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Daily and Circadian Variation in Survival From Ultraviolet Radiation in *Chlamydomonas reinhardtii*

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ABSTRACT

The survival of organisms depends on their ability to adapt to their environment, one important aspect of which is the daily cycle of day and night. During the day, organisms use a variety of strategies to protect themselves from deleterious ultraviolet (UV) wavelengths of sunlight. Among those strategies could be timing of UV-sensitive cellular processes to occur at night to avoid UV-induced damage. We tested whether the unicellular alga *Chlamydomonas reinhardtii* uses this strategy by measuring the survival of cells following exposure to UV radiation at different phases of the day. *Chlamydomonas* cells displayed a rhythm of survival from UV radiation where the most sensitive phases occurred during the end of the day and at the beginning of the night. This phase of sensitivity corresponds to the time of nuclear division. The rhythm continues in constant light indicating control by a circadian clock. The results presented here suggest a hypothesis of how circadian clocks may have evolved; a temporal program whereby light-sensitive processes are timed to avoid sunlight-induced damage would be advantageous and therefore selected.

INTRODUCTION

Adaptation of organisms to the environment includes the anticipation of cyclical events such as the daily rhythm of sunlight and night. Temporal programs reflecting the 24 h cycle of day and night are expressed by most organisms from microscopic cyanobacteria (1) to macroscopic humans (2). This daily temporal organization is not simply driven by the external cycle of day and night, but is regulated by an endogenous biochemical oscillator—a circadian clock—that controls myriad behavioral, physiological and biochemical processes (3,4).

Intuitively, organisms that have adapted to the cyclical 24 h appearance of the sun should have a reproductive advantage. However, only recently has this issue been addressed experimentally. The adaptive significance of circadian clocks has been shown for cyanobacteria under competition conditions (5) and for mammals in nature (6). Both of these studies demonstrated the significance of having a circadian clock, but neither addresses how a circadian clock might have been adaptive in the early phases of its evolution. In photosynthetic organisms, there are at least two plausible reasons why having a circadian clock could have been adaptive (see Johnson and Golden (1) and references therein). First, it could have been advantageous to be able to anticipate dawn so that the organism may prepare its photosynthetic apparatus for the onset of sunlight and thus be able to use every second of the available radiant energy from the sun. Second, an organism may be better adapted if it could temporally organize its cellular physiology so that oxygen-sensitive reactions could be restricted to times when photosynthesis does not occur (*e.g.* the night).

Perhaps more profoundly, the early evolution of circadian clocks could have been driven by the advantage inherent in phasing cellular events that are sensitive to deleterious wavelengths of sunlight so that they occur in the night. This idea has been called the ''escape from light'' hypothesis (7). That speculation seems plausible when one considers the numerous examples of microorganisms with 24 h cell division cycles in which DNA replication and cell division occur during the night (8). Perhaps some of the events of the cell division cycle in these microorganisms are sensitive to sunlight.

One of these organisms, *Chlamydomonas reinhardtii,* replicates nuclear DNA during the early phases of the night and undergoes cytokinesis within the mother cell wall during the later phases of the night. Daughter cells are liberated from the mother cell wall at dawn to begin another round of cell growth and division (9). We used *Chlamydomonas* to determine whether cells were more sensitive to ultraviolet (UV)‡ radiation at certain phases of the 24 h light/dark (LD) cycle. We found that cell survival was attenuated by UV radiation during the late day and early night phases. The sensitivity of cell survival to UV radiation was not regulated directly by the LD cycle, but is modulated by the cell's circadian clock.

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‡*Abbreviations:* CHO, Chinese hamster ovary cells; dim LL, dim continuous light; G1, gap 1 phase; LD, light/dark cycle; LD 12:12 = 12 h of light followed by 12 h darkness, LD 14:10 = 14 h light and 10 h darkness, *etc.*; LDT, a phase of an LD cycle; S, synthesis phase; UV, ultraviolet.

MATERIALS AND METHODS

Cell culture. *C. reinhardtii* strains were obtained from the *Chlamydomonas* Genetics Center, Department of Botany, Duke University (Durham, NC). Stock cultures of two *Chlamydomonas* strains, wild-type (mt+ 137c, CC-125) and a long period mutant, *per1* (mt+, CC-1117), were maintained in continuous light on solid tris-acetate-phosphate (TAP) medium (0.4 g/L NH_4Cl, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $CaCl_2 \cdot 2H_2O$, 0.108 g/L K_2HPO_4, 0.056 g/L KH_2PO_4, 2.42 g/L Tris, 1 mL/L glacial acetic acid and 1 mL/L Hutner's trace elements solution (9), pH ~ 7.4, 1.5% agar). For experiments, cells from stock cultures were inoculated into liquid 0.3 high-salt-medium (HSM) (0.3 HSM = HS medium (9) with the following modifications: 0.15 g/L NH_4NO_3, 0.02 g/L $CaCl_2 \cdot 2H_2O$). Liquid cultures were grown phototrophically on various 24 h LD cycles as indicated for each experiment. Cell growth in liquid cultures was determined by counting the cells with a hemocytometer; a small aliquot (50–100 μL) of liquid culture was removed and added to 0.05 vol of 0.25% (wt/vol) iodine in 95% ethanol and the cells counted under a microscope.

In preparation for the UV experiments, Petri dishes (15 × 100 mm^2) were filled with 30 mL of 0.3 HSM plus 1.5% agar the day before plating. Cells from the liquid culture (10^5–10^6 cells/mL) were diluted with 0.3 HSM to a density of 3000 cells/mL, and 100 μL of cells from the diluted culture was spread onto the 0.3 HSM solid medium in Petri dishes. Typically, cells were plated at the beginning of the LD cycle. Individual colonies could easily be identified on all of the plates after 5–7 days of growth. UV treatments were begun within 8 h of plating before individual cells initiated the first round of mitosis. Cells plated on the solid medium were maintained on the same LD cycle to which the liquid culture had been exposed.

UV treatment. Replicate cultures were exposed to UV-C (254 nm) unless otherwise specified (in particular, for the data of Fig. 4, UV-B light was used). Culture plates were placed on a turntable that rotated 60 cm below a germicidal lamp to give an even exposure to UV. During UV exposure, fluence was simultaneously measured and integrated using a Ushio UIT-150 Unimeter (Tokyo, Japan) with a 254 nm detector that was placed at the same distance from the UV source as the culture plates. Plates were treated with UV from the germicidal lamp until the Ushio meter registered the desired fluence (*e.g.* 40 J/m^2). The duration of the UV-C radiation needed to obtain a fluence of 40 J/m^2 was typically 30–45 s. Following the experimental treatments, the plates were placed in darkness for 18–24 h. After the dark incubation, cultures were placed in continuous light (25–30 $\mu E/m^2/s$) until colonies were visible (about 5–7 days), at which time they were counted. Data were normalized by dividing experimental values by the number of colonies on corresponding control plates.

The effect of blue light was measured on cultures exposed to UV-C followed by 30 min treatment with blue light from a 20 W Toshiba (Tokyo, Japan) bulb (FL20S BLB) that hung 30 cm above the plates. Plates were incubated in darkness for 18–24 h following blue-light treatment, and placed in continuous light until colonies were visible.

Cultures were exposed to UV-B from a Philips bulb (Universal Light Sources, San Francisco, CA) (TL40W/12/RS; peak = 310 nm) covered with one layer of 5 mil cellulose acetate to block the residual UV-C. As with UV-C, culture plates were placed on a turntable that rotated 9 cm below the UV-B bulb. Following UV-B treatment, cultures were incubated in the dark for 18–24 h followed by continuous light until colonies were visible. The intensity of UV-B was measured with a National Biological Corporation UV-B meter (Twinsburg, OH). Eleven measurements of UV-B irradiance were made over a 20 min period. The mean ± standard deviation for irradiance was 2.8 ± 0.13 $J/m^2/s$. Using the Ushio UIT-150 Unimeter with the 254 nm detector, no UV-C could be detected from this UV-B lamp.

RESULTS

Circadian rhythms of UV sensitivity

The deleterious effect of UV on *C. reinhardtii* was tested by measuring the colony-forming ability of cells after they were exposed to UV radiation. Cells that were able to form visible colonies were considered to have survived the UV treatment. Cells plated on solid medium at a dilute concentration were treated with various doses of UV radiation. As shown in Fig. 1a, *Chlamydomonas* cultures were sensitive to UV-C in a dose-dependent fashion. UV-C sensitivity was phase dependent; cells on an LD cycle of 12 h light/12 h dark (= LD 12:12) that were treated 4 h after lights-off (LDT 16) were less viable than those irradiated 4 h after lights-on (LDT 4). (LDT is a phase of an LD cycle. By definition, LDT 0 equals dawn. On LD 12:12, dusk is LDT 12; on LD 14:10, dusk is LDT 14.)

We then tested the effect of UV-C on survival over a 28 h time course at 4 h intervals with a moderate dose (40 J/m^2) of UV-C. There was a striking dependence of survival on the daily phase of irradiation (Fig. 1b). UV-C sensitivity was most pronounced during the late day and early night

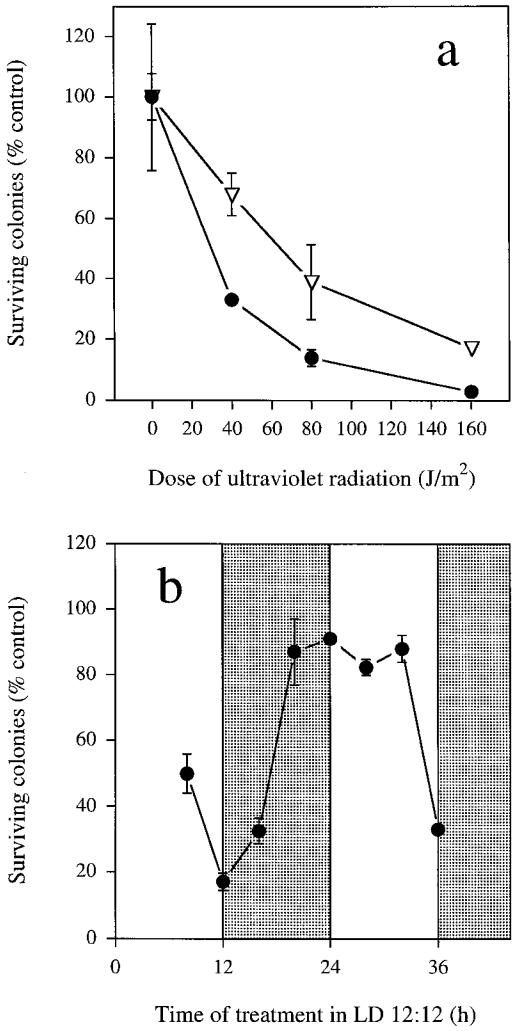

Figure 1. Effect of UV radiation on the survival of wild-type *C. reinhardtii*. Cells maintained on solid medium on LD 12:12 for one day were exposed to UV-C. Survival was measured as the colony-forming ability of cells following treatment. The mean value and standard deviation are plotted for triplicate measurements. (a) The percentage of surviving colonies is plotted against increasing amounts of UV-C radiation. Inverted triangles: UV-C treatment at 4 h after lights-on (LDT 4). Circles: UV-C treatment at 4 h after lights-off (LDT 16 on LD 12:12). (b) Time course of the effect of UV-C exposure at 4 h intervals during the LD 12:12 cycle. Dose of UV-C was 40 J/m^2. Stippled blocks represent the dark intervals of the LD cycle.

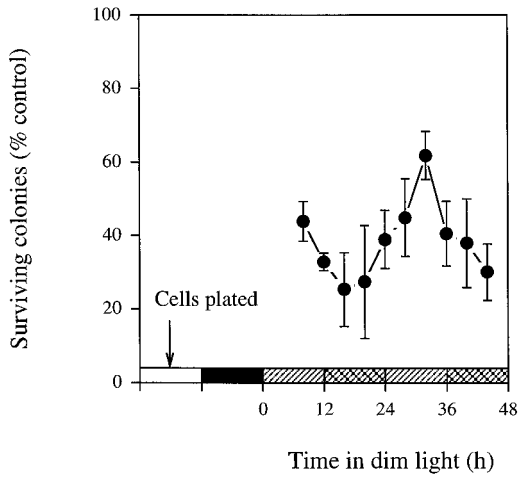

Figure 2. Effect of UV-C exposure of cultures maintained on dim LL. Wild-type cells that were entrained to LD 12:12, while in liquid culture, were plated during the day (arrow) and incubated in bright light (25–30 $\mu E/m^2/s$) for the remainder of the day and in darkness during the night. At dawn, the cultures were placed in dim LL (0.2 $\mu E/m^2/s$) and allowed to free-run. UV-C treatments (1 min exposures to a germicidal lamp; approximately 100 J/m^2) commenced 8 h after the dawn of LL and continued for 32 h. The mean value and standard deviation are plotted for triplicate measurements. Bars at the bottom of the graph indicate day (white), night (black), subjective day (hatched) and subjective night (cross-hatched). Subjective day: 0–12 and 24–36 h. Subjective night: 12–24 and 36–48 h.

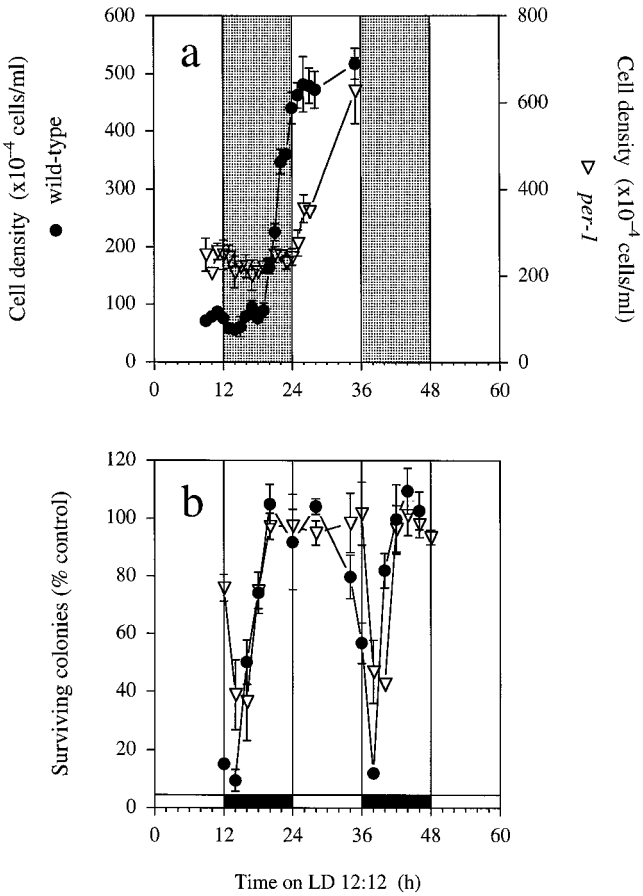

Figure 3. Comparison of hatching rhythms and time course of survival from UV-C exposure in wild-type *versus per1* (long period) cells. (a) The 24 h rhythm of hatching was determined by counting cells from liquid cultures. Wild-type (circles) and *per1* (inverted triangles) cells were grown in HSM and bubbled with humidified air on an LD 12:12 cycle (light intensity was 150 $\mu E/m^2/s$). (b) Daily rhythms of survival after UV-C exposure in wild-type and *per1* cells. The percentage of surviving colonies (mean ± standard deviation; N = 3) is plotted at the phase of UV-C treatment. Circles: wild-type cells; inverted triangles: *per1* cells. Dose of UV-C was 40 J/m^2. Bars on each graph represent the time of light (white bar) or darkness (dark or stippled bars).

phases. Cells were essentially insensitive to UV-C irradiation during the late night and during the day. Cells were slightly less sensitive to UV-C radiation during the second cycle (see also Figs. 3 and 5), possibly reflecting the increased number of cells in a colony following one round of cell division. Because *Chlamydomonas* is a multiple-fission alga, a cell that is growing vegetatively may yield daily two or more daughter cells, all of which need to be incapacitated by UV treatment to prevent colony formation.

The rhythmic variation in survival to UV-C irradiation persists in continuous dim light (dim LL) as a free-running circadian rhythm. In dim LL, the cells were more sensitive to UV-C during the subjective night and less sensitive during the subjective day (Fig. 2). Overall, *Chlamydomonas* was more sensitive to UV-C in dim LL than on LD cycles. To our knowledge, the 24 h periodicity of sensitive and insensitive phases seen in Figs. 1b and 2 has not been previously reported.

Phasing of UV sensitivity and cell division cycle events

Because studies with yeast cells had revealed UV-C sensitivity that was a function of the phase of the cell division cycle (10), and because the timing of cell division is controlled by the circadian pacemaker in *Chlamydomonas* (11,12), we reasoned that the daily rhythm of UV-C sensitivity might be due to circadian phasing of some cell division cycle event(s). To test that hypothesis, we compared the phasing of UV sensitivity in wild-type cells with that of a circadian period mutant of *Chlamydomonas.* In constant light, this mutant (*per1*) has a free-running circadian rhythm of phototaxis with a period of 26–28 h, 3 h longer than wild-type cells which display a phototaxis circadian period of 23–25 h (11,13). In an LD 12:12 cycle, the circadian oscillator of both wild-type and *per1* mutant cells entrains to the LD cycle such that their periods become 24 h. However, circadian oscillator theory would predict that the longer period mutant would entrain to LD 12:12 so that its rhythms could occur at a later phase than those of wild-type cells (14). This is what we found for the diurnal rhythm of one cell division cycle event, the phasing of daughter cell liberation (or ''hatching'' from the mother cell wall). During vegetative growth on LD 12:12, daughter-cell liberation occurred significantly later for *per1* cells than for wild-type cells (Fig. 3a). Wild-type cells began to hatch during the middle of the night (LDT 18–20) while *per1* cells began to hatch later, during the day (LDT 1–4).

Although the liberation of daughter cells is delayed by 5–10 h in *per1* cells, other cell division cycle events, namely nuclear DNA replication and cytokinesis, occurred only slightly later in *per1* cells as compared with wild-type cells (assessed by microscopic inspection of fixed cells treated

with ribonuclease and stained with propidium iodide by the method described in Yee and Bartholomew (15)). *Per1* cells inspected at LDT 9 had one dense spot of staining near the basal body of the flagella and less-intensely stained particles elsewhere. Our interpretation of this image is that the dense stain represents DNA in the nucleus while less-intensely stained particles represent chloroplast DNA. The compactly stained spot swelled and diffused at LDT 14 on LD 12:12, suggesting that nuclear division was underway. This diffuse staining persisted until LDT 17 when two stained spots could be seen near the basal body of some cells. By LDT 18, multiple nuclei could be seen within the mother cell wall of some cells. Some cells contained only two nuclei within the mother cell wall (one mitotic division), others contained four (two mitotic divisions), while rarely there were eight or more nuclei with a single mother cell wall (three or more mitotic divisions). These results were similar to those of wild-type cells on LD 12:12. In wild-type cells harvested and stained at LDT 10 and 12, a compact nuclear fluorescence was visible for many cells. At LDT 14, the fluorescence staining was diffuse, and by LDT 16, stained particles had a compact, stained nucleus for each cell contained within the mother cell wall. We were unable to obtain clear results by labeling of DNA with tritiated adenine or with flow cytometry. Radiolabeled thymidine incorporation only labels chloroplast DNA in *Chlamydomonas* (16).

When compared to wild-type cells, the timing of one cell division cycle event (hatching) is greatly delayed in *per1* cells, while that for an earlier cell division cycle event (nuclear division) is almost the same. When these rhythms are compared with that for UV sensitivity, we found that the timing of maximal UV-C sensitivity of *per1* occurred slightly later than that for wild-type; wild-type at LDT 14, while *per1* was most sensitive at LDT 16 (Fig. 3b; also compare Fig. 5a with b). This timing more closely matches when nuclear division occurs rather than hatching, and implies that the UV sensitivity rhythm might be related to nuclear division or to an early cell division cycle event that is linked with nuclear division.

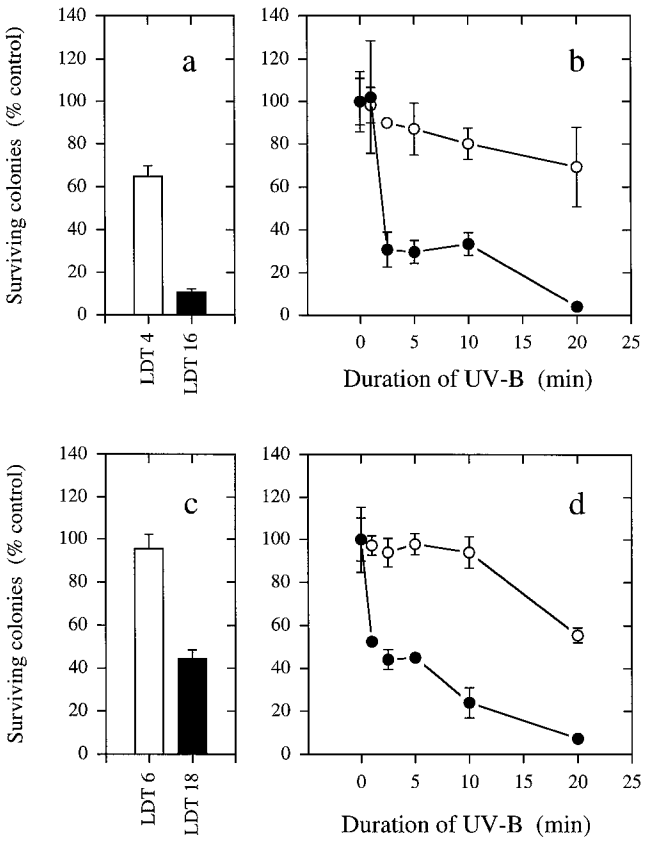

Figure 4. Comparison of UV-C *versus* UV-B survival. Cells were exposed to UV-C (panels a and c) or UV-B (panels b and d) as described in Fig. 1, except half of the number of replicates for each strain (wild-type or *per1*) were entrained to the opposite LD cycle enabling simultaneous treatment of cells from opposite phases. The mean and standard deviation are plotted for triplicate measurements. UV-C exposures were 40 J/m^2 at 254 nm. UV-B exposures were for various durations at an intensity of 2.8 J/m^2/s. (a) Wild-type cells were exposed to UV-C at LDT 4 or 16. (b) Time course of UV-B exposure at LDT 4 (open circles) or LDT 16 (closed circles) for wild-type cells. (c) Long period mutant cells (*per1*) were exposed to UV-C at LDT 6 and 18. (d) Time course of UV-B exposure at LDT 6 (open circles) or LDT 18 (closed circles) for *per1* cells.

Relevance to natural environments: UV-B and photoreactivation

In the natural environment, *Chlamydomonas* cells would probably not be exposed to significant levels of UV-C due to its absorption by the ozone layer in the atmosphere. However, because there is significant penetration of UV-B (wavelengths of 280–315 nm), we wanted to confirm that the results we obtained with UV-C were relevant to *Chlamydomonas* in a natural setting. For this experiment, two cultures were entrained to opposite LD 12:12 cycles for 5 days in liquid 0.3 HSM. Plates of solid 0.3 HSM were inoculated as described for previous experiments at the beginning of the day. At the phases indicated in Fig. 4 (LDT 4 for one set of plates and LDT 16 for the other set of plates), cells were treated simultaneously with UV radiation. A comparison of the daily sensitivity of wild-type cells to UV-C with that to UV-B shows that as with UV-C, wild-type cells are more sensitive to UV-B during the night than during the day. During the day (LDT 4 on LD 12:12), we detected only a slight effect of UV-B and UV-C on cell survival (Fig. 4a,b). In contrast, wild-type cells were much more sensitive to UV-B and UV-C irradiation during the night phases (LDT 16 on LD 12:12). Essentially the same result was obtained with *per1* mutant cells on LD 12:12 (Fig. 4c,d).

The primary difference between UV-B and UV-C was the enhanced cellular sensitivity to the more energetic UV-C. A fluence of 40 J/m^2 for UV-C resulted in a greater than 80% reduction of wild-type cell survival at night, whereas the fluences for UV-B that resulted in a similar reduction in cell survival were greater than 168 J/m^2 (2.8 J/m^2/s $\times$ 60 s). Therefore, the UV-C sensitivity of *Chlamydomonas* cells is relevant to the wavelengths of light to which the cells would be exposed in their natural environment (UV-B); for experimental convenience, we have used UV-C as our testing condition for most of the experiments reported herein.

Our data show that during the late phases of the day, when the cells are still exposed to light, UV radiation can be deleterious to cell survival. However, at least two factors might minimize cell death due to UV during the late day exposure to sunlight. First, measurements of the full spectrum of sun-

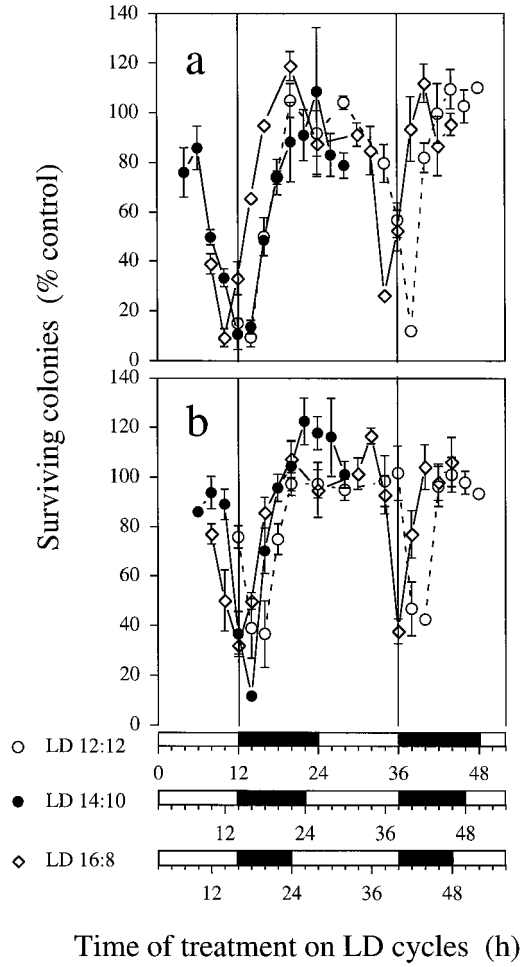

Figure 5. Survival from UV-C exposure for *Chlamydomonas* cells growing on different photoperiods. Cells on three different photoperiods, LD 12:12 (○), LD 14:10 (●) and LD 16:8 (◇), were treated in three separate experiments with UV-C (40 J/m^2). Data were collected over a 36 h period for experiments with cells on LD 12:12 or 16:8. For cells on LD 14:10, only 24 h of data were collected. The phases of light and dark periods are shown as the open and closed bars, respectively, at the bottom of the figure. The scales are plotted such that lights-on is designated as LDT 0 (=circadian convention), but are lined up relative to lights-off; (a) wild-type cells, (b) *per1* cells. The mean value and standard deviation are plotted for triplicate measurements.

light during the course of the day show that the UV component is attenuated in early and late day (17). Therefore, there might not be significant levels of damaging UV in the late afternoon when *Chlamydomonas* cells begin to be UV sensitive.

Second, if the deleterious effect of UV is due to DNA damage, under natural conditions *Chlamydomonas* may be able to reduce the deleterious effect of UV during the daytime when sunlight is present by repairing DNA damage with blue-light activated DNA photolyase (18). The enzyme DNA photolyase directly reverses cyclobutane pyrimidine dimerization, a type of DNA damage caused by UV. To test the hypothesis that DNA photolyase may be active at certain phases of the day, plates of cells were treated with UV-C and half of the replicate plates were subsequently exposed to 30 min of blue light. As with all previous experiments, plates were incubated in darkness for 18–24 h following UV or UV + blue-light treatments to prevent additional activation of DNA photolyase subsequent to the experimental treatment. For the plates treated with UV only, significant decreases in survival between control (no UV treatment) and experimental samples were found at all three phases (Table 1). During the day, cells were better able to survive UV exposure if they were treated with UV followed by blue light; at LDT 10 and 12, the subsequent treatment of UV-treated plates with blue light substantially rescued viability (as can be seen by the lack of a significant difference between the control plates and the UV + blue plates at LDT 10 and 12). On the other hand, at LDT 14 (2 h after lights-off), there was little rescue, by blue light, of the UV-induced decrease in survival.

Therefore, cells exposed to UV during the early night phase were less able to survive UV exposure regardless of whether they received additional blue-light exposure. Because UV levels are low at the end of the day, and because photoreaction appears to be active at the end of the day, the decline in recovery of *Chlamydomonas* cells from UV administered during the late day phase (*e.g.* LDT 12) in the laboratory is not likely to be an important factor in their ability to survive in nature. If UV exposure were to occur just 2 h later into the night, *Chlamydomonas* would not likely survive. This scenario, however, does not occur in nature.

Phasing of UV sensitivity on different photoperiods

The data shown in Figs. 1 and 3 are from cells on LD 12:12. But, *Chlamydomonas* cells in the natural environment

Table 1. Effect of blue light on survival following UV radiation at times near the light-to-dark transition on LD 12:12

Phase of treatment	Surviving colonies (mean ± SD)* Control (no treatment)	Treated with UV only	Treated with UV + blue light
LDT 10	100 ± 10.8%	31 ± 10.5%†	66 ± 21.9%
LDT 12	100 ± 12.9%	23 ± 5.3%†	94 ± 13.4%
LDT 14	100 ± 21.1%	12 ± 3.9%†	26 ± 15.6%†

* At each phase, wild-type cells were treated with UV-C only or UV-C followed by 30 min of blue light at three phases of the LD 12:12 cycle. UV-C dose was 40 J/m^2. Surviving colonies were normalized to the mean of control plates (no treatment) for each phase. ANOVA indicated that the averages were significantly different ($P < 0.005$).

† Significantly different from corresponding control by student's *t*-test ($P < 0.05$).

can be exposed during summer to much longer days. Since the cells are sensitive to UV near the end of the day on LD 12:12, we wondered if UV killing might be enhanced on long summertime photoperiods. Therefore, we tested the survival to UV under a range of photoperiods to which *Chlamydomonas* cells might be exposed in nature: LD 12:12, LD 14:10 and LD 16:8. Wild-type cells on LD 12:12 and LD 14:10 display almost the same phasing of their UV-sensitivity curves (maximum sensitivity during the early night), while the maximum sensitivity to UV occurred during the late day on LD 16:8 (Fig. 5a). The same result was obtained with the *per1* mutant (Fig. 5b), except that, as was shown in Fig. 3, the phasing of UV sensitivity is later for all photoperiods in *per1* as compared with wild-type cells. These results imply that *Chlamydomonas* cells might have more trouble with UV on very long photoperiods, which are reached during the summer at the upper latitudes of the species range (*e.g.* Scotland) (9).

DISCUSSION

UV sensitivity: circadian and cell division cycle rhythms

The unicellular alga *C. reinhardtii* showed a phase-dependent difference in its ability to survive UV irradiation. This phase-dependent sensitivity was detected in colonies maintained on three different LD cycles (LD 12:12, LD 14:10 and LD 16:8) and in dim LL. These data are consistent with the interpretation that some parameter, which confers UV sensitivity, is regulated by the circadian clock in *Chlamydomonas*. What could this parameter be? A likely candidate is the cell division cycle. In the yeast, *Saccharomyces cerevisiae*, and in cultured mammalian cells (CHO cells), UV sensitivity is a function of the phase in the cell division cycle; cells are most sensitive to UV radiation during the gap 1 (G1) and early synthesis (S) phases (10,19).

The timing of cell division in *Chlamydomonas* cells is triggered by the circadian clock (11,12). We found that the phase of maximal UV sensitivity occurred within 1–4 h of the light-to-dark transition; this phasing correlates closely with the timing of DNA synthesis. For *Chlamydomonas* cells synchronized to LD 12:12, increase in nuclear DNA (S phase) occurs during the first 6 h of the night (20–24). Therefore, as with yeast and CHO cells, the time of maximal UV sensitivity for *Chlamydomonas* falls within the time of G1/S phase (during the first 4 h of the night on LD 12:12). *Chlamydomonas* is a multiple-fission alga where DNA synthesis and nuclear division occur sequentially and often at least twice during a cell division cycle (25). Differences in growth conditions (especially light intensity) will allow *Chlamydomonas* cells to divide more than once in a night. Generally, the number of rounds of mitosis a cell will undergo during the night depends on the amount of growth of that cell during the day (21,26). In a population of *Chlamydomonas* cells, there may be cells undergoing one, two or three rounds of cell division during the night. In order for UV irradiation to prevent colony formation, all cells resulting from mitotic events from a single parental cell must be inactivated. It would not be surprising if UV irradiation early in the S phase of *Chlamydomonas* would have the maximal impact because the probability of inactivating two progeny cells at the beginning of cell division would be greater than inactivating all four progeny cells later in the cell division process.

Our experiments suggest that a process that occurs early in the night is sensitive to UV radiation. That process(es) may include DNA synthesis and/or DNA repair mechanisms such as excision repair. Pyrimidine dimers caused by UV radiation can be removed by the dark DNA repair mechanism, excision repair, if the fluence of UV is not very great (27). In *Chlamydomonas*, excision repair was able to completely remove UV-induced pyrimindine dimers formed by UV radiation of 25 J/m^2, but no excision repair activity was detected if the UV fluence was 250 J/m^2 (27). The fluence of UV radiation we used in this study was between these values (in our case, 40 J/m^2). At this UV dose, differences in excision repair activity at different phases of the 24 h day may become measurable.

The variable sensitivity to UV radiation at different phases of the 24 h day is not due solely to an increase in cell number (and hence an increase in the number of hits needed to prevent colony formation). If survival depended only on the number of cells in a colony at the time of UV exposure, one would expect to see a steady increase in the number of colonies surviving as UV treatment was delayed from the time of plating. Instead, we observed a rhythm of survival with a minimal decrease in the number of colonies if UV radiation was given during the day or late night phases, and a maximal decrease in the number of colonies if UV radiation was given during the early night phase on either the first or second 24 h cycle following the plating of cells.

Therefore, our interpretation of the data is that the cells exhibit a daily/circadian rhythm of UV sensitivity because cell division is regulated by the circadian clock. The cells are most sensitive around dusk, which is when the first round of DNA synthesis would be predicted to occur. Different photoperiods did not greatly alter the phase of maximal sensitivity, which occurred at LDT 14 for wild-type cells and LDT 16 for *per1* cells. Even though *Chlamydomonas* cells are sensitive to UV before dusk, they might be able to survive under these conditions because in natural photoperiods the intensity of UV radiation declines before the end of the day (17). At these times, blue-light activated photolyases might be robust enough to repair UV-induced DNA damage since the intensity of the UV is not too great.

Could UV cycles have been instrumental in the evolution of circadian clocks?

Is UV a threat in nature to *Chlamydomonas* cells? Even in the present day when the ozone layer protects the earth from the most energetic UV radiation (UV-C), it might be adaptive for UV-exposed organisms to segregate the UV-sensitive phases of the cell division cycle to the night time. Although the ozone layer blocks solar UV-C radiation, UV-B radiation penetrates the ozone layer, and could have a deleterious effect on cell survival, especially for cells that are exposed to UV. Cells that are naturally exposed to sunlight (and UV-B) such as corneal cells of Japanese quail (28), plants (29) and several protists (8) segregate mitosis to the night.

Protists like *Chlamydomonas*, which live in soil and in

shallow aquatic environments, are exposed to UV-B during most of the daytime. Protists living in the surface layers of soil would obviously be exposed to UV-B. Even in aquatic environments, UV penetration can be significant. One study found that the viability of bacteriophages absorbing solar radiation at the surface of the ocean for 1 day decreased by 96% (30). Even at a depth of 2 m, the bacteriophage viability decreased by 65%. Although UV penetration through pond water is less (absorbance of 312 nm light is 10 times greater in pond water than in ocean water (31)), organisms at the pond water surface would be exposed to similar UV doses.

The consequences of UV irradiation could have been significant to the evolution of circadian clocks, especially since the ozone layer did not exist at the time of the early evolution of cells. Estimations of when the present ozone layer developed are imprecise; the available geological data can only estimate the atmospheric O_2 concentrations that were present 0.5–2 billion years ago to a range spanning 2–3 orders of magnitude. Nonetheless, the oxygenation of the atmosphere is thought to have arisen in at least two major events: the first around 2 billion years ago and the second about 550–600 million years ago. From the present time to 600 million years ago, the range of atmospheric O_2 concentrations lies well within the calculated range of atmospheric O_2 concentrations that could produce an ozone layer that is a biologically effective screen of UV radiation. Between 600 million years ago to 2 billion years ago, the atmospheric O_2 concentration may have been at the threshold level that could produce a biologically effective ozone screen (32).

Eukaryotes appeared 1–2 billion years ago (33). Prokaryotic cyanobacteria evolved as early as 3.5 billion years ago (33), and some present-day species of cyanobacteria are known to possess circadian clocks (1). Therefore, at least these circadian clocks are likely to have evolved under strong selective pressure from the daily cycle of UV-C. Based on the above estimates of when eukaryotes and the ozone layer arose, it is possible that the earliest eukaryotes may have faced life in an environment with damaging UV-C radiation, and certainly with UV-B radiation. These organisms may have employed a number of the same avoidance and DNA repair mechanisms as present-day plants and animals (34–40) to survive under these conditions (41). As suggested by our data, one adaptive strategy may have been the evolution of a circadian program that regulated UV-sensitive events, such as DNA synthesis and nuclear division, to occur in the night, an ''escape from light'' (7). The advent of an effective ozone layer may have allowed organisms that had already developed effective mechanisms to minimize UV-C damage to subsequently expand into new niches undeterred by the weaker UV-B and UV-A radiation.

Therefore, the daily cycle of UV radiation may have created a selective pressure favoring the evolution of circadian clocks. This hypothesis dovetails with the recent discovery of a role for cryptochromes in circadian systems. Cryptochromes are pigmented photoreceptors that are involved in blue-light mediated photomorphogenesis (42) and photoperiodism (43) in plants. Cryptochromes share sequence homology to another blue-light activated protein, DNA photolyase, which uses blue-light energy to catalyze the photochemical reversal of pyrimidine dimerization caused by UV radiation (44). Cryptochromes are photoreceptors that are not involved in DNA repair, but probably evolved from a DNA photolyase. Cryptochromes appear to play a role in the photoentrainment of circadian clocks in *Drosophila* (45,46) and *Arabidopsis* (47), and a nonphotoreceptive role in the central oscillator of mammals (48,49). Possibly, an ancestral protein involved in repair of DNA damaged by a daily cycle of UV may have been enlisted in a photosensitive process that later was incorporated into the mechanisms for biological timing.

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