RECOMBINANTS BETWEEN CLOCK MUTANTS OF *CHLAMYDOMONAS REINHARDI*

VICTOR G. BRUCE

Department of Biology, Princeton University, Princeton, N. J. 08540

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ABSTRACT

Mutants affecting the period length of the biological clock in *Chlamydomonas reinhardi* have been isolated and a start has been made on analyzing the genetics of this system. In four mutants, the long period characteristic seems to be controlled by single genes at separate loci. Crosses between single mutants, as well as crosses involving three or four mutant genes, yielded progeny with periods characteristic of the parents as well as recombinant types, including normal period (wild type) and extra-long periods (double, triple and quadruple mutants). It was found that the period lengthening effect is additive; that is, the period of double mutants is lengthened by the sum of the period lengthening of the single mutants.

IN previous papers (BRUCE 1972, 1973) we described a system for a genetic analysis of the biological clock in *Chlamydomonas reinhardi*. Several clock mutants were described and on the basis of a few preliminary genetic crosses, it was concluded that the period length of the clock-controlled rhythm was a useful genetic marker, whereas the phase or amplitude of the rhythm was not. We have extended these observations and investigated the effect of combinations of independently derived clock mutations on the phenotype. In other genetic studies of biological clocks (KONOPKA and BENZER 1971; FELDMAN and HOYLE 1973; FELDMAN, HOYLE and SHELGREN 1973), most mutations so far described have been in the same gene (or so closely linked that no recombinants are found). This is not the case in *Chlamydomonas*, and it is possible to determine the phenotype of double, triple, and other multiple mutants. From eight clock period mutants we have selected four long period ones for a more detailed study. We have not tried to map the mutations, as technical limitations have prevented the clock-assay of large numbers of cultures. The objective, at this stage of the study, was to get perspective on the genetic system.

MATERIALS AND METHODS

Most of the details describing methods for growing and mutagenizing cultures, carrying out crosses, and assaying for the clock-controlled phototactic rhythm have been described (BRUCE 1972). *Chlamydomonas reinhardi* strain 137c, previously referred to as Lo^+ and Lo^-, was used. Two modifications of techniques are worth noting. Zygotes were allowed to mature on plates containing a lower phosphate concentration than previously used (7.2×10^{-4} molar rather than 4.4×10^{-3} molar). Mating tests were done in small plastic trays (Linbro Disposo trays) by

mixing 2 or 3 drops of the strains to be tested with 2 or 3 drops of a tester strain. The clumping reaction was scored under a dissecting microscope the following day. Phototactic records for most experiments were based on 5-day runs at 21.5° to 22°. Some of the longer period progeny (33 to 40 hrs) were also run for 8 days to get better estimates of the periods. The reproducibility and precision of the system was briefly discussed in an earlier paper (BRUCE 1972). We cannot give a statistical estimate of the variance on period length determinations but for these experiments we estimated periods to the nearest half-hour.

One long period mutant, previously described and designated *Lo-104* (BRUCE 1972), will hereafter be called *per-1* where *per* stands for period. Three other long period mutants have been isolated and tentatively designated *per-2*, *per-3*, and *per-4*. All were obtained from nitrosoguanidine-treated cultures of *Lo mt+* using the procedure previously described (BRUCE 1972). The *per-2* was obtained by random testing of about 50 surviving clones for their period length. The *per-3* and *per-4* strains were obtained by using a procedure designed to enrich for clock mutants as briefly described below.

The basic procedure of the enrichment process was to mutagenize a culture, plate a sample and pick several hundred clones. They were grown in liquid cultures and then pooled to form a mixed culture, in which most cells were normal but presumably a small fraction of the cells were clock period mutants. A free-running phototactic rhythm was initiated in the mixed culture. The rhythms of the small fraction of mutants which had modified periods should have gotten out of phase with the normal cells. Aliquots were removed from the stimulating light region at a time in the cycle when most cells were non-phototactic or were responding minimally. The chance of recovering mutants with non-normal periods was expected to increase by preferentially removing the phototactically active cells.

The apparatus used for the enrichment of mutants was similar to the one used in making the phototactic assay but was provided with a special culture chamber with removable cover. The phototactic rhythm was monitored and aliquots were removed with a Pasteur pipette from the region of the stimulating phototactic test light. Samples were removed during the 3rd and 4th minimum of the cycle, at which time one could expect mutants with 27- or 28-hr periods to be responding maximally. The samples were diluted and plated, and randomly selected colonies were tested for period length.

This procedure has not yet been systematically employed, but the two long-period mutants *per-3* and *per-4* were obtained from pilot experiments.

RESULTS

Nature of the mutants

The period lengths of the four mutants and the wild-type strains at various temperatures are shown in Table 1. All are temperature-compensated in that

TABLE 1

The period lengths in hours of the wild types, four long-period mutants, and one double mutant at different temperatures

Temp.	*Lo+*	*Lo-*	*per-1**	*per-2*	*per-3*	*per-4*	*per-3 per-4*
16°	21.5	22	24	24	24	25	26.5
22°	24.5	24	27	26.5	26.5	28	30
25°	24.5	24.5	27	29	28	28	30
29°	—	—	27†	28†	28†	26†	—

* Previously designated Lo-104 (BRUCE 1972).

† The measurements at 29° are not very reliable as the phototaxis and rhythm become poor at high temperature.

TABLE 2

Crosses involving one mutant

	Cross (Periods of parents)	Normal-period progeny No.	Average period	Range	Long-period progeny No.	Average period	Range
(1)	*Lo*+ × *per-1 mt*⁻ (24.5) (27)	16	24.1	24–24.5	11	26.6	26–27
(2)	*per-1 mt*+ × *Lo*⁻ (27) (24)	9	23.5	23–24	7	27.1	26–28
(3)	*per-1 mt*+ × *per-1 mt*⁻ (27) (27)	0			12	27.4	27–28
(4)	*per-2 mt*+ × *Lo*⁻* (26.5) (24)	9	24.0	23.5–24.5	3	26.3	26–27
(5)	*per-2 mt*+ × *per-2 mt*⁻ (26.5) (27)	0			12	26.1	26–27
(6)	*per-3 mt*+ × *Lo*⁻* (28) (24)	2	23.0	23	4	27.3	26–28
(7)	*per-3 mt*+ × *per-3 mt*⁻ (28) (28)	0			25	27.7	26–28

The progeny from each of seven crosses were classified as normal- or long-period as indicated.
* Only enough progeny of this cross were tested to recover a long period of opposite mating type.

they run faster at 16° than at 22° or 25°, showing a "negative" temperature coefficient. This can be compared with some of the wild-type and mutant strains previously described (BRUCE 1970, 1972), which do not have a "negative" temperature coefficient.

The four mutants were all crossed with wild type and the progeny classified by period length as indicated in Tables 2 and 3. The progeny of three of the four mutants (*per-1*, *per-3*, *per-4*) could be classified into two types, normal-period and long-period (Table 2). It appears that each of these mutants contains a segregating factor which confers a long-period phenotype (26 to 28 hrs). The normal-period progeny of crosses between these three mutants and *Lo*⁻ sometimes were slightly shorter than 24 hrs. When the fourth mutant *per-2* was crossed with *Lo*⁻, short- (22.5- and 23-hr), as well as normal- (24-hr) and long-period progeny were recovered (Table 3, cross 1). However, the cross of *per-2* with a normal-period (24.5-hr) progeny of cross 1 yielded only two clock phenotypes (normal and long), as shown in cross 2 of Table 3.

Long-period progeny of opposite m.t. were recovered from crosses between all four mutants and wild type. These were tentatively identified as *per-1*, *per-2*, *per-3* and *per-4*. As indicated in Tables 2 and 3, backcrosses of each of the four mutants to the long-period opposite mating type yielded only long-period progeny. We inferred that each of the four mutants contains a segregating factor which confers a long-period characteristic.

As noted earlier, the crosses between three of the four mutants and *Lo*⁻ yielded some progeny with periods either slightly shorter than normal (*per-1* and *per-4*), or with periods short enough to fall into a short period class (*per-2*). It seems possible that a factor (or factors) in *Lo*⁻ is responsible for the shortening of the

TABLE 3

Crosses involving the per-2 *mutant*

Cross	Short period No.	Short period Average period	Short period Range	Normal period No.	Normal period Average period	Normal period Range	Long period No.	Long period Average period	Long period Range
1	12	22.8	22.5–23	24	24.0	23.5–24.5	19	25.8	25–27
2		0		14	24.0	24	18	26.8	26–28*
3		0			0		16	27.1	26–28*
4		0			0		8	26.8	26–27*

The progeny from the four crosses indicated below were classified as short, normal or long period as indicated.
Cross 1 *per-2 mt*+ × *Lo mt*−
Cross 2 *per-2 mt*+ mated with a 24.5-hr progeny of Cross 1.
Cross 3 Long-period progeny (27 and 28 hrs) of opposite
Cross 4 mating type from two zygotes of Cross 2.
* 29 out of 42 of these progeny were 27-hr periods.

period of the progeny of certain crosses. Because of the technical limitations of our system, we have not tried to clarify this complication. However, it does appear that each of the four mutants contains a long-period gene, thus justifying the tentative designations *per-1*, *per-2*, *per-3* and *per-4*.

Crosses between mutants

Pairwise crosses were made between the four long period mutants, and recombinant types were obtained from all six of the possible crosses. These four independent mutations seem to be at separate loci. The four progeny from each of ten zygotes of one such cross were identified and classified as either parental or recombinant types. The other five crosses were less extensive, but enough progeny were tested to recover wild-type and double mutant recombinant types. Recombinant types had either normal period (23 to 25 hrs) or extra-long-period (30 to 33 hrs) phenotypes. The extra-long-period progeny were presumed to be double mutants on the basis of their phenotypes. The six possible double mutants were recovered.

The *per-1* × *per-4* cross, shown in Table 4, summarizes the results based on the progeny of ten zygotes. These zygotes were classified as parental di-types (PD), non-parental di-types (NPD), and tetra-types (TT) with respect to these two loci. In making this classification we assumed that all zygotes with four long-period progeny (26- to 28.5-hr) are PD. Zygotes with two normal (23.75- to 25-hr), and two extra-long-period (29- to 33-hr) are NPD. Zygotes with one normal (24- or 24.5-hr), two long (26- to 28-hr), and one extra-long-period (30- to 32-hr) progeny are TT. The ten zygotes whose progeny were analyzed included four PD, four NPD, and two TT zygotes. An intercross between two extra-long-period progeny of this cross yielded only extra-long-period progeny as shown in the footnote to Table 5.

A summary of all of the two-factor crosses is shown in Table 5. These results are based in part on incomplete tetrads and although the results are compatible with the segregation pattern illustrated in Table 4, the data are too limited to

TABLE 4

Zygote classification of the cross between the two long-period mutants, per-1 *and* per-4

Zygote no.	Periods lengths of zygote progeny mt+		mt−		Zygote type
1	26	28	26	28.5	
2	27	27	27	27	Parental
3	27	27	27.5	28	di-types (PD)
4	28	27	27	28	
5	24	25	29	32	
6	23.8	33	24	31	Non-parental
7	24	32	32	24	di-types (NPD)
8	24	33	33	24	
9	30	24.5	27	26.8	Tetra-types
10	24	32	28	26.5	(TT)

The parents were: *per-4 mt*+ (period = 28 hrs) and *per-1 mt*− (period = 27 hrs).
Approximately half of the period length measurements given above are average values of two or more determinations.

permit the classification of zygote type. The progeny were classified phenotypically as either parental or recombinant (wild-type or double mutant). This was done on the basis of the period length. Three of the double mutants were checked by intercrosses and yielded only extra-long-period progeny, as expected (Table 5). Since the parental periods were not all the same, the ranges of periods used to classify parental types as well as double mutants were not the same for each of the crosses. In general, it seems that the period-lengthening effect of the genes is additive, at least to a first approximation. Thus, if one gene lengthens the period by 3 hours and a second legthens it by 2.5 hours, then the period of the recombinant is lengthened by 5.5 hours. Since both normal and extra-long-period progeny were obtained from all crosses, no close linkage is indicated between any of the four loci. The data are too limited to determine whether any of them might be on the same linkage group.

Crosses between double mutants involving three or four genes were carried out and the results are summarized in Table 6. In the first two crosses, where one mutation is common to both parents (*per-1* in the first, and *per-4* in the second) no normal (24–25-hr) progeny were expected and none were found. Also, as expected, some progeny were found with periods longer than that of the double mutants (36 hours in one cross). Some of these progeny are presumed to be triple mutants, but more crosses would be required to positively identify the triple mutants.

In the two crosses which involved four different genes, recombination was expected to yield both normal (24–25-hr) and quadruple mutants as well as many combinations of single, double, and triple mutants. From the range of period lengths observed (25 to 40 hrs) both clock wild-type and probable quadruple mutants were obtained from at least one of these crosses.

In comparison with the two-factor crosses it was generally more difficult to

TABLE 5

Summary results of two-factor crosses

Period of parents	Cross	Parental type (Long period) No.	Parental type (Long period) Average period	Parental type (Long period) Range	Recombinant type (Normal period) No.	Recombinant type (Normal period) Average period	Recombinant type (Normal period) Range	Recombinant double mutant (Extra-long period) No.	Recombinant double mutant (Extra-long period) Average period	Recombinant double mutant (Extra-long period) Range
26.5	*per-2 mt*+ ×	14	26.3	26–27.5	15	24.3	23–25	10	29.2	28–32
27.0	*per-1 mt*⁻									
26.5	*per-3 mt*+ ×	21	26.3	26–27	22	24.6	24–25	10	29.1	28–32
27	*per-1 mt*⁻									
26.5	*per-2 mt*+ ×	14	26.3	25.5–27.5	6	24.5	24–25	9	28.9	28–30
26.5	*per-3 mt*⁻									
28	*per-4 mt*+ ×	20	27.2	26–28.5	10	24.1	23.5–25	10	31.7	29–33
27	*per-1 mt*⁻									
26.5	*per-2 mt*+ ×	11	27.0	26–28	5	24.6	24–25	8	29.6	29–31
28	*per-4 mt*⁻									
26.5	*per-3** ×	7	26.7	26–28	8	24.5	24–25	13	30.0	29–32
28	*per-4*									

For each cross the progeny were classified as recombinant (wild type or double mutant) or parental types. The number of progeny of each class, the average period, and the range of periods, which depends on the parental periods, is given.

* This summarizes two reciprocal crosses.

Three control intercrosses of double mutants have been done and gave the following results:

1) *per-1 per-4 mt*+ (32 hr) × *per-1 per-4 mt*⁻ (32 hr)—17 progeny of average period 31.4 hrs (30–33).
2) *per-3 per-4 mt*+ (30 hr) × *per-3 per-4 mt*⁻ (30 hr)—14 progeny of average period 29.5 hrs (28.5–31).
3) *per-1 per-2 mt*+ (30 hr) × *per-1 per-2 mt*⁻ (32 hr)—14 progeny of average period 30.9 hrs (29–32).

identify the progeny genotype from three- and four-factor crosses because of the large number of possible types which could be obtained. It was observed that the average period of the four progeny of individual zygotes was approximately the same as the average period of the two parental types. This is to be expected if the effect of combinations of genes on period length is additive. Sometimes it was clear how many mutant genes each of the four progeny of an individual zygote had. This is illustrated in Table 6. The progeny of one zygote from each of the four crosses are listed together with the presumed number of mutant genes in each, and the average period of the zygote progeny is compared with the parental average. For example, when a presumed quadruple mutant (40-hr) was obtained, a normal (25-hr) wild type was found from the same zygote.

Growth rate

The mutants grew more slowly than wild type. When cultured photoauto-

TABLE 6

Summary results of 3 and 4 factor crosses

Cross and (parental periods)	No. of progeny	Average period	Range	Presumed number of mutant genes	Results for one selected zygote: Progeny periods	No. of mutant genes	Average period of Zygote progeny	Parents
(1) *per-1 per-3 mt+* (31 hr) × *per-1 per-4 mt−* (32 hr)	8	26.9	26–28	single mutants	28	1		
	11	30.5	29–32	double mutants	31	2		
	6	34.5	33–36	triple mutants	31	2	31.5	31.5
					36	3		
(2) *per-1 per-4 mt+* (32 hr) × *per-3 per-4 mt−* (30 hr)	8	31.8	30–33	all double mutants	30	2		
		.....	or alternatively	.....	31	2		
	5	31.0	30–32	double mutants	32	2	31.5	31.0
	3	33.0	33	triple mutants	33	2		
(3) *per-1 per-2 mt+* (31 hr) × *per-3 per-4 mt−* (30 hr)	11	27.2	26–28	single mutants	30	2		
	15	30.5	30–32	double mutants	31	2		
	11	34.0	33–36	triple mutants	30	2	30.5	30.5
	2	37.5	37–38	quadruple mutants	31	2		
(4) *per-3 per-2 mt+* (30 hr) × *per-4 per-1 mt−* (32 hr)	2	25.0	25	wild type	25	0		
	10	26.7	26–28	single mutants	26.5	1		
	8	30.9	29–32	double mutants	34	3	31.4	31.0
	3	34.7	34–36	triple mutants	40	4		
	1	40.0	40	quadruple mutants				

The progeny for each cross were classified on the basis of the presumed number of mutant genes. The period lengths of the 4 progeny from one selected zygote for each cross are shown at the right, together with the presumed number of mutant genes and average period. However for the main body of the table many of the results are not based on complete tetrads and the presumed phenotypes are based primarily on the period lengths.

trophically, the average doubling time of wild type was 10–12 hours, while that of *per-3* and *per-4* was 13–15 hours. All four mutants grew slower than wild type in the dark on acetate. The doubling time for wild-type was 24–25 hrs, and for the mutants it ranged from 29 hrs for *per-2* up to 59 hrs for *per-3*, which grew consistently slower than the other mutants. The slow growth of the *per-3* mutant seems to be uncorrelated with the clock mutation, however, as was revealed by testing the growth of several progeny carrying the *per-3* gene. Furthermore, doubling times in the dark for five double mutants and one quadruple mutant ranged from 24 to 31 hrs. A second mutation in the *per-4* strain which segregates independently of the clock mutation inhibits growth above 31°. It seems, from these results, that the clock mutations probably do not effect growth rate, but that other mutations have been induced which do.

DISCUSSION

With one exception, the clock-period-length mutants which have been described in other systems are very tightly linked to each other and possibly allelic. For example, the three clock mutants of Drosophila (28-hr and 19-hr periods, and non-rhythmic) all map to the same locus on the *X*-chromosome (KONOPKA and BENZER 1971). Three mutants in Neurospora (16.5-hr, 19.4-hr, and 24.2-hr;

wild type is 21.6 hr) also map to a common locus (FELDMAN and HOYLE 1973). The one exception, a recently discovered long-period mutant in Neurospora, was found to be at a different locus (J. F. FELDMAN, personal communication). These results can be contrasted with our results with Chlamydomonas in which the four mutations are at different loci. It is not clear whether this represents a basic difference between the systems, but it seems probable that more mutants will soon be tested in all three systems and the point may be clarified. Our results indicate that there may be many genes involved in the Chlamydomonas clock both because of the relatively high frequency of clock mutations and because the mutations are at different loci. However, we have no information on how they might be organized genetically, since none of the mutations has been mapped.

The most interesting result of our investigations is the additive effect of period lengthening. If one gene lengthens the period n hours and a second lengthens it by m hours then the period of the double mutant is lengthened by $n+m$ hours. From pairwise crosses between single mutants, one can generally identify the zygotes as PD, NPD or T. For example, with the above parental types one would find: PD (two $24+n$ hrs, two $24+m$ hrs), NPD (two 24 hrs, two $24+n+m$ hrs), T (one 24 hrs, one $24+n$ hrs, one $24+m$ hrs, one $24+n+m$ hrs). The double mutants can thus be identified not only on the basis of the period length but also by showing that one also finds a wild-type recombinant from the same zygote. The four long-period mutants which we have are not so different phenotypically that one can distinguish between the single mutants from the phenotype, but from two factor crosses one can generally identify the double mutants. The six possible double mutants which can be obtained from crosses with the four mutants have been obtained and were found to have periods ranging from 29 to 33 hours. Triple and quadruple mutants have periods that are longer than the double mutants, and as far as we can judge, the additive effect holds up for any combinations of these mutants.

There are various possibilities as to how two genes might combine to affect the phenotype. Could two rhythms persist independently, as has been observed in multicellular higher organisms (BÜNNING 1973, pp. 231–233)? No evidence of a "beat effect" (BÜNNING 1973, pp. 184–185) was observed in any of the crosses between the long-period mutants, and double mutants always seem to have extra-long periods. Therefore, it seems likely that the mutations affect the same rhythmic system rather than some independently self-oscillatory systems which might be normally mutually synchronized but could become uncoupled if the effect of the mutations was to knock out some component system.

The additive effect would be a logical consequence of several types of models or mechanisms for a clock. For example, it is consistent with "tape-reading"-type models which have been proposed (EHRET and TRUCCO 1967, WATSON 1970). The period length in this class of model is proportional to the length of tape, and mutants could be formed by adding or deleting sections of tape. The additive effect is also consistent with many relaxation-type oscillators. As an analogy consider a pipette washer with many separate constant-flow inputs. Interfering with any input would slow the cycle by a fixed amount. Interfering with two would slow the cycle further, and the effects would be additive.

We are currently developing apparatus to permit the clock-assay of relatively large numbers of cultures so that the genetic analysis can be more efficiently carried out. An improved system to enrich and select for clock mutants based on the sticking rhythm (STRALEY and BRUCE 1973) has been worked out. These improvements should enable us to make a more detailed characterization of the genetic aspects of the Chlamydomonas clock.

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