Essential Role of 3′-Untranslated Region-mediated mRNA Decay in Circadian Oscillations of Mouse Period3 mRNA

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Daily oscillations in mRNA levels are a general feature of most clock genes. Although mRNA oscillations largely depend on transcriptional regulation, it has been suggested that post-transcriptional controls also contribute to mRNA oscillations in Drosophila. Currently, however, there is no direct evidence for post-transcriptional regulation of mammalian clock genes. To investigate the roles of post-transcriptional regulations, we focused on the 3′-untranslated region (3′-UTR) of mouse Period3 (mPer3) mRNA, one of the clock genes. Insertion of the entire mPer3 3′-UTR downstream of a reporter gene resulted in a dramatic decrease in mRNA stability. Deletion and point mutation analyses led to the identification of critical sequences responsible for mRNA decay. To explore the effects of the mPer3 3′-UTR-mediated mRNA decay on circadian oscillations, we established NIH3T3 stable cell lines that express luciferase mRNA with wild-type or mutant mPer3 3′-UTR. Interestingly, a stabilizing mutation of 3′-UTR induced a significant alteration in the oscillation profile of luciferase mRNA. Above all, the peak time, during which the mRNAs reached their highest levels, was significantly delayed (for 12 h). In addition, the luciferase mRNA level with mutant 3′-UTR began to increase earlier than that in the presence of wild-type 3′-UTR. Consequently, luciferase mRNA with mutant 3′-UTR displayed oscillation patterns with a prolonged rising phase. Our results indicate that mPer3 3′-UTR-mediated mRNA decay plays an essential role in mRNA cycling and provide direct evidence for post-transcriptional control of circadian mRNA oscillations.

Circadian rhythms, defined as daily behavioral and physiological oscillations, have been observed in a variety of organisms. These oscillations are driven by a self-sustained time-keeping system, the intracellular clock (1, 2). Over the past several years, great effort has been focused on understanding the molecular mechanisms that underlie the circadian clock and indispensable components, called clock genes, have been elucidated (3–8). In addition, it was revealed that the intracellular molecular clock consists of interacting positive and negative transcriptional and translational feedback loops that involve these components (9–12).

Daily oscillations in protein and/or mRNA levels are central features of clock genes (1, 12). As for the underlying mechanism of mRNA cycling, a number of studies have revealed that mRNA oscillations largely depend on transcriptional regulation (10, 13–17). In Drosophila, nevertheless, it has been suggested that post-transcriptional controls also contribute to mRNA oscillations. First, despite the absence of rhythmic transcription, period (per) mRNA showed circadian cycling in transgenic flies carrying promoter-less per gene (18). Second, results of a nuclear run-on assay suggested that post-transcriptional regulation is responsible for mRNA cycling (19).

In mammals, however, much less is known about the relevance of post-transcriptional control to the circadian oscillations of clock gene mRNAs. By computational modeling, it was demonstrated that circadian clocks depend on mRNA stability (20). These authors proposed that the changes in mRNA stability accounted for the time lag between mRNA and protein levels. In the case of the mouse Period1 (mPer1) gene, it was reported that the 3′-UTR of mPer1 mRNA repressed its own expression and that this translational repression could be involved in the generation of the same time lag (i.e. the time lag between mRNA and protein levels) (21). Currently, however, there is no experimental evidence for the involvement of post-transcriptional regulation in the circadian mRNA cycling of clock genes.

To investigate the role of post-transcriptional regulation in circadian mRNA cycling, we focused on the 3′-UTR of mouse Period3 (mPer3) mRNA, one of the mammalian clock genes that shows typical mRNA oscillations (22, 23). To obtain direct evidence for the role of the 3′-UTR, we established NIH3T3 stable cell lines that each express luciferase mRNA with wild-type or mutant mPer3 3′-UTR. Previous studies have shown that circadian clocks exist even in cultured fibroblasts and that dexamethasone or serum shock can synchronize the circadian expression of a variety of clock genes (24–26). Since then, this cell system has been used as an experimental model to
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examine the molecular mechanisms of the mammalian circadian clock (27–29). In the present study, we demonstrate that the mPer3 3′-UTR regulates mRNA stability and that 3′-UTR-mediated mRNA decay plays an important role in circadian mRNA cycling.

EXPERIMENTAL PROCEDURES

3′-Rapid Amplification of cDNA Ends (3′-RACE)—3′-RACE was performed with mouse suprachiasmatic nuclei cDNAs as templates. The sequences of the mPer3 specific forward primers were as follows: F1, 5′-TCATGGGATGTGTTGGCCCGG-3′ and F2, 5′-CGGAATTCCTTGAACACATATACTGTTT-3′. The sequences of the reverse (dT)17-adaptor primer was 5′-GCTCTAGATTCCACTTGACACATATACTGTGT-3′. Putative poly(A) additional signals are shown in Fig. 3A. Numbering begins at the first nucleotide, following the stop codon. The mPer3 3′-UTR was deposited in GeneBankTM with accession number DQ196346.

FIGURE 1. RNA sequence of mouse Period 3′-UTR. The first three nucleotides (boxed) represent the translation stop codon. Putative poly(A) additional signals are underlined. The boundaries of the deleted constructs, shown in Fig. 2A, are indicated by solid triangles. The asterisks represent the mutation sites of each of the mutant constructs, shown in Fig. 2B. Numbering begins at the first nucleotide, following the stop codon. The mPer3 3′-UTR sequence is indicated by underlined nucleotides following the stop codon. The mPer3 3′-UTR sequence is indicated by underlined nucleotides following the stop codon. The mPer3 3′-UTR sequence is indicated by underlined nucleotides following the stop codon. The mPer3 3′-UTR sequence is indicated by underlined nucleotides following the stop codon. The mPer3 3′-UTR sequence is indicated by underlined nucleotides following the stop codon. The mPer3 3′-UTR sequence is indicated by underlined nucleotides following the stop codon.
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(5'-GCTCTAGAGCAGTGGTTTGGCAAGAGGAA-3') and reverse pri-mer (5'-CAGGTCAGCGGATCCAGCAAAAGC-3'). Following XbaI/Sall digestion, the fragment was cloned into XbaI/Sall sites of D-box-Luc/3'-UTR vector. For D-box-Luc/SDM-3'-UTR/Neo vector, mutant 3'-UTR (SDM-A) was used instead of wild-type mPer3 3'-UTR.

Cell Culture—HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C. CHO-K1 cells were cultured in DMEM/nutrient mixture F-12 (DMEM/F-12) supplemented with 10% bovine calf serum and 1% penicillin-streptomycin. NIH3T3 cells were cultured in DMEM supplemented with 10% bovine calf serum and 1% penicillin-streptomycin.

Transient Transfection—HEK 293T cells were seeded in 6-well plates at a density of 2 × 10^5 cells per well 1 day prior to transfection. Transfection was carried out using Metafectene (Biontex) according to the manufacturer's instructions. After incubation for 17 h, cells were harvested or further incubated with 5 μg of actinomycin D/ml for 6 h before harvest. For reporter mRNA decay kinetics, CHO-K1 cells were transiently co-transfected with 0.5 μg of pcNAT control vector and 2.5 μg of reporter plasmid (pcNAT/1–2220, pcNAT/1–2220(SDM-A), or pcNAT/1–2220(SDM-B)) by the electroporation method (30) at room temperature. After incubation for 5 h, cells were harvested or further incubated for indicated time with 5 μg of actinomycin D/ml before harvest.

Generation of Stable Cell Lines and Dexamethasone Shock Procedure—To generate NIH-Luc-WT 3'-UTR and NIH-Luc-SDM 3'-UTR cell lines, D-box-Luc/WT-3'-UTR/Neo vector, and D-box-Luc/SDM-3'-UTR/Neo vector were introduced into NIH3T3 fibroblasts by calcium phosphate precipitation method, respectively. After 2 days, we started selection with 800 μg of G418 (Invitrogen)/ml. Neomycin-resistant clones were isolated by standard procedure. The resulting cell lines were maintained in DMEM supplemented with 10% bovine calf serum, 1% penicillin-streptomycin, and 200 μg of G418/ml.

The dexamethasone shock was done as described previously, with minor modifications (24). In brief, ~1.5 × 10^5 cells were seeded in each well of 12-well plate. When the cells reached confluence, the medium was exchanged with 100 ml dexamethasone containing medium. After 2 h, this medium was replaced with complete medium. At the indicated time, media were harvested and kept at ~70°C until total RNA was extracted from the cells.

Northern Blot Analysis—Northern blot analysis was carried out as described previously (30). In brief, total RNA was isolated by using TRI Reagent (Molecular Research Center). RNA was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (Roche Applied Science) according to the manufacturer's instructions. For detection and quantification, MyiQ™ real-time PCR detection system (Bio-Rad) was used. The sequences of the forward and reverse primers were as follows: endogenous mPer3, 5'-TTGTCAAGGTGGGGCCT- TCTCT-3' and 5'-GGCAGCTCATGAGGTGAG-3'; luciferase, 5'-GAGGTTCATCTGCGAGTGA-3' and 5'-C- ACACATGTTGGCCTTATGGA-3'; TATA-binding protein (TBP), 5'-CAGGCCTTCACTTATGCTC-3' and 5'-TTGCTGCTGCTTCTTGGTT-3'.

 RESULTS

The mPer3 3’-UTR Regulates mRNA Stability—We cloned the 3’-UTR of mPer3 mRNA by 3’-RACE. Sequence analysis of the cloned cDNA showed that the mPer3 3’-UTR was composed of 2,220 nucleotides, contained a peculiar, U-rich region in the middle of the 3’-UTR, and included three putative poly(A) signals (Fig. 1). When the mPer3 3’-UTR sequence was aligned in the corresponding loci of the genome, the sequence agreed with the mouse genome sequence (data not shown). The
mPer3 3'-UTR sequence was deposited in GeneBank™ with accession number DQ196346.

To examine the function of the mPer3 3'-UTR, a reporter construct (pcNAT/1–2220) was created by inserting the mPer3 3'-UTR downstream from the AANAT-coding sequence of the pcNAT control vector (30) (Fig. 2A). HEK 293T cells were transiently transfected with the pcNAT/1–2220 reporter or the pcNAT control vector and treated with actinomycin D for the indicated times to block new transcription. The effects of the mPer3 3'-UTR were determined by Northern blot analysis. As shown in Fig. 2B, the reporter mRNA with the mPer3 3'-UTR insertion (right panel) was rapidly degraded, whereas control mRNA lacking the mPer3 3'-UTR insertion (left panel) was sufficiently stable to maintain consistent mRNA levels, even after 6 h of actinomycin D treatment. This result suggests that the mPer3 3'-UTR mediates mRNA degradation.

**Deletion and Mutational Analyses of a Critical cis-Acting Element**—To identify the region critical for mRNA degradation, various reporter constructs containing serially deleted fragments of the mPer3 3'-UTR were generated (Fig. 2A). The effects of these 3'-UTR fragments were analyzed by Northern blot analysis. Compared with the full-length 3'-UTR (pcNAT/1–2220), truncation of the distal region of the 3'-UTR to nucleotides 1164, 741, or 561 had no significant effect on mRNA stability (Fig. 3A, see pcNAT/1–1164, pcNAT/1–741, and pcNAT/1–561, respectively). Interestingly, the additional removal of nucleotides 382–561 caused a dramatic increase in mRNA stability (see pcNAT/1–381). Greater than 80% of the initial mRNA amount was present even after 6 h of actinomycin D treatment. To address whether the fragment 382–561 itself shows mRNA decay activity, we generated pcNAT/382–561 reporter construct and analyzed the effect on mRNA stability (Fig. 3A, right panel). Although the effect was weaker than that of full-length 3'-UTR (1–2220), the fragment 382–561 still showed mRNA decay activity. That is, more than 40% of the initial mRNA was degraded after 6 h of actinomycin D treatment.

We then sought to identify critical sequences within nucleotides 382–561. Since most of the protein bindings against mPer3 3'-UTR were competed out by homopolymeric poly(U) nucleotides (data not shown), we decided to mutate U-rich sequences (Fig. 3B). The effects of these mutants on mRNA stability were assessed by Northern blot analysis (Fig. 3B). The results indicate that the mPer3 3'-UTR mediates mRNA degradation.

**Effect of mPer3 3'-UTR Fragments on mRNA Stability**

We then sought to identify critical sequences within nucleotides 382–561. Since most of the protein bindings against mPer3 3'-UTR were competed out by homopolymeric poly(U) nucleotides (data not shown), we decided to mutate U-rich sequences between nucleotides 382 and 561. We generated two mutant reporter constructs, pcNAT/1–2220(SDM-A) and pcNAT/1–2220(SDM-B), in which four and six nucleotides of the mPer3 3'-UTR were substituted with cytidines, respectively (Fig. 3B).

**Deletion and mutational analysis of a critical cis-acting element**—To identify the region critical for mRNA degradation, various reporter constructs containing serially deleted fragments of the mPer3 3'-UTR were generated (Fig. 2A). The effects of these 3'-UTR fragments were analyzed by Northern blot analysis. Compared with the full-length 3'-UTR (pcNAT/1–2220), truncation of the distal region of the 3'-UTR to nucleotides 1164, 741, or 561 had no significant effect on mRNA stability (Fig. 3A, see pcNAT/1–1164, pcNAT/1–741, and pcNAT/1–561, respectively). Interestingly, the additional removal of nucleotides 382–561 caused a dramatic increase in mRNA stability (see pcNAT/1–381). Greater than 80% of the initial mRNA amount was present even after 6 h of actinomycin D treatment. To address whether the fragment 382–561 itself shows mRNA decay activity, we generated pcNAT/382–561 reporter construct and analyzed the effect on mRNA stability (Fig. 3A, right panel). Although the effect was weaker than that of full-length 3'-UTR (1–2220), the fragment 382–561 still showed mRNA decay activity. That is, more than 40% of the initial mRNA was degraded after 6 h of actinomycin D treatment.

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To investigate the effects of these mutations, we examined the kinetics of reporter mRNA degradation by actinomycin D chase experiment and Northern blot analysis (Fig. 3, B and C). Consistent with the results shown in Fig. 2B, wild-type reporter mRNA was rapidly degraded ($t_{1/2}$ of ~3 h). However, SDM-A mutant mRNA was significantly stable ($t_{1/2}$ > 8 h), maintaining 58% of its initial levels even after 8 h of actinomycin D treatment. These results demonstrate that the mutated sequences in the SDM-A reporter comprise a cis-acting element important for mRNA decay.

Interestingly, despite the existence of overlapping mutation sites, SDM-B mutant mRNA showed completely different degradation kinetics from that of SDM-A mutant mRNA. The SDM-B mutant mRNA was rapidly degraded ($t_{1/2}$ of ~3.5 h) with a decay rate similar to that of wild-type reporter mRNA.

mPer3 3’-UTR-mediated mRNA Decay Contributes to Circadian mRNA Oscillations—Before analyzing the effects of the mPer3 3’-UTR on circadian mRNA oscillations, we first examined the circadian oscillation profile of endogenous mPer3 mRNA in NIH3T3 mouse fibroblasts. After dexamethasone shock, mPer3 mRNA showed robust circadian rhythms (Fig. 4A). The mRNA levels rapidly decreased immediately after dexamethasone shock and began to increase after 18 h. After reaching their peak level at 30 h, mRNA levels began to decline again.

To explore the roles of mPer3 3’-UTR-mediated mRNA decay in circadian oscillations, we established NIH3T3 stable cell lines that express luciferase mRNA with wild-type or SDM-A mutant mPer3 3’-UTR and designated them NIH-Luc-WT 3’-UTR or NIH-Luc-SDM 3’-UTR cell lines, respectively (Fig. 4B). The expression of the luciferase reporters was regulated by D-box elements of the mPer3 promoter, which were previously verified to be sufficient for the generation of circadian oscillations (10).

As shown in Fig. 4C, luciferase mRNAs with wild-type mPer3...
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3′-UTR showed similar oscillation profiles to endogenous mPer3 mRNA. The levels of luciferase mRNA began to increase after 18 h of dexamethasone shock. After reaching their peak levels at 30 h, mRNA levels started to decline. We confirmed these oscillation profiles in two different NIH-Luc-WT 3′-UTR cell lines (i.e. #1 and #9). Interestingly, the SDM-A mutation of the mPer3 3′-UTR led to dramatic alterations in these oscillation patterns (Fig. 4D, #3 and #4). Above all, the peak time, during which the mRNAs reached their highest levels, was significantly delayed (for 12 h). In addition, the mRNA levels began to increase earlier than that with wild-type 3′-UTR, and the rising phase continued until 42 h. Consequently, luciferase mRNAs with mutant 3′-UTR showed oscillation patterns with a prolonged rising phase. Based on these results, we concluded that the mPer3 3′-UTR-mediated mRNA decay plays an essential role in mRNA cycling.

DISCUSSION

Most clock genes show circadian oscillations in protein and/or mRNA levels (1, 12). Although protein oscillations can be generated without mRNA cycling (32), there is no doubt that the precision of the protein rhythm is accomplished by appropriate control of the mRNA levels (33).

In this study, we sought to obtain direct evidence for the relevance of post-transcriptional regulation to the mRNA oscillations of mammalian clock genes. To study rhythmic regulation of mPer3 mRNA levels, we focused on the function of the 3′-UTR. The mPer3 3′-UTR induced dramatic mRNA decay when inserted immediately downstream from a reporter gene. We cannot exclude the possibility that the mPer3 3′-UTR plays additional roles in other post-transcriptional events, such as splicing or translation. Nevertheless, it is obvious that the 3′-UTR modulates mRNA stability since, despite similar initial levels, reporter mRNA that is fused to the mPer3 3′-UTR (WT) is more rapidly degraded than is NAT control mRNA (Fig. 3B, upper panel).

Although it is clear that the mutated sequences in the SDM-A mutant are important for mRNA decay, it should be noted that the mPer3 3′-UTR could have additional cis-acting elements for the regulation of mRNA stability, given that SDM-A mutant mRNA is still more unstable than NAT control mRNA (Fig. 3B, middle panel, and C). Furthermore, even when nucleotides 1–1164 were deleted, the remaining 3′-UTR exhibited mRNA decay activity, suggesting that nucleotides 1165–2220 contain additional cis-acting elements involved in mRNA stability (data not shown).

The relevance of the 3′-UTR to circadian behavior was previously studied in the Drosophila per gene (33). Replacement of the per 3′-UTR with the tubulin 3′-UTR altered the accumulation profile of per mRNA. Interestingly, most of the per 3′-UTR could be deleted without severely altering the circadian rhythm; the authors suggested that the regulatory elements for post-transcriptional control may be located in a different region of the per mRNA. Supporting this idea, Stanewsky et al. (34) revealed that the per 5′-UTR contains a regulatory element required for post-transcriptional control, although they did not demonstrate which post-transcriptional events are regulated by the per 5′-UTR. Our results suggest that, in contrast to the Drosophila per gene, mPer3 3′-UTR plays an essential role in circadian mRNA oscillations. In view of the finding that a stabilizing mutation of 3′-UTR induced dramatic alterations in mRNA cycling, we propose that precise regulation of mRNA stability is essential for the control of mPer3 mRNA oscillations.

Although the oscillation profile of the luciferase mRNA with wild-type 3′-UTR was similar to that of endogenous mPer3 mRNA, it should be noted that the two profiles were not identical. First, this discrepancy could be the result of a different promoter structure. In our study, transcription of luciferase mRNA is driven by a D-box fused SV40 promoter. While the D-box is sufficient for circadian oscillations (10), we cannot exclude the possibility that additional regulatory sequences are required for precise control of transcription. Second, it is possible that the 5′-UTR or coding region of mPer3 mRNA contributes to mRNA cycling. As mentioned above, 5′-UTR-mediated post-transcriptional regulation is involved in circadian mRNA oscillations of the Drosophila per gene. In this respect, it will be interesting to explore the contribution of the mPer3 5′-UTR to post-transcriptional regulation as well as to circadian mRNA oscillations.

In conclusion, our study demonstrated that the mPer3 3′-UTR-mediated mRNA degradation and that the stabilizing mutation of the mPer3 3′-UTR caused a dramatic change in the oscillations of reporter mRNA. Based on these results, we propose that 3′-UTR-mediated mRNA decay plays an essential role in the circadian oscillations of mPer3 mRNA.

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