THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 13, Issue of March 30, pp. 9800–9807, 2001

Printed in U.S.A.

Polyunsaturated Fatty Acids Suppress Hepatic *Sterol Regulatory Element-binding Protein-1* Expression by Accelerating Transcript Decay*

Received for publication, October 2, 2000, and in revised form, November 17, 2000
Published, JBC Papers in Press, December 21, 2000, DOI 10.1074/jbc.M008973200

Jing Xu‡, Margarita Teran-Garcia‡, Jung H. Y. Park§, Manabu T. Nakamura‡, and Steven D. Clarke‡¶

From the ‡Division of Nutritional Sciences, and the Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712 and the §Division of Life Sciences, Hallym University, Chunchon, 200-702, Korea

The reduction in hepatic abundance of *sterol regulatory element binding protein-1* (*SREBP-1*) mRNA and protein associated with the ingestion of polyunsaturated fatty acids (PUFA) appears to be largely responsible for the PUFA-dependent inhibition of lipogenic gene transcription. Our initial studies indicated that the induction of *SREBP-1* expression by insulin and glucose was blocked by PUFA. Nuclear run-on assays suggested PUFA reduced *SREBP-1* mRNA by post-transcriptional mechanisms. In this report we demonstrate that PUFA enhance the decay of both *SREBP-1a* and *-1c*. When rat hepatocytes in monolayer culture were treated with albumin-bound 20:4(*n*-6) or 20:5(*n*-3) the half-life of total *SREBP-1* mRNA was reduced by 50%. Ribonuclease protection assays revealed that the decay of *SREBP-1c* mRNA was more sensitive to PUFA than was *SREBP-1a*, *i.e.* the half-life of *SREBP-1c* and *-1a* was reduced from 10.0 to 4.6 h and 11.6 to 7.6 h, respectively. Interestingly, treating the hepatocytes with the translational inhibitor, cycloheximide, prevented the PUFA-dependent decay of *SREBP-1*. This suggests that *SREBP-1* mRNA may need to undergo translation to enter the decay process, or that the decay process requires the synthesis of a rapidly turning over protein. Although the mechanism by which PUFA accelerate *SREBP-1* mRNA decay remains to be determined, cloning and sequencing of the 3′-untranslated region for the rat *SREBP-1* transcript revealed the presence of an A-U-rich region that is characteristic of a destablizing element.

Dietary (*n*-6) and (*n*-3) polyunsaturated fatty acids (PUFA)[1] lower blood triglycerides, decrease intra-muscular lipid droplet size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in two ways. First, they induce the transcription of genes encoding proteins involved in lipid oxidation, *e.g. carnitine palmitoyltransferase* (6) and *acyl-CoA oxidase* (7). Second, PUFA suppress the expression of genes encoding proteins involved in lipid synthesis, *e.g. fatty acid synthase* and *acetyl-CoA carboxylase* (8). Genes encoding the oxidative enzymes appear to be regulated by a common PUFA-activated transcription factor, peroxisome proliferator-activated receptor α (9, 10). On the other hand, PUFA appear to coordinately inhibit hepatic lipogenic gene transcription by rapidly reducing the nuclear content of the lipogenic transcription factor, sterol regulatory element binding protein-1 (SREBP-1) (11–14).

There are three members of the SREBP family: 1a, 1c, and 2 (15). SREBP-1 appears to be more involved with the regulation of lipogenic genes, while SREBP-2 may have the greatest influence on the expression of cholesterolgenic genes (16). The SREBPs were identified because of their ability to bind to the sterol regulatory element and confer sterol regulation to several genes involved with cholesterol synthesis (15). SREBPs are synthesized as 125-kDa precursor proteins that contain two transmembrane domains for insertion into the endoplasmic reticulum membrane (15). The N-terminal domain, a 68-kDa helix-loop-helix leucine zipper transcription factor (*i.e.* mature SREBP), is released for nuclear translocation by a sterol-dependent proteolytic cascade (15). The proteolytic release of mature SREBP is also regulated by PUFA (11–14), but this control may be limited to the release of SREBP-1. SREBP-1a and -1c are derived from the same gene, but the N terminus of the SREBP-1a protein is 24 amino acid residues longer because *SREBP-1a* and *-1c* employ different promoter sites (17).

The nuclear abundance of SREBP-1, and hence the rate of lipogenic gene transcription, is determined by the rate of proteolytic release of the mature SREBP-1 and the relative abundance of SREBP-1 precursor (11–15). The synthesis of SREBP-1 precursor is dependent upon the relative abundance of *SREBP-1* mRNA (11). Fasting, diabetes, or the ingestion of PUFA reduce the amount of hepatic *SREBP-1* mRNA. On the other hand, carbohydrate-refeeding or insulin administration increase the abundance of *SREBP-1* mRNA (18–20). The changes in hepatic *SREBP-1* mRNA abundance, and ultimately SREBP-1 precursor protein, associated with fasting, carbohydrate ingestion, diabetes, or insulin administration reflect alterations in *SREBP-1* gene transcription (20). However, nuclear run-on assays indicated that the reduction in hepatic content of *SREBP-1* mRNA resulting from PUFA ingestion may involve post-transcription mechanisms (11). This has led us to hypothesize that PUFA suppress the hepatic expression of *SREBP-1* by accelerating the rate of *SREBP-1* mRNA decay. With this report, we in fact demonstrate that PUFA accelerate the decay of *SREBP-1* mRNA, and that *SREBP-1c* mRNA is more sensitive to PUFA regulation than is *SREBP-1a*.

* This work was supported by National Institutes of Health Grants DK 53872 (to S. D. C.) and DK 09723 (to M. T. N.), the Universidad Nacional Autonoma de Mexico (to M. T. G.), and by the sponsors of the M. M. Love Chair in Nutritional, Cellular and Molecular Sciences at The University of Texas at Austin (to S. D. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: 115 Gearing, The University of Texas at Austin, Austin, TX 78712. Tel.: 512-232-1537; Fax: 512-232-5864; E-mail: stevedclarke@mail.utexas.edu.

[1] The abbreviations used are: PUFA, polyunsaturated fatty acids; SREBP, sterol regulatory element-binding protein; ADD1, adipocyte differentiation and determination factor-1, BSA, bovine serum albumin.

EXPERIMENTAL PROCEDURES

Primary Hepatocyte Culture—Male Harlan Sprague-Dawley rats (150–170 g) were fasted for 24 h prior to hepatocyte isolation. Hepatocytes were isolated and maintained in primary monolayer culture as previously described by Salati and Clarke (21). Briefly, isolated hepatocytes (9×10^6 cells) were plated onto 10-cm tissue culture plates that were previously coated with rat tail collagen (Becton Dickinson Labware). Cells were allowed to attach for 4 h in Waymouth MB 752/1 medium (Life Technology) supplemented with 0.4 mM alanine, 0.5 mM serine, 26 mM sodium bicarbonate, 100 nM insulin (Life Technologies Inc.), 100 nM dexamethasone (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. After the attachment period, medium was changed to a serum-free media and treated with 150 μM albumin-bound 20:4(*n*-6), 20:5(*n*-3), and 18:1(*n*-9) at a fatty acid/albumin ratio of 4:1. Cells not treated with fatty acid received an amount of albumin equal to that provide with the fatty acid-albumin complex. The source of albumin for all studies was essentially fatty acid-free bovine serum albumin (Sigma).

Nuclear Run-on Assay and mRNA Analyses—The impact of dietary PUFA on the transcription of hepatic *fatty acid synthase* and *SREBP-1* was determined using the nuclear run-on assay (11). A rat specific *SREBP-1c*/*ADD1* cDNA (B. Spiegelman) was employed to quantify the amount of nascent *SREBP-1* mRNA synthesized by the nuclei. The abundance of *SREBP-1* and *fatty acid synthase* mRNA in cultured primary rat hepatocytes was determined using total RNA extracted by the phenol-guanidinium isothiocyanate procedure (22). For Northern analysis, total RNA (30 μg per lane) was size-fractionated on a 1% agarose/formaldehyde denaturing gel, and subsequently transferred to a Zeta-probe nylon membrane (Bio-Rad) (11). The mRNA abundance of the respective transcripts was estimated by sequentially hybridizing the membrane with ^{32}P-labeled cDNA probes for *SREBP-1, fatty acid synthase*, and *glyceraldehyde-3-phosphate dehydrogenase*. All probes were labeled with [α-^{32}P]dCTP (PerkinElmer Life Sciences) using random prime labeling (Life Technologies). Hybridization and wash conditions have been described previously (23). Autoradiographic signals were quantified using Instant Imager (Packard). The effect of fatty acid on the abundance and decay of *SREBP-1c* and *-1a* was determined using the ribonuclease protection assay (24). A rat *SREBP-1a* cDNA fragment with sequence corresponding to exon 1 (specific to *SREBP-1a*) and part of exon 2 (common to both *SREBP-1a* and *-1c*) was produced using reverse transcription-polymerase chain reaction amplification and total rat liver RNA as the template. The primers were those described in Ref. 17. The amplified cDNA fragment was then subcloned into the pBluescript vector (Invitrogen) and the plasmid was linearized with *Hin*dIII. The cDNA fragment used to produce *18S* ribosome RNA was purchased from Ambion. Both antisense probes were transcribed using bacteriophage T3 RNA polymerase (RPAIII Kit, Ambion). The probes were radiolabeled with [α-^{32}P]UTP (PerkinElmer Life Sciences) and possessed specific activities of $5–8 \times 10^8$ dpm/μg for *SREBP-1* and $4–10 \times 10^3$ dpm/μg for *18S*. Hybridization was conducted by incubating 10 μg of total RNA with at least 4 M excess of probe at 56 °C overnight. After RNase A/T1 digestion, the protected fragments were separated on a 8 M urea, 5% polyacrylamide gel. The gel was dried and the autoradiographic signals were visualized by exposure to x-ray film, and the abundance of each transcript was quantified using Instant Imager (Packard). The relative level of *SREBP-1a* and *-1c* mRNA was compared upon correcting for the number of ^{32}P-labeled UTP atoms in each protected fragment.

Hepatocyte Abundance of SREBP-1 Protein—Nuclear and microsomal protein extracts were prepared from hepatocyte monolayers as described in Refs. 25 and 26. To prevent proteolysis, all buffers contained 25 μg/ml *N*-acetylleucylleucylnorleucinal, 24 μg/ml Pefabloc, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin. Briefly, hepatocytes from five 10-cm plates were pooled, and the cell pellets were disrupted by Dounce homogenization in 3 volumes of a buffer solution containing 10 mM HEPES-KOH at pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol and protease inhibitors. The homogenate was centrifuged at $3,300 \times g$ for 15 min. The pellets were resuspended and incubated for 1 h in an equal volume of a buffer containing 20 mM HEPES-KOH at pH 7.6, 25% (v/v) glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and protease inhibitors. The nuclei were collected by centrifuging the suspension for 30 min at $15,000 \times g$. Microsomes were isolated by centrifuging the supernatant from the initial $3,300 \times g$ spin for 1 h at $100,000 \times g$. The microsomal proteins were collected by solubilizing the pellet in 10 mM Tris-HCl at pH 6.8, 0.1 M NaCl, 1% (v/v) SDS, 1 mM dithiothreitol plus protease inhibitors. The abundance of mature SREBP-1 (nuclear) and precursor SREBP-1 (microsomal) was determined by Western blotting following the procedure described by Xu *et al.* (11). Immunoreactive SREBP-1 was identified by incubating the blot for 4 h with monoclonal anti-SREBP-1 (IgG-2A4) prepared from hybridoma cells (ATCC, CRL 2121), and the protein visualized using an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech). Bands were quantified for relative intensity using the Ambis imagizing system.

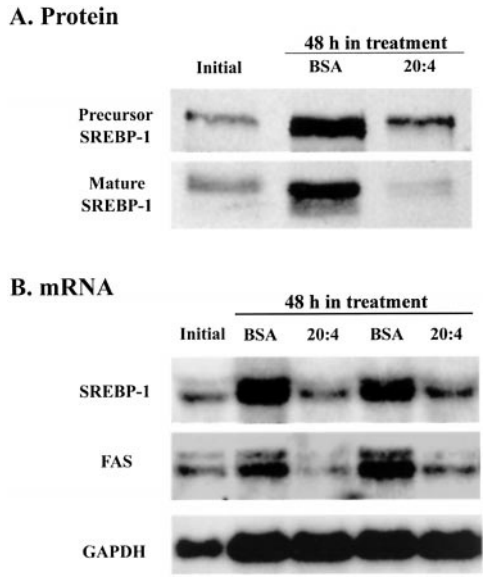

FIG. 1. **Inhibition of the insulin-glucose induction of hepatocyte *SREBP-1* by 20:4(*n*-6).** Hepatocytes were isolated from 24-h fasted rats and maintained in a media containing insulin, 28 mM glucose, and 150 μM albumin-bound 20:4(*n*-6) or 37.5 μM BSA alone. The abundance of membrane bound (*precursor*) and nuclear (*mature*) SREBP-1 protein (*A*) was determined by Western blot analysis using pooled (n = 5 plates) protein extracts prepared from freshly isolated cells (*initial*), or cells treated for 48 h with 20:4(*n*-6) or BSA. The effect of 20:4(*n*-6) on *SREBP-1, fatty acid synthase* (*FAS*), and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA abundance was determined by Northern analysis (*B*) using total RNA (30 μg/lane) extracted from hepatocytes immediately after attachment (initial) or from hepatocytes treated with BSA or 20:4(*n*-6) for 48 h. The Northern blot is a representative experiment showing analyses for replicate plates.

Transcription and Translation Inhibition Assays—PUFA regulation of *SREBP-1a, -1c*, and *fatty acid synthase* mRNA half-lives in primary hepatocyte was evaluated by an α-amanitin transcription inhibition assay (27). Hepatocytes were isolated from fasted rats and maintained in a serum-free media containing 28 mM glucose, 0.1 μM insulin, and 0.1 μM dexamethasone. Following a 44-h incubation to induce *SREBP-1* expression in the hepatocytes, the medium was changed to one containing either albumin-bound 150 μM 20:4(*n*-6) or albumin alone. After a 3-h pretreatment, transcription was arrested by adding α-amanitin (15 μM final concentration). The abundance of *SREBP-1, fatty acid synthase*, and *glyceraldehyde-3-phosphate dehydrogenase* was determined by Northern blot or ribonuclease protection procedures.

To examine the possibility that mRNA translation was needed for *SREBP-1* mRNA to undergo decay, the half-lives of *SREBP-1a* and *-1c* mRNA were determined in the presence of the translational inhibitor, cycloheximide. As described previously, *SREBP-1* expression in isolated hepatocytes was induced by culturing hepatocytes in a media containing glucose and insulin. After 48 h in culture, the cells were treated with cycloheximide (5 μM final concentration) for 2 h, and subsequently treated with 150 μM albumin-bound 20:4(*n*-6) or albumin alone for an additional 6 h. The abundance of *SREBP-1a* and *-1c* mRNA was quantified using the ribonuclease protection assay.

Cloning of the 3′-Untranslated Region of Rat SREBP-1—Total RNA was extracted from rats fed a high carbohydrate diet. The 3′-end of rat *SREBP-1* mRNA was cloned using reverse transcription-polymerase chain reaction methodology. First strand synthesis was accomplished using murine leukemia virus reverse transcriptase and a poly(T) degenerate primer, 5′-TTCTAGTCGACTGAATTCTCTCGAGGCGTTTT-TTTTTTTTTTTTTTTT(G/A/C)(G/A/C/T)-3′ that was linked to an adaptor sequence of 5′-TTCTAGTCGACTGAATTCTCTCGAGGCG-3′.

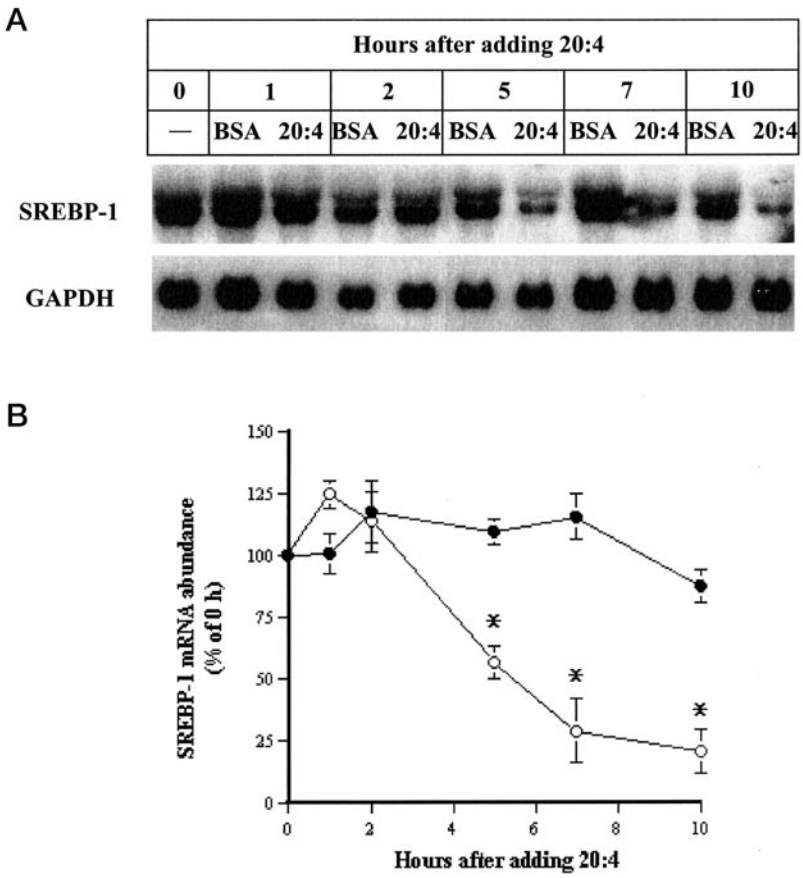

FIG. 2. **The time course for 20:4(*n*-6) inhibition of *SREBP-1* mRNA in rat hepatocytes.** Hepatocytes were isolated from 24-h fasted rats and maintained in a media containing insulin and 28 mM glucose. After 44 h in culture (0 time), 37.5 μM BSA or 150 μM albumin-bound 20:4(*n*-6) was added to the media. Total RNA was extracted from the hepatocytes at the times indicated, and the abundance of *SREBP-1* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA was determined by Northern analysis (*A*). *B* depicts the change in *SREBP-1* mRNA that occurs when hepatocytes were incubated in the presence (*open circles*) and absence (*closed circles*) of 20:4(*n*-6). Data are representative of three experiments and are mean ± S.E., $n = 3$ plates per point. *Asterisk* indicates values that are significantly lower than the BSA-treated cells.

After a 20-min 42 °C reaction, polymerase chain reaction amplification was conducted using the gene-specific primer at position 5′-GAGGAGGGTCTTCCTACATGAGGC-3′ and the adaptor primer sequence 5′-TTCTAGTCGACTGAATTCTCTCGAGGCG-3′. The reaction conditions were comprised of an initial denaturation at 94 °C for 10 min followed by 5 cycles of 94 °C for 30 s and 72 °C for 1.5 min, 5 cycles of 94 °C for 30 s and 70 °C for 1.5 min, and finally 33 cycles of 94 °C for 30 s and 68 °C for 1.5 min. The resulting amplification product was sequenced by the dideoxy chain termination method.

RESULTS

SREBP-1 Gene Transcription Is Not Suppressed by 20:4(n-6)—Earlier work from our group suggested that the hepatic reduction in *SREBP-1* mRNA abundance elicited by dietary polyunsaturated fatty acids was not accompanied by a reduced rate of gene transcription (11). However, this earlier work employed a hamster cDNA for *SREBP-1* to quantify the synthesis of nascent *SREBP-1* transcripts that occurred in rat liver nuclei (11). It is possible that the hamster *SREBP-1* cDNA may have hybridized to a nonspecific nascent transcript produced in the nuclear run-on assay. Such nonspecific hybridization could have masked potential changes in *SREBP-1* gene transcription. Therefore, the effect of dietary (*n*-6) and (*n*-3) polyunsaturated fatty acids on rat liver *SREBP-1* gene transcription was re-evaluated using a cDNA specific for rat *SREBP-1*, *i.e. adipose differentiation* and *determination factor-1* (*ADD-1*). Nuclear run-on assays ($n = 4$ per diet group) continued to indicate that *SREBP-1* gene transcription was not inhibited by dietary fats rich in 18-carbon (*n*-6) or 20- and 22-carbon (*n*-3) fatty acids (data not shown).

20:4(n-6) Accelerates SREBP-1 Decay in Isolated Hepatocytes—The nuclear run-on results cited above led to the hypothesis that polyunsaturated fatty acids reduce *SREBP-1* mRNA and protein abundance by accelerating the decay of the *SREBP-1* transcript. Pursuit of this question required an hepatic cellular model that mimicked intact animal responses. Isolated rat hepatocytes maintained in primary culture fulfill this requirement. As observed in the intact animal (11–13), the amount of mature SREBP-1 found in the nuclei of hepatocytes was positively correlated with the hepatocyte content of *fatty acid synthase* mRNA (Fig. 1). Specifically, culturing hepatocytes isolated from 24-h fasted rats in medium containing insulin and 28 mM glucose resulted in a 3–4-fold increase in the amount of membrane (precursor) and nuclear (mature) SREBP-1 (Fig. 1*A*). The increase in hepatocyte SREBP-1 protein was paralleled by a comparable rise in the abundance of *SREBP-1* mRNA (Fig. 1*B*). Most importantly, treating the hepatocytes with 150 μM albumin-bound 20:4(*n*-6) completely blocked the insulin-glucose dependent induction of *SREBP-1* mRNA and protein (Fig. 1). An examination of the rapidity with which 20:4(*n*-6) exerted its influence on *SREBP-1* expression revealed that a 5- and 10-h exposure to 20:4(*n*-6) reduced the hepatocyte content of *SREBP-1* mRNA 50 and 85%, respectively (Fig. 2). Interestingly, there was at least a 2-h lag before 20:4(*n*-6) exerted its suppressive influence on *SREBP-1* mRNA (Fig. 2). Finally, it is important to note that 20:4(*n*-6) had no effect on either the insulin-glucose induction or the steady state level of *glyceraldehyde-3-phosphate dehydrogenase* mRNA (Fig. 1*B* and Fig. 2). This indicates that the suppression of *SREBP-1* and associated lipogenic genes by 20:4(*n*-6) is specific and comparable to the *in vivo* responses achieved with dietary (*n*-6) or (*n*-3) polyunsaturated fatty acids (11–13, 28).

The influence of 20:4(*n*-6) on the half-life of *SREBP-1* mRNA was examined by pretreating rat liver cell monolayers for 3 h with albumin-bound 20:4(*n*-6) or albumin alone, and subsequently adding α-amanitin to inhibit gene transcription (Figs. 3-5). The abundance of *glyceraldehyde-3-phosphate dehydrogenase* was not affected by either 20:4(*n*-6) or α-amanitin (Figs. 3–5). On the other hand, Northern analyses revealed that 20:4(*n*-6) treatment of rat cell monolayers accelerated the rate

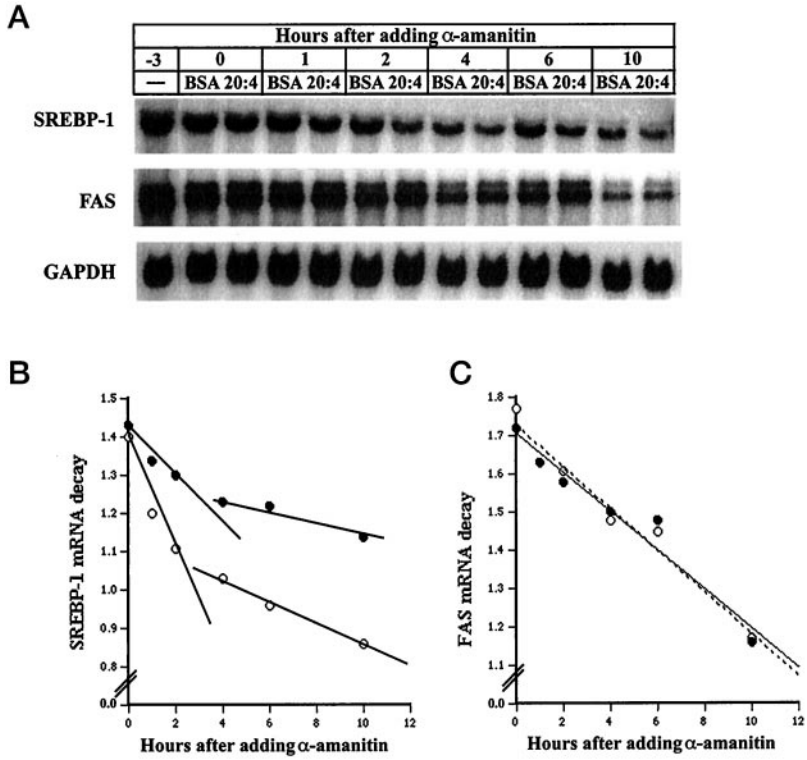

FIG. 3. **20:4(*n*-6) accelerated *SREBP-1* mRNA decay in rat liver cell monolayers.** Rat hepatocytes were isolated from 24-h fasted rat donors and maintained in a media containing insulin and 28 mM glucose. After 44 h in culture, cells were treated with 150 μM albumin-bound 20:4(*n*-6) (*open circles*) or 37.5 μM BSA alone (*closed circles*) for 3 h (−3) prior to the addition of the transcription inhibitor, α-amanitin. *A*, the abundance of *SREBP-1, fatty acid synthase* (*FAS*), and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was determined by Northern analysis (30 μg of total RNA per lane). The logarithmic decay for *SREBP-1* and *FAS* mRNA are depicted in *B* and *C*, respectively. The *SREBP-1* mRNA decay equations for BSA and 20:4(*n*-6) are $y = -0.026x + 1.376$ and $y = -0.047x + 1.274$, respectively. A test of differences revealed a significant effect of 20:4(*n*-6) ($p < 0.05$). *GAPDH* mRNA did not decrease during the 10-h period. Consequently the abundance of *SREBP-1* and *FAS* mRNA is expressed relative to the level of *GAPDH*. The regression equations for *FAS* mRNA decay are $y = -0.026x + 1.655$ and $y = -0.04x + 1.636$ for the BSA and 20:4(*n*-6) treated cells, respectively. Data are representative of two independent experiments.

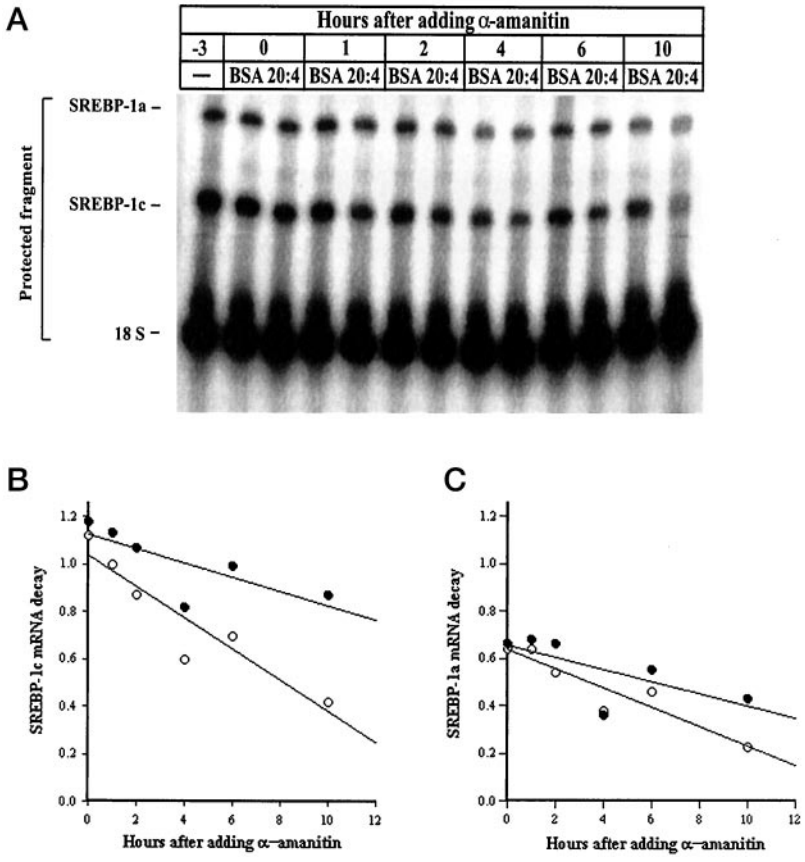

FIG. 4. **20:4(*n*-6) differentially regulates transcript decay for *SREBP-1a* and *SREBP-1c*.** Using the same RNA samples analyzed in Fig. 3, ribonuclease protection assays were conducted to quantify the rate of *SREBP-1c* and *-1a* decay (*A*) depicts a representative assay. The change in *SREBP-1c* (*B*) and *SREBP-1a* (*C*) mRNA half-life observed in hepatocytes treated with 150 μM 20:4(*n*-6) or 37.5 μM BSA was calculated and plotted using the curve-fit of a linear plot. Values were normalized to the abundance of *18S* ribosomal RNA. The regression equations for *SREBP-1c* mRNA decay are $y = -0.066x + 1.036$ *versus* $y = -0.030x + 1.124$ for 20:4(n-6) and BSA, respectively ($p < 0.05$); and the regression equations for *SREBP-1a* mRNA decay are $y = -0.04x + 1.636$ *versus* $y = -0.026x + 1.655$ for 20:4(n-6) and BSA, respectively ($p > 0.05$).

of *SREBP-1* mRNA decay (Figs. 3–5). Regression analysis employing all measurements from 0 to 10 h indicated that 20:4(*n*-6) significantly ($p < 0.05$) shortened the half-life for total *SREBP-1* mRNA from 11.6 h ($r = 0.94$, $p < 0.01$) in the absence of fatty acid to 6.4 h ($r = 0.92$, $p < 0.01$) in the presence of 20:4(*n*-6). Examination of the decay curve suggested that the decay of *SREBP-1* mRNA occurred in two phases, *i.e.* a rapidly decaying pool (0–4 h), and a more slowly decaying pool (4–10 h). However, 20:4(*n*-6) accelerated the decay of both of these putative pools (Fig. 3). Because the cDNA probe utilized in the Northern analyses encoded a sequence that was common to both *SREBP-1a* and *-1c*, we hypothesized that the apparent

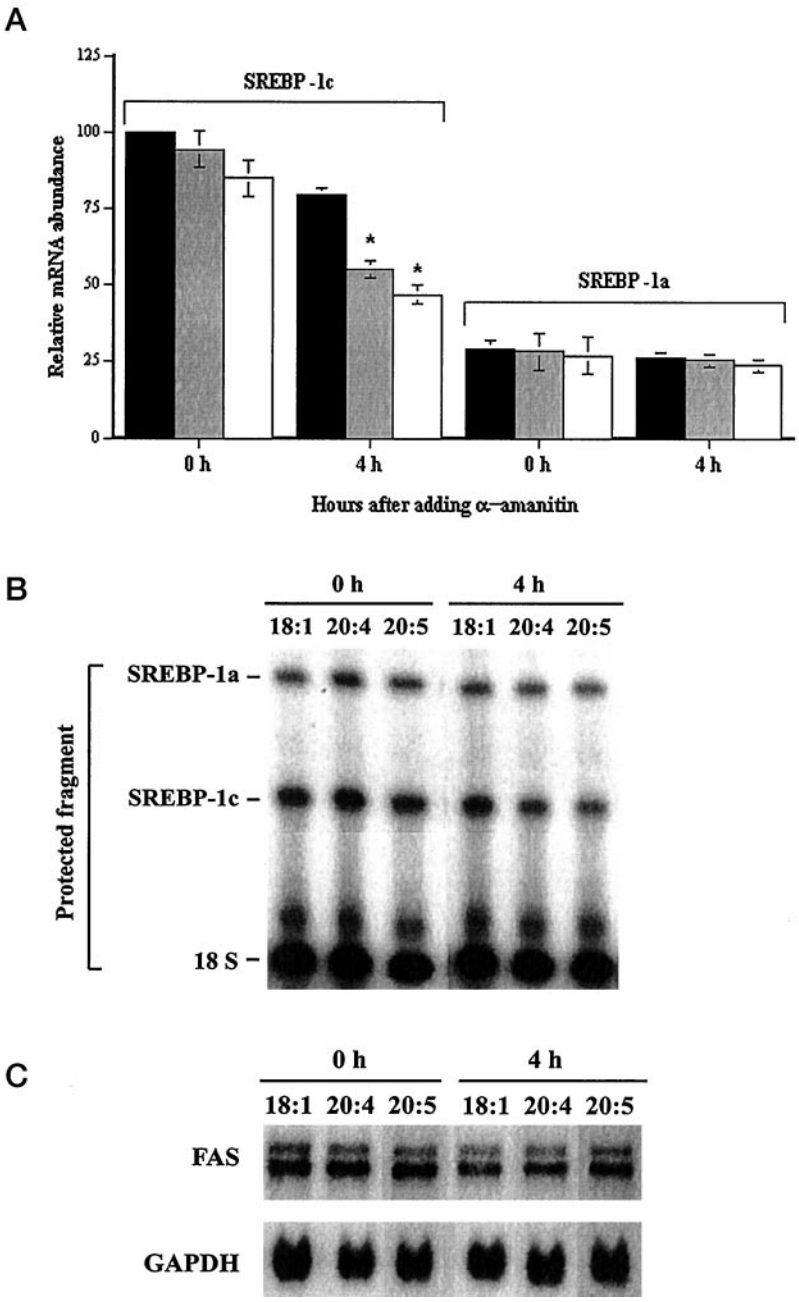

FIG. 5. **The effects of different unsaturated fatty acids on mRNA decay of *SREBP-1c* and *SREBP-1a* in rat hepatocytes.** *A*, total RNA was extracted from rat hepatocyte monolayers that had been pretreated with 250 μM albumin-bound 18:1 (n = 9) (*black bars*), 20:4(n-6) (*gray bars*), or 20:5(n-3) (*white bars*) for 3 h. After the 3-h pretreatment period (0 h), α-amanitin was added to the media to halt transcription, and the relative abundance of *SREBP-1c* and *-1a* mRNA was determined by the ribonuclease protection procedure. Values are expressed relative to 18:1(n-9) at 0 h, and the mRNA values were normalized to *18S* ribosomal RNA. *Asterisk* denotes a significant ($p < 0.05$) reduction from 0 h; *B* depicts a representative ribonuclease protection assay for *SREBP-1a* and *1c*; and *C* is a representative Northern blot demonstrating that the decay of *fatty acid synthase* (*FAS*) and *glyceraldehyde dehydrogenase* (*GAPDH*) mRNA was not affected by 20:4(n-6) or 20:5(n-3).

existence of two decay rates for *SREBP-1*, as determined by Northern analyses, may represent differences in decay between the *SREBP-1a* and *-1c* transcripts. To examine this possibility, a ribonuclease protection assay was employed to separately quantify the effect of 20:4(n-6) on the decay of *SREBP-1a* and *-1c* (Fig. 4). The *SREBP-1c*/*1a* ratio found in isolated hepatocytes maintained in a media containing insulin and glucose was 3/1, which was not dissimilar from the 5/1 ratio observed *in vivo* (29). When hepatocytes were maintained in a fatty acid-free media, the half-life of *SREBP-1c* was similar to that of *SREBP-1a*, *i.e.* 10.0 h ($r = 0.77$, $p < 0.07$) and 11.6 h ($r = 0.71$, $p < 0.10$), respectively (Fig. 4). However, when the hepatocyte monolayers were treated with 20:4(n-6), the half-life of *SREBP-1c* was significantly ($p < 0.05$) reduced to 4.6 h ($r = 0.93$, $p < 0.01$). The half-life of *SREBP-1a* mRNA also appeared to be reduced by 20:4(n-6) but the decrease from 11.6 to 7.7 h ($r = 0.94$) was not statistically significant. Like 20:4(n-6), 20:5(n-3) accelerated the decay of *SREBP-1c* (Fig. 5). The decay of *SREBP-1a* appeared to be unaffected by 20:5(n-3), but this lack of significant decay likely reflects the fact that cells were harvested after 4 h of fatty acid treatment which was well below the 7.7 h half-life of the *SREBP-1a* transcript. Unlike the effect of PUFA, the monounsaturated fatty acid, 18:1(n-9) had no effect on the hepatocyte content of *SREBP-1c* and *-1a* mRNA. This observation was consistent with several dietary studies that have shown that *SREBP-1* expression is only suppressed by (n-6) and (n-3) polyunsaturated fatty acids (8, 11, 23). Finally, the rate of hepatocyte *fatty acid synthase* mRNA decay appeared to be unaffected by either 20:4(n-6) or 20:5(n-3) (Figs. 3 and 5). Specifically, the half-life of *fatty acid synthase* mRNA in monolayers treated with 20:4(n-6) was 5.5 *versus* 5.8 h for cells treated with BSA alone (Fig. 3). This observation is consistent with numerous reports indicating that hepatic *fatty acid synthase* mRNA levels are primarily governed by changes in gene transcription (11, 28).

Decay of SREBP-1c mRNA Requires Translation—In an attempt to ascertain if the PUFA enhancement of hepatic *SREBP-1* mRNA decay involves a short-half-life regulatory

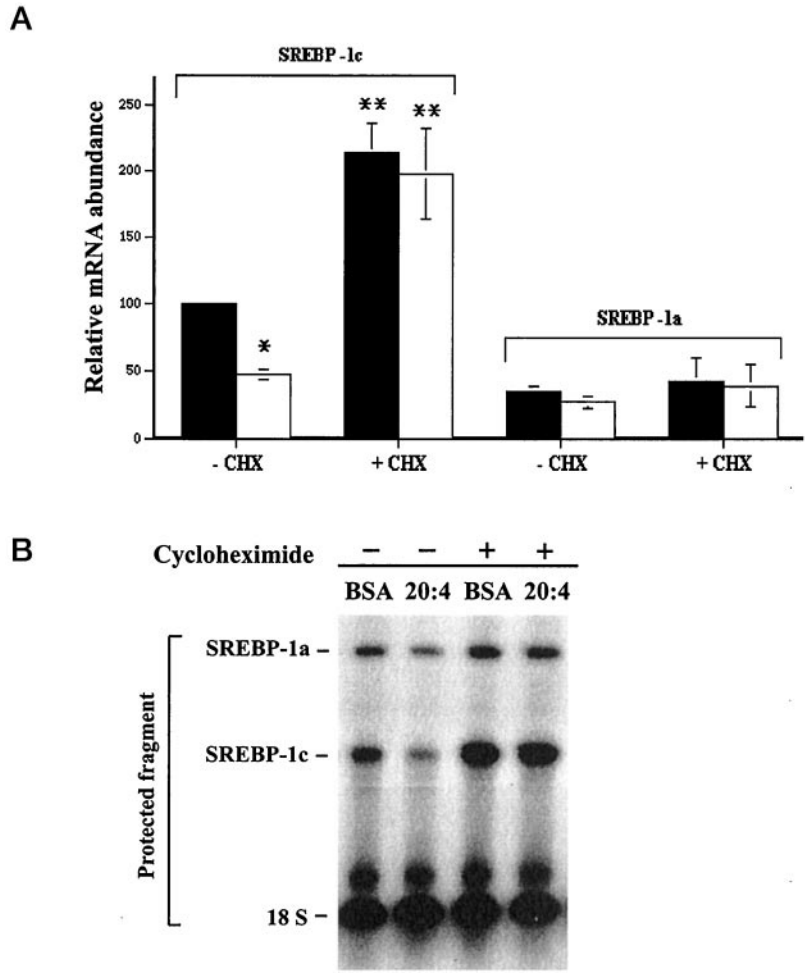

FIG. 6. **Protein synthesis is required for the decay of *SREBP-1*.** After 44 h in culture, hepatocytes were treated with cycloheximide (*CHX*) for 2 h prior to the addition of 150 μM albumin-bound 20:4(*n*-6) or 37.5 μM albumin alone (*BSA*). *SREBP-1a* and *-1c* mRNA abundance was determined 6 h after the addition of 20:4(*n*-6). *A* depicts the relative level of *SREBP-1c* and *-1a* mRNA in the absence (*black bars*) or presence (*white bars)* of 20:4(*n*-6) and with or without cycloheximide. The abundance of *SREBP-1a* and *-1c* mRNA is expressed relative to *18S* mRNA and represent the average of $n = 3$ plates per treatment. *Asterisk* (*) indicates 20:4(*n*-6) significantly ($p < 0.01$) reduced *SREBP-1c* mRNA; *double asterisk* (**) indicates a significant ($p < 0.05$) increase in SREBP-1c mRNA due to CHX treatment; *B,* representative ribonuclease protection assay for *SREBP-1a* and *-1c*.

protein, the influence of 20:4(*n*-6) on *SREBP-1a* and *-1c* mRNA decay was examined in the presence of the translational inhibitor, cycloheximide. Interestingly, cycloheximide treatment of the hepatocyte monolayers increased hepatocyte *SREBP-1c* and *-1a* mRNA abundance (Fig. 6). Moreover, blocking mRNA translation completely eliminated the PUFA-dependent increase in *SREBP-1c* and *-1a* mRNA decay (Fig. 6).

Cloning and Sequence of the 3′-Untranslated Region of Rat SREBP-1—Future pursuit of the mechanisms by which PUFA regulate the decay rate of *SREBP-1* mRNA require knowledge of the complete transcript sequence for rat *SREBP-1*. However, the sequence for rat *SREBP-1* (*i.e. ADD-1*) that was available did not include a termination codon nor did it include the 3′-untranslated region of the rat *SREBP-1* transcript. Using total rat liver RNA, and reverse transcription and polymerase chain reaction amplification, we successfully cloned a 692-base pair cDNA for rat *SREBP-1* that included a stop codon, a poly(A) tail, and a 350-base pair 3′-untranslated region (Fig. 7). Alignment of the rat *SREBP-1* transcript sequence with that for human *SREBP-1* revealed that the 3′-end of the rat SREBP-1 protein is highly homologous with the human, and that the reported sequence for *ADD-1* (*i.e.* rat *SREBP-1*) lacked sequence for 86 amino acid residues (Fig. 7). The 3′-untranslated region contained four A-U regions that may be candidates for the regulation of *SREBP-1* mRNA stability.

DISCUSSION

Supplementing a high carbohydrate diet with oils rich in (*n*-6) and (*n*-3) PUFA results in a rapid (*i.e.* <3 h) and coordinate inhibition of hepatic gene transcription for a wide array of lipogenic enzymes including fatty acid synthase, acetyl-CoA carboxylase, citrate lyase, malic enzyme, and stearoyl-CoA desaturase (8, 11, 23, 30). Dose-response studies indicate that lipogenic gene transcription is suppressed 50% when the diet contains as little as 3–5% of its energy as PUFA (31). The coordinate regulation of gene transcription by PUFA suggested that PUFA may employ a "master switch" type mechanism. The hepatic nuclear factor SREBP-1 may serve this function (11–14). PUFA appear to reduce the nuclear content of SREBP-1 by interfering with the proteolytic release of mature SREBP-1 from its endoplasmic reticulum anchored precursor. In addition, PUFA reduces the amount SREBP-1 precursor by decreasing the abundance of *SREBP-1* mRNA (11–14). *In vivo* and cell culture studies indicate that insulin and glucose increase the hepatic content of SREBP-1 by inducing the *SREBP-1* gene transcription (17, 32). In contrast, glucagon and cAMP suppress *SREBP-1* gene transcription, and consequently block the rise in hepatic *SREBP-1* mRNA and protein elicited by glucose and insulin (20). Like glucagon and cAMP, treating hepatocytes with 20:4(*n*-6) also blocked the insulin-glucose induction of *SREBP-1* expression (Fig. 1). However, unlike glucagon and cAMP, PUFA govern *SREBP-1* expression by post-transcriptional mechanisms (11). Such mechanisms may involve interference with mRNA processing (33) and/or acceleration of mRNA decay (34). In the case of *SREBP-1*, PUFA reduce the hepatic content of *SREBP-1* mRNA by accelerating the rate of mRNA decay.

The liver expresses two forms of SREBP-1, 1a and 1c. SREBP-1 antibody recognizes both proteins. Consequently, the relative abundance of the two proteins cannot be quantified. However, due to differences in splicing, the 5′-end of the

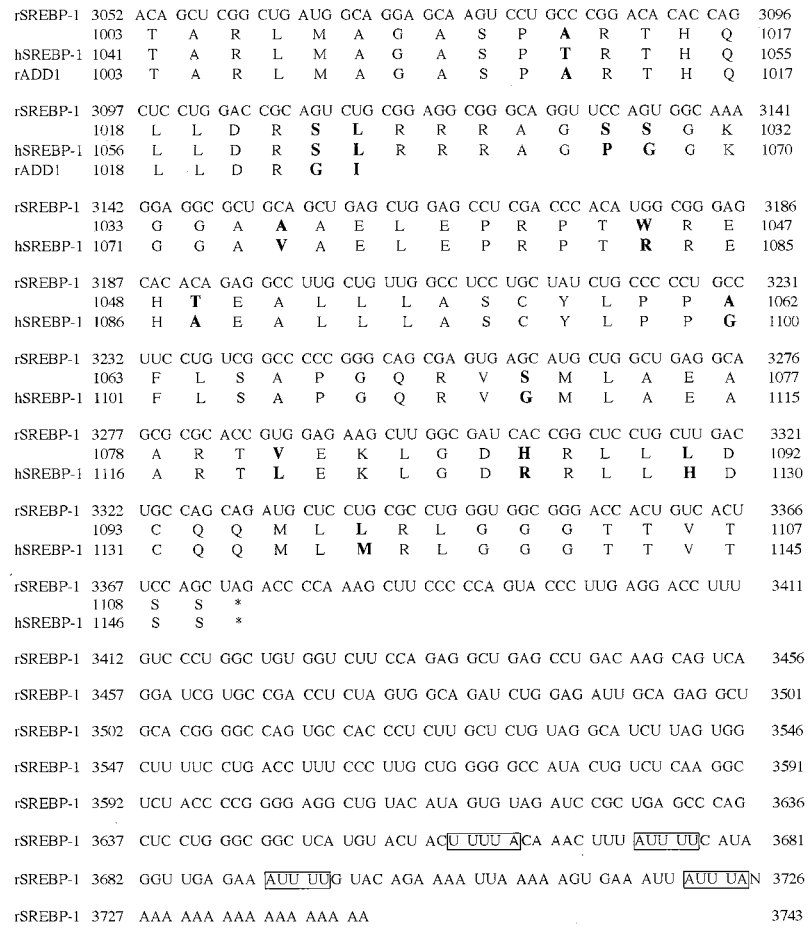

FIG. 7. **Nucleotide sequence for the 3′-end of rat *SREBP-1* mRNA.** The 3′-end of rat *SREBP-1* (*rSREBP-1*) was cloned as described under "Experimental Procedures." The nucleotide sequence and the predicted amino acid sequence for rSREBP-1 is aligned with human SREBP-1 (hSREBP-1) and *rat ADD-1* (*rADD-1*). The differences in predicted amino acid sequence are denoted by *bold letters*. The *asterisk* represents the termination codon. A-U-rich regions are *boxed*. Numbering for the nucleotides and amino acid positions are based upon the sequences previously reported in GenBank™ for hSREBP-1 (accession number P36956) and for rADD-1 protein sequence (accession number P56720) and for *rADD-1* nucleotide sequence (L16995).

SREBP-1c transcript is shorter than the *SREBP-1a* mRNA (29). This difference in size permits the use of a ribonuclease protection assay to examine the influence of PUFA on *SREBP-1a* and *-1c* mRNA abundance and decay. Interestingly, this assay revealed that the *SREBP-1c* transcript was more sensitive to PUFA regulation than was *SREBP-1a*. Specifically, PUFA reduced the half-life of *SREBP-1c* mRNA by 55% while it decreased the half-life of *SREBP-1a* by only 35%. Because the decay of *SREBP-1c* mRNA was more sensitive to 20:4(*n*-6) and 20:5(*n*-3) feedback, the ratio of *SREBP-1c*/*1a* in the hepatocytes treated with PUFA decreased from 3:1 to <1:1. The selective loss of *SREBP-1c* in response to PUFA is reminiscent of what has been observed in streptozotocin-diabetic rats (17, 32), but unlike glucagon and insulin which appear to regulate *SREBP-1* gene transcription, PUFA exert their effects at the level of *SREBP-1* mRNA decay.

The mechanisms by which PUFA enhance *SREBP-1* decay remain to be established. Often times the rate-limiting step in mRNA decay is the shortening of the poly(A) tail (35). The rate of poly(A) tail shortening appears to depend upon the binding of specific proteins to "destabilizing" elements within the mRNA (35). One common destabilizing element is the A-U rich sequences, notably UUAUUUA(U/A)(U/A), located within the 3′-untranslated region of a transcript (35). Such an A-U-rich region was found to be present within the 3′-untranslated region of the rat *SREBP-1* transcript (Fig. 7), but its functional significance in the decay of *SREBP-1* remains to determined. Moreover, many transcripts contain A-U-rich instability elements in their 3′-untranslated regions, but PUFA control of mRNA decay is not a widespread phenomenon. Thus, the control of *SREBP-1* decay by PUFA would appear to involve more than simply the A-U-rich regions. One alternative possibility is that the *SREBP-1* transcript contains a destablizing element(s) in the 5′-untranslated region or within the open reading frame that is targeted by PUFA control mechanisms. In this respect it is interesting to note that cycloheximide not only blocked the ability of PUFA to accelerate *SREBP-1* decay, but actually increased the hepatocyte content of both *SREBP-1c* and *-1a*. These results suggest that *SREBP-1* mRNA translation is required for mRNA decay, and/or that PUFA may modulate the activity of a rapidly turning over protein involved in *SREBP-1* mRNA decay. One additional dilemma is why do the *SREBP-1c* and *-1a* transcripts differ in their response to PUFA-regulated decay. Presumably, *SREBP-1c* and *-1a* both contain the same 3′-untranslated region, but under the influence of PUFA, the rate of *SREBP-1c* mRNA decay is faster than is the decay of *SREBP-1a*. A key difference between the two transcripts is that they are produced by two different promoter sites. This results in the 5′-untranslated region of *SREBP-1c* being 45 nucleotides shorter than *SREBP-1a*. Thus, one possible explanation for the difference in decay rates in response to PUFA is that the 5′-untranslated region of *SREBP-1c* and *-1a* plays a regulatory role in the PUFA-mediated decay of *SREBP-1*.

In conclusion, dietary (*n*-6) and (*n*-3) PUFA have long been known to decrease the expression of hepatic lipogenic as well glycolytic genes (8). While recent data indicate that 18:2(*n*-6) and 18:3(*n*-3) must undergo desaturation by the δ-6 desaturase to exert their inhibitory effects (36), numerous studies have failed to link the PUFA control of gene expression to the production of eicosanoids (31). Moreover, PUFA exercise their effects in an insulin- and glucagon-independent manner (37). Despite our inadequate understanding of the signaling mechanisms employed by PUFA, SREBP-1 is emerging as a transcription factor that is pivotal to the overall understanding of PUFA regulation of hepatic lipogenic gene expression. In this story, PUFA exert two effects. First, PUFA inhibit the proteo-

lytic release of SREBP-1c from its endoplasmic reticulum anchored precursor which in turn results in an immediate suppression of lipogenic gene transcription (11, 14). Second, PUFA accelerate the decay of *SREBP-1c* mRNA which in turn lowers the hepatic content of *SREBP-1* mRNA and the synthesis of SREBP-1 precursor. The outcome is a lower capacity for hepatic lipogenesis, and a decrease in hepatic triglyceride output (11–13). This ability of PUFA to suppress *SREBP-1* expression and thereby exert a strong anti-lipogenic influence not only explains how PUFA function as hypolipidemic agents but may also offer a partial explanation for how PUFA improve glucose metabolism and insulin sensitivity (2–5, 38).

Acknowledgment—We thank Tim Osborne for excellent assistance and insight.

REFERENCES

1. Nestel, P. J. (1990) *Annu. Rev. Nutr.* **10,** 149–167
2. Storlien, L. H., Kraegen, E. W., Chisholm, D. J., Ford, G. L., Bruce, D. G., and Pascoe, W. S. (1987) *Science* **237,** 885–888
3. Baur, L. A., O'Connor, J., Pan, D. A., Kriketos, A. D., and Storlien, L. H. (1998) *Metabolism* **47,** 106–127
4. Perseghin, G., Scifo, P., De Cobelli, F., Pagliato, E., Battezzati, A., Arcelloni, C., Vanzulli, A., Testolin, G., Pozza, G., Del Maschio, A., and Luzi, L. (1999) *Diabetes* **48,** 1600–1606
5. Podolin, D. A., Gayles, E. C., Wei, Y., Thresher, J. S., and Pagliassotti, M. J. (1998) *Am. J. Physiol.* **274,** R840–848
6. Power, G. W., and Newsholme, E. A. (1997) *J. Nutr.* **127,** 2142–2150
7. Baillie, R. A., Takada, R., Nakamura, M., and Clarke, S. D. (1999) *Prostaglandins Leukotriens Essen. Fatty Acids* **60,** 351–356
8. Jump, D. B., and Clarke, S. D. (1999) *Annu. Rev. Nutr.* **19,** 63–90
9. Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4318–4323
10. Xu, H. E., Lambert, M. H., Montana, V. G., Parks, D. J., Blanchard, S. G., Brown, P. J., Sternbach, D. D., Lehmann, J. M., Wisely, G. B., Willson, T. M., Kliewer, S. A., and Milburn, M. V. (1999) *Mol. Cell* **3,** 397–403
11. Xu, J., Nakamura, M. T., Cho, H. P., and Clarke, S. D. (1999) *J. Biol. Chem.* **274,** 23577–23583
12. Mater, M. K., Thelen, A. P., Pan, D. A., and Jump, D. B. (1999) *J. Biol. Chem.* **274,** 32725–33215
13. Yahagi, N., Shimano, H., Hasty, A. H., Amemiya-Kudo, M., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., and Yamada, N. (1999) *J. Biol. Chem.* **274,** 35840–35816
14. Worgall, T. S., Sturley, S. L., Seo, T., Osborne, T. F., and Deckelbaum, R. J. (1998) *J. Biol. Chem.* **273,** 25537–25540
15. Brown, M. S., and Goldstein, J. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11041–11048
16. Shimano, H., Horton, J. D., Shimomura, I., Hammer, R. E., Brown, M. S., and Goldstein, J. L. (1997) *J. Clin. Invest.* **99,** 846–854
17. Shimomura, I., Bashmakov, Y., Ikemoto, S., Horton, J. D., Brown, M. S., and Goldstein, J. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13656–13661
18. Horton, J. D., Bashmakov, Y., Shimomura, I., and Shimano, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5987–5992
19. Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., Lowell, B. B., and Spiegelman, B. M. (1998) *J. Clin. Invest.* **101,** 1–9
20. Foretz, M., Pacot, C., Dugail, I., Lemarchand, P., Guichard, C., Le, L.iepvre, X., Berthelier-Lubrano, C., Spiegelman, B., Kim, J. B., Ferre, P., and Foufelle, F. (1999) *Mol. Cell. Biol.* **19,** 3760–3768
21. Salati, L. M., and Clarke, S. D. (1986) *Arch Biochem. Biophys* **246,** 82–89
22. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
23. Clarke, S. D., Armstrong, M. K., and Jump, D. B. (1990) *J. Nutr.* **120,** 225–231
24. Ausubel, F. M., Brent, R., Kinggston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology,* John Wiley & Sons, Inc., New York
25. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
26. Wang, X., Sato, R., Brown, M. S., Hua, X., and Goldstein, J. L. (1994) *Cell* **77,** 53–62
27. Chen, Y., Weeks, J., Mortin, M. A., and Greenleaf, A. L. (1993) *Mol. Cell Biol.* **13,** 4214–4222
28. Blake, W. L., and Clarke, S. D. (1990) *J. Nutr.* **120,** 1727–1729
29. Shimomura, I., Shimano, H., Horton, J. D., Goldstein, J. L., and Brown, M. S. (1997) *J. Clin. Invest.* **99,** 838–845
30. Ntambi, J. M. (1999) *J. Lipid Res.* **40,** 1549–1558
31. Clarke, S. D., and Jump, D. (1993) *Nutrition and Gene Expression* (Berdanier, C. D., and Hargrove, J. L., eds) pp. 227–245, CRC Reviews, Boca Raton, FL
32. Shimano, H., Yahagi, N., Amemiya-Kudo, M., Hasty, A. H., Osuga, J., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., Harada, K., Gotoda, T., Ishibashi, S., and Yamada, N. (1999) *J. Biol. Chem.* **274,** 35832–35921
33. Stabile, L. P., Klautky, S. A., Minor, S. M., and Salati, L. M. (1998) *J. Lipid Res.* **39,** 1951–1963
34. Sessler, A. M., Kaur, N., Palta, J. P., and Ntambi, J. M. (1996) *J. Biol. Chem.* **271,** 29854–29858
35. Day, D. A., and Tuite, M. F. (1998) *J. Endocrinol.* **157,** 361–371
36. Nakamura, M. T., Cho, H. P., and Clarke, S. D. (2000) *J. Nutr.* **130,** 1561–1565
37. Clarke, S. D., and Hembree, J. (1990) *J. Nutr.* **120,** 625–630
38. Clarke, S. D. (2000) *Br. J. Nutr.* **83,** S59–66