SUB1, an *Arabidopsis* Ca^{2+}-Binding Protein Involved in Cryptochrome and Phytochrome Coaction

Hongwei Guo, Todd Mockler, Hien Duong, Chentao Lin*

Cryptochromes and phytochromes are the major photosensory receptors in plants and often regulate similar photomorphogenic responses. The molecular mechanisms underlying functional interactions of cryptochromes and phytochromes remain largely unclear. We have identified an *Arabidopsis* photomorphogenic mutant, *sub1*, which exhibits hypersensitive responses to blue light and far-red light. Genetic analyses indicate that *SUB1* functions as a component of a cryptochrome signaling pathway and as a modulator of a phytochrome signaling pathway. The *SUB1* gene encodes a Ca^{2+}-binding protein that suppresses light-dependent accumulation of the transcription factor HY5.

Plants rely on multiple photosensory receptors to perceive changes of light quality and quantity and to regulate growth and development. The blue/ultraviolet-A light receptors (cryptochromes) and red/far-red light receptors (phytochromes) are major photoreceptors mediating light responses such as inhibition of hypocotyl elongation and stimulation of anthocyanin accumulation (*1*, *2*). The molecular mechanism of photoreceptor signal transduction, especially that of cryptochromes, remains largely unclear. Recent studies have demonstrated that protein phosphorylation and transcriptional regulation are important mechanisms of phytochrome signal transduction (*3*–*5*). The involvement of calcium homeostasis has also been implicated in the signaling processes of both phytochromes and cryptochromes (*6*, *7*). Although genes encoding phytochromes and cryptochromes appear to be evolutionarily unrelated, these two types of photoreceptors often elicit the same light responses. Moreover, for various light responses in different plant species, phytochromes and cryptochromes often affect each other's function, resulting in synergistic or antagonistic light responses (*9*). Such phenomena, collectively referred to as the coaction of phytochrome and cryptochrome (*9*), have also been found for the photomorphogenic responses in *Arabidopsis* (*10*–*15*). It has been reported that phytochromes and cryptochromes may physically interact to affect each other's activity and that the signaling molecules of one photoreceptor may modulate the function of another photoreceptor (*13*–*18*).

To investigate cryptochrome signal transduction, we sought to identify mutations affecting hypocotyl growth in blue light (*19*). One of the resulting mutants was referred to as *sub1* (short under blue light). However, *sub1* was later found to have a short hypocotyl phenotype not only in blue light but also in far-red light (Fig. 1A). The *sub1* mutant shows no sign of photomorphogenic development in the dark, and it appears to grow normally in red light at fluence rates tested (Fig. 1) (*20*). In addition to hypocotyl inhibition, the *sub1* mutant also exhibits hypersensitive light responses in cotyledon expansion and gene expression (*20*). For example, the blue and far-red light–induced expression of the *CHS* and *CHI* genes, encoding flavonoid biosynthetic enzymes, was elevated to a relatively higher level in the *sub1* mutant than in the wild type (Fig. 1, B and C). The function of SUB1 is dependent on the light fluence rate. The *sub1* mutant demonstrates a more pronounced short-hypocotyl phenotype in blue light or far-red light of relatively low fluence rates (<10 μmol m^{-2} s^{-1}) (Fig. 1, D to G). When grown in light of higher fluence rates (>10 μmol m^{-2} s^{-1}), the relative difference in hypocotyl length between *sub1* and wild-type seedlings diminished (Fig. 1, D to G), suggesting that SUB1 functions primarily in low light.

To study how SUB1 is involved in the cryptochrome function, we examined the genetic interactions of *sub1* with cryptochrome mutants. When grown in blue light of relatively low fluence rates, *cry2* and *sub1* exhibited a long- or short-hypocotyl phenotype, respectively, whereas the *sub1cry2* double mutant showed hypocotyl growth comparable to the *sub1* parent (Fig. 1D). This result indicates that *sub1* is epistatic to *cry2* and that SUB1 is likely to function downstream from the cry2 photore-

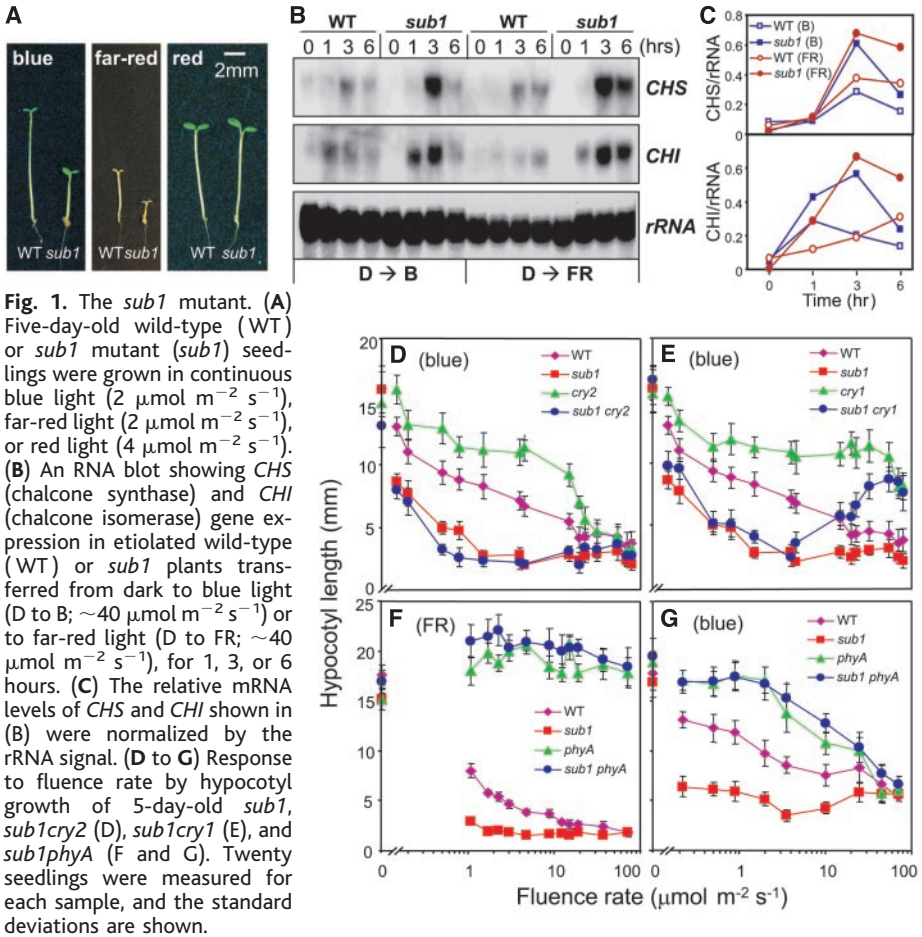

Fig. 1. The *sub1* mutant. (**A**) Five-day-old wild-type (WT) or *sub1* mutant (*sub1*) seedlings were grown in continuous blue light (2 μmol m^{-2} s^{-1}), far-red light (2 μmol m^{-2} s^{-1}), or red light (4 μmol m^{-2} s^{-1}). (**B**) An RNA blot showing *CHS* (chalcone synthase) and *CHI* (chalcone isomerase) gene expression in etiolated wild-type (WT) or *sub1* plants transferred from dark to blue light (D to B; ~40 μmol m^{-2} s^{-1}) or to far-red light (D to FR; ~40 μmol m^{-2} s^{-1}), for 1, 3, or 6 hours. (**C**) The relative mRNA levels of *CHS* and *CHI* shown in (B) were normalized by the rRNA signal. (**D** to **G**) Response to fluence rate by hypocotyl growth of 5-day-old *sub1*, *sub1cry2* (D), *sub1cry1* (E), and *sub1phyA* (F and G). Twenty seedlings were measured for each sample, and the standard deviations are shown.

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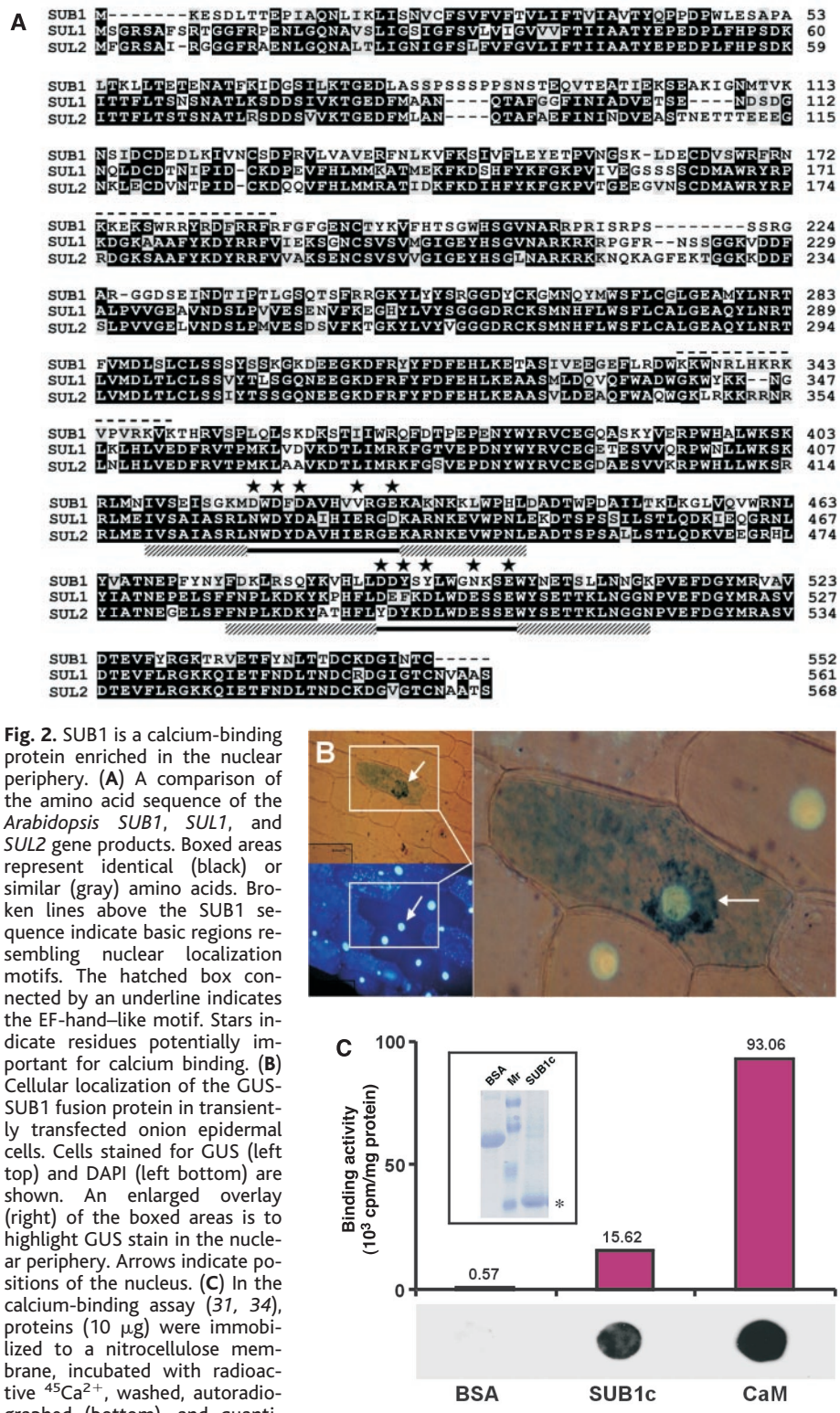

Fig. 2. SUB1 is a calcium-binding protein enriched in the nuclear periphery. (**A**) A comparison of the amino acid sequence of the *Arabidopsis SUB1*, *SUL1*, and *SUL2* gene products. Boxed areas represent identical (black) or similar (gray) amino acids. Broken lines above the SUB1 sequence indicate basic regions resembling nuclear localization motifs. The hatched box connected by an underline indicates the EF-hand–like motif. Stars indicate residues potentially important for calcium binding. (**B**) Cellular localization of the GUS-SUB1 fusion protein in transiently transfected onion epidermal cells. Cells stained for GUS (left top) and DAPI (left bottom) are shown. An enlarged overlay (right) of the boxed areas is to highlight GUS stain in the nuclear periphery. Arrows indicate positions of the nucleus. (**C**) In the calcium-binding assay (*31, 34*), proteins (10 μg) were immobilized to a nitrocellulose membrane, incubated with radioactive $^{45}Ca^{2+}$, washed, autoradiographed (bottom), and quantified for the $^{45}Ca^{2+}$ retained to the membrane by liquid scintillation (top). BSA, bovine serum albumin; SUB1c, purified SUB1 COOH-terminal fragment; CaM, bovine brain calmodulin. Inset shows the purified SUB1c (10 μg) fractionated in a 10% SDS-PAGE. Mr, molecular weight marker.

ceptor. Because cry2 itself functions primarily in low light, presumably because of the degradation of cry2 protein in high light (*21*, *22*), it is not surprising that all three genotypes showed a less pronounced phenotype in high light (Fig. 1D). The *sub1* and *cry1* mutations exhibited a more complex, epistatic relation dependent on fluence rate. When grown in blue light with relatively low fluence rates, the *sub1cry1* double mutant exhibited a short-hypocotyl phenotype just like that of the *sub1* monogenic parent and the *sub1cry2* double mutant (Fig. 1, D and E), suggesting that SUB1 also acts downstream from the cry1 photoreceptor. When plants were grown under blue light of higher fluence rates, the phenotype of the *sub1cry1* double mutant became increasingly like its *cry1* monogenic parent (Fig. 1E). These results are consistent with the hypothesis that, with respect to the hypocotyl inhibition, SUB1 normally functions in low light. The lack of SUB1 activity in high light may result from a light-dependent suppression of the expression or activity of SUB1. The observation that cry1 activity is dependent on SUB1 only in low light suggests that cry1 mediates multiple signaling pathways, resulting in an inhibition of cell elongation, and that the function of SUB1 is associated with a pathway that operates primarily in low light.

Because *sub1* also showed an enhanced response to far-red light and phytochrome phyA is the major photoreceptor mediating far-red light responses (*1*), we next examined the genetic interaction between *sub1* and *phyA* (Fig. 1, F and G). Compared with wild-type plants, the *sub1* and *phyA* mutant seedlings grown in far-red light developed short and long hypocotyls, respectively, but the *sub1phyA* double mutant resembled the *phyA* parent at all the fluence rates of far-red light tested (Fig. 1F). Because phyA is also known to mediate hypocotyl inhibition in blue light (*13*, *23*), especially in low light (Fig. 1G), we further analyzed how *sub1* and *phyA* mutations interacted in blue light (Fig. 1G). In contrast to the *sub1cry2* and *sub1cry1* double mutants, the *sub1phyA* double mutant again showed a hypocotyl length very similar to that of the *phyA* parent in all the fluence rates of blue light tested (Fig. 1G). We conclude that *phyA* is epistatic to *sub1* in both far-red light and blue light. These results suggest that the activity of cry2 and cry1 is dependent, at least partially, on SUB1, whereas the activity of phyA is not dependent on SUB1. Therefore, SUB1 is likely to act as a signal transducer of cry1 and cry2 but as a modulator of phyA signal transduction.

The *sub1* loss-of-function mutation results from a transferred DNA (T-DNA) insertion in the 3′-end untranslated region of the *SUB1* gene, which causes significantly decreased *SUB1* mRNA expression, and consequently, a markedly lower SUB1 protein level in the *sub1* mutant (*19*). Increasing the SUB1 level in *sub1* mutant plants by transgenic expression of the *SUB1* cDNA rescued the defects caused by the *sub1* mutation (*19*). *SUB1* encodes a novel 552-residue polypeptide containing EF-hand-like Ca^{2+}-binding motifs at the COOH-terminal region (Fig. 2A). SUB1 also has two regions enriched in basic residues that resemble nuclear localization signals (Fig. 2A). However, SUB1 does not seem to accumulate in the nucleoplasm. The SUB1-GUS fusion protein expressed in plant cells can be found through-

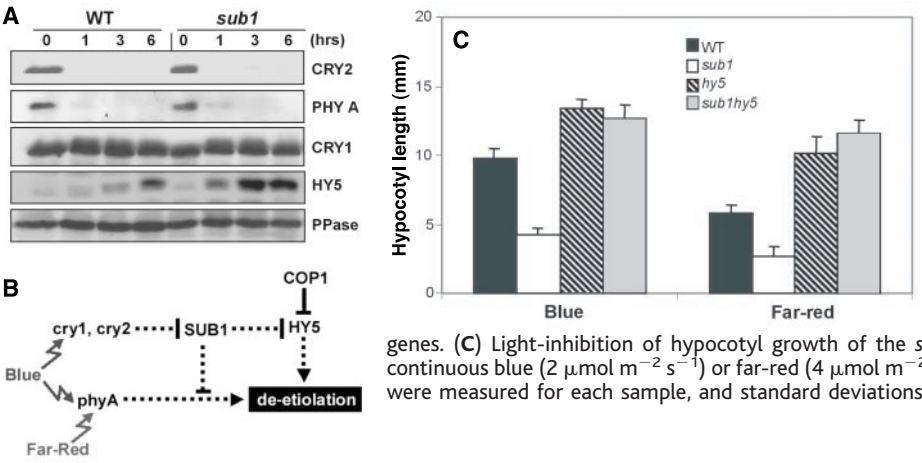

Fig. 3. SUB1 is a negative regulator of HY5. (**A**) Immunoblots showing that the light-regulated expression of HY5, but not PHYA or CRY2, is affected by the *sub1* mutation. Five-day-old seedlings grown in the dark (0 hour) were transferred to blue light (~40 μmol m^{-2} s^{-1}) for 1, 3, or 6 hours, and total protein extracts were analyzed by immunoblots probed with respective antibodies indicated at the right side. (**B**) A working model depicting the function of SUB1 in photoreceptor signal transduction. Arrows and T-bars represent positive or negative effect, respectively. Broken lines indicate that there may be more steps between the two indicated genes. (**C**) Light-inhibition of hypocotyl growth of the *sub1hy5* double mutant. Seedlings were grown in continuous blue (2 μmol m^{-2} s^{-1}) or far-red (4 μmol m^{-2} s^{-1}) for 5 days. Hypocotyl lengths of 20 seedlings were measured for each sample, and standard deviations are shown.

out the cytosol, and it is apparently enriched in the nuclear periphery region surrounding the nucleus (Fig. 2B) (*20*). It is likely that SUB1 may be associated with nuclear envelope or endoplasmic reticular membranes. In addition to *SUB1*, *Arabidopsis* has at least two *SUB1*-like (*SUL*) genes, which we refer to as *SUL1* and *SUL2* (Fig. 2A). The conceptual translation products of the *SUL1 and SUL2* genes are approximately 50% identical to that of *SUB1*. Genes showing high (>50%) amino acid identity to *SUB1* are also found in other plants, including monocotyledons and conifers; but genes similar to *SUB1* were not found in cyanobacteria, yeast, *Caenorhabditis elegans*, or *Drosophila*, for which the genomes have been completely sequenced (*20*). These results suggest that the *SUB/SUL* genes may be unique to terrestrial plants. Like *SUB1*, *SUL1* and *SUL2* also contain EF-hand–like motifs in the COOH-terminal region. The EF hand is a Ca^{2+}-binding motif composed of two α helices connected by a loop that coordinates Ca^{2+} binding (*24*, *25*). To investigate whether SUB1 may be a calcium-binding protein, we expressed and purified a COOH-terminal fragment of SUB1 (SUB1c) that contains EF-hand–like motifs (Fig. 2C). SUB1c indeed showed a calcium-binding activity in an in vitro $^{45}Ca^{2+}$-binding assay, in which proteins bound to a nitrocellulose are allowed to bind to the radioactive $^{45}Ca^{2+}$ (Fig. 2C). Compared with calmodulin, SUB1c has a lower affinity to Ca^{2+} (Fig. 2C). This may not be surprising because, like some other EF-hand proteins that have relatively lower affinity to Ca^{2+}, the primary structure of the EF-hand of SUB1 deviates from that of the canonical EF-hand motifs found in calmodulin (*24*, *25*).

The *sub1* mutation does not affect blue light–induced degradation of cry2 or phyA, nor the level of cry1 protein (Fig. 3A) (*22*, *26*). This is consistent with our hypothesis that SUB1 is a component of the cryptochrome signaling pathway that modulates phyA signal transduction. According to this hypothesis, SUB1 is a negative regulator of photomorphogenesis, whereas the cryptochromes suppress the activity of SUB1 to activate the light response (Fig. 3B). To account for the absence of a mutant phenotype in dark-grown *sub1* plants, our model further predicts that SUB1 acts upstream of another component that is inactive in the dark. A bZIP transcription factor, HY5, appears to be a good candidate for such a component. The *Arabidopsis hy5* mutant exhibits a long hypocotyl when grown in blue, red, or far-red light, but not in the dark (*27*, *28*). It has been shown that HY5 undergoes COP1-dependent degradation in the dark and photoreceptor-dependent accumulation in light, and that the light-induced accumulation of HY5 protein correlates with light inhibition of hypocotyl growth (*29*, *30*). These results indicate that HY5 acts downstream from both phytochromes and cryptochromes and that HY5 is inactive in the dark. To test whether SUB1 acts on HY5 by affecting the expression of HY5, we compared the expression of HY5 protein in wild-type and *sub1* mutant plants. As previously reported, the HY5 protein starts to accumulate when etiolated seedlings are exposed to blue light. However, the light-induced accumulation of HY5 protein occurs much faster in the *sub1* mutant than in the wild type (Fig. 3A). This result is consistent with SUB1's being a negative regulator of the light-induced accumulation of HY5 protein. Furthermore, a comparably low level of HY5 protein is detected in the dark-grown *sub1* mutant and wild-type plants (Fig. 3A), which explains why *sub1* seedlings exhibit normal hypocotyl elongation in the dark. The hypothesis that SUB1 may act upstream of HY5 is further confirmed by an analysis of the *sub1hy5* double mutant. In comparison with the wild type, the *sub1hy5* double mutant showed long hypocotyls in both blue and far-red light, indicating that *hy5* is epistatic to *sub1* (Fig. 3C).

SUB1 defines a point of crosstalk between cryptochrome and phyA signal transduction pathways. The position of SUB1 in a cryptochrome signaling pathway appears to be between photoreceptors and HY5. The finding that SUB1 is a calcium-binding protein suggests that SUB1 plays an important role in photomorphogenic responses resulting from the light-induced changes in ion homeostasis. Elucidation of the biochemical mechanisms of SUB1 would further our understanding of how photoreceptors function in the cell and how signaling from different photoreceptors interacts.

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19. *Arabidopsis* mutants that showed short hypocotyls in blue light (3 μmol m^{-2} s^{-1}) but not in red light (4 μmol m^{-2} s^{-1}) were isolated and investigated. All the data shown here are derived from *sub1-1*, which was isolated from a T-DNA–tagged population of Ws background (NASC, Nottingham, UK). A cosegregation of *sub1-1* and T-DNA was established in the F_2 and F_3 of a cross between *sub1-1* and the wild type. To prepare double mutants, *sub1-1* was crossed to *cry2-1* (*12*), *cry1-304* (*14*), *phyB-211* (*14*), and *phyA-9* (from M. M. Neff and J. Chory). The T-DNA–flanking genomic regions of *sub1-1* were isolated by TAIL-PCR (*32*) and inverse polymerase chain reaction (PCR) methods (*33*), and sequenced. *SUB1* is located in the BAC clone BAC32A17, and mapped to 24.2 cM of chromosome 4. The GenBank accession numbers are AL161512 (*SUB1*), AL049640 (*SUL1*), and AC007213 (*SUL2*) (www.arabidopsis.org/). Sequence alignments and presentation were made using ClustalW (www.hgsc.bcm.tmc.edu/SearchLauncher/) and BoxShade (www.ch.embnet.org/software/BOX_form.html), respectively.

For the results of SUB1 expression and transgenic rescue of the *sub1* mutant, supplementary material is available on *Science* Online at www.sciencemag.org/cgi/content/full/291/5503/487/DC1. The *SUB1* coding region corresponding to residues 267 to 552 of the SUB1 translation product (SUB1c) was amplified by PCR, expressed, purified from *Escherichia coli*, and used to raise polyclonal antibodies against SUB1. The $^{45}Ca^{2+}$ overlay calcium-binding assay was as described (*31*, *34*). Light sources, hypocotyl measurement, genetic and transgenic analyses, RNA blot, immunoblot, and GUS fusion protein cellular localization analyses were as described (*12*, *14*, *21*, *22*, *35*).

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Entrainment of the Circadian Clock in the Liver by Feeding

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Circadian rhythms of behavior are driven by oscillators in the brain that are coupled to the environmental light cycle. Circadian rhythms of gene expression occur widely in peripheral organs. It is unclear how these multiple rhythms are coupled together to form a coherent system. To study such coupling, we investigated the effects of cycles of food availability (which exert powerful entraining effects on behavior) on the rhythms of gene expression in the liver, lung, and suprachiasmatic nucleus (SCN). We used a transgenic rat model whose tissues express luciferase in vitro. Although rhythmicity in the SCN remained phase-locked to the light-dark cycle, restricted feeding rapidly entrained the liver, shifting its rhythm by 10 hours within 2 days. Our results demonstrate that feeding cycles can entrain the liver independently of the SCN and the light cycle, and they suggest the need to reexamine the mammalian circadian hierarchy. They also raise the possibility that peripheral circadian oscillators like those in the liver may be coupled to the SCN primarily through rhythmic behavior, such as feeding.

The light-dark (LD) cycle is the most reliable and strongest external signal that synchronizes (entrains) biological rhythms with the environment. In mammals, LD information is perceived by specialized retinal photoreceptors and conveyed directly to the SCN of the hypothalamus, where it entrains circadian oscillators in what is regarded as the master clock of the organism (*1, 2*). In addition, other cyclic inputs, such as temperature, noise, social cues, or rhythmic access to food, may also act as entraining agents, although the effects of these rhythmic signals on behavior are often weak.

When food is available only for a limited time each day, rats increase their locomotor activity 2 to 4 hours before the onset of food availability (*3*). Such anticipatory behavior also occurs in other mammals and in birds and is often paralleled by increases in body temperature, adrenal secretion of corticosterone, gastrointestinal motility, and activity of digestive enzymes (*4–6*). Entrainment of anticipatory locomotion by restricted feeding (RF) occurs independently of the LD cycle, in constant light, and in SCN-lesioned animals (*7, 8*), suggesting that the circadian oscillators entrained by RF are distinct from those entrained by light.

Using a transgenic rat model in which the mouse *Per1* gene promoter has been linked to a luciferase reporter, we continuously monitored the rhythmic expression of this "clock gene" by recording light emission from tissues in vitro (*9*). We used this model to investigate the effects of RF on rhythmicity in the liver, an organ that is directly involved with food processing, as well as in the SCN and lung.

We first exposed young rats to an RF regimen, in which food was available only for 4 hours during the light portion of a 12-hour:12-hour LD cycle, and recorded their locomotor activity (*10*). Within 3 days, the rats began to increase their wheel-running several hours before food became available, and there was an increase in the amount of nighttime running and also a change in the pattern (Fig. 1A), as has been observed previously (*7, 8*). After 2, 7, or 19 days of RF, we killed the animals; explanted the liver, lung, and SCN; and measured luciferase from each tissue in vitro (*11*) (Fig. 2). Despite the marked effects of this regimen on locomotor behavior, the phase of the SCN rhythm was unaffected and remained phase-locked to the light cycle, even after 19 days of RF (Fig. 3A). This result is consistent with reports that RF does not entrain multi-unit neuronal activity in the SCN (*12*) and supports the general notion that entrainment to cycles of food availability does not directly involve the SCN.

In contrast, the circadian clock in the liver was entrained by the 4-hour RF regimen (Fig. 3A). By the second day of RF, the four liver samples that were measured had already shifted an average of 10 hours, a slightly smaller and somewhat more variable response than the 12-hour shift achieved by liver cultures from rats exposed to RF for 7 or 19 days (Fig. 3A). The large phase shift after only 2 days suggests that the liver may have a unique ability to adapt temporally to changes in the feeding pattern.

Rhythmicity in the lung was also affected by the 4-hour RF regimen. Explants taken from four animals on the second day of RF showed a range of responses: two were arrhythmic, one was rhythmic but with such low amplitude that phase could not be reliably measured, and the fourth was rhythmic but unshifted relative to ad lib–fed controls. By the seventh day of the 4-hour RF regimen, the lung explants were shifted by 6 hours and were not shifted further after 19 days of RF (Fig. 3A).

Because the lung is not directly involved in the response to food and yet was shifted by the 4-hour regimen, we considered the possibility that RF might be acting through a global signal(s) such as the hormonal changes accompanying the stress that this treatment is known to produce [e.g., increases in blood levels of corticosterone (*13*)]. We tested the

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