LARK activates posttranscriptional expression of an essential mammalian clock protein, PERIOD1

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The mammalian molecular clock is composed of feedback loops to keep circadian 24-h rhythms. Although much focus has been on transcriptional regulation, it is clear that posttranscriptional controls also play important roles in molecular circadian clocks. In this study, we found that mouse LARK (mLARK), an RNA binding protein, activates the posttranscriptional expression of the mouse *Period1* (m*Per1*) mRNA. A strong circadian cycling of the mLARK protein is observed in the suprachiasmatic nuclei with a phase similar to that of mPER1, although the level of the *Lark* transcripts are not rhythmic. We demonstrate that LARK causes increased mPER1 protein levels, most likely through translational regulation and that the LARK1 protein binds directly to a cis element in the 3′ UTR of the m*Per1* mRNA. Alterations of m*Lark* expression in cycling cells caused significant changes in circadian period, with m*Lark* knockdown by siRNA resulting in a shorter circadian period, and the overexpression of mLARK1 resulting in a lengthened period. These data indicate that mLARKs are novel posttranscriptional regulators of mammalian circadian clocks.

circadian rhythms | posttranscriptional regulation | RNA binding protein | 3′untranslated region | suprachiasmatic nucleus

Circadian rhythms are generated by endogenous clocks, and the principal circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus in mammals (1, 2). Circadian clocks regulate the daily fluctuations of biochemical, physiological, and behavioral rhythms, and these rhythms persist under constant conditions and are entrained by the environmental light–dark (LD) cycles (3, 4). In mammals, the molecular circadian clock is comprised of interlocking transcriptional-translational feedback loops containing several regulatory steps, transcriptional, posttranslational, and protein–protein interactions (reviewed in ref. 5). These regulatory steps must be coordinated and orchestrated properly for the fine-tuning of the 24-h periodicity. Although less well understood, it is clear that posttranscriptional regulation also plays a very important role, contributing to the phase and amplitude of rhythmic mRNA expression in many organisms (reviewed in ref. 6).

One of several clock genes in mammals, *Period1* (*Per1*) was originally identified as a structural homologue of the *Drosophila* circadian clock gene *per* (7). The transcription of *Per1* is activated by the CLOCK–BMAL1 heterodimer (8, 9) and repressed by a complex containing PER and cryptochrome (CRY) proteins (10), thus comprising one of the core feedback loops. The molecular function of mPER1 is not yet clarified, but m*Per1* is an essential gene for maintenance of circadian rhythms, because loss of m*Per1* in knockout mice results in an altered period (11–13). mPER1 is also thought to be involved in resetting of the circadian oscillator (14). m*Per1* expression is rhythmic, but the phase of the protein rhythm is delayed 6–8 h relative to that of the mRNA in the mouse SCN (15), indicating that mPER1 expression is regulated posttranscriptionally. A similar 6- to 8-h time lag between the expression of *Drosophila per* (d*per*) mRNA and dPER protein has also been observed (16), suggesting that these time lags are important evolutionarily conserved aspects of the clock mechanism.

Posttranscriptional regulation of mRNA stability and translational efficiency are often mediated by cis elements in mRNAs that interact with trans-acting factors such as RNA-binding proteins and/or microRNAs. In most cases, these cis elements reside in the 3′ UTR, and several 3′ UTR motifs have been identified that are critical for mRNA splicing, transport, stability, localization, and translation (17). Mouse *Per1* (m*Per1*) 3′ UTR likely has an important regulatory role, because the human *Per1* and m*Per1* 3′ UTRs have high homology (78.0%) (18). In addition, two studies have shown that m*Per1* is regulated posttranscriptionally via its 3′ UTR, but little is known about the mechanisms (18, 19). In *Drosophila*, d*per* mRNA half-life also appears to be regulated by the circadian clock, resulting in different message stability during accumulating and decay phases (20). Also, the circadian cycling of dPER levels depends on the 3′ UTR of the d*per* mRNA (21).

Here, we identified an RNA-binding protein, called LARK, that interacts with the m*Per1* 3′ UTR and regulates m*Per1* expression in a posttranscriptional manner. Alteration of mouse LARK (mLARK) expression resulted in changes in the circadian period. Thus, we propose that mLARK is a novel posttranscriptional regulator of the circadian clock.

Results

Because our previous work had shown that m*Per1* expression was regulated posttranscriptionally via sequences within its 3′ UTR (18), we began to investigate the potential trans-acting factors that could be responsible for this regulation. At the time we began these studies the only protein that contained putative RNA-binding motifs that had been tied to circadian rhythms was *Drosophila* LARK (dLARK) (22–25). Using a candidate approach, we decided to investigate a possible role for mammalian Lark homologs in posttranscriptional regulation of m*Per1*.

Circadian Expression of mLarks in the SCN. In our analysis, mouse *Rbm4a* (m*Rbm4a*) and *Rbm4b* (m*Rbm4b*) encode polypeptides highly homologous to the N-terminal region of dLARK that contain three RNA binding domains: two RNA recogni-

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The authors declare no conflict of interest.

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Abbreviations: *Per*, *Period*; m*Per*, mouse *Per*; SCN, suprachiasmatic nucleus; DD, constant dark; LD, light–dark; CT, circadian time; ZT, Zeitgeber time; IRES, internal ribosome entry site; Cry, cryptochrome; d*per*, *Drosophila per*; mLARK, mouse LARK; dLARK, *Drosophila* LARK; m*Rbm4*, mouse *Rbm4*; IR, immunoreactive; RMSA, RNA mobility-shift assay; F-luc, firefly luciferase; R-luc, *Renilla* luciferase.

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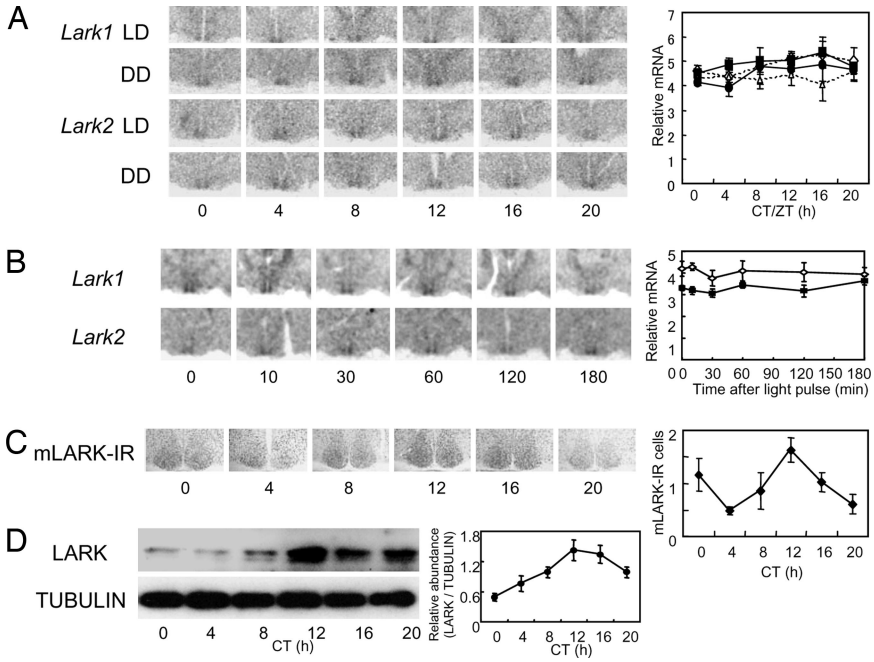

Fig. 1. Expression profiles of m*Larks* in mouse SCN. (*A*) (*Left*) Representative *in situ* hybridization results for m*Lark1* and m*Lark2* mRNAs in mouse SCN harvested at different times of day from mice housed in either LD or DD conditions are shown. (*Right*) The graph shows mean quantification of m*Lark1* (□, LD; ■, DD) and m*Lark2* (□, LD; ●, DD) mRNAs. (*B*) (*Left*) Representative *in situ* hybridization results for m*Lark1* and m*Lark2* mRNAs in SCN harvested from mice after 600 lux of light exposure for 30 min are shown. (*Right*) The graph shows mean quantitation of m*Lark1* (□) and m*Lark2* (■) mRNAs. (*C*) Representative immunohistochemical analysis of mLARKs in mouse SCN (*Left*) in DD and quantitation of mLARK-IR cells (*Right*) are shown. One-way ANOVA revealed a highly significant ($P < 0.0001$) time effect. (*D*) Western blot analysis of mLARK (*Left*) in extracts of microdissected mouse SCNs harvested in DD and quantification of mLARK levels (*Right*) are shown. One-way ANOVA revealed a highly significant effect of time ($P < 0.001$). All data are representative of at least three independent experiments.

tion motifs and a CCHC-type zinc finger motif (22–27) [supporting information (SI) Fig. 7*A*]. The amino acid sequence identity between the protein encoded by m*Rbm4a* and m*Rbm4b* is 86% over the entire protein sequence. Both *Rbm4a* and *Rbm4b* were strongly expressed in the heart, brain, spleen, lung, liver, kidney, and testis, but not in the skeletal muscle (SI Fig. 7*B*) and are independent genes located in adjacent regions on both human and mouse chromosomes 11q13 (26) and 19A, respectively. Therefore, we concluded that *Lark1* and *Lark2* are more suitable nomenclatures than *Rbm4*a and *Rbm4*b, and they are used in this study. It has been shown that human LARK1 and LARK2 (RBM4A and RBM4B) modulate alternative splicing; however, it is not clear whether this is the case for mLARKS (27).

To determine the time dependence of m*Lark1* and m*Lark2* mRNA expression in the SCN, their levels were examined by *in situ* hybridization. cRNA probes were designed to each 3′ UTR to distinguish the expression of the two mRNAs, because the nucleotide sequences of the coding regions of m*Rbm4a* and m*Rbm4*b are quite similar. Strong signals for both m*Lark1* and m*Lark2* were detected in the mouse SCN, but neither m*Lark1* nor m*Lark2* mRNA showed distinct circadian fluctuation in the mouse SCN under either LD or constant dark (DD) conditions (Fig. 1*A*). Zeitgeber time (ZT) 0 is light onset, and ZT12 is light offset in a LD cycle, whereas circadian time (CT) 0 indicates the beginning of subjective day, and CT12 is the beginning of subjective night. Unlike *Per1* and *Per2* transcripts that are rapidly induced by a short exposure to light (28), neither m*Lark1* nor m*Lark2* transcripts showed a response (Fig. 1*B*).

Next, we examined the mLARK protein in the SCN by using an antibody that recognizes both mLARK1 and mLARK2 (SI Fig. 8). mLARK-immunoreactive (IR) cells were detected in the SCN, and the number of mLARK-IR cells were rhythmic in DD conditions, with a peak at CT12 and a trough at CT4 (Fig. 1*C*). We also confirmed the expression levels of mLARKs under DD conditions by Western blot analysis using proteins extracted from microdissected SCNs (Fig. 1*D*). The anti-LARK antibody detected two major bands at ≈42 kDa in the SCN, and both of these bands disappeared by the preabsorption of the antigen peptide (data not shown). We presume that the upper and lower bands corresponded to mLARK2 and mLARK1, respectively, judging from the estimated molecular weights of these two proteins (SI Fig. 8). The expression profiles of the mLARK proteins in the SCN examined by Western blot analysis were consistent with our observations of the mLARK-IR cells. The mLARK expression was also rhythmic in cortex (data not shown). Because protein (but not mRNA) rhythmicity was observed even under DD conditions, it is clear that m*Larks*' expression is under the control of the circadian clock, and that the temporal oscillation of mLARK is regulated at the posttranscriptional level.

Posttranscriptional Regulation of mPER1 Expression by mLARKs. Because the temporal profile of mLARK in the SCN was similar to that of the mPER1 protein, in which the highest expression level was observed at CT12–16 and the lowest at CT0–4 (Fig. 1 *C* and *D*), the effect of mLARK on m*Per1* expression was examined by using two reporter plasmids, pPLS and pPL3 (Fig. 2*A*) (18). These plasmids contain a *luciferase* reporter gene under the control of the m*Per1* promoter, and the *luciferase* gene is followed by either an simian virus 40 poly(A) signal (pPLS) or the m*Per1* 3′ UTR (pPL3). These plasmids were transfected into NIH 3T3 cells along with plasmids expressing mCLOCK and mBMAL1 (activators of the *Per1* promoter). Addition of mLARK1 and mLARK2 expression vectors resulted in ≈2.8- and 5.0-fold inductions of the luciferase activity of pPL3, respectively, whereas no induction was observed in pPLS (Fig. 2*B*). In addition, the induction of pPL3 by mLARK1 was dose-dependent (Fig. 2*C*). Interestingly, mLARK1 did not enhance the transcription of pPL3 nor pPLS, because no significant differ-

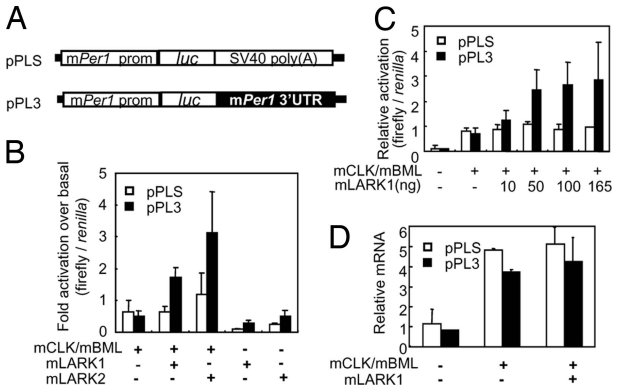

Fig. 2. Posttranscriptional regulation of m*Per1* chimeric reporter genes by mLARKs. (*A*) Structures of chimeric luciferase genes are shown. Both constructs contain a 6.8-kb m*Per1* promoter driving a luciferase reporter gene followed by either the simian virus 40 poly(A) signal (pPLS) or the m*Per1* 3′ UTR (pPL3). (*B*) Relative luciferase activities of pPLS and pPL3 either with or without the presence of mLARK expression plasmids (165 ng) with the transcriptional activators CLOCK and BMAL1 are shown. (*C*) mLARK1 increases reporter gene expression in a dose-dependent manner. The experiment was done as in *B* except with varying levels of mLARK1 plasmid as indicated. (*D*) Relative *luciferase* mRNA levels are not altered by mLARK1. The experiment was done as in *B*, except total RNA was isolated and the level of *luciferase* mRNA was measured by real-time RT-PCR. Data consist of two or three independent experiments.

ence between the mRNA levels of the *luciferase* mRNA in the presence and absence of mLARK1 was detected, whereas similar amounts of the transcripts were induced by mCLOCK/mBMAL1 in both pPLS and pPL3 (Fig. 2*D*). These data indicate that mLARK1 regulates pPL3 posttransciptionally, most likely through the m*Per1* 3′ UTR.

Subsequently, we examined whether mLARK1 also affects the endogenous expression of mPER1 in NIH 3T3 cells. When mLARK1 was overexpressed in NIH 3T3 cells, the endogenous mPER1 protein level was markedly increased; however, the endogenous level of m*Per1* mRNA remained unchanged (Fig. 3). These results are consistent with our observations with the pPL3 reporter gene system and indicate that mLARK1 regulates the endogenous mPER1 expression posttranscriptionally.

mLARK1 Interacts Directly with mPer1 RNA. Because mLARK posttranscriptionally activates mPER1 expression and contains several RNA-binding domains, we tested whether mLARK1 could interact directly with m*Per1* 3′ UTR. RNA mobility-shift assays (RMSAs) were performed by using a series of RNA fragments covering the entire m*Per1* 3′ UTR (Fig. 4*A* and SI Table 2). Specific binding was detected only in RNA fragment 19, which contains nucleotides 559–589 of m*Per1* 3′ UTR (Fig. 4*A*). Specificity of this binding was confirmed by the demonstration that the intensities of the shifted

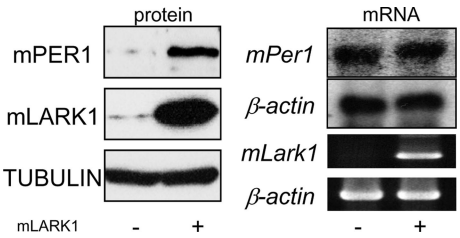

Fig. 3. Posttranscriptional regulation of endogenous mPER1 by mLARK1. Measurement of endogenous mPER1, TUBULIN, and transfected mLARK1 by Western blot (*Left*) and measurement of endogenous m*Per1* and β-*actin* by Northern blot and transfected m*Lark1* and β-*actin* by RT-PCR (*Right*) from NIH 3T3 cells with or without transfected mLARK1 are shown.

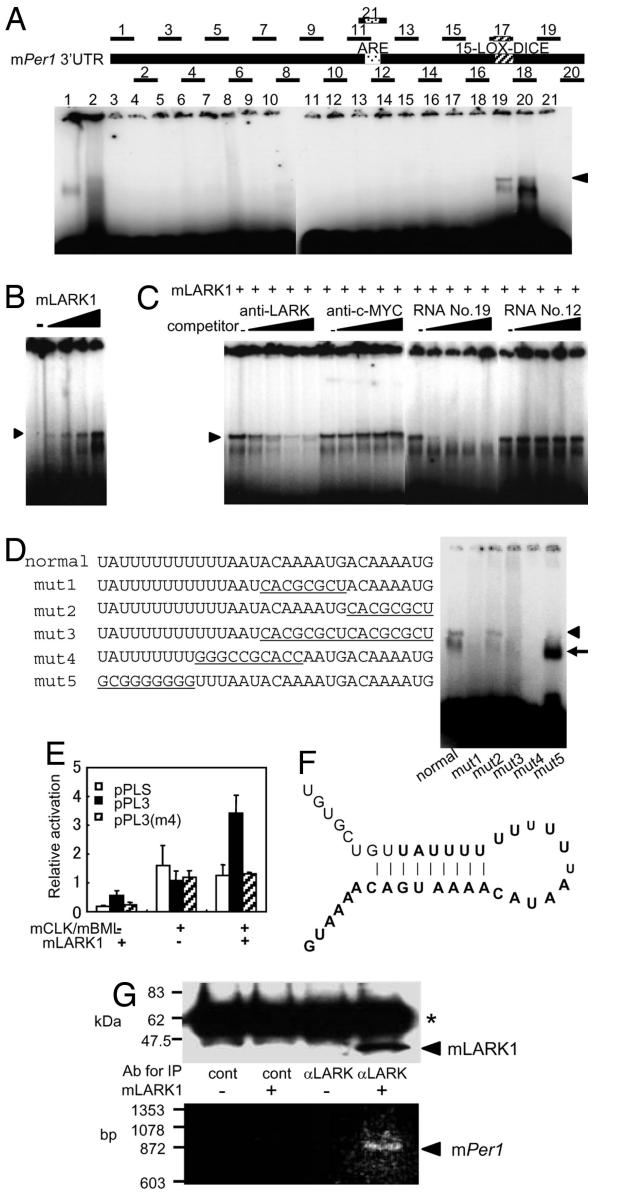

Fig. 4. Direct interaction between mLark1 and m*Per1* 3′ UTR. (*A*) RMSA of His-mLARK1 and RNAs derived from m*Per1* 3′ UTR is shown. The numbers correspond to RNA oligonucleotides that span the entire m*Per1* 3′ UTR as diagrammed at the top (the sequences are listed in SI Table 2). The position of the previously identified ARE and LOX-DICE elements are marked (18). Arrowhead denotes the LARK-specific retarded band. (*B*) Dose–response relationship between His-mLARK1 and RNA 19 is shown. Shown is an RMSA as in *A*, but using only RNA 19 as probe and with increasing doses of His-mLARK1 as indicated. (*C*) Competitive analyses verify specificity of interaction between mLARK1 and RNA. RMSAs were done as in *B* but with the addition of increasing amounts of antibody or cold RNA competitors as indicated. (*D*) RMSA using normal and mutated versions of RNA 19 is shown. Underlined nucleotides are mutated nucleotides. The arrowhead indicates the RNA bands retarded by mLARK1, and the arrow indicates a nonspecific band. (*E*) LARK1 only activates reporter gene expression when the LARK binding site is intact. Reporter analyses were done as in Fig. 2, except an additional reporter construct was included in which the m*Per1* 3′ UTR contains the mutations shown in mut4 (*D*) [pPL3(m4)]. (*F*) The predicted RNA secondary structure is shown. Bold type indicates nucleotides corresponding to RNA fragment 19. (*G*) The direct interaction between LARK1 protein and *Per1* mRNA is shown. Extracts from NIH 3T3 cells with either overexpressed mLARK1 or control plasmid were immunoprecipitated with either anti-LARK or control antibody. Immunoprecipitates were analyzed by Western blot using LARK antibody (*Upper*) or RT-PCR for m*Per1* (*Lower*). Asterisk indicates the IgG heavy chain from the immunoprecipitating antibody.

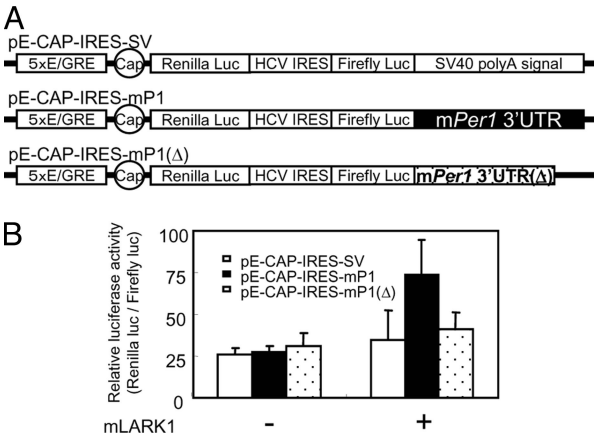

Fig. 5. mLARK1 affects mPER1 translation. (*A*) Schematic representation of bicistronic vectors. The promoter is minimal promoter containing five times ecdysone/glucocorticoid (E/GRE) responsive element (18). See *Results* for further details. (*B*) Luciferase assay of bicistronic vector. The *y* axis represents the ratio of R-luc/F-luc activity. Data consist of three independent experiments.

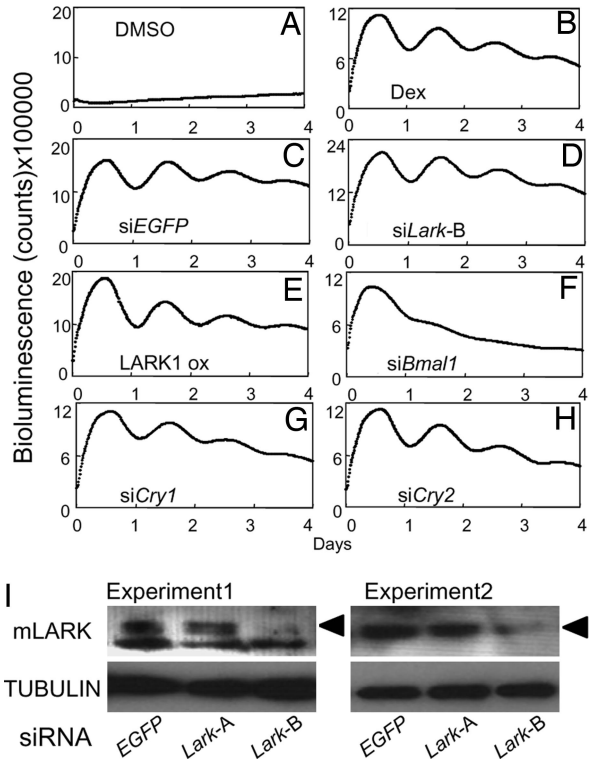

Fig. 6. Circadian rhythms of bioluminescence in NIH-PL cells are affected by LARK levels. Shown are representative recordings of NIH-PL cells over 4 days in culture, measuring bioluminescence as an output of the m*Per1*::*luc* reporter gene. (*A* and *B*) Oscillations are not observed in these cells when treated with DMSO (*A*) but are induced after synchronization with dexamethasone (Dex) (*B*). (*C–H*) Representative recordings of NIH-PL cells as in *B*, but with the addition of siRNAs against *EGFP* (*C*), *Lark*-B (*D*), *Bmal11* (*F*), *Cry1* (*G*), and *Cry2* (*H*) or with overexpression of mLARK1 (*E*). (*I*) The effect of siRNAs on the expression of mLARKs was confirmed by immunoblotting. Extracts of cells containing siRNAs against *EGFP*, *Lark*-A, and *Lark*-B were analyzed on Western blots using antibodies to LARK and TUBULIN.

bands were proportional to the amount of mLARK1 (Fig. 4*B*), and excess amounts of anti-LARK antibodies or cold RNA 19 fragments inhibited this interaction, whereas a unrelated control antibody or other cold RNA (RNA 12) did not (Fig. 4*C*). Although other retarded bands were observed in lanes 1 and 20 in Fig. 4*A*, we concluded that these were nonspecific interactions, because these bands were observed even in the presence of competitors (data not shown).

The sequences involved in this interaction were characterized by the introduction of several mutations into the RNA 19 fragment. When we used these mutated RNAs in RMSA, RNA–protein interactions were detected only in the mut2 RNA (Fig. 4*D*). The secondary structure of this region was predicted to form a stem–loop structure by using the GENETYX-MAC program (Fig. 4*F*), and such a structure would be disrupted in all mutant RNAs except mut2. Therefore, it is possible that mLARK1 recognizes the stem–loop structure rather than specific RNA sequences. To clarify the significance of this protein–RNA interaction for the posttranscriptional activation of m*Per1* expression, we constructed another reporter plasmid pPL3(m4), in which the same mutation as mut4 was introduced into pPL3. The increased luciferase activity of pPL3 by mLARK1 was completely abolished in pPL3(m4) (Fig. 4*E*), indicating that the interaction between the m*Per1* 3′ UTR and mLARK1 is necessary for the posttranscriptional activation of m*Per1* expression.

The direct interaction between mLARK1 and m*Per1* mRNAs in NIH 3T3 cells was examined by a UV cross-linking immunoprecipitation assay (29). Lysates were prepared from cells with or without overexpressed mLARK1 and UV-irradiated before immunoprecipitation. When RNA–protein complexes were analyzed separately by SDS/PAGE and RT-PCR, both mLARK1 protein and m*Per1* mRNAs were detected only in the anti-LARK immunoprecipitate prepared from cells transfected with mLARK1 (Fig. 4*G*), indicating that mLARK1 physically interacts with endogenous *mPer1* RNA.

To investigate whether mLARK1 activates mPER1 expression by modulating translation, we made use of a bicistronic reporter gene system (30). In this system, *Renilla* luciferase (R-luc) and firefly luciferase (F-luc) are under the control of a single inducible promoter, but are translated by using two distinct mechanisms (Fig. 5*A*). R-luc activity represents eIF4G/poly(A) tail-dependent translation, because it is near the Cap structure, whereas F-luc activity represents internal ribosome entry site (IRES)-dependent translation. F-luc activity also serves as an internal control for transfection efficiency. Both R-luc and F-luc translation are affected by 3′ UTRs located downstream of F-luc, therefore, the ratio of the R-luc/F-luc represents the efficiency of Cap/poly(A)-dependent translation. We constructed three bicistronic reporter genes, each of which contained either the simian virus 40 poly(A) signal, the m*Per1* 3′ UTR, or the m*Per1* 3′ UTR lacking the LARK1 binding domain (Fig. 5*A*). Significant induction was observed when mLARK1 was overexpressed with pE-CAP-IRES-mP1, but not with pE-CAP-IRES-SV and pE-CAP-IRES-mP1(Δ) (Fig. 5*B*). These results suggest that LARK1 activates the translation of mPER1 via eIF4G/poly(A) interaction depending on m*Per1* 3′ UTR.

Functions of mLARKs in the Mammalian Circadian Clock System. To analyze the role of m*Lark* in the cellular circadian clock system, we used a NIH 3T3 cell line stably transfected with a m*Per1*::*luc* reporter gene (NIH-PL cells). It should be noted that the pPLS reporter construct that we used did not possess the m*Per1* 3′ UTR (Fig. 2*A*) to observe the circadian property of the cells. Treatment of NIH-PL with dexamethasone induced the circadian fluctuations of bioluminescence with a circadian period of ≈23.9 h, which was close to behavioral rhythm in mice (Fig. 6 *A* and *B*). When we introduced a siRNA of m*Lark* (si*Lark*-B), which targeted and significantly repressed both m*Lark1* and m*Lark2* (Fig. 6*I*), the period of bioluminescence was ≈0.3 h shorter than the control in dexamethasone-induced NIH-PL cells (Fig. 6*D* and Table 1), whereas another m*Lark* siRNA (si*Lark*-A), which targeted only m*Lark1*, did not repress the mLARK protein expression nor

Table 1. Period length of bioluminescent oscillation in NIH-PL cells

DNA or RNA used for transfection	No. of cells tested	Mean period ± SEM, h
Mock transfection	7	23.93 ± 0.26
si*EGFP*	8	23.89 ± 0.19
si*Lark*-A	12	23.85 ± 0.10
si*Lark*-B	12	23.58 ± 0.32*†
si*Bmal1*	8	Arrythmic
si*Cry1*	3	23.23 ± 0.50*†
si*Cry2*	4	24.35 ± 0.10*†
mLARK1 overexpression	25	24.25 ± 0.62*

The length of the period shown is the average for the indicated number of separate wells combined from at least three independent experiments. *, $P < 0.05$ vs. mock transfection; †, $P < 0.01$ vs. si*EGFP* (Student's *t* test).

shorten the period (Fig. 6*I* and Table 1) (31). On the other hand, overexpression of mLARK1 in the cells resulted in a 0.3-h longer period in *Per1* oscillation (Fig. 6*E* and Table 1). The administration of siRNAs against *Bmal1*, *Cry1*, or *Cry2* to the NIH-PL cells resulted in arrhythmic, 0.6- to 0.7 h-shortened, or 0.4- to 0.5-h-lengthened circadian period of the reporter expression, respectively (Fig. 6 *F–H* and Table 1), as expected from the behavioral phenotypes of mutant mice of these genes.

Discussion

A great deal of attention has been paid to transcriptional regulation of the mammalian circadian clock mechanism and rhythmic gene expression, including extensive studies using powerful tools such as DNA microarrays (32, 33). In contrast, very little is known about the mechanisms of posttranscriptional regulation despite accumulating evidence that these mechanisms are important for proper circadian clock function. For example, a recent study (34) revealed that only 50% of genes that encode rhythmic proteins exhibited circadian variation in mRNA levels, indicating that a large number of mRNAs are regulated posttranscriptionally.

The identification of m*Lark*s as m*Per1* 3′ UTR-binding proteins provides mechanistic insight into posttranscriptional control of mammalian clock processes. Interestingly, decreased or increased levels of *lark* in *Drosophila* pupae leads to an early- or late-eclosion phenotype, respectively, presumably through posttranscriptional mechanisms (22–25). In addition, the overexpression of d*lark* in the central clock cells of fly causes behavioral arrhythmicity without affecting the cycling of core clock proteins such as *per* and *timeless* (*tim*) (25). However, interactions of LARK with RNA targets had not been reported to our knowledge. Circadian control of translation has been observed in *Gonyaulax polyedra* and *Chlamydomonas reinhardtii*, where CCTR and CHLAMY1 (35, 36), respectively, regulate circadian translation by interaction with the UG-containing region in corresponding mRNAs. Also in *Arabidopsis*, a nuclear RNA-binding protein (AtGRP7) has been found as a component of the circadian clock system, although its effect on translation has not been clarified (37).

We have shown here that in mammals mLARK protein levels oscillate in the SCN, with a peak at CT12–16 and a minimum at CT0–4 (Fig. 2 *E* and *F*), but m*Lark* mRNA expression remained constant throughout the day. This is also the case for d*Lark*; dLARK protein fluctuates with a peak at ZT6 in pupae, but d*Lark* mRNA is not rhythmic (24). This result suggests that *Lark*s, as well as *Per*s, are under posttranscriptional control in both *Drosophila* and mammalian systems. Our finding that the oscillation profiles of mLARK proteins were in-phase with the mPER1 protein is consistent with our proposed role for mLARK in regulating mPER1 expression.

In humans, RBM4a (human LARK1), appears to have a role as a modulator of alternative splicing of mRNAs (27, 38, 39); however, it is unlikely that mLARK regulates m*Per1* expression at the level of splicing, because mLARK1 directly interacts with the m*Per1* 3′ UTR, not with a splicing junction, and up-regulates mPER1 protein expression. The most parsimonious explanation is that mLARKs act on mPER1 translation, because mLARKs activate mPER1 expression in a Cap/poly(A)-dependent manner (Fig. 5). By increasing efficiency of m*Per1* translation, mLARKs may also contribute to the generation of the mPER1 protein rhythm, which is phase-lagged relative to its mRNA rhythm, although the exact mechanism of this regulation remains to be elucidated.

We previously reported that within m*Per1* 3′ UTR, there are sequences that repress mPER1 expression posttranscriptionally (18), yet the regulatory molecule(s) still remains to be determined. In this study, we found that LARK activates the m*Per1* expression via binding to the m*Per1* 3′ UTR at a distinct cis element. In our current working model, these two molecules could act on m*Per1* at different times of day; during the daytime, when mRNA expression of m*Per1* is high and protein expression is low, the translational repression could be caused by the unidentified molecule(s) bound to the cis element, whereas during late afternoon/early night when m*Per1* mRNA expression decreases but protein expression reaches its peak translational activation by rising LARK levels would become dominant. Thus, there is a possibility that the effect of mLark on m*Per1* expression is to accentuate the amplitude and provide robustness of the molecular cycling of PER1.

The alteration of circadian period as a result of changing mLARK expression levels in NIH 3T3 cells demonstrates the functional relevance of m*Lark*s in the circadian system. In our study, m*Lark* gene knockdown shortened the period length of NIH-PL cells ≈0.3 h, and mLARK1 overexpression lengthened the period ≈0.3 h. Because mLARK levels regulate mPER1 levels, it seems likely that mLARK's effect on the circadian period is through the regulation of m*Per1* expression. This idea is supported by data from mutant animals; *Per1*$^{-/-}$ mice, which lack functional PER1 expression, exhibit a shortened period of locomotor activity (11–13), whereas *Per1* transgenic rats, which overexpress *Per1*, have a 1 h-lengthened period (40). These results indicate that either loss or overexpression of *Per1* does not abolish the circadian oscillation, but does influence the length of its period, and the period of the rhythm may have positive correlation with the expression levels of *Per1*. Thus, we hypothesize that the decreased levels of mLARK upon the application of si*Lark*-B (Fig. 6*I*) could lead to the decreased level of mPER1 and shorten the period length, whereas the increased level of mLARK1 up-regulated the mPER1 protein level (Fig. 3) and lengthened the rhythm in NIH-PL cells.

Although our analysis of mLARK function was done in NIH 3T3 cells, there is abundant evidence that this system is a good correlate for circadian control in general (41, 42). For example, we also tested several other kinds of siRNAs directed against clock genes (*Bmal*, *Cry1*, and *Cry2*) in our NIH-PL cells and observed changes in rhythmicity that correspond closely to reported phenotypes of mutant mice (although the effects of the siRNAs were somewhat milder) (43, 44). The effect of siRNA against *Lark*s was subtle, but statistically significant, and the changes in circadian period in these cells strongly suggest that LARK is important in maintaining normal 24-h rhythmicity in the mammalian clock system. Future analysis of mutant mice lacking the *Lark* genes will help to further clarify the role of LARKs in the regulation of clock gene expression.

We propose that m*Lark* is a novel clock gene that regulates circadian gene expression at a posttranscriptional level. Identification of this regulatory mechanism begins to elucidate the role of posttranscriptional regulation within mammalian circadian clocks. In addition to m*Per1*, it is likely that m*Lark* regulates other genes (perhaps even other core clock genes), and the identification of such genes will be important for understanding the molecular function of mLARK and the role it plays in the circadian system.

Materials and Methods

Isolation and Sequencing of mLarks. m*Lark1* and *Lark2* were isolated from the mouse brain Marathon-Ready cDNA library (Clontech, Mountain View, CA) by a PCR technique. Primer sequences are in *SI Text*. The amplified DNA fragment was cloned into pCI-neo (Promega, Madison, WI) and verified by sequencing.

Animals and Histochemistry. Male BALB/c mice (JACJO, Osaka, Japan) were housed in 12-h light (300 lux)/12-h dark (LD) cycles from 2 to 4 weeks, then subjected to complete darkness (DD) as a continuation of the dark phase of the last LD cycle. For a light-pulse experiment, mice were exposed to a fluorescent light stimulus (600 lux, 30 min) at CT16. *In situ* hybridization and immunohistochemistry was performed as described (28, 45).

Transient Expression Assay. Mouse NIH 3T3 cells (JCBR0615) were obtained from the Health Science Research Resources Bank (Tokyo, Japan), and luciferase assay and real-time RT-PCR analysis were performed as described (18). Construction of pPL3(m4) and bicistronic vectors is described in *SI Text*.

Protein and mRNA Expression Analysis. Polyclonal antisera were raised in rabbits by using a synthetic peptide (MARYEREQY-ADRARYSAF) corresponding to amino acids residues 344–361 of mLARK1. Antisera were purified by using a Mab Trap kit (Amersham, Piscataway, NJ). Other antibodies were commercially available (α*PER1*; ABR; α-Tubulin; Sigma, St. Louis, MO). For Northern blot analysis poly(A)$^+$ RNAs were extracted with a QuickPrep Micro mRNA Purification kit (Amersham). Primers that were used to make probes are described in *SI Text*.

RMSA. Histidine (His)-tagged mLARK was partially purified with the PROEX HT Prokaryotic Expression System (Gibco/BRL, Gaithersburg, MD). m*Per1* 3′ UTR RNA fragments were chemically synthesized (JBios, Saitama, Japan), and RMSAs were performed as described (46) with slight modifications. In a competition assay, an anti-LARK antibody, an anti-c-MYC antibody (Roche, Indianapolis, IN) (0, 1, 2, 4, and 8 μg), or heat-treated nonlabeled RNA fragments (0, 10, 20, 40, and 100 pmol) were preincubated with His-mLARK1 at 30°C for 30 min before the addition of radiolabeled RNA.

***In Vivo* Binding Assay.** Cells were lysed in lysis-binding buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA/150 mM NaCl/10 mM $MgCl_2$/0.05% Nonidet P-40). The lysed samples were collected and UV-irradiated (Funakoshi, Tokyo, Japan) for 15 min. The RNA–protein complexes were immunoprecipitated with the anti-LARK antibody or rabbit IgG (Sigma)–protein G Sepharose 4 fast flow (Amersham), and RNAs were extracted with TRIzol (Gibco/BRL) and reverse-transcribed by using SuperScript II (Gibco/BRL). PCR conditions are described in *SI Text*.

Functional Analysis of mLark Using siRNAs. Stable transfectants of m*Per1*::*luc* reporter gene (pPLS) in NIH 3T3 cells were generated by selecting cells with G418 (Promega), and these reporter cells (designed as NIH-PL cells) were cultured in a 35-mm Petri dish with 2 ml of DMEM supplemented with 10% FBS. The siRNA transfection prodcedure and the sequences are described in *SI Text*. Then bioluminescence was measured with a photomultiplier tube detector assemblies (Hamamatsu, Ichinocho, Japan). Origin 7.0 software (OriginLab, Northampton, MA) was used to calculate of the period length of bioluminescent oscillation.

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