SREBP-1 as a Transcriptional Integrator of Circadian and Nutritional Cues in the Liver

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Abstract The act of feeding in mammals can generate such powerful cues for peripheral organs that, under certain conditions, they can override the entraining signals coming from the clock in the brain. Restricting the feeding time to the inactivity period, for example, can completely and quickly reverse the rhythms of gene expression in the liver. This manipulation does not affect the central oscillator in the suprachiasmatic nucleus, which is phase-locked to the light-dark cycle, but does release the peripheral oscillations in the liver from central control. It seems reasonable to predict the existence of one or more immediate response systems designed to sense the need to acutely reverse the sequence of absorptive and postabsorptive phases in the liver. In this study, the authors monitored the posttranslational activation of the sterol response element binding proteins from a circadian point of view to evaluate the role they might play in the circadian organization of the liver transcriptome as well as in the reversal of hepatic physiology that accompanies diurnal restricted feeding. This study highlights a possible direct link between the immediate effects of food consumption on the level of key membrane and humoral factors and the expression status of a set of coordinately regulated target genes in the liver.

Key words circadian, liver, sterol response element binding protein, feeding behavior

Circadian gene expression has been observed not only in the neurons of the SCN (Weaver, 1998), the central pacemaker in mammals, but also in virtually every peripheral tissue tested. Circadian activity in the liver, for example, is normally driven by the rhythmic expression of a ubiquitously expressed set of clock genes. This machinery, in turn, maintains the rhythmic expression of genes that encode enzymes or regulatory proteins involved in food processing and energy homeostasis. However, it has been found that restricting the feeding to a short time interval during the inactivity period in mice uncouples circadian activity in the peripheral oscillators from the central oscillator in the SCN (Damiola et al., 2000). Large phase shifts in the expression of clock genes in the liver of mice subjected to restricted feeding (RF) during the day can be observed after only 2 days of treatment (Stokkan et al., 2001). Such a rapid shift in gene output has been proposed to evince a remarkable ability of the liver to adapt rapidly to changing meal times. To better understand the signaling pathway(s) responsible for this response, we focused on the sterol response element binding proteins (SREBPs). The SREBP family consists of the SREBP-1a, -1c, and -2 factors, known to differentially regulate a large pool of target genes in the liver (Magaña et al., 2000; Amemiya-Kudo et al., 2002) involved in lipogenesis and cholesterol homeostasis (Horton et al., 2002; Pai

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et al., 1998). SREBP-1c, for example, has emerged as the most important candidate for mediating insulin and nutritional homeostasis in the liver (Zhang et al., 2003; Osborne, 2000).

The SREBPs are activatable by a standby post-translational mechanism linked to food-dependent variables (Brown and Goldstein, 1997; Shimomura et al., 1998; Sakai et al., 1996). Indeed, activation of SREBP-1, either the gene or the protein, by insulin (Zhang et al., 2003; Kim et al., 1998) or sterol depletion (Horton et al., 2002; Osborne, 2000) can be detected within the 1st few hours of treatment. Both variables have been shown to fluctuate under various feeding paradigms (Zhang et al., 2003; Peschke and Peschke, 1998; Mater et al., 1999).

Finally, the DNA binding specificity of SREBPs is not restricted to the sterol response element (SRE: TCACNCCAC) and related sites (Vallett et al., 1996) but extends even further to include a short palindromic repeat known as the E-box (Murre et al., 1994), which is, interestingly, the main hexameric sequence driving circadian control of clock gene transcription (Jin et al., 1999; Muñoz et al., 2002).

Taking this body of evidence in consideration, it would appear that the SREBPs are obvious and ideal coupling devices between starvation/feeding signals and the swift and transient adjustment of physiological oscillations in the liver. Accordingly, we set out to analyze the pattern of SREBP maturation/activation from a circadian point of view, predicting that such analysis could shed new light on the dynamic mechanisms controlling rhythmic gene expression in the liver, a prototypic peripheral oscillator.

MATERIALS AND METHODS

Animal care and experimental protocols. Age-matched male and female C3H/HEN mice (NCI/DCT, Frederick, MD) were housed under a 12:12-h light:dark schedule (lights-on at 1100 h) for 2 weeks before use. For ad libitum feeding studies, mice were fed with a rodent NIH-31 diet (Wafer, Zeigler Bros. Inc., Gardners, PA) for 6 days, and then 2 males and 2 females were sacrificed by cervical dislocation every 4 h for 24 h. The livers were harvested, and 4 identical pieces (~30-40 mg) from each animal were quick-frozen in separate tubes on dry ice. For restricted feeding studies, mice that had been fed ad libitum were subjected to 18 h of food deprivation. Animals were then divided into 2 treatment groups. One group underwent restricted feeding for 4 h during the night (activity phase in rodents) from ZT 17 to ZT 21 (NF), while a 2nd group received food for 4 h during the day (inactivity period) from ZT 5 to ZT 9 (DF). After 1 day of RF, 2 males and 2 females from each group were sacrificed by cervical dislocation every 3 h for 24 h. Livers were harvested as described above. All the experiments were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Animal Welfare Act regulations, following experimental protocols that were approved by the Animal Care and Use Committee and met the National Institute of Health guidelines.

Immunoblotting. Protein extracts were prepared by homogenizing individual quick-frozen liver fragments in buffer C (20 mM HEPES [pH 7.9], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM sodium fluoride, 5 μM sodium orthovanadate, and 25% glycerol). Aliquots containing 35 μg of total protein were subjected to electrophoresis in 4% to 12% NuPage™ Bis-Tris gels (Invitrogen Life Technologies, Carlsbad, CA) and then transferred to Immobilon-P™ transfer membranes (Millipore, Bedford, MA). For Immunoblot analysis, membranes were blocked in Blotto (5% nonfat dry milk, 10 mM Tris-HCl [pH 8.0], and 150 mM NaCl), followed by overnight incubation at 4 °C with either 0.5 μg/mL of monoclonal SREBP-1 IgG (BD Biosciences, San Diego, CA) or 1 μg/mL of polyclonal SREBP-2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in Blotto containing 0.05% thimerosal. After incubation with primary antibody, the blots were washed in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 8.0] and 150 mM NaCl) containing 0.05% Tween-20 before incubating with horseradish-peroxide-conjugated donkey antimouse IgG (Jackson Immuno-Research Laboratories Inc., West Grove, PA) diluted 1:15,000 in Blotto for 1 to 2 h at room temperature. After washing, antibody/protein complexes on the membranes were detected using SuperSignal™ West Femto Maximum Sensitivity Substrate (Pierce Biotechnology Inc., Rockville, IL), diluted 5-fold. After detection, membranes were stripped in a solution of 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.7), and 2% sodium dodecyl sulfate (SDS) at 50 °C for 20 min before incubation with 14.3.3 ε antibody (a generous gift from D. Klein, National Institute of Child Health and Development [NICHD]/National Institutes of Health [NIH]; Roseboom et al., 1994) for 1

Table 1. Primer Pairs Used for RT-PCR Amplification of the Indicated SREBP Target Genes in Mouse Liver cDNA

SREBP Target	*GenBank Accession Number*	*Forward Primer 5′-3′*	*Position*	*Reverse Primer 5′-3′*	*Position*
ACC1	XM_109883	GCTATGGAAGTCGG CTATGGAAATTG	3785-3810	TCAGGAAGAGGCG GATGGGAATTG	3889-3866
FAS	BC046513	CAAGATGAAGGTGG CAGAGGTGCTG	3780-3804	GTCGGTGGCTGTG TATTCCAGTTG	3888-3865
ACS4	NM_019477	ACTGACTACACTACT GGAAGAGTT	1581-1604	ACCGATCACAATCT CACCTCTGG	1706-1684
G3PAT	NM_008149	GGCGAGAGGCGTTA TCAGAATGC	1302-1324	AACAGTGCTTGCTC CAGAGAAAGG	1445-1422
HMG-CoAR	BC034317	CTATGGTTCCCTGG CTTCTGTCCTG	1071-1095	CTGGTGTGGCGTC TTGTGTGAC	1207-1186
CYP51	BC031813	GGCAAGACCTTCAC TTACCTTCTG	421-444	GCAACTCCCTTCC CAAACACAGGTG	548-524

NOTE: RT-PCR, reverse-transcriptase PCR; SREBP, sterol response element binding protein.

to 2 h at room temperature, followed by washes and incubation with secondary antibody as described above. This antibody/protein complex was detected using SuperSignal™ West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc.). Chemiluminescence on the blots was visualized on CL-Xposure™ film (Pierce Biotechnology Inc.). Bands were quantified by scanning with a Kodak Image Station 440 System (Eastman Kodak Company, Rochester, NY).

Northern blot analysis. Total RNA was extracted from individual quick-frozen liver pieces using TRIzol™ Reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. Aliquots of 8 μg -10 μg of denatured RNA were subjected to electrophoresis on a 1% agarose-formaldehyde gel and transferred to Nytran™ SuPerCharge nylon membranes (Schleicher & Schuell, Keene, NH) and visualized by hybridization with radiolabeled PCR fragments. Probes were labeled by random priming with ^{32}P-dCTP (Amersham Biosciences Corp., Piscataway, NJ) using the MegaPrime™ DNA labeling kit (Amersham Biosciences Corp.). Probes were obtained through a reverse-transcription PCR (RT-PCR) amplification procedure. Briefly, total mouse liver RNA (1 μg) was reverse transcribed using oligo dT primers (Invitrogen Life Technologies), and cDNA products from mouse ACC1 (acetyl-coenzyme A carboxylase 1; XM_109883), FAS (fatty acid synthase; BC046513), ACS4 (acyl-CoA synthetase long-chain family member 4; NM_019477), G3PAT (glycerol-3-phosphate acyltransferase; NM_008149), HMG-CoAR (3-hydroxy-3-methylglutaryl-coenzyme A synthase 1; BC034317), and CYP51 (cytochrome P450; BC031813) were then amplified by PCR using the primer pairs described in Table 1. After hybridization, the blots were visualized using a STORM 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Blots were stripped by incubating them for 2 min in 0.1% SDS at 97 °C (repeated 3-5 times, as necessary) and reprobed with an 18S ribosomal RNA oligo probe for normalization. Densitometric analysis was performed using ImageQuant™ V5.2 software (Molecular Dynamics, Sunnyvale, CA).

Data analysis. When amplitude or phase was required, a fitting technique was applied. Data were fitted by the following function: $c + a\cos[2\pi(t - ø)/24]$, where c is a baseline, a is the amplitude of the cosine wave, t is time in hours, and ø is the phase in hours from ZT 0 in the imposed light cycle. The phase is, in general, only used to calculate a phase difference between the day and night feeding conditions. The fitting was performed in the NonlinearRegress routine of the Statistics package of Mathematica 5 (Wolfram Research, Champaign, IL). The routine also estimates the standard error of the fit parameters. The standard error arises from scatter in the data and from deviations of the data from cosine form. Note that the frequency was not fit but was taken as the 1 cycle per 24 h of the light regime. The standard error of a phase difference was calculated as the square root of the sum of the squared standard errors of the differenced phases.

RESULTS

Daily patterns of SREBP posttranslational activation. To test whether SREBP-1 and/or SREBP-2 in the liver follow a daily pattern of activation, as well as whether these patterns might be gender specific, groups of 4 male and 4 female mice were sacrificed around the

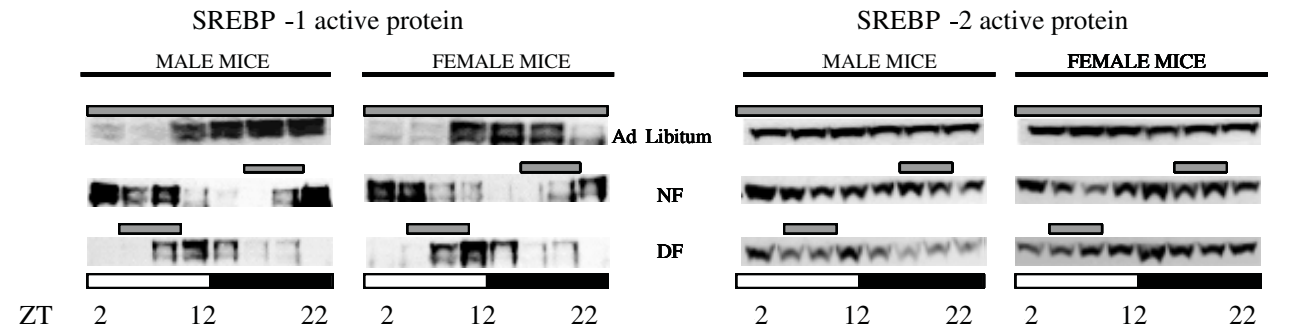

Figure 1. Effect of restricted feeding (RF) on circadian activation of sterol response element binding protein (SREBP) protein. Expression of hepatic SREBP-1 and SREBP-2 active protein (60-70 kDa) in male or female mice fed ad libitum (gray bar in top panels), during the night (NF: ZT 17-21, gray bar in middle panels) or during the day (DF: ZT 5-9, gray bar in bottom panels). Bands from representative Western blots are shown. Experiments were carried out loading 35 μg of total liver protein in each lane from samples taken every 4 h in the case of mice fed ad libitum or every 3 h in mice under RF. White/black bar represents day/night cycles, respectively.

clock. Whole-cell protein extracts were prepared from the liver as described in the Methods section and subjected to SREBP-1 and SREBP-2 immunoblotting (Fig. 1, top panels). The results revealed that the proteolytic activation of SREBP-1 (60- to 70-kDa fragment) follows a robust diurnal rhythm, with activation beginning around dusk and persisting for the remainder of the dark phase. A similar pattern was seen in both genders. In contrast, no significant rhythmicity was observed, in either gender, in the activation of SREBP-2 protein.

Restricted feeding during the day can acutely shift the peak of SREBP-1 activation. Providing nocturnal rodents with access to food only during the day (inactive phase of the cycle for mice) has been found to reverse the phase of many cycling transcripts (Damiola et al., 2000; Stokkan et al., 2001). To test the responsiveness of the SREBP activation pathway to an acute reversal in the feeding schedule, we subjected mice to an RF paradigm (Fig. 1). We found that restricting feeding to a 4-h window during the middle of the night (ZT 17-21) or the middle of the day (ZT 5-9) had 2 noticeable effects on the pattern of SREBP-1 activation. First, the rhythm of activation seen in male mice fed ad libitum had consolidated, peaking at the end of the night/beginning of the day, while SREBP-1 activation occurred at the beginning of the day in female mice (Fig. 1; SREBP-1, middle panels). Second, and more important, the peak of SREBP-1 activation was significantly shifted by about 12 h in male and female mice fed during a diurnal 4-h window, relative to the peak detected in mice fed during a nocturnal 4-h window (Fig. 1; SREBP-1, bottom panels). Restricted feeding did not affect activation of SREBP-2 protein. These observations are consistent with the hypothesis that SREBP-1 is an important contributor to the orchestration of circadian gene expression in the liver. The extent to which this hypothesis may be true was evaluated by assessing the degree of overlap between the set of cycling liver transcripts and the set of SREBP-1 gene targets.

Key metabolic SREBP targets display a circadian expression pattern. The availability of a comprehensive circadian database of liver transcripts (Panda et al., 2002), together with the recently published microarray analysis of putative SREBP targeted genes in vivo (Horton et al., 2003), allows for a direct assessment of the potential for SREBP-1 involvement in the organization of the circadian liver transcriptome. To evaluate that potential, we compared these 2 databases and noted the normal expression profile of liver genes induced after SREBP-1 overexpresssion. The results of this meta-analysis yielded 25 putative SREBP-1 targets that were present in the liver database (Table 2).

Further analysis indicated that 6 genes were arrhythmic, while the remaining genes displayed 1 of 2 distinct patterns of expression. These 2 groups consist of strongly circadian transcripts with peaks of expression between dusk and the end of the night or transcripts with a narrow set of morning acrophases. Interestingly, genes in the 1st group function mostly in fatty acid metabolism, whereas the majority of the genes with an early morning peak are involved in cholesterol synthesis and uptake. The group of arrhythmic genes contains mostly those belonging to various other functional categories (Table 2).

Table 2. List of SREBP-1 and/or SREBP-2 Targets Present in the Mouse Liver Transcriptome

Gene ID/GenBank Accession Number	SREBP 1	SREBP 2	Acrophase (Circadian Time)	Functional Classification
1. 3-hydroxy-3-methylglutaryl-coenzyme A reductase/M62766	+++	+++	4-8	Cholesterol metabolism
2. 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1/AW124932	++	+++	4-8	Cholesterol metabolism
3. Isopentenyl-diphosphate D-isomerase like/AA716963	++	+++	4-8	Cholesterol metabolism
4. Lanosterol 14 α-demethylase (CYP51)/AW122260	++	+++	4-8	Cholesterol metabolism
5. Squalene synthase/D29016	++	+++	4-8	Cholesterol metabolism
6. Sterol-C5-desaturase/AB016248	+	+	4-8	Cholesterol metabolism
7. Short-chain aldehyde reductase/AB030505	++	++	4-8	Miscellaneous
8. Cysteine sulfinic acid decarboxylase/AW120896	+	–	4-8	Miscellaneous
9. Glutathione S-transferase μ 6/AI326397	+	–	4-8	Miscellaneous
10. SREBP-1/AI843895	+	+	4-8	Fatty acid metabolism
11. Fatty acid coenzyme A ligase, 5/AI838021	+	–	4-8	Fatty acid metabolism
12. ELOVL6/ AI839004	+++	+	14	Fatty acid metabolism
13. Malic enzyme/J02652	+++	+	14	Fatty acid metabolism
14. Mac 30/AI852985	+	+	14	Miscellaneous
15. L-specific multifunctional β-oxidation protein/AJ011864	+	–	18-24	Fatty acid metabolism
16. Fatty acid synthase/X13135	+++	++	18-24	Fatty acid metabolism
17. Glycerol-3-phosphate acyltransferase/U11680	+	–	18-24	Fatty acid metabolism
18. Acyl-CoA synthetase long-chain 4/31560423	+	–	18-24	Fatty acid metabolism
19. Cold inducible glycoprotein 30 U97107	–	–	18-24	Fatty acid metabolism
20. Stearoyl-CoA desaturase 1/M21285	++	–	Arrhythmic	Fatty acid metabolism
21. Pyruvate dehydrogenase kinase 1/AA691492	+	–	Arrhythmic	Miscellaneous
22. μ-immunoglobulin/V00817	+	–	Arrhythmic	Miscellaneous
23. Q8/9d peptide/D90146	+	–	Arrhythmic	Miscellaneous
24. Squalene epoxidase/D42048	+	++	Arrhythmic	Cholesterol metabolism
25. Unknown/AI844396	+	+	Arrhythmic	Unclassified

NOTE: Genes are identified by their common name and GenBank accession number, their acrophase (circadian time) if rhythmic, and their functional classification. Approximate fold increase of each gene's expression after SREBP-1 or SREBP-2 overexpression in vivo (Horton et al., 2003) is indicated by plus signs; – (0- to 2-fold), + (2- to 5-fold), ++ (5- to 10-fold), and +++ (> 10-fold). SREBP, sterol response element binding protein.

SREBP-1 is ideally positioned to mediate a shift in the expression of lipogenic and cholesterogenic transcripts in the RF paradigm. The above observations led us to examine the temporal expression of known SREBP target genes during the RF paradigm. Six genes codifying key rate-limiting enzymes in fatty acid (ACC1, FAS), triglyceride (ACS4, G3PAT), and cholesterol (HMG-CoAR and CYP51) metabolism were selected from the intersection between the SREBP "targetome" (Horton et al., 2003) and the liver transcriptome (Panda et al., 2002). The expression patterns of these 6 SREBP target genes were analyzed in the livers of male and female mice throughout a 24-h period by Northern blot analysis (Figs. 2A and 3A). Under NF conditions, 5 of the 6 genes (the exception being CYP51) displayed similar patterns of expression to those reported in the circadian database for male mice fed ad libitum (Panda et al., 2002; http://expression.gnf.org/cgi-bin/circadian/index.cgi).

Our results, analyzed by cosine analysis, showed that mRNA levels of lipogenic FAS and G3PAT genes peaked at the end of the night/beginning of the day in the livers of both male and female mice fed during the night (Fig. 2). The temporal profile of these 2 genes was rapidly shifted, by about 12 h, in diurnally fed male and female mice when compared to those that received food during the night period, with both genes being very sensitive to acute reversal in the schedule of food consumption. ACC1 displayed a similar response in male mice; however, a much weaker shift was seen in female mice after the DF. Similarly, the shift seen in ACS4 was about 15 h in males and 7 h in females. Despite obvious gender differences, these results are consistent with a causal relationship between the nutritional regulation of the SREBP-1 pathway and the rapid resetting of the circadian phase of expression of its target genes in the liver.

The circadian expression of the cholesterogenic genes, HMG-CoAR and CYP51 (Fig. 3), displayed a more complex pattern. Maximal expression of HMG-CoAR occurred around the beginning of the day in male mice fed during the night but peaked at the end

of the day/beginning of the night, reaching a significantly higher amplitude in the liver of female mice under the same feeding regime. Feeding the animals for 4 h during the day caused a 14-h (±1.2 h) phase shift in the temporal profile of HMG-CoAR only in male mice. No significant phase shift in HMG-CoAR expression was detected in female mice under the same RF protocol. The expression pattern of HMG-CoAR revealed additional evidence not only of sexual dimorphism under NF conditions but also of a lower sensitivity to the phase-shifting effects of the restricted feeding.

While no significant rhythmicity was detected in the expression of CYP51 under NF conditions, the cosine analysis revealed a daily oscillation in the diurnally fed males and females.

DISCUSSION

The transcription factors known as SREBPs have been the focus of intense study aimed at understanding the links between nutrition and the regulation of genes controlling liver physiology. Early studies in cell culture have shown that the SREBPs regulate lipid biosynthesis. For example, SREBP-1 can induce transcription of genes in fatty acid synthesis pathways (Bennett et al., 1995; Lopez et al., 1996), while overexpression of SREBP-2 leads to the accumulation of triglycerides and cholesterol (Jackson et al., 1996). It is now widely accepted that SREBPs control the transcription of a number of genes involved in the cellular synthesis and uptake of cholesterol and unsaturated fatty acids in the liver.

Since a significant fraction of the metabolic transcriptome in the liver is strongly circadian (Panda et al., 2002), it seems peculiar that more focused attention has not been paid to the evaluation of SREBP factors, well established as global lipid synthesis regulators (Shimano, 2001), and their potential involvement in the circadian organization of gene expression in the liver. This could be particularly relevant for SREBP-1, a factor that undergoes a very robust mode of circadian activation that can be phase-shifted dramatically and acutely by a short pulse of RF (Fig. 1).

We predicted that overlapping the SREBP targetome with the circadian transcriptome in the liver might reveal important information regarding the global control of transcription in the liver and provide valuable leads for dissecting the temporal uncoupling between central (SCN) and peripheral (e.g., liver) circadian oscillators that can be observed when animals are subjected to RF during the inactive phase of the photoperiod (Damiola et al., 2000; Stokkan et al., 2001).

Such analysis showed that most (64%) putative SREBP-1 targets present in the liver database are indeed circadian. The distribution of temporal patterns—that is, diurnal, nocturnal, and arrhythmic (Table 1 and Fig. 2)—suggests that SREBP is likely to exert its effects through interactions with at least 3 different transcriptional networks, likely the result of different promoter architectures and their function in the context of different transcriptional modules.

The protein PPARα is a good example of a transcription factor likely to be involved in the differential configuration of such modules. It has been shown that the normally oscillatory transcription of the FAS and HMG-CoA genes becomes completely arrhythmic in PPARα-deficient mice (Patel et al., 2001), a result that provides a tantalizing clue to the complexity underlying the organization of transcriptional networks in the liver.

The results of our analysis of 6 selected key rate-limiting enzyme genes with specific roles controlling fatty acid (ACC1 and FAS), triglyceride (ACS4 and G3PAT), and cholesterol (HMG-CoAR and CYP51) metabolism are consistent with studies in hamsters (Rowland, 1984) showing that the diurnal phase is characterized by inactivity and fuel storage (maximal enzymatic activity in fatty acids and triglycerides synthesis), whereas the night phase is one of exercise and fuel mobilization (fatty acids oxidation and sterols synthesis).

When mRNA levels of the same metabolic genes in male and female mice fed under the RF paradigm were analyzed, a daily rhythmicity, confirmed by cosine analysis, was observed in the expression level of lipogenic ACC1, FAS, ACS4, and G3PAT genes. FAS and G3PAT showed the same pattern in both males and females, peaking at the end of the night/beginning of the day and contributing in this way to the anabolic activity during the inactivity phase. RNA levels of ACC1 and ACS4 showed a differential expression throughout the day, peaking at the beginning of the day in the male liver while reaching their maximum at the end of the day in the female liver. This temporal profile was rapidly reversed in the diurnally fed animals, indicating the presence of a very sensitive mechanism capable of integrating nutritional and circadian

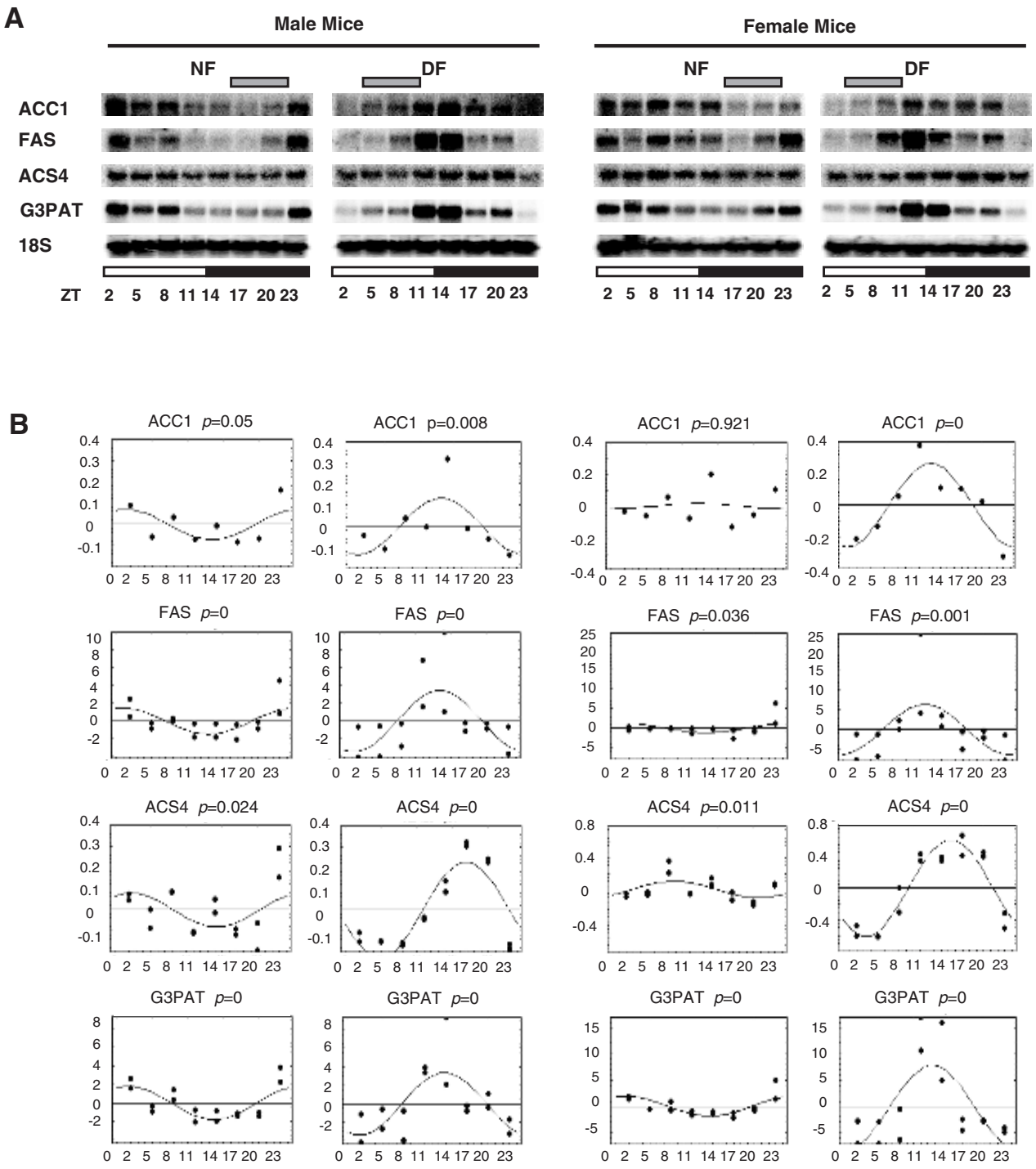

Figure 2. Rhythmic expression of sterol response element binding protein (SREBP) target genes. (A) Rhythmic expression of lipogenic rate-limiting enzymes. mRNA levels of ACC1, FAS, ACS4, and G3PAT genes were determined by Northern blot analysis. Autoradiographs from representative Northern blots are shown. Samples were taken every 3 h from male and female mice exposed to restricted feeding during the night (NF, gray bar) or restricted feeding during the day (DF, gray bar). 18S ribosomal RNA, whose expression is constant throughout the day, was included as a control. White/black bars at the bottom represent day/night cycles, respectively. (B) Average cosinor fit for mRNA rhythmic expression of ACC1, FAS, ACS4, and G3PAT genes. Curves represent normalized mRNA levels versus zeitgeber times.
***$p \leq 0.05$ indicates detection of a rhythm.**
****$p = 0$ indicates $p < 0.0005$.**

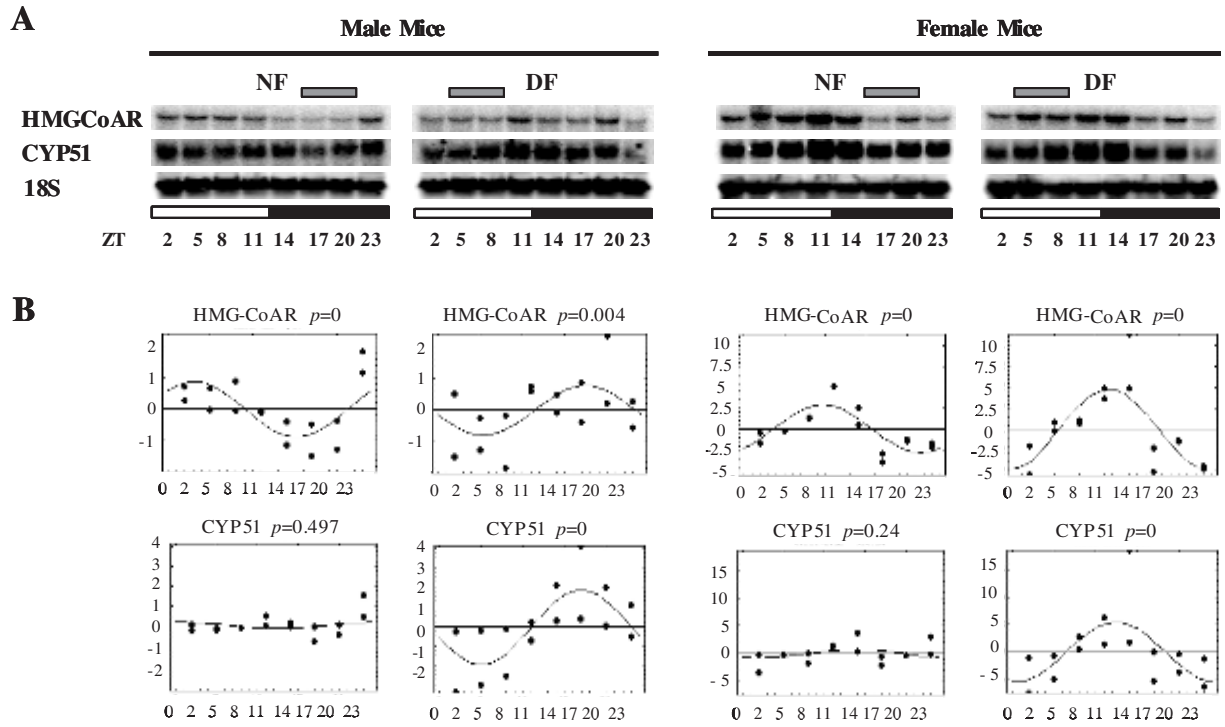

Figure 3. Rhythmic expression pattern of cholesterogenic sterol response element binding protein (SREBP) target genes. (A) Rhythmic expression of cholesterogenic rate-limiting enzymes. mRNA levels of HMG-CoA reductase and CYP51 genes were determined by Northern blot analysis. Autoradiographs from representative Northern blots are shown. Samples were taken every 3 h from male or female mice exposed to restricted feeding during the night (NF, gray bar) or restricted feeding during the day (DF, gray bar). 18S ribosomal RNA, whose expression is constant throughout the day, was included as a control. White/black bars at the bottom represent day/night cycles, respectively. (B) Average cosinor fit for mRNA rhythmic expression of HMG-CoA reductase and CYP51 genes. Curves represent normalized mRNA levels versus zeitgeber times.
***$p \leq 0.05$ indicates detection of a rhythm.**
****$p = 0$ indicates $p < 0.0005$.**

clues in the liver and making rapid decisions regarding the needed expression levels of select genes. Interestingly, FAS, which provides the cells with fatty acid precursors for more complex lipids, and G3PAT, which functions as a source of triglycerides for energy storage, were the most sensitive to the acute reversal in the schedule of food consumption in both male and female mice. This mechanism could perhaps contribute to increase energy storage, preparing the body for or anticipating a period of food deprivation.

The significant increase in the level of expression of many lipogenic genes under diurnal feeding conditions (Fig. 2) is consistent with the reported rapid switch of hepatic fatty acid metabolism from oxidation to synthesis and a concomitant hyperactivation of lipogenesis upon refeeding of diurnally fed rats (Moir and Zammit, 1993).

The different magnitude of phase shifts observed after RF suggests that lipogenic genes are more sensitive to the nutritional zeitgeber than cholesterogenic ones (Figs. 2 and 3). On the other hand, circadian expression of cholesterogenic rate-limiting HMG-CoAR and CYP51 genes displayed a more complex pattern, characterized by marked sexual dimorphism and less sensitivity to the RF protocol.

It has been shown that SREBPs can regulate cholesterol and fatty acid metabolism on the basis of differential DNA binding affinities toward classic SRE, SRE-like, and E-box sequences, with SREBP-1c showing the highest affinity for SRE-like and E-box sequences in the lipogenic gene promoters and SREBP-2 binding to the classic SRE site in cholesterogenic promoters (Shimano et al., 1996; Jackson et al., 1996). In addition, recruitment of co-regulators and/or arrangement of SREBP binding sites to the promoters of metabolic target genes is critical for their differential regulation (Magaña et al., 2000; Magaña and Osborne, 1996; Dooley et al., 1998; Ericsson et al., 1996). Based on our results, it seems unlikely that SREBP-2 plays a defining role in establishing the circadian pattern of regu-

lating cholesterogenic genes; rather, other gender-related factors, such as GH or sex steroids, are better poised to control the sexually dimorphic expression pattern of cholesterogenic genes and their response to the RF challenge seen in male and female mice (Waxman et al., 1991; Schwartz et al., 2002; Mode et al., 1982; Gustafsson et al., 1977).

Rhythm Integration in the Liver

Clock gene activation is required for the circadian expression of rhythmic patterns in the central SCN. In peripheral clocks such as the liver, a coordinated action to integrate circadian clock and physiological activities is necessary. It has been shown that an RF schedule phase-shifts liver rhythms of clock components, including Per1 in the rat and Per1 and Per2 in the mouse (Damiola et al., 2000; Stokkan et al., 2001; Hara et al., 2001). Interestingly, preliminary results from our laboratory indicate that the strongly circadian mPer1 promoter, which contains 3 SRE-like and 3 E-box sites, could be transactivated when co-transfected with SREBP-1a or SREBP-1c expression vectors in hepatic AML12 cells (data not shown).

Taken together, our results prompt us to hypothesize a model of transcriptional integration in the liver whereby acute reversal of SREBP-1 activation might play a central role in controlling the circadian variations in the metabolism of its target genes. We cannot formally rule out at this point the possibility that the reversal of phase we observed is oscillator mediated; additional experiments will need to be performed in our system to assess dynamic changes in clock gene transcription throughout the RF paradigm. However, the well-established pace of true resetting in circadian oscillators typically occurs over a period of days and through a series of "transients," a phenomenon that also applies to the resetting in the rhythms of individual clock components (Damiola et al., 2000). It has also been suggested that, in contrast to the central oscillator, circadian oscillators in peripheral tissues may remain responsive to phase resetting throughout the day (Balsalobre et al., 2000), revealing the existence of powerful shifting mechanisms. The present observations are likely to reveal an important component of such powerful mechanisms, capable of driving a transient (acute) uncoupling of selected transcriptional rhythms in the liver from the effects of central oscillator input. Accordingly, we propose that the processes leading up to SREBP activation could play an important role in the SCN-independent resetting of the peripheral clock in the liver (Hirota and Fukada, 2004; Schibler et al., 2003).

Further research into SREBP biology could be a rich source of information on the pathways that integrate metabolism and circadian rhythmicity in the liver. The well-studied SREBP activation pathway provides a unique opportunity to test and identify the critical humoral factors that are generated upon feeding and their role in providing an integrated signal to the robustly circadian systems in the liver.

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