

CONDITIONAL MUTANTS IN
CHLAMYDOMONAS REINHARDTII BLOCKED
IN THE VEGETATIVE CELL CYCLE

I. An Analysis of Cell Cycle Block Points

STEPHEN H. HOWELL and JAY A. NALIBOFF

From the Department of Biology, University of California, San Diego, La Jolla,
California 92037

ABSTRACT

Conditional "cycle-blocked" (cb) mutants of *Chlamydomonas reinhardtii* have been detected and isolated. These mutants exhibit normal vegetative growth at permissive temperature but are unable to complete a cell cycle (or a specified number of cell cycles) at restrictive temperature. A simple technique has been devised to determine the cell cycle stage in each mutant when the defective gene product, which ultimately affects cell division, completes its function. This stage is called the "block point", and is determined by scoring the residual cell division in an exponentially growing population after shift to temperature restrictive conditions. In the cb mutants isolated so far, block points representing many stages throughout the cell cycle have been found. Two categories of cb mutants are described here: one set which prevents the subsequent cell division when the cell encounters the block point after a shift to restrictive temperature, and another set which permits an additional round of cell division after the block point is encountered. The general applicability of block point analysis to other cell systems is presented.

INTRODUCTION

The cell cycle in eucaryotic microorganisms is marked by the periodic expression of discrete cytological and biochemical events. In *Chlamydomonas reinhardtii*, the timing of cell cycle events has been studied in light-synchronized cultures. A partial list of events limited to specific cell cycle intervals in *C. reinhardtii* include nuclear and chloroplast DNA synthesis (Chiang and Sueoka, 1967), synthesis of chlorophyll and other components of the photosynthetic system (Armstrong et al., 1971), synthesis of certain autoregulated enzymes (Kates and Jones, 1967), periods in which the subsequent cell division is sensitive to cycloheximide or chloramphenicol inhibition (Mihara and Hase,

1971), and a period of gametogenic potency (Schmeisser et al., 1972).

The temporal control of cell cycle activities is generally considered to be the product of the time-ordered expression of gene-controlled events (see reviews by Tauro et al., 1969; Mueller, 1971). Hartwell et al. (1970) have strengthened this notion by their studies of cell division cycle mutants in yeast. These mutants are a special class of conditional mutants which are defective in gene products that perform indispensable functions at single cell cycle stages. To pinpoint the timing of such functions, Hartwell et al. (1970) developed a technique to determine cell cycle stages in which

the mutant genes would normally complete their functions at permissive temperatures. This critical cell cycle stage in each mutant was termed the "execution point." It was determined by recording photomicroscopically the cell cycle progress of individual cells after shifting to restrictive temperature. The concept of the execution point taken from the yeast studies has been valuable to an understanding of the genetic control of the cell cycle, but the techniques employed by Hartwell et al. (1970) for the detection of cell division cycle mutants and determination of execution points are not easily adaptable to other cell systems. Their techniques are most readily applied when the cell cycle stage of mutant cells can be accurately identified as it can in budding yeast where the relative size of the parent cell and bud is used as a measure of cell cycle progress.

We have selected for a more general class of mutants in *C. reinhardtii* which share many features of the cell division cycle mutants in yeast. These mutants are called cell "cycle-blocked" (cb) mutants. These mutants can carry out normal vegetative cell divisions at permissive temperature but cannot complete a cell cycle (or a specified number of cell cycles) at restrictive temperature. We have designed a simple technique to determine the cell cycle stage when the defective gene products in these mutants would normally complete their functions at permissive temperature. We have called this critical stage the "block point" and have operationally defined it for cb mutants to be the last cell cycle stage in which a shift to temperature-restrictive conditions can block the subsequent cell division(s). The block point is determined essentially by scoring the extent of cell division in an exponential culture of mutant cells after a shift to restrictive temperature.

We have applied block point analysis to temperature-sensitive cb mutants in *C. reinhardtii*. In this paper we describe two sets of these mutants: one set which blocks cell division within one cell cycle after shift to restrictive temperature, and another set which blocks cell division within two cell cycles.

MATERIALS AND METHODS

Media and Growth Conditions

Cells were grown autotrophically on high salt medium, $\frac{3}{10}$ HSM (Sueoka et al., 1967), or mixotrophically on YA medium, $\frac{3}{10}$ HSM, enriched with

0.1% yeast extract and 0.1% sodium acetate. Autotrophic liquid cultures were constantly bubbled with filtered 3% CO₂ in air.

For asynchronous growth, 150-ml cultures in 250-ml erlenmeyer flasks were grown autotrophically at 21°C. Flasks were swirled at 150 rpm on a New Brunswick Scientific Co., Inc. G76 gyratory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, N. J.) cooled by a refrigerated water bath circulator. Cultures were illuminated from above by fluorescent lighting at a light intensity (measured from the surface of the culture) of 4,000 lux. To shift temperature, 75 ml of the culture was transferred to a 250-ml flask in an identical water bath shaker set up for incubation at 33°C. Cultures attained a temperature of 33°C in 4.5 min after transfer.

Synchronous growth was carried out according to the general procedure described by Surzycki (1971). 300-ml cultures in 1-liter flasks were grown autotrophically with a 12 h dark-12 h light illumination cycle. Cultures were maintained in a 21°C incubator and illuminated from above by fluorescent lighting with a light intensity of 3,000 lux. Cultures were constantly mixed with stirring bars driven by thermally isolated magnetic mixers. To shift temperature, 50-ml samples were transferred to 125-ml flasks and placed at 33°C in the gyratory water bath shaker described above.

Stock cultures were maintained throughout the experiments on YA agar slants in a 21°C illuminated incubator. For photomicrograph analysis, mutant cells were grown on $\frac{3}{10}$ HSM agar plates (1.5% agar) and incubated at 33°C in an illuminated incubator.

Parental Strain and Mutant Induction

Parental haploid strains used in this study were derivatives of *C. reinhardtii* Dangeard strain 137C (obtained from W. T. Ebersold) mating type (mt) + and -, with the mt⁻ strain bearing an unmapped Mendelian marker for actidione sensitivity, (act⁸¹). Mutants were generated by UV mutagenesis at an irradiation dose (3.72×10^6 ergs/cm²) giving 90-95% survival. After 12-24 h in the dark to prevent photoreactivation, irradiated cells were spread on YA plates and grown mixotrophically at 21°C. Within 4-5 days clones were replica plated on YA plates and the replicas subjected to mixotrophic growth at 33°C. Replica clones unable to form macroscopic colonies were selected. Each selected clone was retested by the same replica plating procedure.

Cell Concentration Determinations

Cell concentration in liquid cultures was determined by duplicate hemacytometer counting. Cells having completed division and containing multiple daughter cells in the mother cell wall were scored as single cells until release (cell separation).

RESULTS

Selection of Cycle-Blocked (cb) Mutants

The object of our selection procedure was to find conditional mutants unable to complete one or a few cell cycles at restrictive temperature. To obtain these mutants in mass selection procedures, we picked "tight" temperature-sensitive mutants or clones which rapidly terminated growth at 33°C. Such selections were carried out using replica plating procedures. The fractional yield of no- or slow-growing clones at restrictive temperature ranged from $1-5 \times 10^{-4}$.

The selected mutant clones were subjected to a more stringent testing of their growth capacity at restrictive temperature. Clones to be tested were dispersed in liquid suspension and spread on YA agar plates (400 cells/plate) in order to follow colony formation from single cells. At 33°C non-mutant cells normally form four to eight daughter cells from a single cell at each division and produce macroscopic colonies by 72 h. We observed mutant cells microscopically at 3-4 days of growth and selected those clones in which single cells completed no more than one round of cell division, i.e., produced no more than eight daughter cells. About 20% of the clones selected by replica plating passed this test, giving a final fractional yield $2-10 \times 10^{-5}$ for cell cycle mutants. In the following results, we will report on the general properties of nine phenotypically distinguishable cb mutants and specific properties of four which show block points at different cell cycle stages.

Temperature Shift-Up During Asynchronous Growth

In order to determine the block point for each cell cycle mutant, we carried out temperature shift experiments during asynchronous exponential growth. It was expected that mutant cells growing at permissive temperature would rapidly cease cell division activity upon shifting to restrictive temperatures, and the amount of cell division observed after temperature shift, then, could be used as an indicator of the position of the block point in the cell cycle.

To examine this possibility, we first determined growth parameters of nonmutant cultures (strain 137C mt⁺) subjected to temperature shift. Either at 21°C or after shift to 33°C, autotrophic cultures of nonmutant cells grow exponentially with nearly

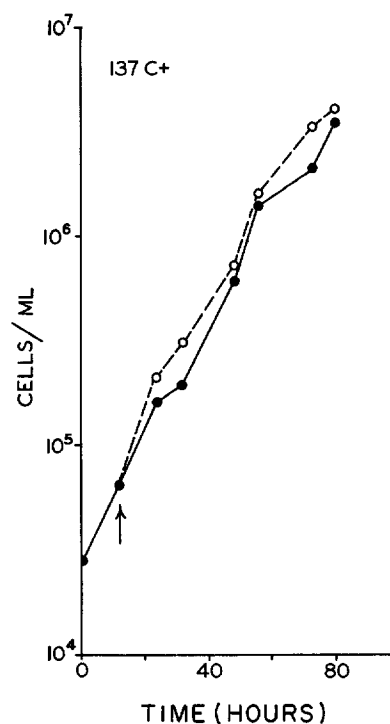


FIGURE 1 Growth of parental strain (137C mt⁺) at 21° and 33°C. A 150-ml culture was grown autotrophically at 21°C. At the time indicated by the arrow (↑), the culture was split into equal portions. Half of the culture was retained at 21°C and the other half shifted to 33°C. Growth at 21°C ●—●; growth after shift to 33°C ○- -○.

the same doubling time of 11 h or a generation time of 22 h (Fig. 1). Under these growth conditions, a single cell always produced four daughter cells at each division. From repeated experiments it was determined that stationary phase is generally reached at 1×10^7 cells/ml at 21°C and $5-8 \times 10^6$ cells/ml at 33°C.

At 21°C all mutant cultures grow at nearly the same rate as the nonmutant parental strains (Fig. 2). After a temperature shift to 33°C, mutant cultures show a complete cessation of growth within one cell generation. The extent to which any culture increased in cell concentration after temperature shift was characteristic for the mutant tested and independent of the cell concentration at the time of temperature shift. We have shown here (Fig. 2) four mutants which display quite different increases in cell concentration after temperature shift, ranging from nearly a quadrupling in ts10003 to virtually no increase in ts10021.

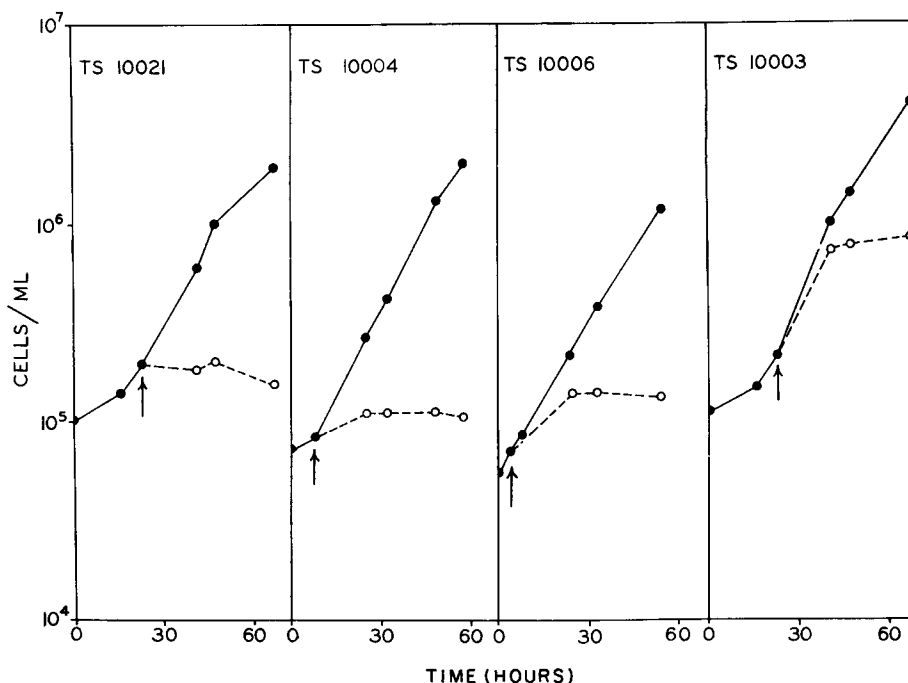


FIGURE 2 Growth of mutant strains at 21° and 33°C. Conditions are the same as in Fig. 1. Growth at 21°C ●—●; growth after shift to 33°C ○—○.

To determine the cycle block points from these data, we assumed that the gene product for which each mutant is conditionally defective completes its function at a constant point in the cell cycle. We further assumed that mutant cells which encounter this point (or the previous interval in which the defective gene would normally function) at restrictive temperature will be blocked in progressing through the cell cycle before the next cell division. In a later section we will consider those mutants which undergo one or more cell divisions after encountering the block point.

In an asynchronous exponentially growing culture, cells will be distributed throughout the cell cycle at the time of temperature shift in accordance to an age distribution function derived by Cook and James (1964). That age distribution is represented by the equation:

$$y = kA^{(1-x)}, \quad (1)$$

where y is the relative number of cells at cell cycle stage x (varying from 0 to 1), A is the number of daughter cells produced by one parent cell each cell cycle, and k is a normalization factor such that $\int_0^1 y dx = 1$. Then $k = (\ln A)/(A - 1)$, and

$$y = \frac{\ln A}{A - 1} A^{(1-x)}. \quad (2)$$

The features of this equation as they relate to the *C. reinhardtii* cell cycle are illustrated in Fig. 3. Here, the cell cycle is represented as a clock which advances from zero time, the time at cell separation, to 1. During exponential growth, it is shown that cells are not distributed uniformly around the cell cycle clock, but that there are more younger (earlier in the cell cycle) than older cells, because one single old cell becomes four progeny cells at division. (For *C. reinhardtii* grown under the described conditions, $A = 4$.)

If, then, an exponentially growing mutant culture is shifted to temperature-restrictive conditions, cells which have passed the block point at the time of temperature shift will complete another round of cell division before encountering a division block, while cells which have not passed the block point at the time of temperature shift will not divide. The proportion of cells in a culture that are past the block point at the time of temperature shift is an indicator of the position of the block point in the cell cycle. This proportion, therefore, can be determined by measuring the extent to

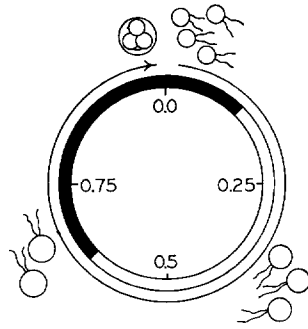


FIGURE 3 Diagrammatic representation of the vegetative cell cycle in *Chlamydomonas*. Cell cycle progress is measured on a scale from 0 to 1. Zero time is defined as the stage of cell separation when four daughter cells are released from the mother cell. The number of cells shown at various cycle stages is a rough representation of the age distribution of cells during exponential growth. The relationship of cell cycle stages in synchronous growth to the 12 h light- 12 h dark illumination cycle is shown in the inner circle (light period, open bar; dark period, closed bar).

which cells will increase after shift to restrictive temperature.

If the block point for a given conditional mutant is at a unique cell cycle stage x_1 , then according to the age distribution Eq. 2, the cell population, I_1 , which has not passed x_1 is:

$$I_1 = \frac{\ln A}{A-1} \int_0^{x_1} A^{(1-x)} dx, \quad (3)$$

and the cell population, I_2 , which has passed x_1 is:

$$I_2 = \frac{\ln A}{A-1} \int_{x_1}^1 A^{(1-x)} dx. \quad (4)$$

The final cell population achieved at the cessation of growth at restrictive temperature will depend on the number of cell divisions, n , a mutant cell can undergo after it has encountered the block point at restrictive temperature. This is easily assessed by determining the extent of cell division of a mutant culture after temperature shift. The extent of cell division is represented by N/N_0 , where N is equal to the final cell concentration at restrictive temperature and N_0 equals the cell concentration at the time of shift. The number of cell divisions, n , is therefore the lowest whole integer in n_{\max} that results from the equation:

$$n_{\max} = \frac{N/N_0}{A}. \quad (5)$$

Also, the final cell population at growth cessation, I_F , will depend upon the relative size of the two populations at temperature shift—the one which has not passed the block point (I_1) and the one that has (I_2). The population which has passed the block point, x_1 , at the time of temperature shift will undergo a round of cell division before encountering x_1 . Thus, I_2 always divides one more time than I_1 and so:

$$I_F = A^n I_1 + A^{(n+1)} I_2. \quad (6)$$

Substituting Eq. 3 and 4 into Eq. 6 we obtain:

$$I_F = \frac{\ln A}{A-1} [A^n \int_0^{x_1} A^{(1-x)} dx + A^{n+1} \int_{x_1}^1 A^{(1-x)} dx]. \quad (7)$$

Evaluating the integral and simplifying gives:

$$I_F = A^n A^{(1-x_1)}. \quad (8)$$

If we consider the relative increase (N/N_0) in cells after shift to restrictive temperature, we can let $I_F = N/N_0$. Then,

$$N/N_0 = A^n A^{(1-x_1)}. \quad (9)$$

Therefore, the equation in general form for determining the block point (x_1) is

$$x_1 = 1 + n - \frac{\ln (N/N_0)}{\ln A}. \quad (10)$$

To demonstrate the features of this equation we have calculated block points for a set of hypothetical *C. reinhardtii* mutants assigned N/N_0 values as shown in Fig. 4. It is considered that these hypothetical mutants would normally quadruple at each cell division ($A = 4$), but would not divide after encountering their respective block points at restrictive temperature ($[N/N_0]/A < 1$, and hence, $n = 0$). Therefore Eq. 10 reduces in this case to

$$x_1 = 1 - \frac{\ln (N/N_0)}{1.386}. \quad (11)$$

From this equation it can be seen that mutants with block points early in the cell cycle will nearly quadruple in cell concentration at restrictive temperature while mutants with late blocks points increase minimally.

Table I lists block points calculated from Eq. 11

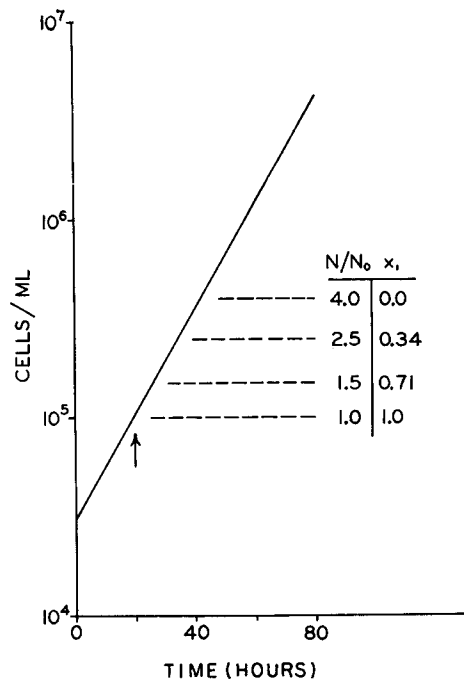


FIGURE 4 Family of growth curves for hypothetical mutants grown at 21°C and shifted to 33°C. Temperature shift is performed at the time indicated by the arrow (\uparrow) and the cell density at growth cessation for each mutant is represented by ---. Block points (x_1) are calculated from values of N/N_0 , the extent of cell growth at restrictive temperature, according to Eq. 11.

for nine cell cycle mutants which do not more than quadruple at restrictive temperature. This table includes those mutants for which growth curves are shown in Fig. 2. It should be noted that block points for these mutants are generally spread throughout the cell cycle with a greater number of mutants isolated so far showing block points in the middle of the cell cycle. Mutants listed here with similar block points can be distinguished phenotypically by their resultant morphologies or macromolecular synthesis capabilities at restrictive temperature. These phenotypic differences are currently under study.

Temperature Shift-Up During Synchronous Growth

To validate the described technique for block point determination, the same temperature-shift procedure was applied to synchronous cultures. In these experiments mutant cultures were grown synchronously at permissive temperatures, and

TABLE I
Summary of Block Points for Mutants Unable to Complete One Cycle at Restrictive Temperature

Mutant designation (cb)	Block point	
	From exponential growth (x_1)	From synchronous growth (interval containing x_1)
ts10003	0.01	0.00-0.08
ts10001	0.30	0.31-0.40
ts50040	0.32	0.55-0.59
ts10006	0.42	0.37-0.46
ts10002	0.46	0.54-0.62
ts10009	0.50	ND*
ts10004	0.79	0.54-0.61
ts10010	0.80	ND
ts10021	0.96	0.56-0.81

* Not determined.

portions of the culture were shifted at various times in the cell cycle to restrictive temperatures. Again, it was assumed that if the synchronous cell population had passed the block point (x_1) at the time of temperature shift, then nearly all cells would divide once and only once. If the synchronous population had not passed the block point at the time of temperature shift, then none of the cells would divide. Thus, by shifting samples of a synchronous mutant culture to restrictive temperature at successive cell cycle stages, one can determine a time interval which includes x_1 .

Mutant or nonmutant cultures growing at 21°C with 12 h light- 12 h dark illumination will divide synchronously at 8-11 h into the dark period. Nonmutant cells shifted to 33°C at almost any point in the cell cycle and kept under constant illumination divide nearly when expected with only some loss in cell division synchrony. Therefore, in order to score the increase in cell concentration after temperature shift in mutant cultures, we determined the cell concentration of the cultures 6-8 h after the expected cell division. In no mutant cultures did we observe any further cell division after this period.

For four cb mutants, the synchronous growth curves at permissive temperature and the increase in cell concentration after temperature shift are shown in Fig. 5. It can be seen for each mutant that there is a single, critical cell cycle stage (the block point) beyond which the cell population can divide. To estimate this cell cycle stage, we have determined the interval which includes the block

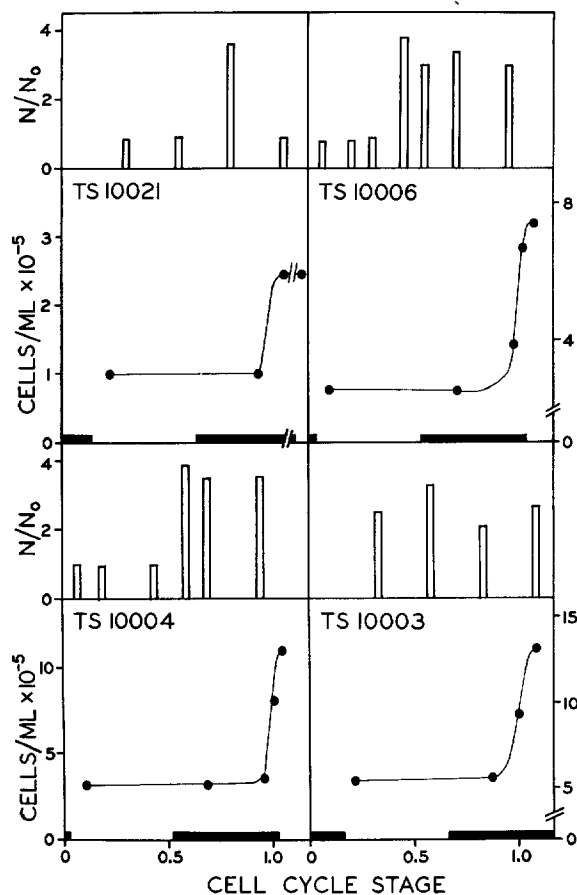


FIGURE 5 Temperature shift-up of mutant cultures during synchronous growth. In the lower panel for each mutant is shown the growth curve for cultures grown autotrophically at 21°C and subjected to a 12 h light (open bar)- 12 h dark (closed bar) illumination cycle. Time axis is expressed in terms of cell cycle stage where 0 and 1 are the times when the culture reaches midpoint cell density during synchronous division. 50-ml samples from each 600-ml synchronous culture were shifted to 33°C at times indicated by the position of the bars on the upper panel for each mutant. Bar heights indicate the extent of cell growth (N/N_0) at growth cessation for each sample shifted at different cell cycle stages.

point (x_1). This is done by recording the cell cycle stages, at the time of temperature shift, spanning the period during which the ability to divide at restrictive temperature is acquired. Cell cycle stage values (x) are assigned to synchronous cultures by considering the start (or end) of each cell cycle to be the time of cell separation or when the cell concentration has increased to 50% of its final concentration for that cell cycle. (Note that the start of the cell cycle is not the beginning of the light period but 1-4 h before that time as shown in Fig. 3.). So, for example, the cell cycle interval in

cb mutant ts10006 which includes the block point is $x = 0.37-0.46$.

Table I compares the intervals containing the block point determined from synchronous growth with the block points determined from exponential growth. The degree of correspondence between the techniques is surprising when one considers the possible differences in growth conditions between an exponential culture in balanced growth and a light-induced synchronous culture. Nonetheless, the results from synchronous growth support the validity of the technique for the determination of

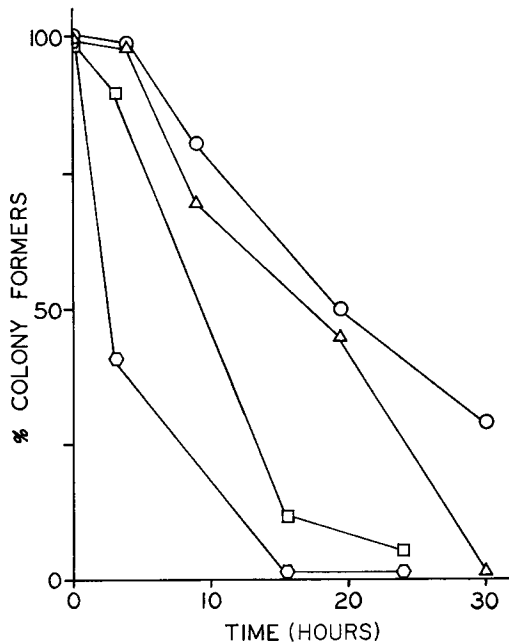


FIGURE 6 Colony-forming ability of cells incubated at restrictive temperature. Cells growing in asynchronous autotrophic culture at 21°C were plated on 3/10 HSM agar and subjected to 33°C in the light for various periods of time. The number of colonies formed per plate relative to the control (incubated for 0 h at 33°C) is expressed as % colony formers. Ts10021, Δ — Δ ; ts10004 \circ — \circ ; ts10006, \square — \square ; ts10003, \diamond — \diamond .

block point from temperature shift of cultures in asynchronous exponential growth.

Temperature Shift-Down During Asynchronous Growth

To determine if mutant cells will recover after exposure to temperature-restrictive conditions, temperature shift-down experiments were performed on asynchronous cultures incubated for varying periods of time at 33°C. This is a measure of the recovery of colony-forming ability only and not a measure of survival, since certain vital signs (motility, etc.) are observed in cultures that can no longer form colonies.

The recovery of colony-forming ability is shown in Fig. 6. It can be seen that these cb mutants vary in this ability after exposure to restrictive temperature. The only generalization that can be drawn from these results is that these mutants generally do not show fully reversible cell division inhibition characteristics and therefore would be

difficult to use for temperature shift-down experiments (such as cell synchronization by temperature shift). It is curious to see the rapid loss of reversibility of ts10003, for example. Ts10003 has a block point early in the cell cycle and, therefore, nearly each cell in asynchronous growth will quadruple after temperature shift to restrictive conditions, but apparently cell division cannot proceed further for these cells even after return to permissive temperature.

Photomicrograph Analysis of Growth at Restrictive Temperature

The growth of individual cells on agar plates at restrictive temperature was monitored for all mutant clones. Cells to be observed were taken from autotrophic exponentially grown cultures at 21°C and spread on 3/10 HSM plates for autotrophic growth at 33°C. At various times after temperature shift, a portion of the agar plate was overlaid with a clover slip and photographed.

For nonmutant cells a constant increase in colony size is observed during incubation at 33°C (Fig. 7). The cells retain a morphology that is characteristic of their growth at 21°C on agar plates. Few nonmutant cells are observed in a divisional state as is seen in some mutants blocked by restrictive temperatures.

The appearance of cb mutants grown at 33°C (Fig. 8) contrasts considerably to that of nonmutant cells. In no case, does any cell divide more than once, if at all, at restrictive temperature, i.e., no cell produces more than four to eight daughter cells. The number of cells which do divide once at restrictive temperature depends upon the position of the block point in the cell cycle. The mutants are arranged in Fig. 8 (from top to bottom) with block points at increasingly earlier cell cycle stages. Thus, for ts10021 which has a late block point, virtually no cells divide at restrictive temperature, while for ts10003 with a very early block point, nearly all cells divide once.

Other morphological differences can be observed in mutant cells grown at 33°C. Most cells seen in Fig. 8 continue to increase in size up to 21–42 h after temperature shift. For example, ts10004 increases nearly threefold in average cell diameter (more than tenfold in average cell volume) by 42 h at 33°C. Also, most cells begin to show degenerative changes by 42 h. The cells pictured here become more vacuolate and begin to lyse at this time.

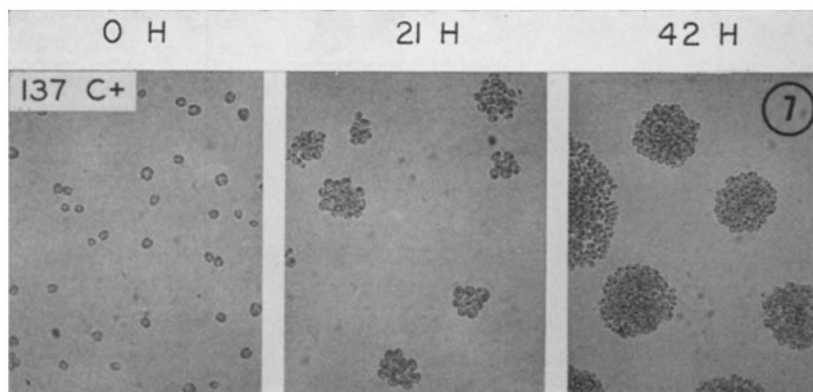


FIGURE 7 Photomicrographs of nonmutant cells grown at restrictive temperature on agar plates. Cells growing in asynchronous autotrophic culture at 21°C were spread on 3/10 HSM agar and subjected to 33°C in the light for the time intervals indicated. Random fields containing cells were overlaid with a cover slip and photographed. $\times 200$.

Mutants Able to Complete More than One Cycle at Restrictive Temperature

Several mutants we have studied complete more than one cell cycle at restrictive temperature. These mutants were selected because when spread out as single cells on agar plates and incubated at restrictive temperature they produced more than four to eight cells but less than a macroscopic colony in 5–7 days of growth.

We carried out temperature shift-up experiments on asynchronous exponentially growing cultures of these mutants. Growth curves for two of these mutants, ts50033 and ts10022, are shown in Fig. 9. The growth curves bear out our expectations—that the cells more than quadruple, but show a reproducible incremental increase in cell concentration at restrictive temperature. In applying block point analysis to these growth curves, we assumed that these mutants can carry out an additional round of cell division after they first encounter the block point. Another possibility is that there is a delay in the expression of the lesion in the defective gene product after temperature shift and, hence, the block point would not actually be encountered until one or more cell cycles after temperature shift. We presently tend to discount the second possibility because some important macromolecular synthesis functions, which are presently under study, are rapidly shut down in these cells.

To calculate the block point for these mutants, we can solve general Eq. 10 for the case in which a mutant will divide once after encountering

the block point at restrictive temperature ($1 < n_{\max} < 2$ and, therefore, $n = 1$). In this case the equation reduces to:

$$x_1 = 2 - \frac{\ln(N/N_0)}{1.386}. \quad (12)$$

Table II lists block points of mutants which divide once after encountering the block point. The same divisional behavior and block point was observed when one of these mutants, ts50033, was tested by shift-up during synchronous growth procedure (Fig. 10). At early cell cycle stages in this mutant, most cells undergo one round of cell division after shift to restrictive temperature, but beyond a critical interval in the cell cycle (an interval containing the block point) cells complete more than one round (but less than two) of cell division before growth cessation. This experiment suggests, then, that such mutants can carry out one additional cell division after they first encounter the block point.

DISCUSSION

In this study we have described a new class of conditional mutants in *C. reinhardtii* called cycle-blocked (cb) mutants. These mutants are unable to complete a cell cycle (or a specified number of cell cycles) at restrictive temperature. We have determined for each mutant a block point, which we operationally define as the last time in the cell cycle in which a shift to temperature-restrictive conditions can block subsequent cell division(s). The block point is interpreted to be the cell cycle

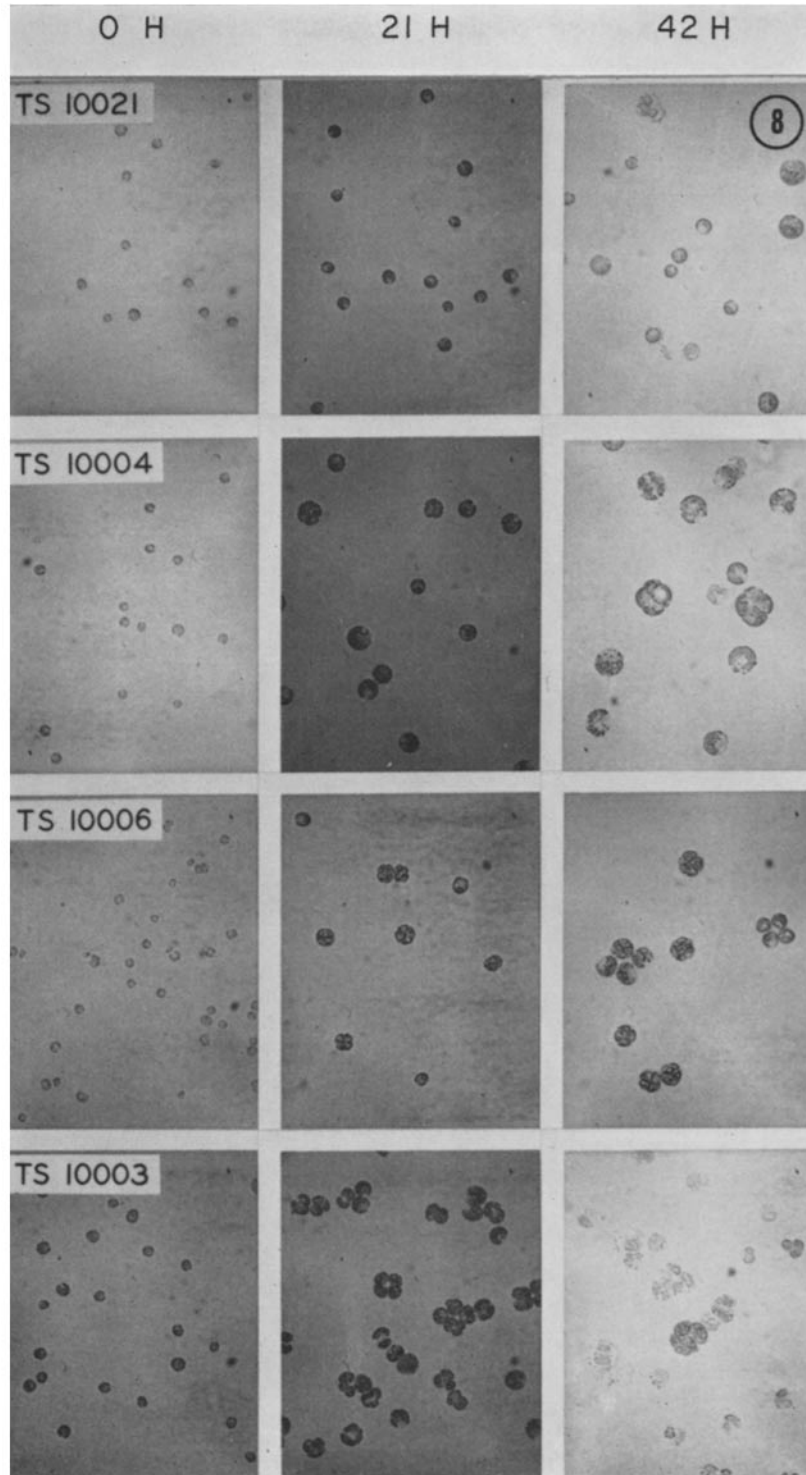


FIGURE 8 Photomicrographs of mutant cells grown at restrictive temperature on agar. Conditions are the same as in Fig. 7. $\times 200$.

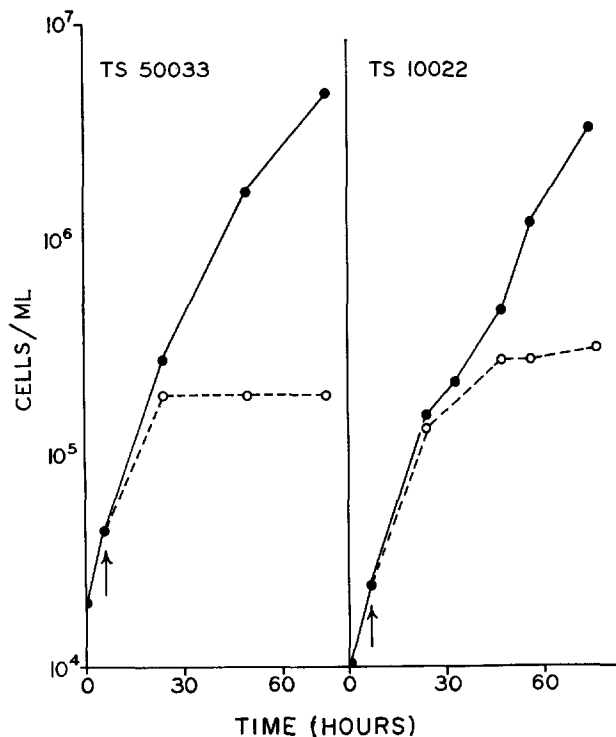


FIGURE 9 Growth of mutant strains at 21°C and 33°C. Conditions are the same as in Fig. 1. Growth at 21°C ●—●; growth after shift to 33°C ○—○.

TABLE II
Summary of Block Points for Mutants Able to Complete
More than One Cycle at Restrictive Temperature

Mutant designation (cb)	Block point	
	From exponential growth (x_1)	From synchronous growth (interval con- taining x_1)
ts10022	0.52	ND*
ts10005	0.82	ND
ts10016	0.88	ND
ts50033	0.94	0.75-0.98

* Not determined.

stage when a defective gene product which can ultimately affect cell division completes its function. Two important features characterizing cb mutants are demonstrated in this study. First, the mutants are defective in gene products essential for cell cycle progress and second, the defective gene products complete their functions at unique points in the cell cycle at permissive temperature.

The class of cb mutants includes, but is more general than, the class of cell division cycle (cdc)

mutants described in yeast (Hartwell et al., 1970). Yeast cdc mutants are purported to be defective in genes which would normally function at a single cell cycle stage, while cb mutants are defective in gene products which, in some cases, might normally function over longer intervals of the cell cycle. Recently, however, Hartwell (1971) has broadened the definition of yeast cdc mutants to include cells in which the defective gene products function over a limited interval in the cell cycle and not just at a single cell cycle stage. DNA synthesis mutants which can block DNA replication any time throughout S-phase are included in this category. Because the defective genes in some cb mutants of *C. reinhardtii* might normally function over longer cell cycle intervals, it could be argued that these mutants may simply be defective in general growth and metabolic activities and not in cell cycle stage-specific processes. It should be pointed out, however, that in *C. reinhardtii* many apparent general metabolic activities are not required to function continuously in order to permit completion of a full cell cycle. An example of this point is the effect of blocking cytoplasmic or

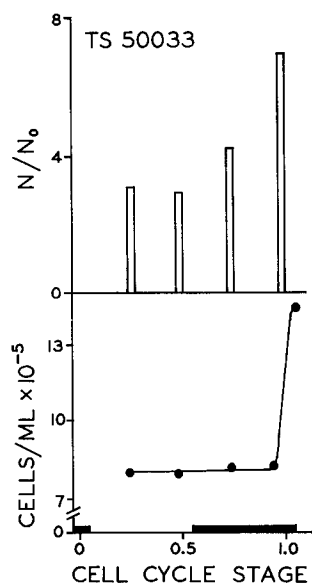


FIGURE 10 Temperature shift-up of mutant culture during synchronous growth. Conditions and experimental procedure are the same as in Fig. 5.

organellar protein synthesis observed by Mihara and Hase (1971). They showed that there were discrete intervals in the cell cycle in which the subsequent cell division was sensitive to cycloheximide or chloramphenicol.

Our experiments have demonstrated that block points are found at nearly all cell cycle stages in *C. reinhardtii*. The completion of gene-controlled functions required for cell division were found at rather unexpected times in the cell cycle, including the very beginning and end. It is notable that in all cycle-blocked mutants we have analyzed, including the ones described in detail in this paper, each displays a very discrete block point or a single cell cycle stage in which the defective gene product completes its function. This finding argues that cell cycle mutants are not simply defective in providing a rapidly depleted gene product which functions at all stages in the cell cycle.

It is of interest that the block point is not necessarily the stage when a cell will be arrested in the cell cycle. This is clearly illustrated by the fact that some mutant cells can complete more than one cell cycle at restrictive temperature. Hartwell et al. (1970) recognized this situation for several yeast mutants and, therefore, distinguished between the execution point and the termination point, the cell cycle stage at which mutant cells accumulate at restrictive temperature. We have not determined

termination points for the *C. reinhardtii* mutants because we have no continuous measure of cell cycle stage. Instead we have assumed, for cells which no more than quadruple at restrictive temperature, that these cells accumulate at cell cycle stage(s) before cell division, while for those cells which more than quadruple, we assume that they accumulate at some cell cycle stage(s) after the first cell division. Some cycle-blocked mutants which accumulate before the first cell division after the block point still continue to grow in cell size (some up to 12 times normal volume). These mutants which continue to grow or divide in the absence of an important cell cycle function suggest that not all gene-controlled functions are tightly interlocked in the cell cycle. Hartwell (1971) has clearly demonstrated that certain cell cycle events can be uncoupled from others in some yeast mutants. For example, one mutant defective in DNA synthesis at restrictive temperature continues bud initiation at regular cell cycle intervals.

The technique employed in this study to determine the block point for a cycle-blocked mutant is simple and generally applicable to almost any cell system, not just to *Chlamydomonas*. Furthermore, it would seem possible to use this technique not only to determine the block point for conditional cb mutants but also to find, for example, the cycle stage at which a division-inhibiting drug acts. The technique requires only that an exponential culture be obtained (or a culture in which the cell cycle age distribution is known) and that the residual cell division after introduction of the division-inhibiting drug can be determined. This technique, of course, is most sensitive for cells which produce larger (but constant) numbers of daughter cells at cell division.

The experiments described here are the beginning steps in generating a temporal map of gene functions which control the cell cycle. We have shown that defective gene products which ultimately block cell division are found at many different cell cycle stages. The mutants we have isolated are unselected in a biochemical or nutritional sense, and, therefore, may represent lesions in a broad spectrum of cellular activities. Coupling this isolation procedure with experiments to determine defective gene product functions, we hope to learn what gene-controlled events tightly regulate the progress of the cell cycle.

We thank Dr. D. W. Smith for his help in formulating the mathematical relationships described in this

paper, Ms. D. M. Baumgartel for her helpful discussions, and L. L. Walker for her excellent technical assistance.

This work was supported by grants from the National Science Foundation, GB-30237 and GB-27386x

Received for publication 16 November 1972, and in revised form 29 January 1973.

REFERENCES

- ARMSTRONG, J. J., S. J. SURZYCKI, B. MOLL, and R. P. LEVINE. 1971. Genetic transcription and translation specifying chloroplast components in *Chlamydomonas reinhardi*. *Biochemistry*. **10**:692.
- CHIANG, K.-S., and N. SUEOKA. 1967. Replication of chloroplast DNA in *Chlamydomonas reinhardi* during vegetative cell cycle: Its mode and regulation. *Proc. Natl. Acad. Sci. U. S. A.* **57**:1506.
- COOK, J. R., and T. W. JAMES. 1964. Age distribution of cells in logarithmically growing cell populations. In *Synchrony in Cell Division and Growth*. E. Zeuthen, editor. Interscience Publishers, Inc., New York. 485.
- HARTWELL, L. H. 1971. Genetic control of the cell division cycle in yeast. II. Genes controlling DNA replication and its initiation. *J. Mol. Biol.* **59**:183.
- HARTWELL, L. H., J. CULOTTI, and B. REID. 1970. Genetic control of the cell-division cycle in yeast. I. Detection of mutants. *Proc. Natl. Acad. Sci. U. S. A.* **66**:352.
- KATES, J. R., and R. F. JONES. 1967. Periodic increases in enzyme activity in synchronized cultures of *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta.* **145**:153.
- MIHARA, S., and E. HASE. 1971. Studies on the vegetative life cycle of *Chlamydomonas reinhardi* Dangeard in synchronous culture. II. Effects of chloramphenicol and cycloheximide on the length of the cell cycle. *Plant Cell Physiol.* **12**:237.
- MUELLER, G. C. 1971. Biochemical perspectives of the G₁ and S intervals in the replication cycle of animal cells: A study in the control of cell growth. In *The Cell Cycle and Cancer*. R. Baserga, editor. Marcel Dekker, Inc., New York. 269.
- SCHMEISSER, E. T., D. M. BAUMGARTEL, and S. H. HOWELL. 1972. Gametic differentiation in *Chlamydomonas reinhardi*. Cell cycle dependency and rates in attainment of mating competency. *Dev. Biol.* In press.
- SUEOKA, N., K.-S. CHIANG, and J. R. KATES. 1967. Deoxyribonucleic acid replication in meiosis of *Chlamydomonas reinhardi*. *J. Mol. Biol.* **25**:47.
- SURZYCKI, S. 1971. Synchronously grown cultures of *Chlamydomonas reinhardi*. In *Methods in Enzymology*. A. San Pietro, editor. Academic Press Inc., New York. **XXIII**:67.
- TAURO, P., E. SCHWEIZER, R. EPSTEIN, and H. O. HALVORSON. 1969. Synthesis of macromolecules during the cell cycle in yeast. In *The Cell Cycle. Gene-Enzyme Interactions*. G. M. Padilla, G. L. Whitson, and I. L. Cameron, editors. Academic Press Inc., New York. 101.