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A bioluminescence resonance energy transfer (BRET) system: Application to interacting circadian clock proteins

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ABSTRACT **We describe a method for assaying protein interactions that offers some attractive advantages over previous assays. This method, called bioluminescence resonance energy transfer (BRET), uses a bioluminescent luciferase that is genetically fused to one candidate protein, and a green fluorescent protein mutant fused to another protein of interest. Interactions between the two fusion proteins can bring the luciferase and green fluorescent protein close enough for resonance energy transfer to occur, thus changing the color of the bioluminescent emission. By using proteins encoded by circadian (daily) clock genes from cyanobacteria, we use the BRET technique to demonstrate that the clock protein KaiB interacts to form homodimers. BRET should be particularly useful for testing protein interactions within native cells, especially with integral membrane proteins or proteins targeted to specific organelles.**

Interactions between proteins play a role in many biological processes. Current techniques to identify and characterize these interactions include *in vitro*-binding assays, library-based methods, and genetic methods (1). In this report, we introduce a method for assaying protein–protein interactions that takes advantage of a phenomenon that occurs in nature, namely, the Förster resonance energy transfer between a light-emitting luciferase and an acceptor fluorophore (2–5). The technique is related to an existing method for assessing protein–protein interaction, fluorescence resonance energy transfer (FRET). In this process, one fluorophore (the "donor") transfers its excited-state energy to another fluorophore (the "acceptor"), which usually emits fluorescence of a different color. FRET efficiency depends on the spectral overlap, the relative orientation, and the distance between the donor and acceptor fluorophores. Generally, FRET occurs when the donor and acceptor are 10–100 Å apart (4), so it can be used to assay protein–protein proximity by attaching the donor and acceptor fluorophores to the candidate proteins. By using mutants of the green fluorescent protein (GFP; M_r = 27 kDa), it is possible to genetically attach donor and acceptor fluorophores to proteins (6–8). This GFP-based FRET assay allows protein interactions to be observed in the native organism under physiological conditions (9, 10). Moreover, compartmentalization of these interacting proteins is visible in the microscope (9, 10).

As with any fluorescence technique, however, photobleaching and autofluorescence can limit the usefulness of FRET. It also can be complicated by direct excitation of the acceptor fluorophore. Furthermore, FRET may be impractical in tissues that are easily damaged by the excitation light or that are photoresponsive (e.g., retina). Our protein interaction assay, which we call bioluminescence resonance energy transfer (BRET), offers the advantages of FRET but avoids the consequences of fluorescence excitation. BRET is a naturally occurring phenomenon. For instance, when the photoprotein aequorin is purified from the jellyfish, *Aequorea,* it emits blue light in the absence of GFP, but when GFP and aequorin are associated as they are *in vivo*, GFP accepts the energy from aequorin and emits green light (2, 5). In BRET, the donor fluorophore of the FRET technique is replaced by a luciferase. In the presence of a substrate, bioluminescence from the luciferase excites the acceptor fluorophore through the same Förster resonance energy transfer mechanisms described above. We show that by choosing the proper luciferase/GFP mutant combination, BRET can be used to measure protein interactions both *in vivo* and *in vitro*.

We have applied the BRET method to assay interactions between proteins encoded by the circadian clock genes *kaiA* and *kaiB* from the cyanobacterium *Synechococcus* sp. strain PCC 7942 (11, 12). We found that the protein encoded by the *kaiB* gene self associates to form homodimers. In eukaryotes, several recent investigations report that protein interactions play key roles in the circadian mechanism (13–18). Other assays of Kai protein interactions (H. Iwasaki, T. Kondo, M. Ishiura, personal communication) and the data reported herein suggest that protein interactions are apparently a characteristic of circadian clock proteins in both prokaryotic cyanobacteria as well as in eukaryotes.

MATERIALS AND METHODS

Construction of RLUC●EYFP Fusion Expression Cassette. Based on the DNA sequence for pRL-null (Promega), two primers were designed for cloning of the *Renilla* luciferase-(RLUC) coding region without its stop codon TAA but with a T7 promoter by using Pwo DNA polymerase (Boehringer Mannheim). The amplified fragment with an *Nde*I and an *Apa*I linker was then inserted into the *Nde*I/*Apa*I site of the vector pEGFP-N1 (CLONTECH) containing an enhanced GFP gene (*Egfp*) to give the plasmid pT7/*Rluc*●*Egfp*. To make the *Rluc*●*Eyfp* gene fusion, the EGFP-coding region in the pT7/*Rluc*●*Egfp* was replaced with the *Bam*HI/*Not*I fragment containing the enhanced yellow fluorescence protein-coding sequence (*Eyfp*) from the vector pEYFP (CLONTECH) to produce the plasmid pT7/*Rluc*●*Eyfp*, in which the EYFP ORF is in frame with that of RLUC. There are 11 codons between *Rluc* and *Eyfp* in this gene fusion, as shown in Fig. 1*A*. To create the pT7/*Rluc* plasmid, the EYFP-coding region was removed

Abbreviations: BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; *Egfp*, DNA coding for enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; *Eyfp*, DNA coding for enhanced yellow fluorescent protein; RLUC, *Renilla* luciferase; *Rluc*, DNA coding for *Renilla* luciferase; KaiB, protein encoded by the *kaiB* gene; LB medium, Luria–Bertani medium; IPTG, isopropyl β-D-thiogalactoside.

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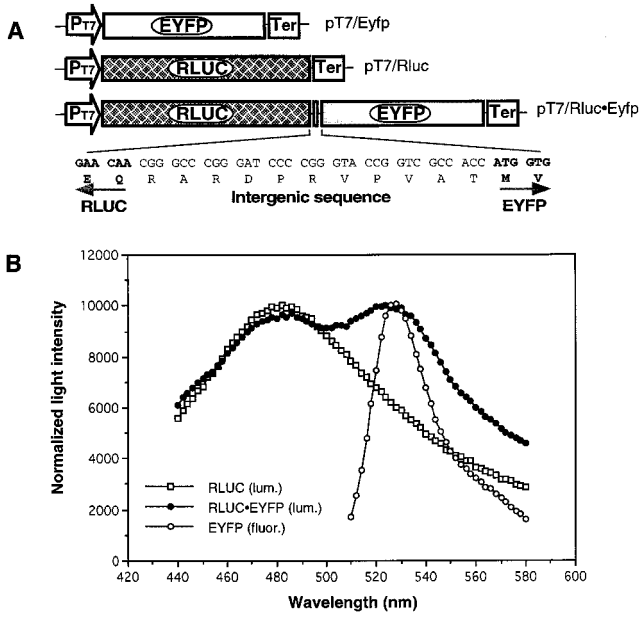

FIG. 1. *In vivo* BRET in *E. coli* cells expressing the RLUC–EYFP fusion protein. (*A*) Diagrams of the expression cassettes of pT7/*Eyfp*, pT7/*Rluc*, and pT7/*Rluc*•*Eyfp*. P_{T7}, T7 promoter region; Ter, transcription terminator region. The intergenic linker sequence of 11 amino acid residues between *Rluc* and *Eyfp* in the *Rluc*•*Eyfp* fusion construct is shown below the pT7/*Rluc*•*Eyfp* diagram. (*B*) Luminescence (lum.) or fluorescence (fluor.) emission spectra from transformed strains expressing the proteins RLUC, EYFP, or the fusion protein RLUC•EYFP. Luminescence reactions were initiated by the addition of 1 μM coelenterazine. All spectra were normalized.

from pT7/*Rluc*•*Eyfp* by digestion with *Bam*HI and *Not*I, end-blunting and self-ligation.

Cloning of Cyanobacterial Clock Protein-Coding Regions. Plasmid p44N carries the clock genes *kai*A, *kai*B, and *kai*C from the cyanobacterium *Synechococcus* sp. strain PCC 7942 (12). We used p44N as the template for cloning *kai*-coding regions. To fuse the coding region sequences for KaiA or KaiB in frame with the His-Tag-binding domain of the pRSET-B vector (Invitrogen), the following two pairs of primers were designed and include the underlined linker sequences: 5′-(*Bgl*II) AGATCT**ATG**CTCTTGGAGTATGC-3′ and 5′-(*Hin*dIII) AAGCTTCGAGCTTAAGACCTCCT-3′ (for cloning of *kai*A); 5′-(*Bgl*II) AGATCT**ATG**AGCCCTCGTAAAACCTAC-3′ and 5′-(*Hin*dIII) AAGCTTGCAGTTAACGACAGTTAGAAG-3′ (for cloning of *kai*B). The resulting *kai*B fragment and a slightly 5′-truncated *kai*A fragment were digested and each cloned into the *Bgl*II/*Hin*dIII site of the expression vector pRSET-B to produce plasmids pHis•*kaiA* and pHis•*kaiB*, respectively.

Fusion of the Coding Sequences for Cyanobacterial Clock Proteins to *Rluc* or *Eyfp*. The *Hin*dIII (blunt end)/*Bam*HI fragment containing the full length *kai*B-coding region from pHis•*kai*B was used to replace the *Not*I (blunt end)/*Bam*HI fragment containing the EYFP-coding region in pT7/*Rluc*•*Eyfp* to produce pT7/*Rluc*•*kaiB*, in which the *kai*B-coding region is in frame with that of RLUC. An EYFP-coding sequence without stop codon was synthesized by designing the following two primers and including the underlined linker sequences: 5′-(*Nde*I) CAT**ATG**GTGAGCAAGGGCGA-3′ and 5′- (*Bgl*II) AGATCT**CTT**GTACAGCTCGTCCA-3′. The EYFP fragment was inserted into the *Nde*I/*Bgl*II sites of pHis•*kaiA* and pHis•*kaiB*, respectively. This insertion removed the His-Tag and enterokinase site sequences of pRSET and produced the two plasmids pT7/*Eyfp*•*kaiA* and pT7/*Eyfp*•*kaiB*. The *kaiB*-coding region in pT7/*Eyfp*•kaiB was removed by digestion with *Bgl*II and *Hpa*I and ligated to make the plasmid pT7/*Eyfp*.

Escherichia coli strain BL21 (DE3) was used as a host strain (Novagen) to express all gene fusions controlled by the T7 promoter. Constructs were designed with either kanamycin or ampicillin resistance to allow cotransformations. All constructs and transformants were confirmed by DNA sequence analysis, luminescence, and/or fluorescence assay, and the correct molecular weights of the fusion proteins also were confirmed.

Bioluminescence and Fluorescence Assay *in Vivo*. *E. coli* strains were grown overnight in Luria–Bertani (LB) medium containing 50 μg/ml of kanamycin and/or 100 μg/ml of carbenicillin (for ampicillin resistant strains) at 37°C. Because LB medium has background fluorescence, the *E. coli* cultures were washed and resuspended in M9 minimal salts medium before assay. To assay EYFP fluorescence, 1.5 ml of *E. coli* cells resuspended in M9 medium (OD_{600} = 0.8–1.0) was transferred to a 2-ml cuvette. Fluorescence excitation and emission measurements were performed on a SPEX Fluorolog spectrofluorometer with a 250 W xenon arc lamp (Edison, NJ). EYFP fluorescence was excited at 470 nm, and its emission was scanned from 505 to 580 nm. For the luminescence assay, the lamp was shuttered off, and the cuvette was gently bubbled with air to supply sufficient oxygen for the *Renilla* luciferase reaction after the addition of coelenterazine (BioSynth, Naperville, IL) to a final concentration of 1 μM. Bioluminescence emission spectra were collected between the wavelengths 440 and 580 nm.

***In Vitro* BRET Assay for KaiB–KaiB Interaction.** Two expression strains, RLUC•KaiB and EYFP•KaiB, were used to examine KaiB–KaiB interaction *in vitro*, and the RLUC•EYFP fusion strain was used as a positive control. A single colony of each strain was inoculated into LB medium with 50 μg/ml of kanamycin or 100 μg/ml of carbenicillin and grown overnight at 37°C. The cells were washed once with fresh LB medium and resuspended in the same medium containing 1 mM isopropyl β-D-thiogalactoside (IPTG). After a 3-hr incubation at 37°C, the cells were collected and washed once in assay buffer (50 mM KCl/50 mM NaCl/2.5 mM $MgCl_2$/2 mM EDTA/5 mM DTT/0.2% Nonidet P-40/100 μg/ml phenylmethylsulfonyl fluoride/2 μg/ml leupeptin/2 μg/ml aprotinin/20 mM Hepes, pH 8.0). The cells were then resuspended in fresh assay buffer at 4°C containing 10 mg/ml of lysozyme and kept on ice for 30 min. Microcentrifuge tubes containing the cells were put into a −70°C ethanol bath to quickly freeze the cells and then were placed on ice. An equal volume of fresh assay buffer was added into the cell lysates. After the mixtures were spun for 5 min in a microcentrifuge at 8,000 rpm (4°C), the supernatants were tested for fluorescence and luminescence as above except without air bubbling. For monitoring KaiB–KaiB interaction *in vitro* by BRET, RLUC•KaiB extracts were mixed 1:1 with EYFP•KaiB extracts and thereafter incubated at room temperature; luminescence spectra were taken at 0 min, 30 min, and 120 min after mixing.

BRET Imaging of *E. coli* Cultures. *E. coli* cultures (1.5 ml) that had been grown overnight at 37°C in LB medium with appropriate antibiotics were washed twice with M9 medium. The cell pellet was resuspended in 300 μl of M9 medium containing 3 μM coelenterazine. Five microliters of the suspension was immediately added to each well of a Nunc-Microwell plate. The small volume of the sample allowed good gas exchange and so bubbling was not required. Eight duplicates were made for each strain, which were then divided into two groups. One group was visualized through a 480-nm ($\pm$5 nm) interference filter and the other through a 530-nm ($\pm$5 nm) filter (Ealing Electro-Optics, Holliston, MA). Images were captured with a cooled-CCD camera (TE/CCD512BKS,

Princeton Instruments, Trenton, NJ) under the control of and analyzed by custom software created by Takao Kondo (19).

RESULTS AND DISCUSSION

Detection and Calibration of BRET. As the donor luciferase, we chose *Renilla* luciferase (RLUC; MW = 35 kDa) (20), because its emission spectrum is similar to the cyan mutant of *Aequorea* GFP ($\lambda_{max} \approx 480$ nm), which has been shown to exhibit FRET with a red-shifted mutant of *Aequorea* GFP, called EYFP (8). The substrate for RLUC, coelenterazine, is a hydrophobic molecule that is able to permeate cell membranes. As the acceptor fluorophore, we used the enhanced yellow mutant of GFP, EYFP (21). In nature, both RLUC and GFP are coupled via BRET to other proteins (a *Renilla* GFP and aequorin, respectively). As described above, *Aequorea* GFP naturally participates in a BRET mechanism with the photoprotein aequorin (2, 5). A similar interaction occurs in the sea pansy, *Renilla*, between the blue-emitting RLUC and *Renilla* GFP (3, 20). Despite the *in situ* oligomerization of aequorin and the GFP of *Aequorea*, and of RLUC and the GFP of *Renilla*, it is important to note that RLUC and EYFP do not naturally interact with each other. Because *Aequorea* and *Renilla* are not closely related, it is unlikely that EYFP and RLUC intrinsically dimerize with each other. However, the spectral overlap between RLUC and EYFP is similar to the spectral overlap of EYFP and the enhanced cyan mutant of GFP, ECFP, which yields a critical Förster radius (R_0) for FRET of ≈50 Å (9). Thus, we would expect significant BRET between RLUC and EYFP, with an R_0 for BRET of ≈50 Å.

We expressed a protein fusion of a blue-emitting luciferase (RLUC) and an acceptor fluorophore in *E. coli*. Initially, we tried EGFP as the acceptor, but as previously reported by Wang *et al.* (22) with a similar fusion protein, only a barely detectable shoulder at 508 nm was added to the RLUC emission spectrum (data not shown). We then tested EYFP as an acceptor fluorophore (21), with the constructs shown in Fig. 1*A*. Both the excitation and fluorescence spectra of the EYFP-expressing *E. coli* are red-shifted from EGFP, with an excitation peak at 513 nm and fluorescence peak of 527 nm. Although the excitation peak of EYFP is not perfectly matched to the emission peak of RLUC, the emission spectrum of RLUC is sufficiently broad to provide good excitation of EYFP (Fig. 1*B*). Bioluminescence and fluorescence spectra of *E. coli* containing RLUC, EYFP, and RLUC●EYFP constructs under the control of the T7 promoter are shown in Fig. 1*B*. The luminescence profile of the *E. coli* cells expressing the RLUC construct displayed a typical emission spectrum for *Renilla* luciferase with a single peak at 480 nm (Fig. 1*B*). However, the luminescence spectrum from cells expressing the RLUC●EYFP fusion construct yielded a bimodal spectrum, with one peak centered at 480 nm (as for RLUC), and a new peak centered at 527 nm (as for EYFP fluorescence). These data suggest that a significant proportion of the RLUC energy was transferred and emitted at the characteristic wavelength of EYFP.

Because RLUC and EYFP appear to be a good BRET pair for a protein–protein interaction assay, we wanted to confirm that they do not spuriously associate with each other. After coexpression of separate RLUC and EYFP (the top two constructs in Fig. 1*A*) in the same *E. coli* cells (without IPTG induction), the luminescence spectrum looks like an unaltered RLUC spectrum—there is no evidence of a second peak (Fig. 2*B*, ○). However, when EYFP and RLUC were overexpressed by IPTG induction, we sometimes did observe a small second peak, which suggests that there may be a weak BRET signal that can be forced by overexpression. Because we were able to achieve sufficient signal levels by using the T7 promoter without IPTG addition, we could use a regime in which RLUC and EYFP do not associate by themselves. It is easy to determine from the intensity of RLUC luminescence and EYFP fluorescence the approximate level of RLUC and EYFP expression and choose samples with optimal expression. All further experiments reported in this paper were performed under conditions in which expression was controlled such that no second peak developed in the RLUC- and EYFP-expressing cells.

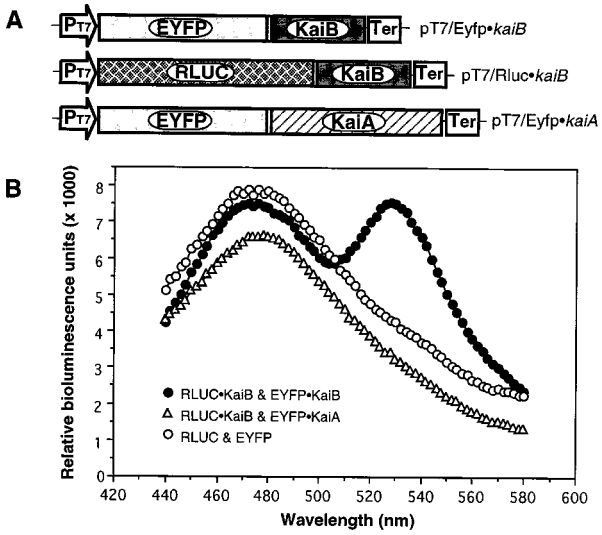

FIG. 2. *In vivo* BRET assay for protein interaction in *E. coli* cells. (*A*) Diagrams of the gene fusion expression cassettes of pT7/*Eyfp●kaiB*, pT7/*Rluc●kaiB*, and pT7/*Eyfp●kaiA*. (*B*) Luminescence emission spectra from the transformed strains expressing unfused RLUC and EYFP, fusion proteins RLUC●KaiB and EYFP●KaiB, and fusion proteins RLUC●KaiB and EYFP●KaiA. Luminescence reactions were initiated by the addition of 1 μM coelenterazine.

Currently, the yeast two-hybrid system is the most popular technique for identifying protein interactions (23). This method has proven useful for identifying potential protein interactions, but it is limited in that the interaction must occur in the yeast cell nucleus. This means interactions that depend on cell-type specific processing and/or compartmentalization will not be detected. Because the yeast two-hybrid system is a selection technique, potential interactors can be identified rapidly without laborious screening. Other than this single (but admittedly important) advantage, optical energy transfer techniques (i.e., FRET and BRET) are potentially superior to the yeast two-hybrid system for assaying protein interactions.

For example, FRET or BRET interactions can be assayed in different cellular compartments of the native cells by appropriate targeting of the labeled constructs (9, 10). This feature is particularly important in the case of interacting membrane proteins that are unlikely to yield a positive result with the two-hybrid assay. Moreover, interactions dependent on cell-type specific post-translational modifications that do not occur in yeast may be uncovered by FRET or BRET. Moreover, after a possible interacting pair is found with the yeast two-hybrid assay, further characterization by analytical techniques is necessary. On the other hand, FRET and BRET can be both screening and analytical techniques. Finally, it may be possible in some cases to use FRET/BRET to assay the kinetics of changes in protein interactions *in vivo*. All of these advantages will be particularly useful for investigations of membrane protein interactions, interactions that occur within specific organelles, and high-throughput screening (as described below).

The resonance energy transfer methods have some limitations, however. For example, Förster resonance energy transfer is dependent on proper orientation of the donor and acceptor dipoles. Conformational states of the fusion proteins

may fix the dipoles in a geometry that is unfavorable for energy transfer. Further, because the fluorophore/luciferase tags are fused to ends of the potentially interacting molecules, it is possible that some parts of the candidate molecules are interacting without allowing the fluorophore/luciferase tags to be close enough for energy transfer to occur. Consequently, two proteins might interact in a way that is blind to the FRET/BRET technique. In other words, a negative result with a resonance transfer technique does not prove noninteraction. (Of course, this problem is not unique to FRET/BRET-negative results in the two-hybrid assay also do not prove noninteraction.) However, this apparent limitation can be used to advantage: by testing different combinations of N-terminal and C-terminal fusions in the BRET assay, it may be possible to learn the orientation in which candidate proteins interact. Another consideration in the use of GFP variants as fluorophore tags is that GFP does not turn over rapidly. First, it takes at least 1 hr for the GFP molecule to fold properly (24–25). Second, once folded, GFP is remarkably resistant to proteolysis in foreign cells. Therefore, in some cases, the slow kinetics of GFP turnover may hamper measuring the kinetics of interaction. On the other hand, *Renilla* luciferase does not suffer these same disadvantages in turnover rate. Therefore, if one of the interacting proteins turns over more rapidly than the other, it might be useful for kinetic estimates to fuse the more unstable protein to RLUC.

Because BRET does not require the use of excitation illumination, it has potential advantages over FRET. BRET should be superior for cells that are either photo-responsive (e.g., retina or any photoreceptive tissue) or damaged by the wavelength of light used to excite FRET (typically near-UV) (10). Cells that have significant auto-fluorescence also would be better assayed by BRET than by FRET. In addition, although photobleaching of the fluorophores can be a serious limitation of FRET, it is irrelevant to BRET. A more subtle, but significant, advantage of BRET over FRET is that FRET may be prone to complications due to simultaneous excitation of both donor and acceptor fluorophores. Specifically, even with monochromatic laser excitation, it is impossible to excite only the donor without also exciting the acceptor fluorophore to some degree. Therefore, specific acceptor-only control experiments must be performed for FRET. In contrast, because BRET does not involve optical excitation, all the light emitted by the fluorophore must result from resonance transfer. In this respect, BRET is theoretically superior to FRET for quantifying resonance transfer.

BRET is not without its own limitations, however. RLUC requires a substrate, coelenterazine. Coelenterazine is hydrophobic and permeates many cell types (*E. coli*, yeast, plant seedlings, mammalian cell cultures), although it is possible that there are some cell types that will not be permeable to coelenterazine. Coelenterazine also can exhibit auto-luminescence in certain media; however, we measured no coelenterazine auto-luminescence in the simple salt media used here. Finally, lack of sensitivity may hinder the use of BRET in some cases. Depending on the expression level of RLUC, the luminescence can be dim, and for ratio imaging, only a small proportion of the total light is collected. Therefore, a sensitive light-measuring device is necessary.

Application of BRET to Detect the Interaction of Cyanobacterial Circadian Clock Proteins. The *kaiABC* gene cluster encodes three novel proteins that are essential for circadian clock function in cyanobacteria. Point mutations in each of the three *kai* genes result in circadian clock mutants of various phenotypes, especially short and long free-running periods, arhythmia, and disrupted waveform (12, 19). Deletion of the entire *kai* cluster or of any individual *kai* gene abolishes rhythmicity but does not affect growth rate (12). These data support the conclusion that these genes encode proteins whose functions are circadian clock-specific. The KaiA, KaiB, and KaiC proteins do not show homology to any known proteins, including eukaryotic clock proteins. The only clue to their biochemical activity from the sequence analysis is that one (KaiC) has a nucleotide-binding motif, and there is no information for the other two proteins (KaiA and KaiB) from their sequences.

Genetic experiments have provided some information on the roles of KaiA and KaiC in the circadian feedback loop. In particular, pulses of KaiC expression reset the phase of the circadian rhythms in cyanobacteria, and KaiA expression enhances the activity of the promoter which drives *kaiB* and *kaiC* expression. Therefore, there appears to be negative feedback control of *kaiC* expression by KaiC that is intimately involved in the generation of circadian oscillations, whereas KaiA acts to sustain the oscillation (12). H. Iwasaki and coworkers have used the yeast two hybrid and *in vitro* binding assays to discover that Kai proteins interact in various ways (personal communication). KaiB is encoded by the same gene cluster as KaiA and KaiC, and *kaiB* shares its promoter with *kaiC*. Furthermore, there are two different mutations in this locus (B21a and B22a) that confer short period phenotypes, and deletion of the *kaiB* locus causes arhythmicity (12). Therefore, the *kaiB* gene is crucial for circadian rhythmicity.

Based on the example of interacting clock proteins (13–18), we decided to test whether the BRET assay could be used to discover interactions among Kai proteins. Thus, we made fusion constructs of *kaiB* to the *Eyfp* and *Rluc* genes, respectively, (Fig. 2*A*) and measured the luminescence spectra of *E. coli* expressing these constructs. As shown in Fig. 2*B* (●), a second peak emerges from cells expressing both RLUC●KaiB and EYFP●KaiB. The similarity between the spectrum depicted in Fig. 1*B* for the fusion construct and the RLUC●KaiB and EYFP●KaiB combination in Fig. 2*B* strongly suggests that interaction among KaiB molecules has brought the RLUC and EYFP into close proximity such that energy transfer occurs for ≈50% of the RLUC luminescence. To demonstrate that this bimodal spectrum does not occur nonspecifically, we fused EYFP to a slightly truncated KaiA and coexpressed it with RLUC●KaiB. In this case, there is no second luminescence peak, and thus no indication of interaction between KaiA and KaiB (Fig. 2*B*).

We also can observe KaiB interactions *in vitro* by BRET. Fig. 3 shows spectra derived from mixing *E. coli* extracts from two different cultures, one expressing RLUC●KaiB and the other expressing EYFP●KaiB. Immediately after mixing, there is no evidence of a second peak. After room temperature incubation for 30 min, a significant shoulder has emerged on the spectrum. At the 30-min incubation time point, the intensity of the RLUC luminescence has decreased, probably because of proteolysis within the extract. After 2 hr of room temperature incubation, the luminescence exhibited a clear bimodal spectrum, even though the overall luminescence intensity has decreased further. These changes in the spectra are not due to coelenterazine oxidation, because fresh coelenterazine was added at each assay time point. The development of a shoulder followed by an obvious bimodality indicates that KaiB–KaiB interaction can be assayed *in vitro* by BRET.

Ratio Imaging of BRET between KaiB Interactors. In Figs. 1–3, we measured the extent of BRET by measuring emission spectra. For applications such as microscopic imaging and high-throughput screening, it would be more convenient to measure the ratio of luminescence intensities at 480–530 nm to determine the magnitude of BRET and to correct for differences in overall levels of expression of RLUC and EYFP fusion proteins. We therefore collected images with a cooled-CCD camera of *E. coli* cultures expressing our KaiB fusion constructs. Fig. 4*A* shows images of liquid *E. coli* cultures as seen through 10-nm bandpass interference filters centered at 480 nm and 530 nm, respectively. In the cultures containing a control strain (RLUC alone) or a noninteracting combination

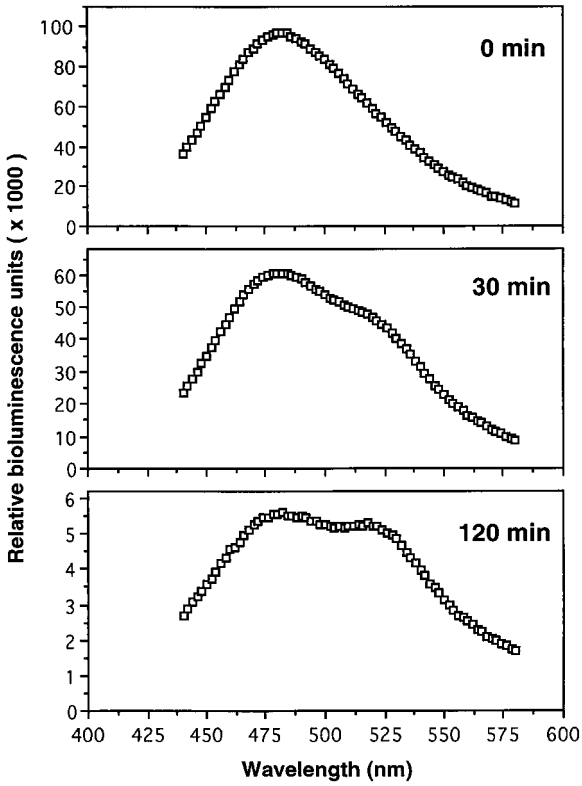

FIG. 3. KaiB–KaiB association *in vitro* revealed by BRET. Extracts containing fusion proteins EYFP●KaiB were mixed in equal proportions with those containing RLUC●KaiB. Bioluminescence emission profiles were measured at the indicated times after the extracts were combined and incubated at room temperature. Fresh coelenterazine (1 μM) was added at the beginning of the reaction at each time point.

(RLUC●KaiB and EYFP●KaiA), there is much less light emitted at 530 nm than at 480 nm. In the fusion construct (RLUC●EYFP) or the interacting combination (RLUC●KaiB and EYFP●KaiB), the amount of light emitted at 480 nm and 530 nm are roughly equal, as would be predicted from the spectra depicted in Figs. 1*B* and 2*B*. These images can be quantified, as shown in Fig. 4*B* and the ratios calculated as in Fig. 4*C*. The 530 nm/480 nm ratios of luminescence for the RLUC control and the noninteracting KaiB and KaiA couple are low (approximately 0.4), whereas the 530 nm/480 nm ratios for the RLUC●EYFP fusion construct and for the interacting KaiB fusion proteins are above 0.9.

The data of Fig. 4 suggest a relatively simple scheme for designing an *in vivo* library screening system for protein–protein interaction by using BRET. By measuring the light emission collected through interference filters, the 530 nm/480 nm luminescence ratio of *E. coli* (or yeast) colonies expressing a "bait" protein (e.g., a Kai protein) fused to RLUC and a library of "prey" molecules fused to EYFP (or vice versa) could be measured. Colonies that express an above-background ratio could be saved and the "prey" DNA sequence further characterized. With appropriate instrumentation, high-throughput screening by using BRET is a possibility. By using an imaging instrument similar to the one we used here, it would be possible to screen colonies of bacteria or yeast on agar plates. On the other hand, a photomultiplier-based instrument designed to measure luminescence of liquid cultures in 96-well plates could be adapted to high-throughput BRET screening by insertion of switchable 480- or 530-nm filters in front of the photomultiplier tube.

The data reported in Figs. 2–4 indicate that KaiB proteins can form homodimers (or perhaps even larger complexes). By using other techniques, H. Iwasaki and coworkers have discovered various interactions among Kai proteins (personal communication). These observations are reminiscent of the cases of clock proteins from *Drosophila* and mammals that interact with each other in a process mediated by PAS binding domains (13–18). Taken together, these data support the model suggested for the clock mechanism of the cyanobacterial clock that includes Kai protein interactions as a crucial component of the oscillatory mechanism (12). Some studies have identified the specific role that protein interactions serves in transcriptional control in eukaryotes (16, 17). Our analysis of the mechanistic interplay among the clock gene products of cyanobacteria is in the nascent stages (12)—we do not yet know whether the interaction of KaiB proteins is directly involved in transcriptional control. Nevertheless, even though there are no similarities in the sequences of the three cyanobacterial clock genes with other known clock genes (12), it appears that common themes emerge in the comparison of the

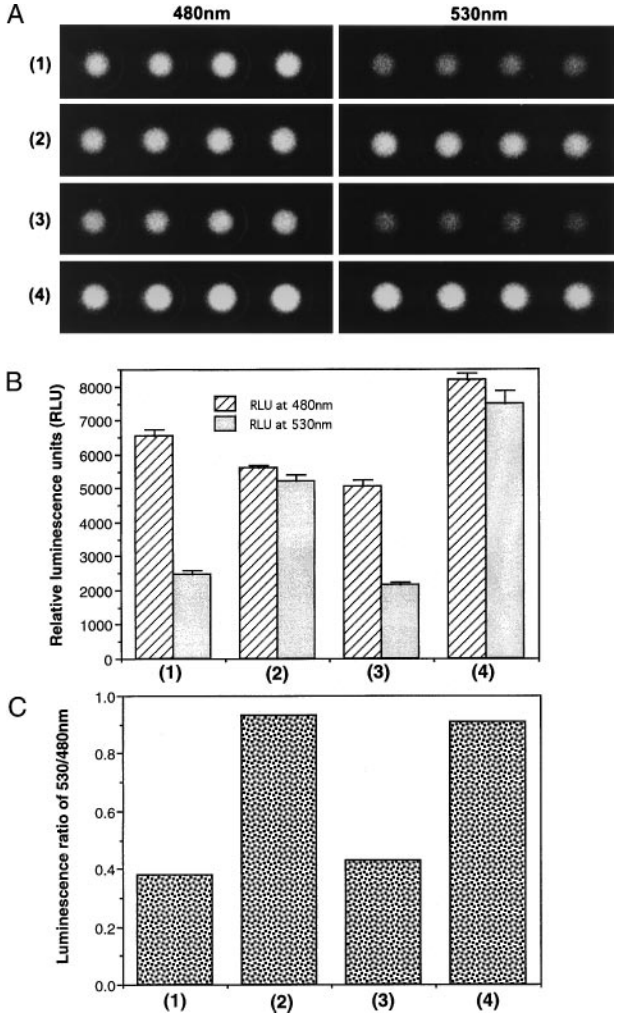

FIG. 4. Imaging and quantification of BRET luminescence from transformed *E. coli* strains. (*A*) Bioluminescence visualized through interference bandpass filters transmitting light of 480 ± 5 nm (left side) or 530 ± 5 nm (right side). Row 1 is the strain producing only RLUC; row 2 is the strain expressing the RLUC●EYFP fusion protein; row 3 is the strain expressing the fusion proteins RLUC●KaiB and EYFP●KaiA; and row 4 is the strain producing the fusion proteins RLUC●KaiB and EYFP●KaiB. Luminescence reactions were initiated by the addition of 3 μM coelenterazine. (*B*) Quantification of luminescence intensity in relative light units at 480 nm and 530 nm of the cultures imaged in *A*. (*C*) Calculation of the 530 nm/480 nm ratios for the data of *B*. For *B* and *C*, strains are labeled as in *A*: (1) RLUC, (2) RLUC●EYFP, (3) RLUC●KaiB and EYFP●KaiA, and (4) RLUC●KaiB and EYFP●KaiB.

circadian clockworks of prokaryotes versus eukaryotes: protein–protein interaction may be essential to the mysterious biochemistry of circadian timekeeping that drives a temperature compensated oscillation with the surprisingly long time constant of approximately 24 h.

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1. Phizicky, E. M. & Fields, S. (1995) *Microbiol. Rev.* **59,** 94–123.
2. Johnson, F. H., Shimomura, O., Saiga, Y., Gershman, L. C., Reynolds, G. T. & Waters, J. R. (1962) *J. Cell. Comp. Physiol.* **60,** 85–103.
3. Ward, W. W. & Cormier, M. J. (1978) *Methods Enzymol.* **57,** 257–267.
4. Wu, P. & Brand, L. (1994) *Anal. Biochem.* **218,** 1–13.
5. Wilson, T. & Hastings, J. W. (1998) *Annu. Rev. Cell Dev. Biol.* **14,** 197–230.
6. Heim, R., Prasher, D. C. & Tsien, R. Y. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12501–12504.
7. Heim, R. & Tsien, R. Y. (1996) *Curr. Biol.* **6,** 178–182.
8. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M. & Tsien, R. Y. (1997) *Nature (London)* **388,** 882–887.
9. Mahajan, N. P., Linder, K., Berry, G., Gordon, G. W., Heim, R. & Herman, B. (1998) *Nat. Biotechnol.* **16,** 547–552.
10. Periasamy, A. & Day, R. N. (1998) *J. Biomed. Opt.* **3,** 1–7.
11. Johnson, C. H., Golden, S. S., Ishiura, M. & Kondo, T. (1996) *Mol. Microbiol.* **21,** 5–11.
12. Ishiura, M., Kutsuna, S., Aoki, S., Iwasaki, H., Andersson, C. R., Tanabe, A., Golden, S. S., Johnson, C. H. & Kondo, T. (1998) *Science* **281,** 1519–1523.
13. Gekakis, N., Saez, L., Delahaye-Brown, A. M., Myers, M. P., Sehgal, A., Young, M. W. & Weitz, C. J. (1995) *Science* **270,** 811–815.
14. King, D. P., Zhao, Y., Sangoram, A. M., Wilsbacher, L. D., Tanaka, M., Antoch, M. P., Steeves, T. D. L., Vitaterna, M. H., Kornhauser, J. M., Lowrey, P. L., *et al.* (1997) *Cell* **89,** 641–653.
15. Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. & Sakaki, Y. (1997) *Nature (London)* **389,** 512–516.
16. Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steeves, T. D. L., Weitz, C. J., Takahashi, J. S. & Kay, S. A. (1998) *Science* **280,** 1599–1603.
17. Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S. & Weitz, C. J. (1998) *Science* **280,** 1564–1569.
18. Hogenesch, J. B., Gu, Y.-Z., Jain, S. & Bradfield, C. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5474–5479.
19. Kondo, T., Tsinoremas, N. F., Golden, S. S., Johnson, C. H., Kutsuna, S. & Ishiura, M. (1994) *Science* **266,** 1233–1236.
20. Cormier, M. J. (1978) *Methods Enzymol.* **57,** 237–244.
21. Ormö, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y. & Remington, S. J. (1996) *Science* **273,** 1392–1395.
22. Wang, Y., Wang, G., O'Kane, D. J. & Szalay, A. A. (1997) in *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons*, eds. Hastings, J. W., Kricka, L. J. & Stanley, P. E. (Wiley, New York), pp. 419–422.
23. Fields, S. & Song, O. K. (1989) *Nature (London)* **340,** 245–246.
24. Heim, R., Cubitt, A. B. & Tsien, R. Y. (1995) *Nature (London)* **373,** 633–633.
25. Chalfie, M. & Kain, S. R. (1998) *GFP: Green Fluorescent Protein Strategies, Applications, and Protocols* (Wiley, New York).