Supporting Information

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SI Materials and Methods

Proteins. Recombinant KaiA was expressed as described in Iwasaki et al. (1) and purified according to a previously described method (2). Recombinant KaiB was expressed and purified as described previously (1, 3, 4). Full-length KaiC and KaiC-CI, in which the CII domain is truncated, were expressed and purified as hexamers according to the previously described methods (1, 2, 4, 5). Protein concentrations were determined by the Bradford method using BSA as the standard.

Reconstitution of Circadian Oscillation in Vitro. The phosphorylation rhythm of KaiC was reconstituted and detected as previously reported (6), with slight modifications. Briefly, 0.5 mg/mL KaiC was incubated at 30 °C in the presence of 0.1 mg/mL KaiA, 0.1 mg/mL KaiB, and 2 mM ATP in MD-2 buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, and 2 mM DTT). Aliquots of the reaction mixture were taken every 4 h and subjected to SDS/PAGE; gels were stained with Coomassie brilliant blue (CBB) staining. The amount of phosphorylated KaiC relative to total KaiC was plotted against time.

Detection and Quantitation of Nucleotides Bound to KaiC Containing Mutations at the Phosphorylation Sites. KaiC-AA and KaiC-DE were expressed and purified as previously described (1, 2, 4, 5). In KaiC-AA, S431 and T432 are replaced with alanine; in KaiC-DE, S431 and T432 are replaced with aspartate and glutamate, respectively. These proteins were monomerized, and the nucleotide-bound state was analyzed as described in *Materials and Methods*.

Chase of Prebound Nucleotides on KaiC Hexamers in the Presence of KaiA and KaiB. KaiC hexamers (1.0 mg/ ml) were prepared from monomers in the presence of 2 mM [α-^{32}P]ATP (3.0 GBq/mmol), and then incubated at 30 °C with KaiA (0.2 mg/mL) and KaiB (0.2 mg/mL). After 4 or 8 h of incubation, unbound radioactive nucleotides were removed using Micro Bio-Spin P-30 columns (BioRad) equilibrated with MD-2 buffer supplemented with 2 mM ATP. The eluate was further incubated at 30 °C, and aliquots were collected at the indicated time points and passed twice through Micro Bio-Spin P-30 columns (BioRad) to remove unbound nucleotides. The signals from the KaiC-bound ^{32}P-labeled nucleotides were quantitated as described in *Materials and Methods*.

Nonlinear Regression Analyses. The data shown in Fig. 2*A* were subjected to nonlinear regression analyses to determine the kinetic parameters. We modeled the process of ATP hydrolysis, followed by the release of the resultant ADP, in the absence of KaiA, and we fit the data presented in Fig. 2*A* to the rate equations derived from Eqs. **S1**–**S3**. We assumed that KaiC contains two ATPase active sites: site 1 and site 2. ADP bound to site 1, generated by the hydrolysis of ATP, is released immediately after the reaction completed. k_1 is the overall rate constant of the reaction, including ATP hydrolysis and the release of ADP

$$\text{Site } 1 \cdot \text{ATP} \xrightarrow{k_1} \text{Site } 1 + \text{ADP}. \qquad \textbf{[S1]}$$

We hypothesized that the ATPase cycle at site 2 is paused before ADP is released. k_2 is the rate constant for the hydrolysis of ATP, and k_3 is the rate constant for the release of ADP

$$\text{Site } 2 \cdot \text{ATP} \xrightarrow{k_2} \text{Site } 2 + \text{ADP} \qquad \textbf{[S2]}$$

$$\text{Site } 2 \cdot \text{ADP} \xrightarrow{k_3} \text{Site } 2 + \text{ADP}. \qquad \textbf{[S3]}$$

We performed analyses using Global Kinetic Explorer (7), a program that allows for the numeric integration of rate equations and the fitting of experimental data based on nonlinear regression analysis.

1. Iwasaki H, Taniguchi Y, Ishiura M, Kondo T (1999) Physical interactions among circadian clock proteins KaiA, KaiB and KaiC in cyanobacteria. *EMBO J* 18(5):1137–1145.
2. Nishiwaki T, et al. (2004) Role of KaiC phosphorylation in the circadian clock system of *Synechococcus elongatus* PCC 7942. *Proc Natl Acad Sci USA* 101(38):13927–13932.
3. Iwasaki H, Nishiwaki T, Kitayama Y, Nakajima M, Kondo T (2002) KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria. *Proc Natl Acad Sci USA* 99(24):15788–15793.
4. Nishiwaki T, et al. (2007) A sequential program of dual phosphorylation of KaiC as a basis for circadian rhythm in cyanobacteria. *EMBO J* 26(17):4029–4037.
5. Terauchi K, et al. (2007) ATPase activity of KaiC determines the basic timing for circadian clock of cyanobacteria. *Proc Natl Acad Sci USA* 104(41):16377–16381.
6. Nakajima M, et al. (2005) Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science* 308(5720):414–415.
7. Johnson KA, Simpson ZB, Blom T (2009) Global kinetic explorer: A new computer program for dynamic simulation and fitting of kinetic data. *Anal Biochem* 387(1):20–29.

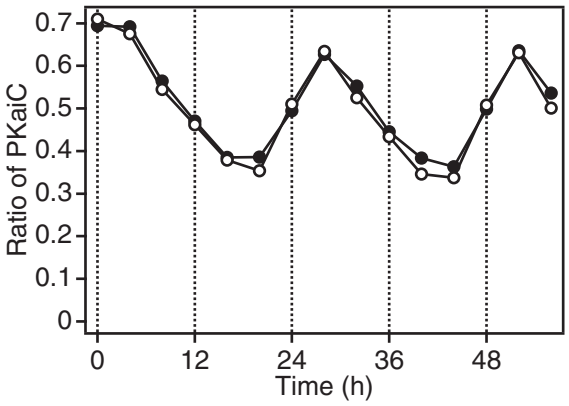

Fig. S1. KaiC phosphorylation rhythms observed from the two separate experiments shown in Fig. 3. The open and the closed circles represent phosphorylation rhythms observed in the experiments shown in Fig. 3 *A* and *B*, respectively. These two rhythms were not significantly different.

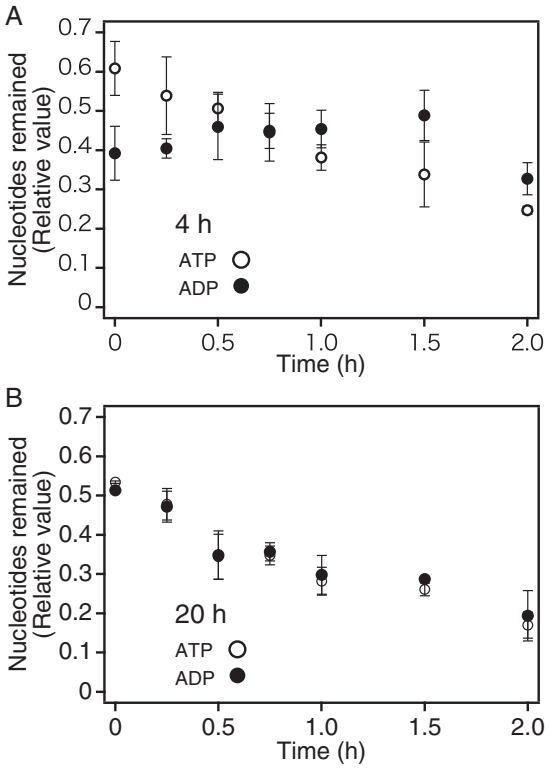

Fig. S2. Diurnal changes in the release of nucleotides prebound to KaiC. To test whether the release of KaiC-bound nucleotides exhibit diurnal changes, we reconstituted KaiC phosphorylation rhythm and chased prebound radioactive nucleotides with nonradioactive ATP, starting from the two different time points. KaiC hexamers (1.0 mg/mL) were prepared from monomers in the presence of 2 mM [α-^{32}P]ATP, KaiA (0.2 mg/mL) and KaiB (0.2 mg/mL) were added, and the mixture was incubated at 30 °C. After 4 (*A*) or 20 h (*B*), unbound radioactive nucleotide was exchanged with nonradioactive ATP, and the reaction mixture was further incubated for 2 h at 30 °C. At the indicated time points, aliquots were removed, and KaiC-bound radioactive signals were quantitated. The amount of KaiC-bound radioactive ATP (○) and ADP (●) is expressed relative to the total radioactive nucleotides initially bound to KaiC. The data represent the means ± SD from three experiments.

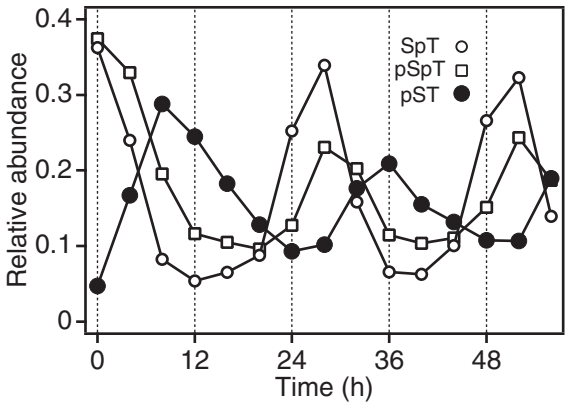

Fig. S3. Accumulation profiles of different phosphorylation forms of KaiC. The samples prepared in the experiment shown in Fig. 3*A* were subjected to long distance SDS/PAGE to separate different phosphorylation forms of KaiC. Open circles, closed circles, and open squares represent T432-phosphorylated (SpT), S431-phosphorylated (pST), and doubly phosphorylated (pSpT) forms of KaiC, respectively. The ATP incorporation profile (Fig. 3*A*) was in antiphase with that of the accumulation of S431-phosphorylated KaiC.

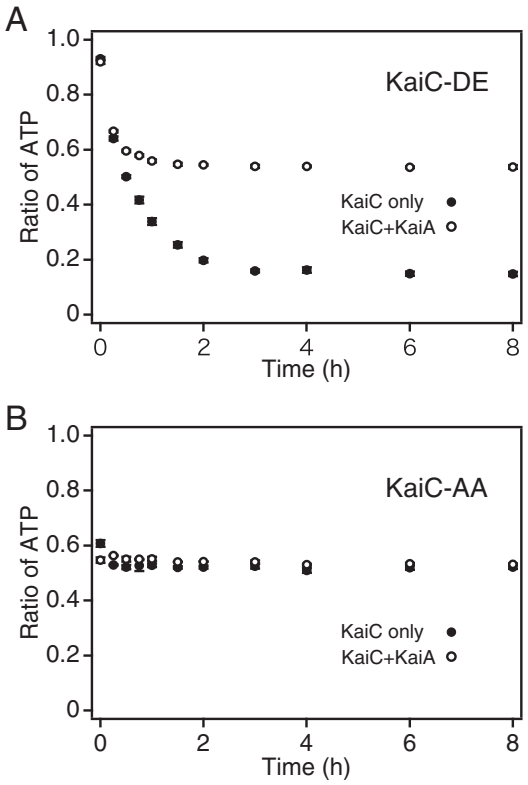

Fig. S4. Nucleotide-bound states of KaiC proteins containing mutations in the phosphorylation sites. The nucleotide-bound states of KaiC-DE (*A*) and KaiC-AA (*B*), which, respectively, mimic constitutively phosphorylated and dephosphorylated KaiC, were monitored in the same manner as for WT KaiC (*Materials and Methods*). In KaiC-DE, S431 and T432 are replaced with aspartate and glutamate, respectively. In KaiC-AA, S431 and T432 are replaced with alanine. The data were plotted as the ratio of ATP to total bound nucleotides in the presence (○) or absence (●) of KaiA. The data represent the means ± SD from three experiments.