczynski and Sacchi (32), was size-fractionated by electrophoresis through denaturing 1% formaldehyde-agarose gel and transferred to Hybond N+ nylon membrane. The RNA blots were hybridized with three α-32P-labeled probes corresponding to nucleotides 459–1421 of rat liver TCC cDNA (33), nucleotides 91–280 of rat liver adenine nucleotide translocase isof orm 2 (ANT2) cDNA (GenBankTM accession number NM_057102), and nucleotides 613–726 of the ubiquitously expressed rat liver mitochondrial outer membrane channel, was used as a control, because its expression is not affected by thyroid hormones (34). The bound antibody was revealed by peroxidase-conjugated anti-rabbit IgG antibody using 3,3′-diaminobenzidine and hydrogen peroxide as substrates. The blots were evaluated by densitometric analysis with Molecular Analyst software.

**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. (8). After isolation, hepatocytes were washed twice with cold phosphate-buffered saline and then 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, 0.5% (v/v) Nonidet P40) by homogenization with a Dounce homogenizer. The crude nuclei were purified by centrifugation through a 2.0 M sucrose cushion as described by Siculella et al. (28) and stored at −80 °C in aliquots (210 μl) of storage buffer (50% (v/v) glycerol, 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.1 mM EDTA, 10 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride) containing ~1 × 108 nuclei each for analysis later. Nuclei suspension was used in a nuclear run-on assay as described by Liu et al. (35). The newly transcribed RNA was extracted as indicated above and hybridized to Hybond N+ nylon membranes as reported by Siculella et al. (28). Hybridization signals were quantified as described above.

**mRNA Turnover Assay**—Hepatocytes from hypothyroid and euthyroid rats were maintained on plastic Petri dishes (60 mm) until monolayer formation (8), and after 2-h plating, 4 μg/ml actinomycin D in Ham’s F-12 medium was added. At different times, 10 plates (~4 × 106 cells/plate) from each group were
washed with cold phosphate-buffered saline, and total RNA was extracted as described above. For each time point, aliquots of 10 μg of RNA were loaded on a 1% formaldehyde-agarose gel, electrophoresed, and transferred onto nylon membranes. 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 and 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 mM guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 100 mM β-mercaptoethanol, and 0.5% N-lauroylsarcosine). RNA isolation was carried out as described by Chomczynski and Sacchi (32).

Probe Designed for the Ribonuclease Protection Assay—Several probes were designed for use in the ribonuclease protection assay. They were obtained by PCR amplification of a genomic clone p5B8 containing the TCC gene (data not shown) using specific primers as previously reported (36). Briefly, the first designed probe (see Fig. 5A), intron 3-exon 4 (I3-E4), was amplified using the following primers: rp1, 5'-GAAATTCT-GCTGAGGGACACACACATAC-3' and rp2, 5'-AGCTCA-CGGTCTCCATGGG-3'. The second designed probe, exon 7-intron7, was amplified using the following primers: rp3, 5'-GAAATTCTGAGACACACCAACAGGC-3' and rp4, 5'-AAGCTTCTGCAGGCGGACAGCAGCC-3'.

The third designed probe, exon 8-intron 8 (E8-I8) (see Fig. 5A) was obtained using the following primers: rp5, 5'-GAATT-TCCGGCCTTGGGACACACACATAC-3' and rp6, 5'-AAGCTTCTGGGTAGAGCAGAGAGCC-3. For subcloning purposes, an EcoRI site was added at the 5'-end of the primers rp1, rp3, and rp5, whereas a HindIII site was added at the 5'-end of the primers rp2, rp4, and rp6 (underlined). The amplified products were subcloned into pBluescript II vector. After linearization, the recombinant plasmids were used in the in vitro transcription reactions. The β-actin probe, which was used as a control for RNA loading, was made from the pTRI-β-actin-125-Rat antisense control template (Ambion). When this plasmid was transcribed with T3 RNA polymerase antisense, transcripts of 160 nt are produced, and their hybridization to rat total RNA protects a 126-nt fragment derived from exon 5 of rat β-actin. The fourth TCC probe spans the polyadenylation/cleavage site in the 3'-untranslated region (UTR) so that it can detect the abundance of polyadenylated RNA after cleavage, as well as uncleaved RNA in a RNase protection assay. DNA was amplified by PCR of a genomic clone p5B8, using the following primers: rp7, 5'-CTGAGGAGCTTACCAGCTCCAGGG-GCTATAG-3'; rp8, 5'-TAAAGGAGCTTACGATCGG-3'. The amplified sequence was ligated into pBluescript II vector, and the plasmid was linearized prior to use in the riboprobe synthesis reaction.

Ribonuclease Protection Assay—Antisense RNAs were synthesized by an in vitro transcription reaction as reported in Ref. 36. RNA samples were subjected to ribonuclease protection assay using the RPAIII kit and following the instructions given by the supplier. Nuclear RNA (10 μg) was hybridized with 2 × 10^5 counts/min of 32P-labeled-specific antisense probe in 20 μl of hybridization solution at 50 °C for 16 h. For the normalization, a 2 × 10^6 counts/min of antisense β-actin 32P-labeled RNA probe with a 100-fold lower activity was added in each hybridization reaction to obtain a signal comparable with the test mRNAs. Probes were also hybridized with 10 μg of yeast RNA used as a control for testing the RNase activity (data not shown). After digestion with RNase A/T1, the protected fragments were separated onto a 6% denaturing polyacrylamide gel. Gels were dried, exposed for autoradiography, and the intensity of the bands was evaluated by densitometry with Molecular Analyst software.

RNase H Blot—RNase H analysis was carried out according to Ref. 37. Briefly 15 μg of nuclear RNA was mixed with 50 pmol of a TCC-specific oligonucleotide (5'-CTATTGTATCTGT-GTCCAGCTGGCCGT-3', nucleotides located 2517 bp downstream ATG codon of the rat genome sequence). The mixture was heated to 70 °C and then, in 10 min, cooled to room temperature (37). Digestion with RNase H was for 20 min at 37 °C. The samples were electrophoresed through denaturing 1% formaldehyde-agarose gel, transferred to Hybond N nylon membrane, and hybridized with an amplified probe (366 nt) corresponding to the 3'-end of the TCC mRNA, precisely from 2526 to 2892 bp downstream of the ATG codon of the rat genome sequence.

Statistical Analysis—All data are presented as means ± S.E. 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 Course of Citrate/Malate Exchange—Fig. 1 shows the time course of [14C]citrate exchange by malate-loaded mitochondria from the livers of euthyroid and hypothyroid rats. Citrate/malate exchange was strongly reduced in liver mitochondria from hypothyroid versus euthyroid animals. As shown in this figure, during the linear range (15–30 s) of the reaction, the decrease of the transport activity was reproducibly found to be ~50% in hypothyroid versus euthyroid animals.

Effect of Hypothyroid State on TCC mRNA and Protein Levels in Rat Hepatocytes—To establish whether hypothyroidism affected TCC mRNA and protein levels, Northern and Western blot analyses were carried out.

Quantiﬁcation of the signals showed that the hypothyroid state caused an ~30% reduction of the hepatic TCC mRNA level compared with euthyroid rats (Fig. 2A). This figure also shows that, in agreement with a previous report (12), the expression of the housekeeping gene for β-actin was unmodified by hypothyroidism. Furthermore, the hepatic abundance of the mRNA for ANT2, the ubiquitous adenine nucleotide translocase isoform 2, was lowered in liver from hypothyroid rats, as reported by Dummier et al. (38), whereas in the liver from the same group of animals, no alteration in the expression of VDAC1 mRNA, coding for porin, was observed. Interestingly, the immunodecoration reported in Fig. 2B revealed also that the TCC and ANT2 protein levels of mitochondria from hypothyroid rats were lowered by ~35 and 45%, respectively, as compared with euthyroid animals. By contrast, the amount of...
porin, the mitochondrial outer membrane channel, coded by VDAC mRNA and used as a control, was unaffected by the hypothyroid state. The immunoelectrophoretic results for TCC and ANT2 protein content correlate well with the observed alterations in the corresponding mRNA levels.

**Turnover of TCC mRNA**—We then asked whether the reduction in the hepatic TCC mRNA accumulation in hypothyroid rats could reflect changes in the TCC mRNA stability. To address this question, the apparent half-life of TCC mRNA was estimated. Fig. 3 shows the semilog plot representing the decay curve of TCC mRNA (upper panel) and \( \beta \)-actin mRNA (lower panel) from hypothyroid and euthyroid rat hepatocytes. The apparent half-life of TCC mRNA from both groups of rats was similar (10.7 ± 1.1 h in hypothyroid versus 11.2 ± 0.7 h in euthyroid rats). In the same RNA preparation, the relative rate of degradation of the \( \beta \)-actin mRNA remained constant.

**Run-on Assay**—To investigate whether the decrease in the amount of TCC mRNA observed in the hypothyroid state was controlled at the transcriptional level, a nuclear run-on assay was carried out. Nuclei isolated from hypothyroid and euthyroid rat hepatocyte suspensions were allowed to incorporate \( \alpha\)-\[^32\]P\]UTP. The labeled RNA was extracted and hybridized to dots (5 \( \mu \)g) of TCC cDNA, \( \beta \)-actin cDNA, and pUC19 applied to the filters. \( \beta \)-actin and pUC19 were used for normalization and as a negative control, respectively. Incorporation of \( \alpha\)-\[^32\]P\]UTP into TCC and \( \beta \)-actin transcripts was specifically suppressed by \( \alpha \)-amanitin (4 \( \mu \)g/ml) (data not shown), confirming that the labeled RNAs were

**FIGURE 1.** Time course of citrate exchange in rat liver mitochondria. Malate-loaded mitochondria from euthyroid (□) or hypothyroid (●) rats were incubated with 0.5 mM \(^{14}\)Ccitrate for the indicated times. The exchange reaction was started by adding labeled citrate and terminated by the addition of 12.5 mM 1,2,3-benzenetricarboxylic acid. The data, expressed as nmol of \(^{14}\)Ccitrate exchanged/mg of protein, are means ± S.E. of six different experiments. a, \( p < 0.001.\)

**FIGURE 2.** TCC mRNA accumulation and protein content in rat liver. A, 15 \( \mu \)g of hepatic RNA from the pooled livers of three euthyroid (Eu) or three hypothyroid (Hy) rats was analyzed by Northern blot analysis and hybridized with TCC, ANT2, VDAC, \( \beta \)-actin fragments as described under "Materials and Methods." The radiolabeled blots were exposed to x-ray film, and the intensity of the resulting bands was evaluated by densitometry with Molecular Analyst software. The bars represent an optical scan of the autoradiogram for TCC mRNA. Data are means ± S.E. of five independent experiments. a, \( p < 0.05.\) B, liver mitochondrial proteins from euthyroid and hypothyroid rats were immunodecorated with antisera against either a C-terminal peptide of the rat TCC, ANT2, VDAC1 cDNAs, or \( \beta \)-actin fragments, or \( \beta \)-actin and porin. The bound antibody was revealed by peroxidase-conjugated anti-rabbit IgG antibody using 3,3′-diaminobenzidine and hydrogen peroxide as substrates. The content of mitochondrial TCC, ANT2, and porin was quantified by photodensitometric analysis of blots. Data are means ± S.E. of five independent experiments. b, \( p < 0.001.\)
transcribed by RNA polymerase II. The dot blot hybridization revealed a decrease of \(~40\%\) in the transcriptional rate of TCC mRNA from hypothyroid versus euthyroid rats (Fig. 4). The transcriptional rate of β-actin remained constant in the two groups of rats.

**Processing of TCC Precursor RNA**—To study the effects of the hypothyroid state on the splicing of TCC RNA, we compared the amount of unspliced and spliced TCC RNA in the nuclei of hepatocytes from hypothyroid versus euthyroid rats. For this purpose, we used a ribonuclease protection assay and three probes (I3-E4, E7-I7, and E8-I8) to investigate the processing of the precursor-mRNA (pre-mRNA) (Fig. 5A). The three probes represent discrete locations within the primary transcript and permit quantitative analysis of the amount of pre-mRNA. I3-E4 hybridized across a 3′-splice site, and E7-I7 and E8-I8 hybridized across a 5′-splice site. RNase digestion of each hybrid of probe and target RNA resulted in two types of protected fragments, the longer fragments (intron3-exon4, exon7-intron7, and exon8-intron8) corresponding to unspliced RNA that contains both the exon and the intron sequences and the smaller fragments (exons 4, 7, and 8) corresponding to spliced RNA that contains only the exon sequences. The terms “unspliced” and “spliced” RNA refer to an RNA mix containing one or more of all of the TCC introns, and each probe provides information about the splicing of only 1 of the 8 introns in the TCC gene. Using the E8-I8 probe in the nuclei from hypothyroid rats, the amount of spliced RNA (E8-protected fragment) was \(~2.5\)-fold greater than the amount of unspliced RNA. To note that, in the nuclei from euthyroid rats, the amount of spliced RNA was \(~5\)-fold greater than that of unspliced RNA (Fig. 5). Furthermore, the reduction in the amount of spliced RNA in the nuclei of hypothyroid rats compared with euthyroid animals was \(~30\%\), which is similar to the decrease in the accumulation of the mature TCC RNA measured in the cytoplasm (Fig. 2A). By contrast, the amount of unspliced RNA was similar in both rats, despite the \(~40\%\) reduction in the transcriptional rate observed in nuclei of hypothyroid rats (Fig. 4). Vice versa, using the I3-E4 and E7-I7 probes, we found not only the same amount of unspliced RNA but also a similar ratio of spliced/unspliced RNA in both rats (Fig. 5).

**Effect of Hypothyroidism on the 3′-End Processing of TCC mRNA**—Further events that could regulate in the nucleus the processing of pre-mRNA include 3′-end formation by cleavage and polyadenylation. To investigate whether the hypothyroid state could influence this regulatory step, we measured by RNase protection assay the accumulation of uncleaved versus polyadenylated TCC RNA in nuclei from euthyroid and hypothyroid rats. A probe to the TCC 3′-UTR was hybridized with nuclear RNA from euthyroid and hypothyroid rats. The 3′-UTR probe resulted in the detection of two protected fragments, the 284-nt-protected fragment corresponding to uncleaved and therefore unpolyadenylated pre-mRNA and the 144-nt-protected fragment representing the polyadenylated mRNA (Fig. 6). We found that not only the amount of uncleaved, unpolyadenylated TCC RNA was lower than the amount of TCC polyadenylated RNA in the nuclei of both euthyroid and hypothyroid rats but also that the ratio (\(~1.6\))
polyadenylated/unpolyadenylated TCC RNA was similar in both rats.

Furthermore, to verify whether hypothyroidism could destabilize TCC mRNA by shortening the poly(A) tail, the length of the poly(A) tail was measured. Nuclear RNA isolated from euthyroid and hypothyroid rats was subjected to RNase H analysis (Fig. 7). The size of the 3' fragment resulting from RNase H digestion was ~675 nt. Of this sequence, 375 nt corresponds to the TCC 3'-UTR, and the remaining 300 nt represent the poly(A) tail.

**DISCUSSION**

It has been demonstrated that changes in fatty acid synthesis, due to prolonged thyroid hormone administration, occur at the level of gene expression as seen by the variation in the amount of ACC, FAS, and S14 protein (11, 12). Severalfold increases in the levels of the mRNAs for these proteins were observed in hypothyroid compared with euthyroid rats, showing that most of the T3 effect on hepatic lipogenesis can be explained at the pretranslational level (12). In most cases, T3 regulation of lipogenic enzymes is transcriptional (11). In some cases, a transcriptional and post-transcriptional regulation by T3 has been reported, suggesting that the molecular mechanism by which the hormone regulates the expression of a lipogenic enzyme can vary, depending on the enzyme (11). Moreover, much effort has been made during the last few years to clarify the mechanisms of T3-mediated regulation of gene expression, including the interaction of T3 with the thyroid hormone receptors (which are transcription factors), the characterization of the target sequences on the promoter regions, and the identification of co-activators or co-repressors (3). Although the effect of thyroid hormone administration has been quite extensively investigated, much less information is so far available on the hypothyroid state. Recently, by DNA microarray experiments, a complex T3-induced gene expression pattern in hypothyroid rats has been reported, and various signal transduction pathways of T3 action have been suggested (39).

In this study, we report the effect of hypothyroidism on the hepatic activity and expression of TCC, a mitochondrial transport protein also known as citrate carrier. This carrier protein, by transporting from mitochondria to the cytosol acetyl-CoA, condensed with oxaloacetate in the form of citrate, plays a key role in the intermediary metabolism. In fact, by the action of ATP-citrate lyase, cytosolic citrate provides acetyl-CoA for both fatty acid and cholesterol synthesis as well as oxaloacetate for gluconeogenesis (22, 23). Moreover, it supplies NADPH for glycolysis and NADPH for lipid biosynthesis (22). Here we found that the rate of citrate transport across the mitochondrial inner membrane is significantly reduced in the livers of hypothyroid as compared with euthyroid rats. Thus, the decreased enzyme activities of fatty acid synthesis (ACC and FAS) observed in the livers of hypothyroid rats (10) might be ascribed, at least in part, to the reduced TCC activity, which in turn leads to a lesser amount of cytosolic acetyl-CoA, substrate for both fatty acid and cholesterol synthesis. In this context, it is relevant to note that cytosolic citrate is also an activator of ACC, the first enzyme in the de novo fatty acid synthesis (40). Therefore, these findings suggest that TCC activity is regulated by hypothyroidism in coordination with the activities of other lipogenic enzymes. In fact, in addition to the above-mentioned ACC and FAS, activities of the oxidative enzymes of the pentose phos-

**FIGURE 5. Processing of TCC precursor RNA.** A, probes and predicted fragments for the ribonuclease protection assay. The boxes and the lines depict exons and introns, respectively. The protected fragments detected with each probe in the ribonuclease protection assay are indicated. The longer protected fragments contain the intron, and the shorter protected fragments contain only the exon, as the intron has been spliced. The 13-E4 probe (intron 3-exon 4) protects a 201-nt fragment of pre-mRNA and a spliced transcript of 139 nt without intron 3. The E7-17 (exon 7-intron 7) probe reveals a 198-nt fragment of pre-mRNA and a spliced RNA of 116 nt. The E8-18 probe (exon 8-intron 8) protects a 179-nt fragment containing intron 8 and also a spliced transcript of 74 nt without the intron. B, nuclear RNA (10 µg) was analyzed using a ribonuclease protection assay and the three TCC-specific probes. U, undigested control (hybridization of yeast RNA (10 µg) with the probes without subsequent RNase digestion); Eu, and Hy, hybridization of probes to 10 µg of nuclear RNA isolated from the livers of euthyroid and hypothyroid rats, respectively, followed by RNase digestion. The protected fragments are depicted to the right. C, quantitative results of four independent experiments with two rats/experiment (means ± S.E.). The hybridization signal of each protected fragment from the TCC 3'-UTR, and the remaining 300 nt represent the poly(A) tail.
phate pathway, ATP-citrate lyase, malic enzyme, and the lipogenic enzyme “Spot 14” protein are markedly decreased by thyroid hormone deficiency (11, 41). In addition, the decrease of TCC activity adds further support to the gluconeogenesis reduction observed in hypothyroid rats (42, 43) and in hepatocytes from thyroidectomized rats (44). We therefore decided to investigate the molecular mechanism(s) underlying the control of TCC expression in rat liver mitochondria of hypothyroid rats.

Western blot for immunodetection of the TCC protein and Northern blot analysis showed that thyroid hormone deficiency led to a reduction in the rate of TCC synthesis, which resulted from a proportionate decrease of the specific mRNA (Fig. 2). Therefore, these data provide evidence for a pretranslational regulation of TCC expression in the hypothyroid state.

In this respect, it is worthwhile underlining that the expression of another mitochondrial carrier, the ANT2 isoform, is sensitive to the animal thyroid state (38). We therefore decided to investigate the molecular mechanism(s) underlying the control of TCC expression in rat liver mitochondria of hypothyroid rats.

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In this respect, it is worthwhile underlining that the expression of another mitochondrial carrier, the ANT2 isoform, is sensitive to the animal thyroid state (38). In agreement with Dummler et al. (38), we observed in hypothyroid rats a significant reduction of ANT2 mRNA abundance correlated with a decreased ANT2 protein content (Fig. 2). Unlike TCC and ANT2, no alterations in the amount of both protein and mRNA for porin, a protein of mitochondrial outer membrane, were observed in hypothyroid rats. Therefore, our results indicated that not all of the nuclear encoded mitochondrial proteins are down-regulated by thyroid hormone deficiency.

It is well known that the pretranslational control of TCC expression can result from transcriptional and/or post-transcriptional events. To distinguish among the possible levels of regulation, transcriptional activity, mRNA stability, pre-mRNA processing and 3'-end formation (cleavage and polyadenylation) were analyzed. The transcriptional rate of TCC mRNA was reduced by ~40% in nuclei from hypothyroid as compared with euthyroid rats (Fig. 4). The fact that the transcription rate for β-actin mRNA was unchanged indicated that the effect of hypothyroidism was gene-specific. Although the TCC transcription regulation we observed in hypothyroid rats could account for the reduced TCC mRNA level, we investigated whether any post-transcriptional process also occurred in hypothyroid rat liver. Our results clearly show that the cytoplasmic TCC mRNA stability is not the process involved, as the turnover assay demonstrated (Fig. 3); thus, TCC gene regulation in hypothyroid rats must occur in the nucleus. Nuclear post-transcriptional regulation can involve changes in the RNA degradation rate or regulation of the primary transcript proc-
essing or block of mRNA transport from the nucleus to the cytoplasm. In this study, we show that the efficiency of RNA splicing is a step regulating the TCC pre-mRNA accumulation in nuclei of hypothyroid rat liver. In fact, the reduced rate of the amount of spliced to unspliced RNA in hypothyroid, when compared with euthyroid rats (see Fig. 5), indicated that the splicing reaction itself is inhibited in hypothyroidism. Furthermore, these data suggest that the hypothyroid state does not affect the splicing of all of the introns from TCC precursor RNA, as shown by using I3-E4 and E7-I7 probes. Probably hypothyroidism exerts its inhibitory effect through a cis-acting element, which is supposed to map to exon 8. Interestingly, the reduction of spliced RNA observed by using the E8-I8 probe in the nuclei from hypothyroid rat was similar to that of TCC mRNA accumulation measured in the cytoplasm, thus suggesting that (i) TCC regulation by the hypothyroid state occurs in the nucleus and (ii) the nuclear cytoplasmic transport of TCC mRNA does not seem to be regulated. Experiments are in progress in our laboratory to further elucidate the splicing pathway using probes representing all of the other exon-intron junctions, even if the preliminary results seem to indicate that the splicing of the other introns is not affected by the hypothyroidism (data not shown). To note, the rate of degradation of a mRNA can be regulated by both the presence of a poly(A) tail and the length of this tail. The accumulation of uncleaved versus polyadenylated RNA measured by RNase protection assay (Fig. 6) and the length of the poly(A) tail, determined by RNase H analysis (Fig. 7) in euthyroid and hypothyroid rats, showed that the rate of formation of the 3′-end of TCC RNA was not affected by the hypothyroid state. Thus, once fully processed, TCC mRNA was stable in the nucleus, and no change in its rate of degradation was detectable in this hormonal disorder. The activities of other mitochondrial carrier systems have been shown to be reduced in the hypothyroid state (45), whereas enhanced TCC activity has been observed in hypothyroid rats (26). It has been proposed that one of the possible factors responsible for the above-mentioned changes is the modification of the lipid environment surrounding the carrier molecule. In particular, these changes in the physical-chemical characteristics of the mitochondrial inner membrane lipids have been ascribed to a variation in the cardiolipin content, which in turn results from an alteration in the cardiolipin synthase activity (46). Even though we cannot rule out this indirect effect of the hypothyroid state on TCC activity, our results clearly indicate that regulation of transcription and splicing represents a direct control on TCC expression exerted by thyroid hormone deficiency.

In conclusion, the present data show that hypothyroidism causes a reduction of TCC activity that results from a decrease of TCC mRNA and protein levels. The investigations on the molecular mechanisms underlying this phenomenon indicate that the hypothyroid state leads to changes not only in the transcriptional rate of the TCC gene but also in the processing of the nuclear precursor for TCC mRNA. These findings led us to postulate that the modulation of TCC expression observed in the livers of hypothyroid rats occurs at both the transcriptional and post-transcriptional level. Regulating gene expression by both transcriptional and post-transcriptional mechanisms would result in a more rapid response to hormonal (47) or nutritional (48) changes. Regulation of the efficiency of splicing may be a common mechanism for controlling lipogenic gene expression (47, 48) and may account for the discrepancies between changes in the transcriptional rate and changes in mRNA abundance for several of these genes.

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J. Biochem. 26, 587–594
32. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
33. Kaplan, R. S., Mayor, J. A., and Wood, D. O. (1993) J. Biol. Chem. 268, 13682–13690
34. Yehuda-Shnaidman, E., Kalderon, B., and Bar-Tana, J. (2005) Endocrinology 146, 2462–2472
35. Liu, Y., Sun, L., and Jost, J. P. (1996) Nucleic Acids Res. 24, 2718–2722
36. Siculella, L., Damiano, F., Sabetta, S., and Gnoni, G. V. (2004) J. Lipid Res. 45, 1333–1340
37. Amir-Ahmady, B., and Salati, L. M. (2001) J. Biol. Chem. 276, 10514–10523
38. Dummler, K., Muller, S., and Seitz, H. J. (1996) Biochem. J. 317, 913–918
39. Weitzel, J. M., Hamann, S., Jauk, M., Lacey, M., Filbry, A., Radtke, C., Iwen, K. A., Kutz, S., Lizardi, P. M., and Seitz, H. J. (2003) J. Mol. Endocrinol. 31, 291–303
40. Goodridge, A. G. (1973) J. Biol. Chem. 248, 4318–4326
41. Brown, S. B., Maloney, M., and Kinlaw, W. B. (1997) J. Biol. Chem. 272, 2163–2166
42. Muller, M. J., and Seitz, H. J. (1987) Biochem. Pharmacol. 36, 1623–1627
43. Llobera, M., and Herrera, E. (1980) Endocrinology 106, 1628–1633
44. Comte, B., Vidal, H., Laville, M., and Riou, J. P. (1990) Metabolism 39, 259–263
45. Paradies, G., Ruggiero, F. M., and Dinoi, P. (1991) Biochim. Biophys. Acta 1070, 180–186
46. Paradies, G., Ruggiero, F. M., Petrosillo, G., and Quagliariello, E. (1997) Biochim. Biophys. Acta 1362, 193–200
47. Tao, H., Szczel-Fedorowicz, W., Amir-Ahmady, B., Gibson, M. A., Stabile, L. P., and Salati, L. M. (2002) J. Biol. Chem. 277, 31270–31278
48. Walker, J. D., Burmeister, L. A., Mariash, A., Bosman, J. F., Harmon, J., and Mariash, C. N. (1996) Endocrinology 137, 2293–2299