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Correlation of the Period Length of Circadian Rhythms with the Length of Immaturity in *Paramecium bursaria*

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ABSTRACT—The circadian photoaccumulation rhythm of thirty strains of *Paramecium bursaria* collected at different places in Japan and China were measured with a microcomputer assisted data collection apparatus. Although most strains showed a period of 23–26 hours in LL, we found two strains of conspicuously different periods; a short period strain (UK1, 21.8 hr) and a long period strain (T316, 28.7 hr). F1 progeny from a cross between the short and the long period strains showed an intermediate period of about 24.7 hours (range 22.5–25.8 hr). The character was not distributed in a Mendelian ratio among the F1 progeny. We isolated a mutant (E2) with short period (21.8 hr) from the stock strain Kz1 by treatment with nitrosoguanidine (MNNG). The progeny of crosses between E2 and UK1, and between E2 and T316 exhibited the short period and the normal period phenotype respectively. Moreover, the progeny from a cross between E2 and a wild type strain (Sj2w) became sexually mature about 25 fissions after conjugation. This length of immaturity is much shorter than that of the progeny from wild type strains (about 50 fissions). This early maturation character was inherited to progeny in a Mendelian ratio. Homozygotes for the early maturation allele (EM2) exhibited mating ability about 15 fissions after conjugation. These data suggest that there is a correlation between the period length of the circadian rhythm and the length of immaturity after conjugation in *Paramecium bursaria*.

INTRODUCTION

Circadian rhythms have been found at all levels of biological organization including both eukaryotes and prokaryotes [10, 12] and their period lengths are usually about 24 hours in constant conditions. The oscillating mechanism which controls these rhythms is often called a "Circadian clock". Cells of the unicellular organism, *Paramecium bursaria*, show several kinds of circadian rhythms, for instance, mating reactivity, swimming velocity and negative gravitaxis [14, 17]. Further, cells of *P. bursaria* accumulate in a lighted area in the day time than at night. This phenomena is called photoaccumulation [4] and is controlled by a circadian oscillator [9], in a manner similar to that for *Euglena* photoaccumulation [3].

In addition, an exconjugant clone of *Paramecium*, as well as many other ciliates, has a well-defined life cycle, consisting of the sequence of immaturity, adolescence, maturity and senility [7, 20, 23]. The duration of each phase of the life cycle is measured by the number of mitotic fissions [10, 15, 24] and has been shown to be controlled genetically [21, 25]. Some mutants with altered length of immaturity also have been discovered in *Tetrahymena pyriformis* [1] and in *P. caudatum* [16]. Mating type inheritance and determination in *P. bursaria* are controlled by pairs of alleles at two independently assorting loci. The two mating type loci are expressed sequentially; one locus is expressed in the adolescent phase and both loci are expressed when the cells become mature [22]. Thus, the phenomenon of immaturity in *Paramecium* is a suitable model for studying biological timing measured by a "Developmental clock."

Is there a relationship between the time-keeping mechanisms of the "circadian clock" and "developmental clock"? In the present article, we will report the first evidence of a relationship between the period length of circadian rhythms and the length of sexual immaturity, using an early maturation mutant induced by nitrosoguanidine.

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MATERIALS AND METHODS

Strain and Culture

The strains of *Paramecium bursaria*, syngen 1 used in the experiment of measuring the period length of photoaccumulation rhythm were collected at different places in Japan and China. Most of these strains were obtained from Dr. Kosaka at Hiroshima University. We used especially following strains for cross: Kz1 (mating type III, collected at Kouzan in Hirosima), Sj2 (mating type I, collected at Sinji in Shimane), T316 (mating type IV, collected at Tsukuba in Ibaraki), UK1 (mating type I, collected at Urizura in Ibaraki). Strain Sj2w (Chlorella-free white cells) have been induced from Sj2 (Chlorella-containing stock) by rapid growth in the dark. Strain E2 was an early mature mutant induced from Kz1 by treatment with nitrosoguanidine (2 μg/ml for 5 hours).

All strains were cultured in fresh lettuce juice medium, which had been inoculated with *Klebsiella pneumoniae* one day before use [6]. Cultures were kept at 25°C under the light/dark cycle (LD 12:12 hr, 1,000 lux of cool-white fluorescent light).

Photoaccumulation rhythm

The photoaccumulation rhythm was measured in constant light (LL) as described in the previous paper [9]. The apparatus used to assay this rhythm of *Paramecium* was modified from that described by Bruce [2]. Assay of this rhythm, expressed as the decrease of transmittance in percent of the initial transmittance, is easily auto-

mated by a microcomputer-assisted data collection apparatus. The trough or peak of the photoaccumulation rhythm was used as the phase reference point. The time of the phase reference point was calculated by a computer program that was made by Kondo [11], which fitted a parabola to the data of each peak and trough. For calculation of period, the time of the phase reference points were subjected to a least-squares regression.

Mating reactivity rhythm

Mating reactivity of the green cells was tested by mixing them with white "tester" cells of complementary mating type as described in the previous paper [14]. Cells of *P. bursaria* are usually green, containing symbiotic *Chlorella* in the cell. *Chlorella*-free white cells can be derived from green cells. White cells were used as tester cells, since the green cells were easily distinguished from the white tester cells in the mating clumps.

Induction of short period mutant

Concentrated log phase cells of Kz1 (green cells, mating type III) were treated with 2 μg/ml nitrosoguanidine for 5 hours. Cells were washed 3 times with K-DS solution. After about 10 fissions, cells were mixed with Sj2w (white cells, mating type I) for induction of cytogamy to get homozygotes. Cytogamy can be induced with osmotic shock by treatment with 1% Methyl Cellulose for 2 hours at the 3rd micronuclear meiotic division [18]. The conjugation pairs were collected by nylon mesh. At first barium resistant green cells were selected with 9 mM $BaCl_2$ for 15 minutes 5–6 fissions after cytogamy. Then barium resistant cells were assayed for a photoaccumulation rhythm.

Determination of the duration of immaturity

The number of fissions after conjugation was calculated by using the daily isolation culture in depression slides [13]. The cells remaining in the depression slide after daily isolation were transferred into small test tubes containing 1 ml of culture medium, then 2 ml and 4 ml of fresh culture medium were added to the test tubes for two days successively. The cells divide 6 times during the culture of these test tubes. Tests of mating reactivity were performed every day with the cells in the test tubes starved for two days. The cells were mixed with four different mating types (I-IV) of tester cells. We excluded clones that showed the same mating type to parents at the first mating reactivity test (immature test). We used clones whose mating ability did not appear at the first test as a true progeny. When the cells of progeny get to adolescence they can mate with cells of two mating types, and when they get to maturity they can mate with cells of three mating types (all except their own mating type).

RESULTS

Genetic analysis of the period of photoaccumulation rhythm

The photoaccumulation rhythms of 30 strains collected at different places in Japan and China were measured automatically for 7 days in LL. Although most of the strains showed 23–26 hour periods of the photoaccumulation rhythm, two strains of conspicuously different period length were isolated, one strain UK1 showing shorter period (21.8 hr) and another strain T316 showing longer period (28.7 hr), as shown in Fig. 1. To know the period of another circadian rhythm, the mating reactivity rhythm of these two different strains was assayed in LL. As shown in Fig. 2, these two strains exhibited a similar period length for mating reactivity as for the photoaccumulation rhythm.

Next we isolated 32 F_1 progeny from a cross of UK1 and T316, and their photoaccumulation rhythm was assayed after the immature test. As shown in Table 1, they showed about 24.7 hours mean period. The range from the shortest to the longest period was 22.5–26.8 hours. Then we isolated 28 F2 progeny from the clones showing a period of medium duration (about 24 hours). Mean period of F2 progeny was 23.8 hours. These period lengths did not segregate into two or three major groups, and they were distributed almost equally between 21.8 to 26.7 hours. In the back cross between F1 progeny and parents, the two groups of period were not segregated and the range of periods spread equally. However, the mean period in the progeny from back cross with T316 was longer than that from the back cross with UK1 (Table 2). These results suggest that the difference of periods between UK1 and T316 is controlled polygenetically or by the ecotype that is influenced by the ecosystem.

Induction of mutation

To get a typical mutant clone of the circadian rhythm, we treated cells of Kz1 with nitrosoguanidine (MNNG). Cells were mutagenized as described in Fig. 3 and were screened with 9 mM $BaCl_2$ for 15 minutes. We could obtain 24 barium resistant cells after performing this protocol 5 times. The photoaccumulation rhythms of these clones were measured. Almost all clones showed a period of about 26.0 hours, which is nearly the same as the period of Kz1. But one clone (E2) showed a short period rhythm of 21.8 hr (Fig. 4), and we found no clones which exhibited a long period rhythm or an arrhythmic pattern. As listed in Table 3, the progeny from a cross between E2 and UK1 showed a short period, and that from a cross between E2 and T316 showed a slightly longer period. But the range of period length of these progeny did not so wide. Progeny from E2 and Sj2w showed an intermediate period length. During this experiment, we noticed that the progeny of E2 matured early.

Genetic analysis of early mature mutant

An exconjugant clone of *Paramecium* has a period of immaturity, during which cells cannot mate even when they enter the stationary phase. The length of immaturity depends upon the number of fissions undergone since conjugation. In the case of *P. bursaria*, exconjugants become adolescent after about 40 fissions and mature about 55 fissions after conjugation. They never show mating reactivity after less than 30 fissions. For genetic analysis of the early maturation character, E2 (mating type III) was mated with Sj2w (mating type I). Almost all progeny were able to mate about 25 fissions after conjugation and showed mating type I or IV. The appearance of maturity of F2 progeny clones (from SE11$\times$SE14, which are F1 progeny from Sj2$\times$E2) segregated into three groups, about 15, 22, 52 fissions after conjugation (Fig. 5). Number of F2 progeny is too small for χ^2-statistical test, but its ratio (3:10:4) was close to 1:2:1

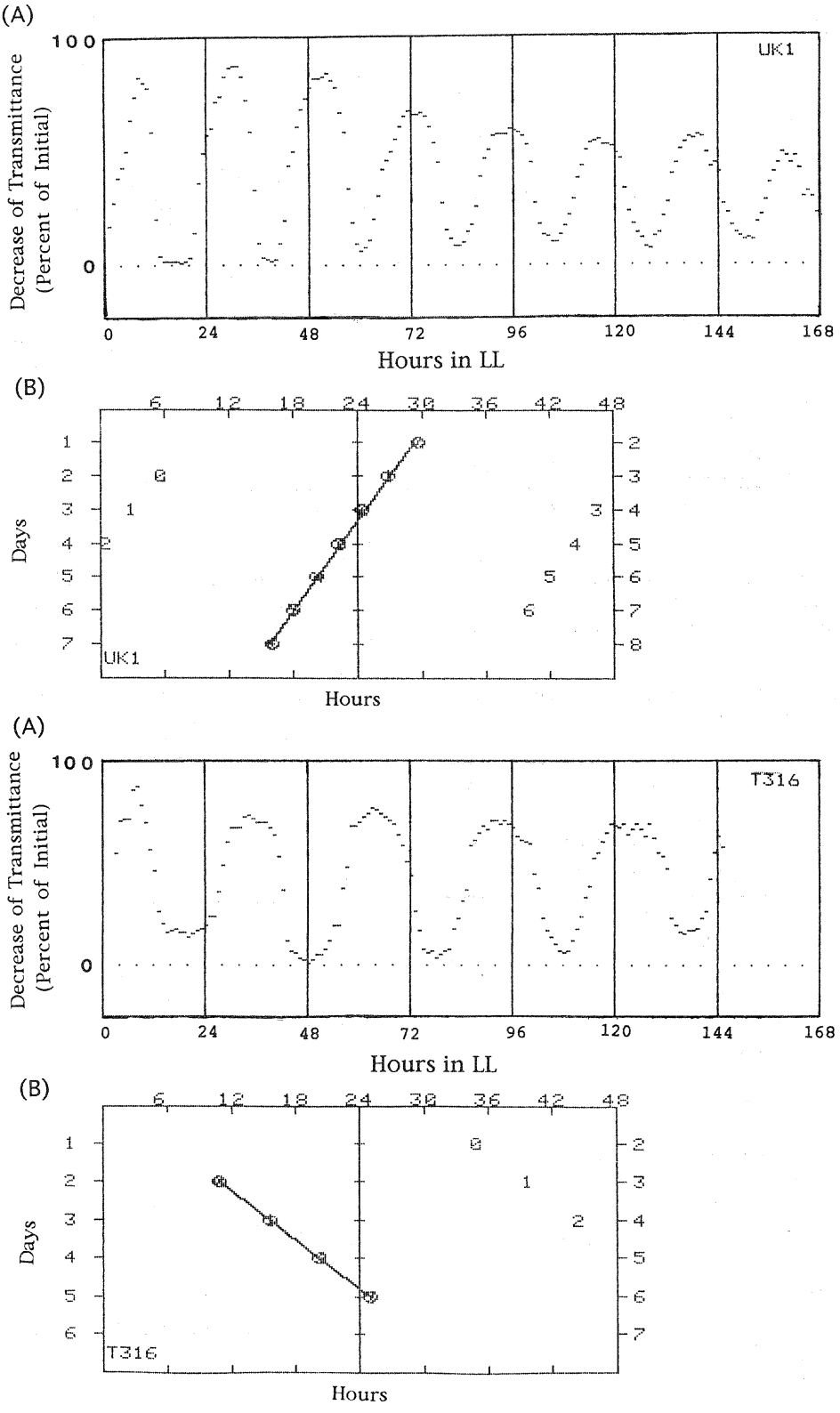

FIG. 1. Photoaccumulation rhythms of short period strain (UK1, 21.8 hr) and long period strain (T316, 28.7 hr) in LL at 25°C. (A): photoaccumulation (ordinate) was assayed every hour automatically by a microcomputer assisted data collection apparatus, and was expressed as the decrease of transmittance in percent of the initial transmittance. (B): The period of rhythm was calculated by a computer program that performs a least-squares regression using the phase reference points of peak values. Points of peaks are presented as a double plot.

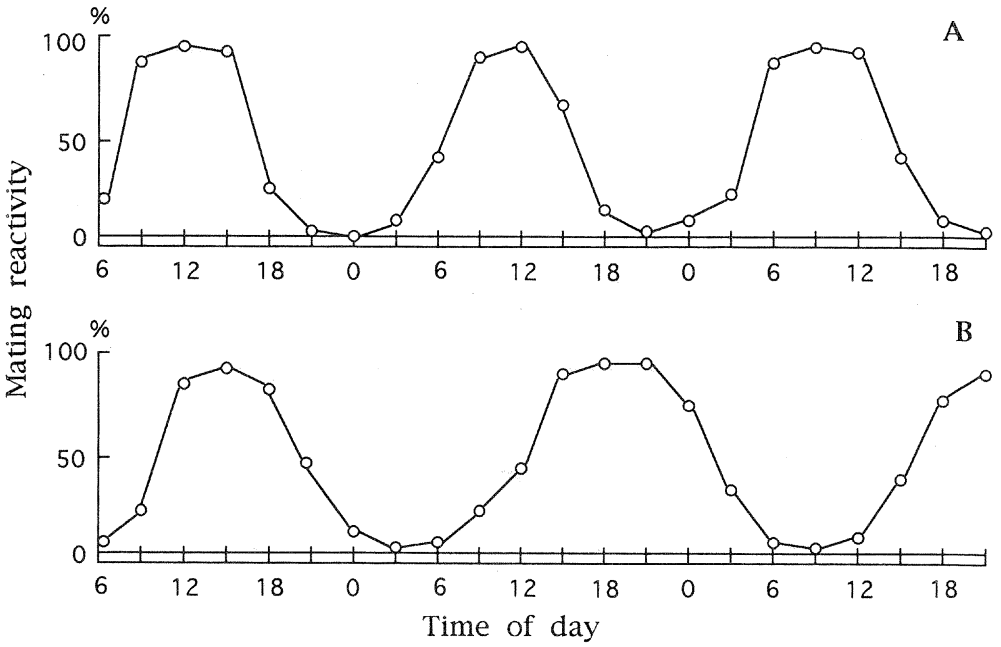

FIG. 2. Mating reactivity rhythms of the short period strain (UK1, A) and the long period strain (T316, B) in LL. Mating reactivity of cell population was tested every 3 hours by mixing them with white tester cells of complementary mating type. The percentage of mating reactive green cells clumping with white tester cells was assayed.

TABLE 1. Period of F1 and F2 progeny from a cross between a short period strain and a long period strain in photoaccumulation rhythm

Cross	Generation	No. of clone tested	Mean period (hr±SD)	Range (hr)
UK1×T316 (I) (IV)	F1	32	24.7±1.1	22.5–26.8
	F2	28	23.8±1.6	21.8–26.7

TABLE 2. Period of photoaccumulation rhythm in the progeny from backcross between F1 and parents

Cross	No. of clone tested	Mean period (hr±SD)	Range (hr)
20a×UK1 (IV) (I)	33	24.5±1.0	23.0–27.1
1a×T316 (I) (IV)	36	25.4±1.8	22.3–28.4

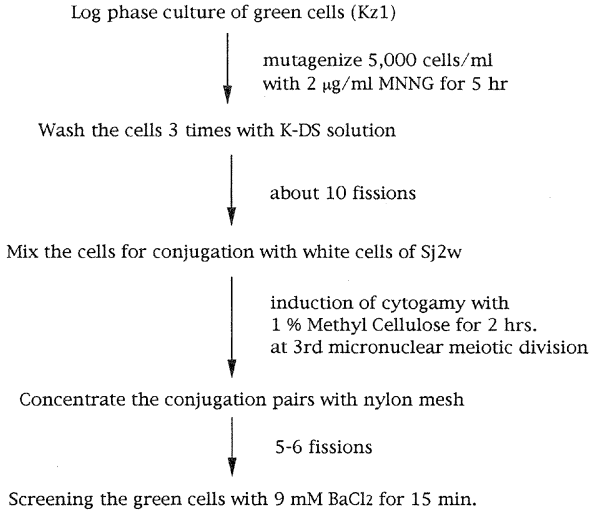

FIG. 3. Schedule of mutagenesis with nitrosoguanidine (MNNG) in *P. bursaria*, stock Kz1.

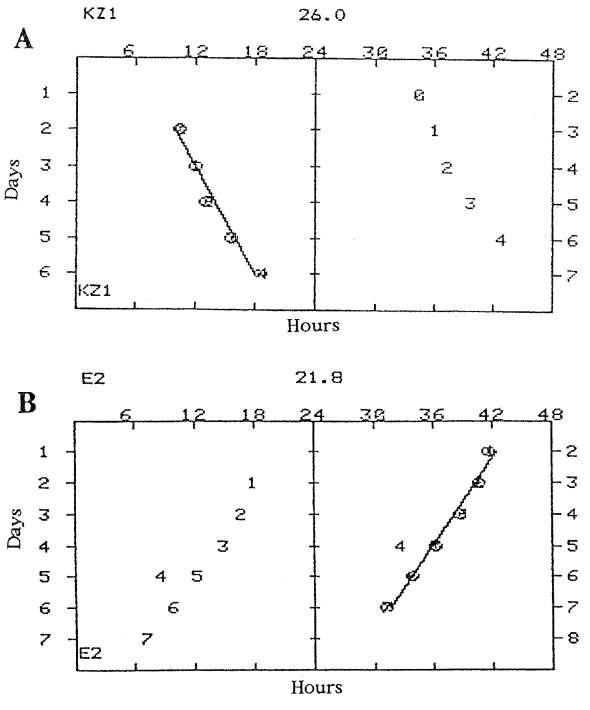

FIG. 4. Period length of photoaccumulation rhythms in natural strain Kz1 (A) and short period strain E2 (B) that was induced from Kz1 by treatment of nitrosoguanidine. The phase reference points of peak were analyzed as in Fig. 1.

TABLE 3. Period of photoaccumulation rhythm in the progeny of the short period strain (E2) induced with nitrosoguanidine

Cross	No. of clone tested	Mean period (hr±SD)	Range (hr)
E2×UK1 (III) (I)	24	21.9±0.6	20.1–23.5
E2×T316 (III) (IV)	27	23.3±0.7	21.0–25.6
E2×Sj2W (III) (I)	32	22.1±0.5	20.6–23.8

TABLE 4. Segregation of early maturation clones and mating types in the F1 and F2 progeny from a cross between E2 and Sj2W

Progeny*	No. of clones matured Early 15	Early 22	Normal 52	Mating type I	II	III	IV	Survival (%)
F1	0	28	0	16	1	0	11	46.7
F2	3	10	4	6	2	1	9	28.3

* F1: from a cross between E2(III) and Sj2w(I)
F2: from a cross between SE11(I) and SE14(IV)

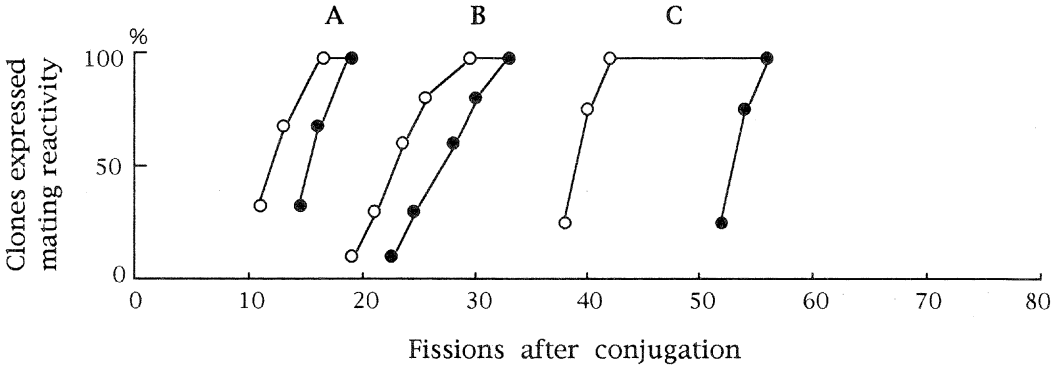

FIG. 5. Maturation curves of F2 progeny clones from a cross between early maturation strain E2 and natural strain Sj2w. The length of F2 progeny clones segregated into three groups (A-C) as shown in Table 4. A included 3 clones, B included 10 clones, C included 4 clones. Mating reactivity of progeny clones was tested every day after growth in a small test tube by mixing with four mating types tester cells (I-IV). Onset of adolescence was determined the day that cells mate with two mating type tester cells. When the cells mate with three mating types tester cells (all except their own mating type), they became mature. ○—○: onset of adolescence, ●—●: onset of maturity.

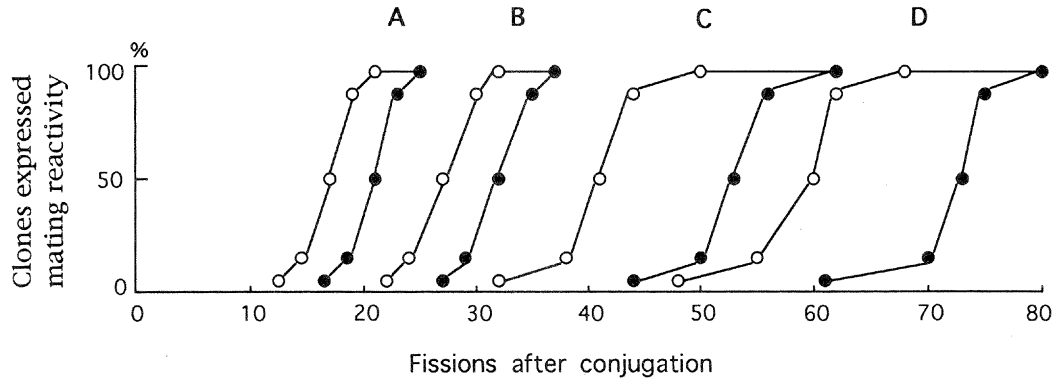

FIG. 6. Maturation curves of the progeny clones from crosses between various strains. Mating reactivity of progeny clones was tested as in Fig. 5. A: early maturation strain E2 (III) crossed with short period strain UK1 (I). B: E2 (III) crossed with long period strain T316 (IV). C: natural strain Kz1 (III) crossed with UK1 (I). D: Kz1 (III) crossed with T316. ○—○: onset of adolescence, ●—●: onset of maturity.

(Table 4). It seems that strain E2 is early mature mutant. The length of adolescence of the two early maturation groups was shorter than that of late maturation group (Fig. 5). The period length of the circadian photoaccumulation rhythm in LL of these three groups of F2 clones was, on the average, 20.8±0.5 (range 20.3–21.2 hr), 22.0±0.9 (range 20.9–23.6 hr) and 25.6±1.0 (range 24.2–26.7 hr) hours. The early maturation groups showed a short period rhythm.

Further, to investigate the correlation between the period length of circadian rhythms and the length of immaturity, E2 (early mature mutant) and Kz1 (wild type) were mated with short period strain (UK1) and long period strain (T316) in photoaccumulation rhythm, respectively. As shown in Fig. 6, the progeny of short period strains became mature earlier than that of longer period strains. The length of adolescence of the progeny from the early mature strain (E2) was shorter than that of the progeny from the wild type strain (Kz1).

DISCUSSION

A short period strain and a long period strain in photoaccumulation rhythm which were isolated from natural stocks also exhibited a short period and a long period rhythm of the

mating reactivity rhythm. It is most simple to conclude that both rhythms of photoaccumulation and mating reactivity are controlled by the same oscillator mechanism in these cells. However, recently two circadian oscillators have been found in one cell of the unicellular alga *Gonyaulax polyedra* [19]. If other rhythms, for instance, locomotor activity or negative gravitaxis can be compared in the same strain of *P. bursaria*, perhaps different oscillators related to different period lengths may be detected.

In genetic analysis of shorter period and longer period strains, the progeny from a cross between natural strains UK1 and T316 did not segregate with a Mendelian ratio, but the range of period spread widely. Therefore this phenotype of shorter and longer period is probably controlled polygenetically or by the ecotype which is influenced by the ecosystem. It is supposed that polygenetic control has much more possibility than ecotype, because stocks UK1 and T316 were collected at near place.

On the other hand, a mutant strain E2 induced by treatment with nitrosoguanidine showed the Mendelian segregation of immature period in F2 progeny. It seems that E2 has a semidominant gene involved in determing the length of immaturity. We called this semidominant gene EM2. The periods of the photoaccumulation rhythm of F2 progeny from a cross between E2 and Sj2w were 20.8 hours in homozygotes (EM2/EM2), 22.0 hours in heterozygotes (EM2/+), and 25.6 hours in wild types (+/+). It seems that the early mature mutant gene (EM2) is somehow related to the short period of the rhythm. Some genes related to the period length of circadian rhythms (short, long, and arrhythmic phenotypes) have been isolated in *Drosophila, Neurospora crassa* and *Chlamydomonas* [5]. Therefore it is expected that mutants of the long period and the arrhythmic will be isolated in *P. bursaria* and the genes will be analyzed in the future.

It is an interesting problem whether the period length of circadian rhythms is related to the length of immaturity. Exconjugant clones of *Paramecium* have a well-defined life cycle, consisting of the sequence of immaturity, maturity and senility [23]. During the immaturity period, cells cannot mate, even under otherwise appropriate conditions. The duration of the immaturity period is measured in terms of the number of fissions after conjugation [15, 24]. It is known that the exconjugant clones of *P. bursaria* became adolescent about 40 fissions and mature about 55 fissions after conjugation. Exconjugant clones of wild-type never exhibit mating reactivity before 30 fissions after conjugation.

The present experiments demonstrated that progeny from the mutant of short photoaccumulation rhythm became adolescent about 20 fissions after conjugation and became mature about 24 fissions after conjugation, and thus the length of adolescence was about 4 fissions. The homozygote of F2 progeny became mature about 15 fissions later. On the other hand, when the early mature mutant strain (E2) was mated with a short period strain (UK1) and a long period strain (T316) in photoaccumulation rhythm, the progeny from UK1 became mature earlier than that from T316. The length of adolescence was about 4 fissions in both crosses. Also in crosses of the natural stocks Kz1 and UK1, Kz1 and T316, the progeny from UK1 became mature earlier than that from T316. But the length of adolescence was about 15 fissions in those crosses. Shortened adolescence appeared only in the progeny from an early mature mutant (E2). Generally, the progeny from a short period stock matured earlier, and the progeny from a long period stock matured later. It seems that maturation in *Paramecium* depends on both of the number of fissions after conjugation (a "Developmental clock") and the period length of the circadian rhythm (a "Circadian clock"). Therefore, it may be important to clarify the relationship between the rate of cell division and the period length of circadian rhythms.

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