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The Transcriptional Repressor STRA13 Regulates a Subset of Peripheral Circadian Outputs*

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Central and peripheral mammalian circadian clocks regulate a variety of behavioral and physiological processes through the rhythmic transcription of hundreds of clock-controlled genes. The circadian expression of many transcriptional regulators suggests that a major part of this circadian gene network is indirectly regulated by clock genes. Here we show that the basic helix-loop-helix transcriptional repressor *Stra13* is rhythmically expressed in mouse peripheral organs. The circadian transcription of *Stra13* is mediated by a response element recognized by the CLOCK-BMAL1 heterodimer and located in the proximal promoter region. CLOCK-BMAL1-dependent activation of *Stra13* is strongly repressed by CRY1 and also by STRA13 itself. To determine putative *Stra13* output genes, we performed microarray analyses of differential gene expression in the liver between wild type and *Stra13*$^{-/-}$ mice and identified 42 target genes including a subset of 20 previously known as clock-controlled genes. Importantly, we demonstrate that circadian gene expression of the serum protein insulin-like growth factor-binding protein 1 and of the NKG2D receptor ligand retinoic acid early transcript was suppressed in *Stra13*$^{-/-}$ mice. These biochemical and genetic data establish a role for the basic helix-loop-helix repressor STRA13 as a circadian output regulator in the periphery.

Circadian rhythms in physiology and behavior are observed in most organisms. They are generated by a self-sustained endogenous circadian clock that is reset by external time cues such as light and temperature (1). This mechanism is believed to provide organisms with an anticipatory adaptive mechanism to the daily predictable changes in their environment. Biochemical and genetic studies in various model systems have identified a molecular oscillator generated by transcriptional/translational feedback loops. In mammals, the main loop involves the E box-mediated transcriptional activation of the *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2* clock genes by the CLOCK-BMAL1 heterodimer. Then PER and CRY proteins form complexes that enter into the nucleus in a phosphorylation-dependent manner to repress the CLOCK-BMAL1-dependent transcription of their own genes, thereby generating a ~24-h period molecular oscillator (2). This loop controls also the rhythmic expression of the repressor REV-ERBα, which is required for rhythmic *Bmal1* transcription, comprising a second loop thought to be important for the overall robustness of the oscillator (3). In mammals, the master oscillator is present in the suprachiasmatic nuclei (SCN)[1] of the hypothalamus, which orchestrates autonomous oscillators in peripheral organs. Surprisingly, this oscillator can be observed in synchronized cultured cells *ex vivo*. The SCN oscillator is directly reset by light perceived and transmitted via the retinohypothalamic tract and is believed to entrain peripheral oscillators via ill-defined neurohormonal pathways. Peripheral oscillators also appear to be reset by hormonal signals and the feeding schedule (4, 5). The mechanisms by which central and peripheral oscillators regulate physiological and behavioral output pathways has remained poorly understood. To address this issue, genome-wide analyses of circadian clock-controlled gene (CCG) expression in the SCN, peripheral organs, and cultured cells have recently been performed by several groups (6). Several hundred rhythmic transcripts were identified regulating a variety of key biological processes in a coordinated and tissue-specific manner (7–12). Interestingly, a significant proportion of transcriptional regulators have been found among these CCGs, suggesting that many physiological outputs may be indirectly or not exclusively controlled by the circadian oscillators in mammals as previously proposed in *Drosophila* (13). Accordingly, response elements for several rhythmically expressed transcription factors have been identified in the promoter region of CCGs (12). One of these clock-controlled transcription factors is *Stra13* (also known as *Dec1*, *Sharp2*, or *Bhlhb2*), a member of the basic helix-loop-helix (bHLH) family of transcription factors, which are important regulators of cell growth, differentiation, and apoptosis. *Stra13* is a transcriptional repressor that is up-

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[1] The abbreviations used are: SCN, suprachiasmatic nuclei; bHLH, basic helix-loop-helix; CCG, clock-controlled gene; CT, circadian time; DD, dark/dark; LD, light/dark; SAM, significance analysis of microarrays; ZT, Zeitgeber time; EST, expressed sequence tag; IGF, insulin-like growth factor; RPA, RNase protection assay.

regulated by multiple cell growth arrest triggers and plays a critical role in the immune system (14–16). Here we provide biochemical and genetic evidence supporting a role for *Stra13* as a clock-controlled transcription factor that regulates a subset of circadian physiological outputs in peripheral organs.

MATERIALS AND METHODS

Animals—Eight-week-old C57BL/6J mice were purchased at IFFA-Credo (Lyon, France), housed in a 12 h of light and 12 h of dark cycle (LD12:12) with the lights on (defined as Zeitgeber time (ZT) 0) at 7:00 a.m. in a temperature- and humidity-controlled environment, and fed *ad libitum*. After 2 weeks in LD 12:12, the mice were transferred to constant darkness (DD) and sacrificed during the DD first cycle under red dim light at various circadian times (CT). The tissues were collected, rapidly frozen in liquid nitrogen, and stored at −80 °C. *Stra13*$^{-/-}$ mice in the C57BL/6J-Sv129 background have been described and were kept under the same conditions as above (16). Littermates were used as controls. The animal procedures were in accordance with Mount Sinai Institutional animal guidelines.

Plasmid Constructs—cDNA fragments for mouse *Stra13* (NM_011498), *Per2* (NM_011066), *Per3* (NM_011067), *Bmal1* (NM_007489), *Clock* (NM_007715) *Rev-erbα* (NM_145434), *Dbp* (NM_016974), *Sharp-1* (NM_024469), *Alas1* (NM_020559), and *Igfbp1* (NM_008341) were reverse transcription-PCR-amplified from total liver RNA using the following primers: 5′-TAAGCAAGAATCCGAAGAGCCC-3′ and 5′-GGTGGGATGAGATAGAAGGGAAGG-3′ (*Stra13*); 5′-AACACCATAGTTTTCTGGCG-3′ and 5′-ACTGGAGAACTCAGGGCAGC-3′ (*Per2*); 5′-AAGTTGCCAGCACCTGTGGA-3′ and 5′-ATACTGCTGGCACTGCTTCC-3′ (*Per3*); 5′-CATTGATGCCAAGACTGGACTTCC-3′ and 5′-GCCTTCCAGGACATTGGCTAAAAC-3′ (*Bmal1*); 5′-CCAGCTCCTTAATGAGGACA-3′ and 5′-CCTTGTCATCTTCTTCCACC-3′ (*Clock*); 5′-ATGCTTGCCGAGATGCA-3′ and 5′-CATAGAGAAGTCTTCCCA-3′ (*Rev-erbα*); 5′-TCCAGGCCATGAGACTTTTGACCC-3′ and 5′-AT-GACGTTCTTCGGGCACCTAGCT-3′ (*Dbp*); 5′-TATGTGTAAACCCAAAAGGAGC-3′ and 5′-ATTATCTTCTGATGCTGCTGCT-3′ (*Sharp-1*); 5′-GAGGTCCATGCAGTGGGGC-TCTAT-3′ and 5′-ACGGTGTCGATCAGCAAACTCGTG-3′ (*Alas1*); and 5′-CGTCTTCTCATCTCTCTCGTA-3′ and 5′-CTGTGTGAGACGATGAGGAAT-3′ (*Igfbp1*). The fragments were cloned into pKSII^{+} Bluescript and sequenced. The *36B4* probe has been described elsewhere (8). The *Stra13* promoter deletion constructs Stra13Δ595::Luc, Stra13 Δ540::Luc, and Stra13 Δ312::Luc were generated by digesting the pGL3KN luciferase reporter plasmid (15) with KpnI and either ApaI or PmlI or BpU10I (partial digest), respectively, followed by fill-in with Klenow and religation. The Stra13(E3E4)3x::Luc, Stra13(E3E4)m3x::Luc, Per1(E)3x::Luc, and Per1(E)m3X::Luc reporters were made by annealing phosphorylated oligonucleotides containing wild type or mutated E box elements from the *Stra13* (E3 and E4) or *Per1* (proximal E box) promoter and ligating three copies of the resulting double strand fragments into a TATA box containing pGL2 reporter plasmid. The following oligonucleotides were used: 5′-GATCTGAGCGTTGTCCAA**CACGTG**AGGCT**CATGTG**ATG-3′ and 5′-GATCCATCACATGAGCCTCACGTGTTGGACAACGCTCA-3′ (Stra13(E3E4)3x::Luc); 5′-GATCTGAGCGTTGTCCAA**CATTGC**-AGGCT**CATTGC**ATG-3′ and 5′-GATCCATGCAATGAGCCTGCAATGTTGGACAACGCTCA-3′ (Stra13(E3E4)m3x::Luc); 5′-GATCCAGCACCCAAGTC**CACGTG**CAGGGATGTGTGA-3′ and 5′-GATCTCACACATCCCTGCACGTGGACTTGGGTGCTG-3′ (Per1(E)3x::Luc); 5′-GATCCAGCACCCAAGTC**CAATTG**CAGGGATGTGTGA-3′ and 5′-GATCTCACACATCCCTGCAATTGGACTTGGGTGCTG-3′ (Per1(E)m3X::Luc) (E box elements are in bold, and mutated nucleotides are underlined). To construct expression vectors for CLOCK and BMAL1, the respective open reading frames were PCR amplified from pBKS-Clock (17) and pCR2-Bmal1 (18) using the following primers: 5′-CAAGACGGATCCATAATCCACCATGGTGTTTAC-3′ and 5′-GAGAGGAAGCTCGAGTGCTACTGTGGCTGGACC-3′ (*Clock*); and 5′-GATGCCGGATCCGAGCCACCATGATTAATATAG-3′ and 5′-CAAAGCAACCTCGAGTGTTTACAGCGGCCATGG-3′ (*Bmal1*), digested by BamH1 and XhoI and ligated into pcDNA3.1 to generate pcDNA-Clock and pcDNA-Bmal1. All of the constructs were verified by sequencing. The pSG5-Stra13 and pcDNA-Cry1 expression vectors have been described (14, 19).

RNase Protection Assay—The plasmids were linearized with the appropriate restriction enzyme, and antisense *Stra13* (+1050 to +1205) *Per2* (+4544 to +4852), *Per3* (+2437 to +2683), *Bmal1* (+657 to +829), *Clock* (+1761 to +1999), *Rev-erbα* (+1207 to +1400), *Dbp* (+1041 to +1457), *Sharp-1* (+157 to +405), *Alas1* (+1330 to +1487), *Igfbp1* (+1126 to +1287), and *36B4* (+24 to +143) probes were synthesized with T7 or T3 RNA polymerase using [α-^{32}P]UTP (3000 Ci/mmol). RNase protection assays (RPA) were performed with 30 μg of total liver RNA using the RPA III kit (Ambion) according to the manufacturer's recommendations. Hybridization was carried out at 55 °C overnight. The signals were quantified with a Storm 840 PhosphorImager and ImageQuant software (Molecular Dynamics).

Real Time PCR—Quantitative PCR was performed with a Lightcycler (Roche Applied Science) using SYBR green I dye detection according to the manufacturer's recommendations. The following primers were used: 5′-GCTGATGGGCAAGAACACCA-3′ and 5′-CCCAAAGCCTGGAAGAAGGA-3′ (*36B4*); 5′-ATGGCATAGCCTTCAGCAGC-3′ and 5′-GTGGGGTCAATGAAAGCACC-3′ (*Cyp2a4*); and 5′-AAGGCAGCAGTGACCAAGCG-3′ and 5′-TGGGGTAGGAGCCTTGATGG-3′ (*Raet1c*). Liver cDNA from wild type and *Stra13*$^{-/-}$ mice or calibrator (dilution 1:100) was added to a reaction mixture (Faststart DNA SYBR Green I; Roche Applied Science) with appropriate primers at 0.5 μM each and amplified using the following PCR conditions: 10 min at 95 °C; 30 s at 95 °C, 10 s at 58 °C, and 10 s 72 °C for 45 cycles; and then melting curve analysis. The expression levels were normalized to the levels of *36B4*. Relative mRNA abundance was calculated using a standard curve method.

Western Blotting—Western blot analysis was performed according to standard procedures using 50 μg of liver nuclear extract from mice kept in DD conditions. STRA13 was detected using a specific affinity-purified rabbit polyclonal antibody (Sigma GenoSys) that was raised against a C-terminal peptide[2] and used at a dilution of 1:100 followed by chemiluminescence detection according to the manufacturer's recommendations (Amersham Biosciences). Equal loading was checked by reprobing the membrane with an EF-1α polyclonal antibody (Upstate Biotechnology Inc.). The signals were quantified with ImageQuant software (Molecular Dynamics).

Cell Culture and Transient Transfections—NIH 3T3 cells and COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) at 37 °C in 5% CO_2. NIH 3T3 cells were serum-shocked as described (20). COS-7 cells were seeded at a density of 10^5 cells/well in 12-well multidishes. On the following day, the cells were cotransfected with reporter vector (40 ng) and expression vector (200 ng) for 6 h using LipofectAMINE (Invitrogen). The cells were then incubated for 48 h in fresh medium and lysed in 50 mM Tris, pH 7.8, 1 mM dithiothreitol, 0.1% Triton X-100 lysis buffer, and luciferase assays were performed using a Trilux luminometer (Wallac). The activities were normalized to the total protein concentrations determined with the Bradford method.

DNA Microarrays Experiments—For each genotype, the livers from two (CT4) or three (CT12) animals were dissected, and total RNA was prepared. cDNA synthesis, biotin labeling of cRNA, and hybridization to murine U74Av2 Genechips (one chip/sample) were performed according the recommendations of the manufacturer (Affymetrix). The .cel files were condensed using the robust multiarray average algorithm (21). To identify differentially expressed genes, we used the significance analysis of microarrays (SAM) method with a maximal false discovery rate of 20% and a fold change of at least 1.5 for genes called significant by SAM (22). Fold change was calculated as the mean from two (CT4) or three (CT12) knockout animals/mean from two (CT4) or three (CT12) wild type animals. Functional annotation was done using NetAffx (www.affymetrix.com/analysis/index.affx; released August 2003). The resulting data sets were compared with publicly available liver data sets of rhythmic transcripts (7, 9–12).

RESULTS

Circadian Expression of Stra13 in Peripheral Tissues—The *Stra13* gene is expressed in many embryonic and adult tissues with high levels in the liver, kidney, and lung (14). Here we show using a quantitative RNase protection assay that *Stra13* mRNA exhibits a robust rhythmic expression pattern in the liver of mice kept in a LD12:12 cycle, with a ~5-fold amplitude and trough and peak levels at ZT4 and ZT8-ZT12, respectively (Fig. 1*A*). Interestingly, this rhythmic expression pattern was also observed in zebrafish liver, suggesting that *Stra13* oscillating expression plays a physiologically important role in the vertebrate circadian system (data not shown). Under constant darkness conditions a similar circadian expression pattern was observed in liver as well as in several other peripheral tissues

[2] S. Azmi and R. Taneja, unpublished data.

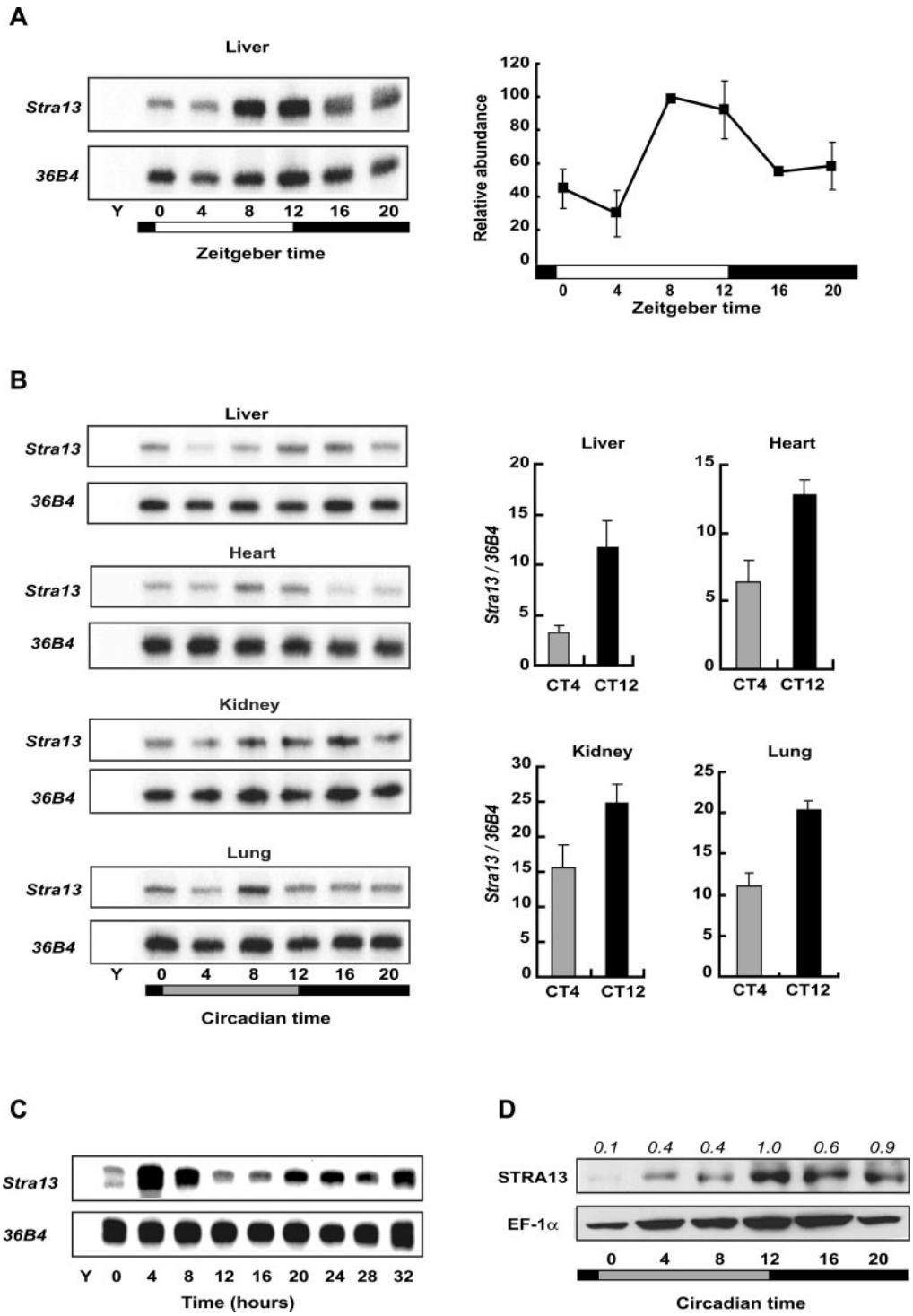

FIG. 1. **Circadian regulation of *Stra13* expression in peripheral organs and cultured cells.** *A*, RPA analysis of *Stra13* mRNA expression in liver from mice entrained to a LD12:12 cycle. The *left panel* shows a representative experiment. The *right panel* shows the quantification of two experiments with data expressed relative to the peak value, which was given the arbitrary value of 100 and presented as the mean ± S.D. *Black* and *white bars* represent night and day, respectively. *B*, RPA analysis of *Stra13* mRNA expression in peripheral organs from mice kept in constant darkness. *Left panels* show a representative experiment for each organ. *Right panels* show means ± S.D. of normalized peak and trough values from four individual mice. *C*, RPA analysis of *Stra13* mRNA expression in serum-shocked NIH 3T3 fibroblasts. For all RPA experiments shown in *A–C*, yeast (*Y*) RNA was included as a negative control, and the constitutively expressed *36B4* mRNA was used for normalization. *D*, Western blot analysis of the STRA13 protein in liver nuclear extracts from mice kept in constant darkness. EF-1α was used as a control for loading. A representative from two independent sets of samples is shown; the values at the *top* of the panel indicate the STRA13/EF-1α signal ratio. The *black* and *gray bars* in *B* and *D* represent the subjective night and day, respectively.

including the heart, kidney, and lung, indicating that *Stra13* cyclic expression is driven by endogenous circadian oscillators in these organs (Fig. 1*B*). A 2-h serum shock of cultured cells followed by serum starvation has previously been shown to trigger circadian gene expression *in vitro* (20). This treatment was also able to induce *Stra13* oscillating expression in mouse NIH 3T3 fibroblasts (Fig. 1*C*). Western blot analysis of STRA13 protein expression in mouse liver nuclear extracts showed a circadian pattern consistent with the mRNA expression profile with a peak at CT12 (Fig. 1*D*). Together these expression data

indicate that *Stra13* is under a circadian regulation in mouse peripheral tissues, in accordance with two recent microarray analyses of the liver gene expression (10, 12). Notably the peak of *Stra13* mRNA expression corresponds to the known maximal activity of the CLOCK-BMAL1 heterodimer in liver, and *Stra13* mRNA levels are depressed in the livers of *Clock*/*Clock* mice (10).

The CLOCK-BMAL1 Heterodimer Activates the Stra13 Promoter—The components of the positive limb of the main feedback loop of the circadian oscillator, CLOCK and BMAL1, have been shown to directly regulate the expression of several clock genes and CCGs through E box elements generally localized in the proximal promoter region or the first intron (23–25). Four such E box sequences were identified within the proximal 5′-flanking region of the mouse *Stra13* gene, two of them (E3 and E4) being also conserved in the puffer fish (fugu) and human promoters (Fig. 2*A*). When a luciferase reporter construct containing the mouse *Stra13* proximal promoter region (Stra13Δ595::Luc construct) and expression vectors for CLOCK and BMAL1 were cotransfected in COS-7 cells, a significant (~3-fold) induction over basal activity level was observed (Fig. 2*B*). Deletion constructs lacking either part of the E box element E4 (Stra13Δ540::Luc construct) or both E box elements E3 and E4 (Stra13Δ312::Luc construct) were unresponsive to CLOCK-BMAL1 (Fig. 2*B*), demonstrating that element E4 is critical for CLOCK-BMAL1 responsiveness. Interestingly the region including elements E3 and E4 had a configuration very close to that recently defined as being optimal for mediating rhythmic transcription, a perfect class B E box followed by a divergent E box (Fig. 2*A*) (26). To test whether this composite element was sufficient to confer responsiveness to the CLOCK-BMAL1 heterodimer, we cloned this sequence in triplicate in front of minimal promoter driving the luciferase reporter gene. This construct was strongly stimulated by the CLOCK-BMAL1 heterodimer, whereas a mutated version was inactive (Fig. 2*C*). As expected this activation could be totally inhibited by coexpressing CRY1, a strong repressor of the negative limb of the circadian oscillator (27). Interestingly, cotransfection of a STRA13 expression vector also resulted in a potent repression of CLOCK-BMAL1-dependent activation as previously described for the *Per1* promoter (28). These functional data provide a direct and simple mechanism for the rhythmic transcriptional regulation of *Stra13* by circadian oscillators and furthermore suggest a role for *Stra13* in negative autoregulation of this mechanism.

Stra13 Is Not Required for Normal Circadian Oscillator Activity—The transcriptional activators CLOCK and BMAL1 contain bHLH DNA-binding domains also present in the STRA13 repressor protein. Consequently, the role of STRA13 in the circadian system could be to negatively regulate the circadian oscillator through DNA binding interference with the CLOCK-BMAL1 heterodimer as previously suggested from *in vitro* studies (28). To genetically test this assumption, we analyzed wild type and STRA13-deficient mice kept in constant darkness for the expression of *Per2*, *Per3*, *Rev-erbα*, and *Dbp* whose transcription is known to be under the control of the CLOCK-BMAL1 heterodimer (3, 25, 29). The liver circadian expression profiles of these four genes were nearly identical in mice from both genotypes (Fig. 3*A*). Consistently, expression patterns of *Bmal1* and *Clock*, which are targets of *Rev-erbα*, were not altered in *Stra13*$^{-/-}$ mutant mice. These data suggest that *Stra13* is not a critical regulator of peripheral circadian oscillators. Alternatively the disruption of *Stra13* in mutant mice may be compensated by its paralog *Sharp-1* (also known as *Dec2*), which exhibits a robust circadian expression pattern in the rat SCN and whose promoter is down-regulated *in vitro* by STRA13 in human cells (28, 30). Analysis of *Sharp-1* mRNA expression in the liver from wild type mice kept in DD showed weaker expression, oscillating with a peak at CT8–CT12 (Fig. 3, *B* and *C*). Expression levels of Sharp-1 were increased by ~2-fold at all time points in *Stra13*$^{-/-}$ mutant animals, consistent with a role for *Stra13* in *Sharp-1* expression (30). We conclude from these observations that *Sharp-1* is a *Stra13* target gene *in vivo* and that *Stra13* is not required for circadian oscillator activity in the presence of *Sharp-1*.

STRA13 Regulates a Subset of CCGs in Liver—To further investigate the physiological role of *Stra13* in the mammalian circadian clock, we sought to identify circadianly expressed STRA13 target genes. To this end, we performed a microarray analysis of liver gene expression at CT4 and CT12 in wild type and *Stra13*$^{-/-}$ mice using DNA GeneChips (Affymetrix) and compared the resulting data sets with previously established lists of rhythmic transcripts in liver. The CT4 time point was selected to minimize the possible redundant activity of *Sharp-1* as *Sharp-1* expression level remained low while the STRA13 protein was present at CT4 (Figs. 1*D* and 3*C*). CT12 was analyzed as the time point of maximum STRA13 protein accumulation (Fig. 1*D*). Using the recently developed robust multiarray average algorithm for normalization followed by a SAM statistical procedure for the detection of differentially expressed genes in mutant *versus* wild type mice, we identified 42 STRA13 target genes showing at least a 1.5-fold change in expression, of which 20 were known as CCGs in liver (Table I) (7, 9–12). Notably a majority (66%) of the identified targets were down-regulated in *Stra13*$^{-/-}$ mice, suggesting that indirect mechanisms are often involved in STRA13-dependent transcriptional regulation. Another general observation is that no clock component was present in both data sets, confirming our RPA analysis data (Fig. 3*A*). Putative STRA13 target genes (26 genes) are involved in several key biological processes including metabolism, detoxification, serum protein production, cell growth, immunity, and proteolysis. With no exception, each of these functional clusters contained one or several CCGs.

De novo cholesterol biosynthesis is a key liver metabolic function, and the rate-limiting step in this pathway is controlled by *Hmgcr* (3-hydroxy-3-methylglutaryl-CoA reductase) that converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate. *Hmgcr* activity and gene expression are clock-regulated (10, 51 1). *Hmgcr* was found to be down-regulated in *Stra13*$^{-/-}$ mice, suggesting a role for *Stra13* in cholesterol metabolism. Regulating the balance between branched chain amino acid catabolism and utilization for protein synthesis as essential amino acids is another important liver metabolic function. We found that the E1β subunit (*Bckdhb*) of the large mitochondrial multimeric enzymatic complex regulating this process is clock-controlled and up-regulated in *Stra13*$^{-/-}$ mice.

Detoxification and steroid catabolism are essential hepatic functions that are under circadian control through the rhythmic transcription of cytochromes P-450 and phase II enzymes (UDP-glucoronosyl transferases and glutathione *S*-transferases) (7, 9–12). STRA13 was found to regulate three circadianly expressed genes coding for such detoxifying enzymes at CT4, *Cyp2c70* (cytochrome 2c70), *Cyp2a4* (steroid 15 α-hydroxylase), and Gstt2 (glutathione *S*-transferase theta 2). Interestingly, *Alas1* (δ-aminolevulinic acid synthase 1), which catalyzes the rate-limiting step in the biosynthesis of heme that is required for cytochrome P-450 function, is also a CCG that was down-regulated at CT4 in *Stra13*$^{-/-}$ mice.

The liver produces many different serum proteins, some of which are under circadian regulation such as *Igfbp1* (insulin-like growth factor-binding protein 1), which plays a role in cell growth and metabolism (32). We observed that *Igfbp1* was

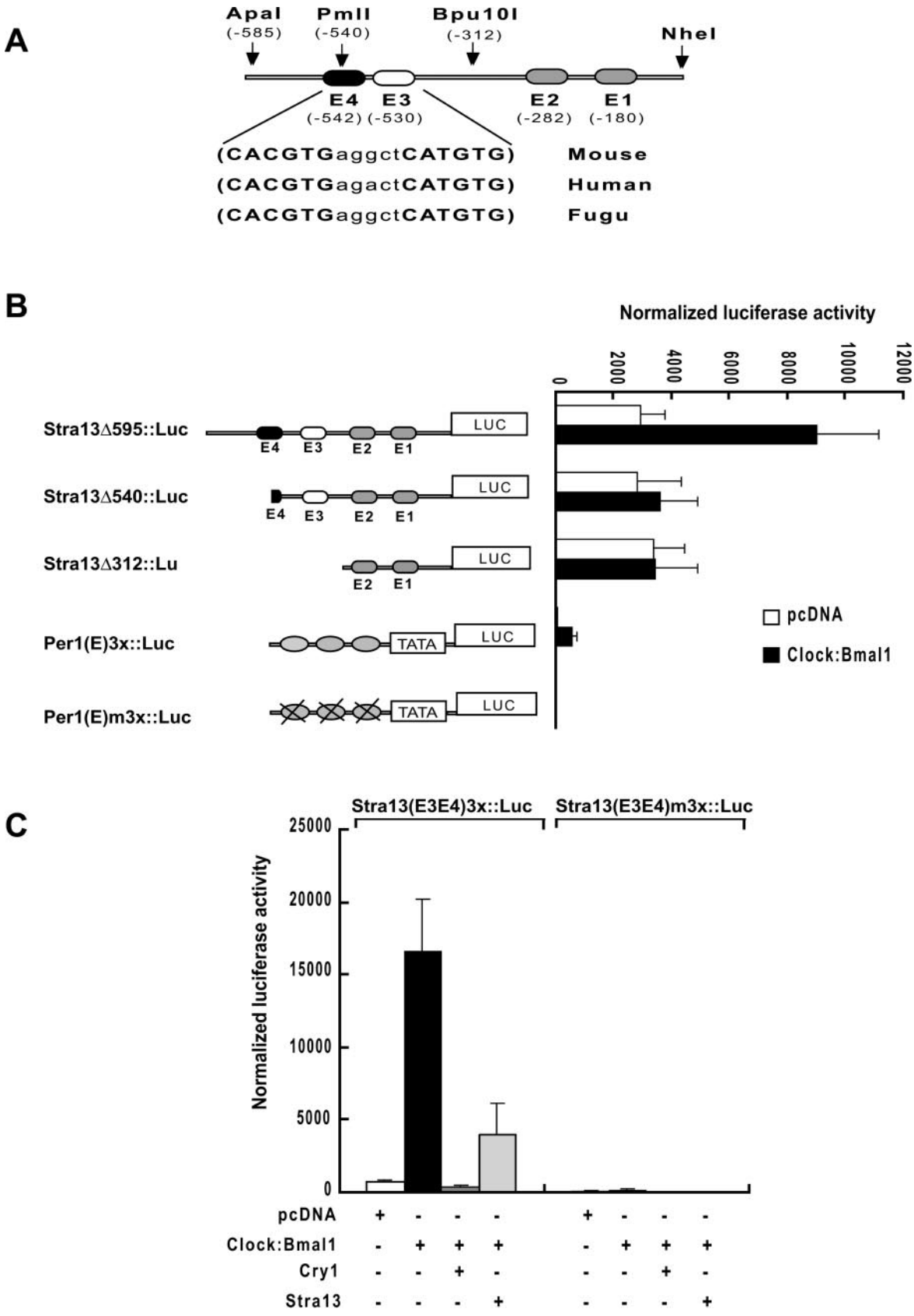

FIG. 2. **The *Stra13* promoter is regulated by circadian oscillator components.** *A*, schematic of the mouse *Stra13* proximal promoter region. *Boxes E1–E4* denote E box response elements. The *black box* denotes a perfect class b E box sequence (CACGTG), and the *white boxes* denote imperfect E box sequences. Sequences of the mouse, human, and puffer fish (fugu) E3 and E4 elements illustrate the evolutionary conservation of these sequences in vertebrates. *B*, transient cotransfection of COS-7 cells with luciferase reporter constructs driven by either the full-length sequence (Stra13Δ595::Luc) or 5′ deletions (Stra13Δ540::Luc and Stra13Δ312::Luc) of the *Stra13* proximal together with the empty pcDNA or Clock and Bmal1 expression vectors. *C*, transient cotransfection of COS-7 cells with luciferase reporter constructs containing a minimal promoter driven by either wild type (Stra13(E3E4)3x::Luc) or mutated (Stra13(E3E4)m3x::Luc) E3 and E4 box elements together with empty pcDNA or the indicated combination of Clock, Bmal1, Cry1, and Stra13 expression vectors. The values are normalized as the means ± S.D. of at least three independent transfections.

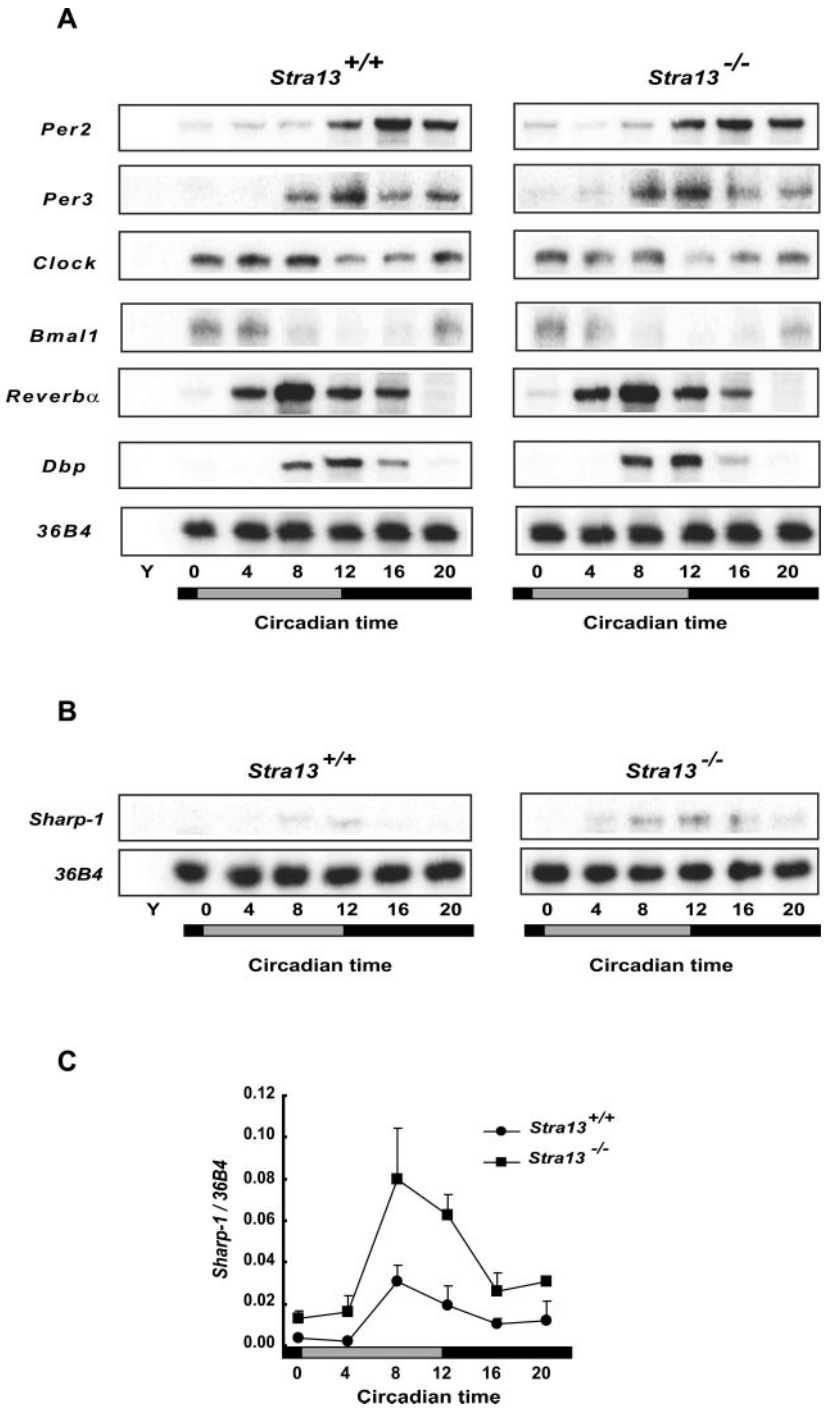

FIG. 3. **Normal circadian oscillator function and up-regulation of *Sharp-1* in *Stra13*$^{-/-}$ mice.** *A*, RPA analysis of *Per2*, *Per3*, *Clock*, *Bmal1*, *Reverbα*, and *Dbp* mRNA expression in the liver of wild type and *Stra13*$^{-/-}$ mice kept in DD conditions. A representative of experiment is shown. *B*, RPA analysis of *Sharp-1* mRNA expression in the liver of wild type and *Stra13*$^{-/-}$ mice kept in DD conditions. *C*, quantification of the *Sharp-1* expression data shown in *B*. The data are normalized as the means ± S.D. from two or three different animals of each genotype. The *black* and *gray bars* represent the subjective night and day, respectively. Yeast (*Y*) RNA was included as a negative control and the constitutively expressed *36B4* mRNA was used for normalization.

significantly down-regulated in *Stra13*$^{-/-}$ mice at CT4, suggesting an indirect role for *Stra13* for its normal expression.

The immune system is known to show circadian variations at different levels, and a number of CCGs involved in the immune response have been recently identified in *Drosophila* and mammals (9–12, 33, 34). Because STRA13 is required for normal immune function as a key regulator of T lymphocyte activation, we investigated the expression of several genes involved in immune function (16). Our data show that the CCG retinoic acid early transcript γ (*Raet1c*), which encodes a membrane bound protein that functions as a ligand for the activating NKG2D receptor from NK and T lymphocyte cells (35), was down-regulated in *Stra13*$^{-/-}$ mice at both CT4 and CT12. Interestingly, *Ctss* (cathepsin S), a cysteine protease recently shown to be involved in antigen processing (36), is clock-controlled with a phase similar to that of *Raet1c* and down-regulated in *Stra13*$^{-/-}$ mice at CT4, suggesting a coregulation of these two functionally related genes by STRA13.

A direct link between circadian gene regulation and cell division in liver has been recently provided by the observation that liver regeneration is retarded in cryptochrome-deficient mice (37). Because STRA13 is a known regulator of cell growth and differentiation (14, 15), we investigated the expression changes of genes involved in these processes in *Stra13*-deficient mice. This analysis revealed a down-regulation of the epidermal growth factor receptor *Erbb3* in *Stra13*$^{-/-}$ mice, suggesting a pathway through which STRA13 may play a role in the clock-dependent control of cell growth.

Finally, expression of two CCG involved in the regulation of transmethylation (*S*-adenosylhomocysteine hydrolase) and in

TABLE I

Microarray analysis of STRA13 target genes in mouse liver

Liver gene expression was analyzed in wild type and *Stra13*$^{-/-}$ mice at CT4 and CT12 using GeneChip Affymetrix high density oligonucloeotide microarrays. Differentially expressed genes in *Stra13*$^{-/-}$ mice with at least a 1.5-fold change were identified following robust multiarray average normalization and SAM analysis of microarray data. Chip refers to the CT4 (4) or CT12 (12) microarray experiment. The *q* value represents the lowest false discovery rate at which a gene is called significant in the SAM analysis. For each CCG, the peak of expression determined in previous microarray experiments is indicated. Fold change (FC) refers to as the mean *Stra13*$^{-/-}$/mean wild type signal ratio. For *Raet1c*, the *q* value and FC at CT4 are given in the table and were 8.94 and 0.53 at CT12, respectively. Affy ID indicates the Affymetrix probe set identification code.

Gene name	Symbol	Accession	Affy ID	Chip	Peak	*q* value	FC
Metabolism							
3-Hydroxy-3-methylglutaryl-coenzyme A reductase[a]	*Hmgcr*	M62766	104285_at	12	12	12.32	0.48
Hydroxysteroid dehydrogenase-6, Δ_5-3-β	*Hsd3b6*	NM_013821	102729_f_at	12		7.75	0.63
Arylacetamide deacetylase	*Aadac*	NM_023383	95439_at	4		13.76	0.65
Enoyl coenzyme A hydratase 1	*Ech1*	NM_016772	93754_at	4		15.65	0.66
Tyrosine aminotransferase	*Tat*	NM_146214	96326_at	4		13.76	1.54
Ornithine aminotransferase	*Oat*	NM_016978	92848_at	4		15.65	1.79
Branched chain ketoacid dehydrogenase E1, β[a]	*Bckdhb*	L16992	102302_at	4	10	4.66	1.84
Detoxification							
Cytochrome P-450 2c70[a]	*Cyp2c70*	NM_145499	95043_a	4	20	4.66	0.63
Glutathione *S*-transferase, θ2[a]	*Gstt2*	NM_010361	104603_at	4	10	4.66	1.61
Cytochrome P-450 2a4[a]	*Cyp2a4*	NM_009997	102847_s_at	4	12	15.65	2.03
Aminolevulinic acid synthase 1[a]	*Alas1*	NM_020559	93500_at	4	12	4.66	2.35
Serum proteins							
Insulin-like growth factor-binding protein 1[a]	*Igfbp1*	NM_008341	103896_f_at	4	2	4.66	0.37
Coagulation factor XII (Hageman factor)	*F12*	NM_021489	92509_at	12		16.82	1.65
Serum amyloid A 2	*Saa2*	NM_011314	103465_f_at	4		4.66	3.13
Cell growth							
v-*erb*-b2 viral oncogene homolog 3 (avian)[a]	*Erbb3*	At006228	96771_at	4	6	4.66	0.62
Enhancer of rudimentary homolog (*Drosophila*)	*Erh*	NM_007951	94040_at	12		13.85	0.64
B-cell translocation gene 3	*Btg3*	NM_009770	96146_at	4		15.65	0.66
Immunity							
Retinoic acid early transcript γ[a]	*Raetc*	NM_009018	102649_s_at	4/12	8	4.66	0.58
CD59a antigen	*Cd59a*	NM_007652	101516_at	4		4.66	0.58
Proteolysis							
Cathepsin S[a]	*Ctss*	NM_021281	98543_at	4	2	16.82	0.58
Ubiquitin-conjugating enzyme E2E 1	*Ube2e1*	NM_009455	92660_f_at	12		11.44	0.63
Cathepsin E	*Ctse*	NM_007799	104696_at	12		7.75	2.87
Renin 1 structural	*Ren1*	NM_031192	98480_s_at	12		7.75	3.26
Miscellaneous							
S-Adenosylhomocysteine hydrolase[a]	*Ahcy*	NM_016661	96026_at	4	18	4.66	0.48
RNA-binding motif protein 3[a]	*Rbm3*	NM_016809	96041_at	12	14	13.85	0.63
Aquaporin 4	*Aqp4*	U88623	102704_at	4		4.66	2.14
Unknown							
Erythroid differentiation regulator	*Edr*	NM_133362	98525_f_at	4		4.66	0.48
EST[a]		AW060549	160799_at	4	10	4.66	0.50
RIKEN cDNA 0610005C13 gene[a]	*0610005C13Rik*	AI042964	104617_at	12	8	11.44	0.54
EST		M17551	93907_f_at	12		7.75	0.55
EST[a]	*D9Wsul8e*	NM_138584	95430_f_at	12	6	7.75	0.55
EST		AI553024	92202_g_at	12		15.80	0.57
EST[a]		M26005	103562_f_at	4	10	4.66	0.59
EST		AI153421	96215_f_at	4		13.76	0.62
EST		X16672	101787_f_at	4		15.65	0.63
EST		AA755234	104327_at	4		4.66	0.64
RIKEN cDNA 2410004N09 gene[a]	*2410004N09Rik*	AA688938	104513_at	12	10	7.75	0.64
Kidney expressed gene 1[a]	*KegI*	NM_029550	96938_at	12	16	7.75	0.65
RIKEN cDNA 9430020E02 gene	*9430020E02Rik*	NM_145393	94895_at	12		7.75	0.66
Mus musculus 12 days embryo spinal cord cDNA[a]		NM_025374	93268_at	4	4	16.82	1.52
RIKEN cDNA 3110038L01 gene[a]	*3110038L01Rik*	NM_02652	95135_at	4	16	16.82	1.67
RIKEN cDNA 1810073K19 gene	*1810073K19Rik*	AI255961	104588_at	4		4.66	2.26

[a] Genes previously identified as clock-controlled.

RNA binding (RNA-binding motif 3) was found to be lower in *Stra13*$^{-/-}$ mice, suggesting that these genes may be indirect targets of this transrepressor.

To independently determine the importance of STRA13 in the regulation of these subset of CCGs, we analyzed the liver circadian expression of *Igfbp1*, *Raet1c*, *Alas1*, and *Cyp2a4* in wild type and *Stra13*$^{-/-}$ mice. *Igfbp1* was expressed according to a robust circadian rhythm in wild type mice with peak levels at CT0–CT4 (Fig. 4*A*). The peak of *Igfbp1* mRNA circadian expression was severely blunted in *Stra13*$^{-/-}$ mice as compared with wild type mice, resulting in a constitutive low expression pattern (Fig. 4*A*). Similarly the circadian expression of *Raet1c* with peak levels at CT8 was dramatically reduced in *Stra13*$^{-/-}$ mice (Fig. 4*B*). These data demonstrate that *Stra13* is an essential regulator of *Igfbp1* and *Raet1c* rhythmic expression, presumably through indirect mechanisms as STRA13 protein functions as a transcriptional repressor. A high amplitude oscillation of *Alas1* and *Cyp2a4* mRNA with peak and trough values at CT12–CT16 and CT0–CT4, respectively, was observed in control animals (Fig. 4, *C* and *D*). The lack of STRA13 in mutant mice resulted in elevated trough levels (CT0–CT4) but normal peak levels, indicating that STRA13 is a repressor of *Alas1* and *Cyp2a4*, contributing to the high amplitude rhythmic expression of these two target genes (Fig. 4, *C* and *D*). Together these data show that STRA13 is an important regulator of a subset of CCGs in mouse liver.

DISCUSSION

The genetic control of circadian behavior and physiology in mammals involves the rhythmic transcription of hundreds of

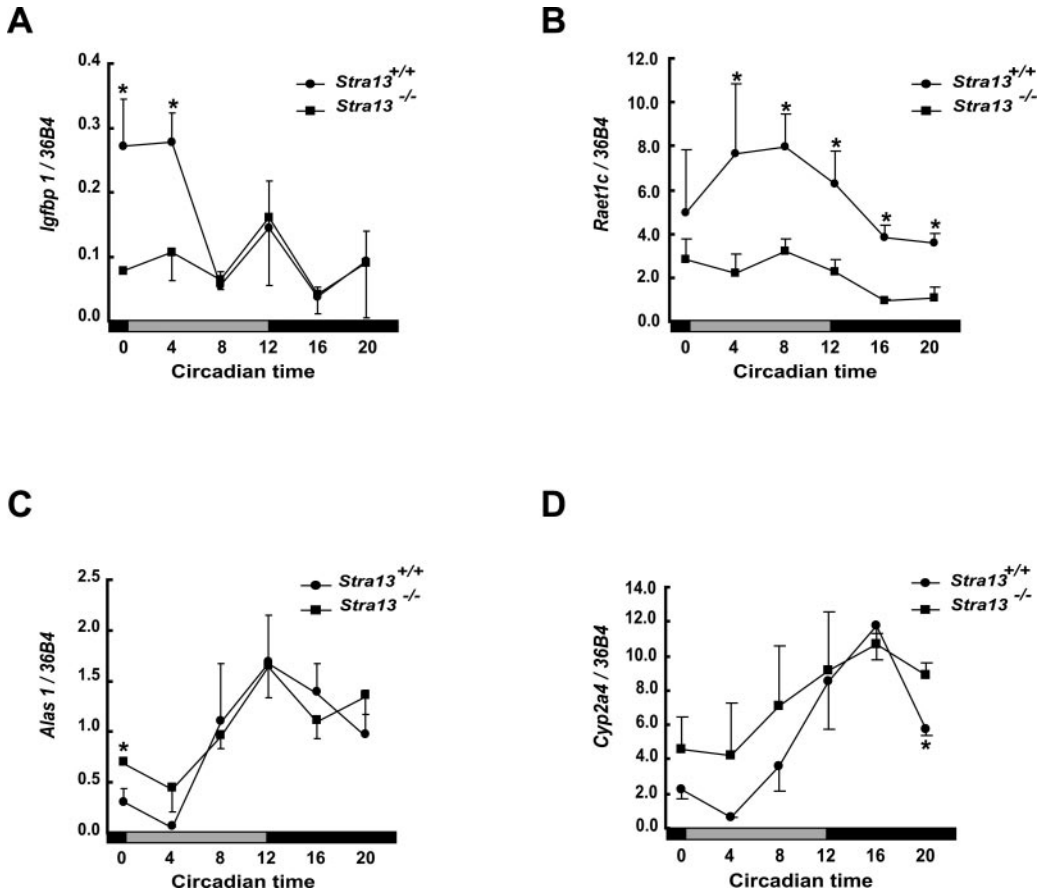

FIG. 4. **Altered clock-controlled gene expression in *Stra13*$^{-/-}$ mice.** Analysis of *Igfbp1* (*A*), *Raet1c* (*B*), *Alas1* (*C*), and *Cyp2a4* (*D*) mRNA circadian expression in the liver of wild type and *Stra13*$^{-/-}$ mice kept in DD conditions. RPA was used for *Igfbp1* and *Alas1* and real time PCR for *Raet1c* and *Cyp2a4*. The constitutively expressed *36B4* mRNA was used for normalization. The data are normalized as the means ± S.D. from two or three different animals of each genotype. The *asterisk* indicates statistically significant difference between mean values of *Stra13*$^{-/-}$ *versus* wild type mice, with a Student *t* test *p* value <0.05; some *error bars* are too small to be visible. The *black* and *gray bars* represent the subjective night and day, respectively.

circadian output genes in the SCN and peripheral organs (7, 10–12). How central and peripheral circadian oscillators orchestrate the regulation of this extensive gene network is therefore an important issue for the understanding of the mammalian circadian system. Recent work in *Drosophila* showed that only 9 of 128 CCGs were under the direct control of dCLOCK (13). Along this line, only a small number of CCGs in mammals were found to contain conserved canonical E box elements within their proximal promoter regions (10). This suggests that a majority of CCGs are indirectly regulated by circadian oscillators most likely via transcriptional cascades. This was further supported by the identification of more than 50 transcription factors with a circadian expression pattern in different mouse tissues (6). In a given tissue, these rhythmically expressed transcription factors oscillate with different phases, thus allowing clock genes to coordinate through these regulators a large repertoire of clock-controlled outputs throughout the 24-h cycle.

In this report, we have analyzed the mechanism and the physiological role of the circadian regulation of *Stra13*, a bHLH transcriptional repressor expressed in numerous peripheral tissues and playing a critical role in cell growth arrest (14–16). Our expression and biochemical data, together with the previous observation that *Stra13* was down-regulated in *Clock*/*Clock* mutant mice, strongly suggest that *Stra13* is a direct transcriptional output of peripheral circadian oscillators (10). *Stra13* has also been shown to be up-regulated by retinoic acid, cAMP, transforming growth factor-β, prostaglandin E2, serum starvation, and hypoxia (14, 15, 38–41). *Stra13* appears therefore to be the target of multiple signaling pathways that may cooperate or interfere depending on the physiological context and time. For instance, retinoic acid, which was recently shown to reset peripheral oscillators in the vascular system (42), could also modulate specific CCGs through its potent stimulatory effect on *Stra13* expression.

To get insight into the physiological role of STRA13, we hypothesized that this bHLH transcription factor could regulate either the molecular oscillator itself or downstream physiological outputs or both. Our extensive analysis of clock gene expression in *Stra13*$^{-/-}$ mouse liver demonstrated that STRA13 was dispensable for normal circadian clock function. Consistent with this observation, no significant behavioral or SCN gene expression phenotypes were observed in mutant mice.[3] Based on expression in the rat SCN, biochemical, and light response experiments, *Stra13* and its paralog *Sharp-1* were recently proposed to be core components of circadian oscillators, and both genes were also shown to be mutually regulated *in vitro* (28, 30, 43). Our expression data showing that *Sharp-1* is a target of *Stra13 in vivo* suggest that the absence of phenotype at the peripheral oscillator level may be caused by redundancy between *Stra13* and *Sharp-1*. The development of double mutant mice should help to clarify this issue. Nevertheless, the identification in liver of CCGs involved in various physiological processes such as metabolism, detoxification, cell growth, immune response, and proteolysis or of

[3] T. K. Sato and J. B. Hogenesch, submitted for publication.

unknown function among a total of 42 STRA13 target genes indicates that this redundancy may only be partial or specific to clock genes. Half of these putative *Stra13* target genes are also cycling in liver, indicating that a principle function of *Stra13* is circadian transcriptional regulation. Interestingly, *Stra13*-regulated CCGs did not cluster into a single functional group but were instead involved in different biological processes in analogy to what was observed at the genomic scale (10–12).

This diversity is exemplified by the finding that altered circadian expression was further confirmed by *Igfbp1*, *Raet1c*, *Alas1*, and *Cyp2a4*. Surprisingly, a majority of these targets were elevated in *stra13*$^{-/-}$ animals including *Igfbp1* and *Raet1c* were elevated in *Stra13*$^{-/-}$. Because *Stra13* is a transcriptional repressor, we postulate that indirect mechanisms are likely responsible. For example, *Stra13* may repress transcription of other repressor proteins such that its removal results in indirect transcriptional activation. Consistent with this hypothesis, we find *Sharp-1/DEC2* mRNA levels elevated in *Stra13*$^{-/-}$ mice. However, the similarity of phase and biochemical properties between *Stra13* and *Sharp-1/DEC2* suggests that this putative mechanism may operate through an unidentified factor.

Furthermore, our results suggest a role for *Stra13* in mediating important circadianly regulated physiological outputs in liver. For example, IGF-binding protein 1 is a liver-specific secreted protein that belongs to a family of six binding proteins that are important modulators of insulin-like growth factor I and II (IGF-1 and IGF-2) biological activity while having also intrinsic activities independent of IGF receptor signaling (32). Circadian and antiphasic oscillations of serum-free IGF-1 and IGF-binding protein 1 concentrations have been described in humans, whereas the total IGF-1 level was found unchanged over time (44, 45). Notably, besides its effects on somatic growth, an important biological effect of IGF-1 is to decrease circulating glucose levels, suggesting a possible role for the STRA13-dependent up-regulation of *Igfbp1* at CT0–CT4 in the moderation of hypoglycemia during the resting phase by decreasing IGF-1 bioavailability. In addition, our data also show that STRA13 is a critical positive regulator of *Raet1c* circadian expression. *Raet1c* is a member of a family of five genes (*Raet1 α-e*) that encode stimulating NKG2D receptor ligands (35). These membrane proteins have been shown to be mainly expressed in tumor cells, and they are believed to play a major role in tumor surveillance (46). Our data provide experimental evidence that STRA13 is a potent regulator of important components of the innate immune system in the liver. We speculate that it may be advantageous for the liver to express NKG2D ligands to suppress the growth or survival of cells that may become tumoral as hepatocytes are regularly exposed to damaging agents during the feeding phase.

Alas1 and *Cyp2a4* are two target genes whose circadian pattern of expression was specifically altered between the peak (CT12) and trough (CT4) levels. In terms of gene regulation, this suggests that these functionally related genes are regulated in a coordinated manner by several circadian transcription factors acting negatively and positively to achieve a high amplitude oscillation. This assumption is supported by recent data showing that *Alas1* and *Cyp2a4* are respectively the targets of the constitutive androstane receptor (CAR/NR1I3) and albumin D box-binding protein (DBP), which are both clock-controlled transcriptional activators (11, 47, 48). Thus, STRA13 may act as a common negative circadian regulator of these two genes whose combinatorial actions with positive regulators such as CAR and DBP determine the observed composite circadian expression profile.

Physiologically, the requirement for a high amplitude circadian oscillation of *Alas1* may be a mechanism by which the cellular metabolism reduces the deleterious effects of δ-aminolevulinic acid and heme during the resting phase while providing higher levels when liver metabolism is fully activated. In line with this hypothesis, *Alas1* was also found to oscillate in *Drosophila*, a diurnal species, with a peak preceding the activity phase (34). Because of the potential toxicity of δ-aminolevulinic acid and heme as a pro-oxidant and iron chelator, respectively, we hypothesize that the up-regulation of *Alas1* exclusively at CT0–CT4 in *Stra13*$^{-/-}$-deficient liver suggests that a role for STRA13 is to prevent the production of an excess of reactive oxygen compounds by the heme biosynthetic pathway during the resting phase (49). Similarly, the down-regulation of *Cyp2a4*, which participates in the catabolism of testosterone and estrogens, at CT20–CT4 may reveal a mechanism for maximizing the bioavailability for these steroid hormones during the resting phase.

In conclusion this present work shows that the bHLH transcriptional repressor *Stra13* is a transcriptional output of peripheral circadian oscillators regulating a subset of CCGs. Previously, *Dbp*, a leucine zipper transcription factor, was shown to be an output of circadian oscillators and to control the circadian regulation of three liver P-450 cytochromes *Cyp7a* (cholesterol 7-α hydroxylase), *Cyp2a4* (steroid 15 α-hydroxylase), and *Cyp2a5* (coumarin 7-hydroxylase) (25, 48, 50). Collectively, these observations support a circadian gene regulatory network in which clock-controlled genes rather than *bona fide* clock components initiate transcriptional cascades that underlie observed circadian physiological outputs.

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