STUDIES ON THE CELL CYCLE IN

**PARAMECIUM TETRAURELIA**

by

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Abstract

Several aspects of the cell cycle in *Paramecium* were studied using the temperature-sensitive mutations *ccl* and *cc2*, and the recessive gene mutation *tamG*.

Cell cycle progression can be blocked by the *ccl* defect up to 0.72 of the cell cycle. There was no progression through the cell cycle at the restrictive temperature, nor excess delay of cell division once cells were returned to permissive conditions. The *ccl* defect also reversibly inhibits macronuclear DNA synthesis throughout the cell cycle. Macronuclear DNA synthesis is not required for cell division past 0.72 of the cell cycle.

Downward regulation of cell mass was examined. Cell mass is regulated by the concerted action of two mechanisms. First, the rate of growth becomes limited by gene dosage when cell mass increases. Second, the cell cycle is shortened when initial cell mass is increased, at the expense of G1. Cells with an initial cell mass greater than 120% of normal initial mass have a cell cycle duration equal to the normal length of S + nuclear division.

Both cell mass and parental DNA content influence the timing of DNA synthesis initiation. As initial cell mass increases, the length of G1 decreases. When initial cell mass exceeds the normal value at the start of S, DNA synthesis begins immediately after fission, without a G1 period. G1 DNA content has no effect on the timing of DNA synthesis initiation. However, cells with low parental DNA content appear to enter S phase earlier than cells with high parental DNA content.
A simple computer simulation model has been developed which is consistent with the experimental observations. The model is based on the unstable inhibitor model of Ycas et al. (1965).
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CHAPTER I.

Regulation of the Cell Cycle in Eukaryotes
The cell cycle is a fundamental unit of development in all organisms and can itself be considered as a developmental pathway, the completion of which depends on many interrelated events. Development in higher organisms depends on two things, growth and organization. Without growth, organisms cannot increase in size or number. However, growth in itself is insufficient. Growth must be organized so that the relationships between cells and tissues allow normal development and maintenance of the organism. Study of the cell cycle has provided information on how cells grow, and on how growth is organized to create a functional cell.

The most important aspect of the cell cycle is the ability of cells to coordinate their rates of growth and cell division. The control of proliferation is especially important in multi-cellular organisms, where tissues depend on precise organization of cells to perform specific functions. In these cases the strict regulation of cellular proliferation is a vital component of developmental organization. For example, the primary feature of oncogenesis is the apparent loss of control over the rate of cell proliferation. Thus, the most important decision made by a cell is between cycling or quiescence. Once this decision has been made, the cell is irreversibly committed to completing the cell cycle. The cell cycle itself consists
of a number of coordinated processes with discrete control points.

This chapter reviews cell cycle control in the yeasts *Saccharomyces* (budding-yeast) and *Schizosaccharomyces* (fission-yeast), and in mammalian tissue culture cells. The major emphasis has been placed on the points of cell cycle control identified so far, and the models which have been devised to explain the experimental observations. This serves as a background for the subsequent experimental work with *Paramecium*.

**Coordination of cell growth and DNA replication**

A proliferating cell must duplicate both its cytoplasmic and nuclear components. Not only must the cell ensure that daughter cells are qualitatively and quantitatively equal, but that each is a functional representative of the mother. Under most circumstances, growth occurs continuously while DNA synthesis is periodic, and doubling of cell mass requires more time than DNA replication. Thus, for cycling cells the major problem is the coordination of cell growth with DNA synthesis. Without coordination, successive rounds of DNA replication would quickly outstrip the cell's capacity to grow, thereby leading to a rapid decrease in cell mass in subsequent generations. This does not occur. Consequently it may be concluded that all cells coordinate these important cell cycle events.

**General view of the cell cycle**

The eukaryotic cell cycle consists of two interdependent cycles; the DNA-division cycle and the cell-growth cycle (Mitchison, 1971). The DNA-division cycle is separable into three parts. $S$ phase, the
period of DNA replication, G2 a period where the cell awaits suitable conditions for initiation of nuclear division, and nuclear division, the partitioning of the nucleus to each daughter cell. In contrast, the cell-growth cycle is not considered divisible since growth continues throughout the cell cycle.

**Experimental systems for cell cycle study**

Several organisms have proven useful for studying cell cycle events. Yeasts, slime molds, ciliates and mammalian tissue culture cells have been the systems favoured. Several features of the biology of the lower eukaryotes make them particularly advantageous for studying cell cycle problems. First, the culture conditions for the cells are very similar to the natural situation. Thus, disruption due to growth conditions is minimal. Second, the rate of cell division is high, and cells typically have cell cycles of less than 6 hours. Yeasts have the advantage of being useful for biochemical and genetic studies, while ciliates, because of their large size, can be used to examine the effects of perturbations on individual cell lineages.

Another important consideration is the ease with which cells can be synchronized. To examine stage-specific processes adequate synchronization is crucial. Synchronization methods are generally of three types: natural, by selection or by induction. Natural synchrony is the ideal situation because there is no experimental perturbation. There are some cell types that possess natural mitotic synchrony (e.g., *Physarum*, and other myxomycetes), but organisms of this type are rare and usually have atypical cell cycles. There are several methods of selection synchrony, usually based on size (e.g. filtration, membrane
elution, sedimentation) or some aspect of morphology (e.g. hand-selection of dividing cells, mitotic selection). These methods usually give excellent synchrony, with a minimum of perturbation to cells. The yields, however, are characteristically low. Synchrony by induction produces the largest yields, but also the greatest disruption to the cell. There are several methods for inducing synchrony in cell cultures, e.g. thymidine block, heat shock, or specific inhibitors (reviews; Mitchison, 1971; Lloyd et al., 1982).

The cell cycle in Saccharomyces cerevisiae

Saccharomyces cerevisiae reproduces by formation of a bud from the side of the mother cell. The cell has a mass-doubling time of 75 minutes under optimal conditions (Carter, 1981), and a typical cell cycle organization (Figure 1-1A). There are several, well-defined landmarks throughout the cell cycle (e.g. bud emergence, DNA synthesis initiation, nuclear division and cell division), and cell cycle stage can be estimated by the ratio of bud to parent cell volume (Carter, 1981).

Genetically, the cell cycle of Saccharomyces is the best studied to date. This is due to the availability of cell-division-cycle (cdc) mutations, originally described by Hartwell et al. (1970). These temperature-sensitive (ts) mutants can divide at the permissive temperature (23 C) but are unable to complete the cell cycle at the restrictive temperature (36 C) and arrest at a characteristic stage. Originally, 148 mutations of 32 different genes were isolated and characterized (Hartwell et al., 1973). Each gene has been shown to have a
characteristic execution point (transition point). The execution point is the stage after which the mutation no longer blocks cell division in the current cell cycle (Hartwell et al., 1970). There are now a total of 50 cdc genes that have been characterized (review; Pringle & Hartwell, 1982). From studies of the cdc mutants, a functional sequence map of gene events necessary for completion of the cell cycle has been developed.

Start

In terms of cell cycle regulation, the most important regulatory point in the Saccharomyces cell cycle is the start function. Start was originally defined as the step mediated by the cdc28 gene product. The execution point of cdc28 is in G1 (Hartwell, 1974).

The G1 period in Saccharomyces is quite variable, with a minimum duration of ten minutes (Tyson et al., 1979). In contrast, S + G2 + M is of fairly constant length for cell cycle durations from 75 minutes to 9 hours (Jagadish & Carter, 1977). Cells in G1 have a variety of alternate developmental fates. Haploid cells can traverse the cell cycle, remain in stationary phase or conjugate. Diploid cells, in addition to going through a cell cycle can undergo meiosis. Each of these fates are determined before the completion of start (Hereford & Hartwell, 1974; Shilo et al., 1978). Execution of the cdc28 step (start) commits the cell to completion of the cell cycle (Hartwell, 1974).

Since the execution of start is prerequisite to cell cycle initiation, it is considered an important regulatory event in the yeast cell cycle. Several factors are required for the completion of start.
1) completion of the previous nuclear division; 2) presence of
nutrients; 3) presence or absence of mating factor and 4) attainment of
a critical cell mass. Under optimal conditions, start is executed when
cell volume exceeds 27 μm (Hartwell, 1974; Johnston et al., 1977). Both
bud emergence and DNA synthesis initiation are dependent on start
(Hartwell, 1978).

Coordination of Growth and DNA synthesis

Under stable growth conditions, cells tend to maintain a constant
size from one cell division to the next. Thus, on average, mass
easily doubles during the course of each cell cycle and cell cycle
duration remains constant. However, when growth conditions decline
(e.g. nitrogen starvation in yeast) cell cycle length increases. In
Saccharomyces, most of this lengthening occurs in G1 before start
(Jagadish & Carter, 1977). Since execution of start appears to depend on
reaching a critical cell mass (Johnston et al., 1977), cells below this
mass remain in G1 until sufficient growth allows completion of start.
Thus, the length of G1 is a function of initial cell mass.

Some observations can not be accounted for by the simple
assumption that the rate of cell division is controlled entirely at
start. At slow growth rates, the duration of the budded interval
increases at a rate of 1.7 minutes for every 10 minute increase in
generation time, suggesting that some dependent event in the cell cycle
other than start may become rate-limiting at low growth rates (Pringle
& Hartwell, 1982). The volume at which cells complete start also
varies with growth rate and age. At slow growth rates the size at bud
emergence is less than in cells grown under optimal conditions.
(Johnston et al., 1979; Lorincz & Carter, 1979). Thus, the criteria for start can be altered according to nutritional state. In mother cells, volume at bud emergence increases about 17% with each successive cell cycle (Johnston et al., 1979). Therefore, mother cells have a growth requirement for start, even though their volume at division is the same as the cell volume at the previous start event (Pringle & Hartwell, 1982). All of these observations indicate that cells are not strictly monitoring cell size, but some parameter correlated with size.

The mechanism which executes start is unknown. Earlier evidence suggested that completion of start depends on attaining a certain cell volume. However, recent studies indicate that a stronger correlation exists between start and accumulation of stable mRNA (Bedard et al., 1980). It has also been proposed that the doubling of the spindle pole body (SPB) commits cells to the cell cycle and demarcates the completion of start by the accumulation of a set number of SPB (Pringle & Hartwell, 1982). This suggests that the rate of SPB subunit synthesis might control entry through the cell cycle, and that SPB production should be correlated with cell mass. It has been shown that in diploids, which have 2 times as many SPB’s, the mean cell mass is also approximately twice the haploid amount (Byers, 1982).

**Nature of G1 in** *Saccharomyces*

As in most eukaryotes, G1 is the most variable portion of the cell cycle in *Saccharomyces*. In addition to being a period of general growth for the cell, G1 contains specific functions required for completion of the cell cycle. Start is one such function. This step always occurs after the end of the previous DNA-division cycle (nuclear
division) and before the next (onset of S phase) and is therefore not part of the chromosome cycle. There are two other genes that function specifically in G1. The \textit{cdc4} and \textit{cdc7} genes act after start but before DNA synthesis and are required for completion of the cell cycle (Hartwell, 1978). Therefore, unlike bacteria, which have no G1-specific functions (Cooper, 1979), there are at least three such events in \textit{Saccharomyces}.

\textbf{The cell cycle in Schizosaccharomyces pombe}

\textit{S. pombe} is the best understood organism in terms of cell cycle regulation. This organism divides by transverse fission rather than by budding. It grows in length during the entire cell cycle, while the width does not change appreciably. Thus, cell length can be used as an indicator of both cell mass and cell cycle stage (Mitchison, 1970). Under optimal conditions, the cell cycle is about 90 minutes. \textit{S. pombe} has a typical cell cycle organization (Figure 1-1B). One unusual feature is that DNA synthesis initiation occurs coincident with cytokinesis (Nurse, 1975).

Several \textit{ts cdc} mutations have also been isolated in \textit{S. pombe} (Nurse et al., 1976). A total of 90 mutations defining 26 genes have been isolated to date. In addition, there is a class of mutants called \textit{wee}, which divide at a smaller size than wild-type cells at the restrictive temperature (Nurse, 1975).

\textbf{Regulation of cell mass and mitosis}

Of the 52 \textit{wee} mutant alleles recovered, all but one map to a
single locus, wee1. At the restrictive temperature, wee causes mitosis

to be initiated at a reduced cell mass (Nurse, 1975). It has been

suggested that the wee defect alters the size threshold for initiation

of mitosis. Cells normally initiate mitosis when cell mass exceeds 20

pg/cell of protein. In the wee mutant, however, mitosis begins when cell

mass exceeds 10 pg/cell of protein (Nurse, 1975).

In addition to the effect on mitosis, wee also delays initiation

of DNA synthesis in the subsequent cell cycle. DNA synthesis normally

occurs concurrent with cell division, but is delayed by 0.29 of a cell

cycle by the action of the wee mutant (Nurse, 1975). This observation

can be explained by the existence of a cryptic size threshold for

initiation of DNA synthesis. Under normal conditions cells complete the

size requirement before the end of mitosis, and enter S after a minimum G1.

However, wee cells, because they initiate mitosis and divide at a

smaller than normal mass, must grow an extra 0.29 of a cell cycle in

order to reach the size threshold (7 pg/cell protein) (Nurse, 1975). Cells

from stationary phase, upon refeeding, do not initiate DNA

synthesis until they pass a similar size threshold (Nurse & Thuriaux,

1977).

Thus, there are two regulatory points in the S. pombe cell

cycle. Cells with an initial mass greater than 15 pg are regulated by

the size threshold at mitosis, followed by a minimum G1 of 0.1 cell

cycles. In cells with an initial mass less than 15 pg, the cell cycle is

regulated by the size threshold for initiation of DNA synthesis

(Nasmyth et al., 1979). Also, in G2 there is evidence for a RNA

synthesis rate doubling step that is prerequisite for completion of the

cell cycle (Barnes et al., 1979).
Start

In *S. pombe* there is a function that operates the same as the start function described in *Saccharomyces* (Nurse & Bissett, 1981). The *cdc2* and *cdc10* genes are required in G1 for commitment to cell division. *S. pombe* cells deprived of nitrogen behave the same as *Saccharomyces* cells treated the same way. Proliferation stops, and cells accumulate in G1. If conditions allow, the cells will undergo conjugation and sporulation. Most *cdc* mutations which block in G1 are unable to conjugate at the restrictive temperature. However, *cdc2* and *cdc10* cells are able to conjugate at the restrictive temperature and therefore must block before commitment to cell division (Nurse & Bissett, 1981). Both *cdc2* and *cdc10* are required for cells to pass start. Recently, the *cdc28* gene from *Saccharomyces*, and the *cdc2* gene from *Schizosaccharomyces* have been cloned (Beach et al., 1982). These two genes appear to be both functionally and structurally homologous. The *cdc28* gene is able to complement *cdc2* but the reverse is not true (Beach et al., 1982).

Nature of G1 in *Schizosaccharomyces*

In rapidly growing cultures, cells have a minimum G1 of 0.10 cell cycles. As growth rate declines, the length of G1 increases. However, the length of S + G2 remains constant over a 6-fold range of generation times (Nasmyth, 1979). Thus, the length of the G1 period is largely determined by cell mass. The start function observed in *S. pombe* demonstrates the existence of G1-specific functions as previously demonstrated for *Saccharomyces*. At present, yeasts are the only organisms in which G1-specific functions have been clearly demonstrated.
Unlike lower eukaryotes, tissue culture cells are studied in an unnatural environment. Generation times of these cells are also much longer than those of yeast and ciliates. In spite of these limitations, tissue cultures fulfill a pressing need, namely the understanding of cell cycle regulation in animals.

As in other systems, ts-mutations have been used to approach the underlying problems of the cell cycle. Mutants with a variety of defects in S phase entry, DNA synthesis, mitosis and cell division have been isolated (reviews; Basilico, 1977; Lloyd et al., 1982). The general method for isolation has been to grow mutagenized cultures at the restrictive temperature in the presence of agents that kill proliferating cells. Cells are then shifted to permissive conditions to allow the putative ts mutants to grow (Lloyd et al., 1982). The largest fraction of mutations recovered are blocked in G1, although mutations representative of other cell cycle phases have also been obtained (Hirschberg et al., 1980). This suggests that there are many genes which act during G1 and make it amenable to mutational dissection.

Several general conclusions can be drawn from available studies. DNA synthesis and cell division are dependent on completion of mitosis. Mutants which block in M cease DNA synthesis and cytokinesis. However,
all the steps of mitosis are not required since some cells continue to synthesize DNA even though mitosis is incomplete. In addition, DNA synthesis and cell division must become unlinked for endoreduplication to occur.

**Coordination of growth and DNA synthesis**

Like all cells, mammalian cells tend to maintain a constant size from one cell cycle to the next, when growth conditions are stable. Therefore, higher eukaryotes must also possess a means of coordinating growth and DNA synthesis. Studies of a wide variety of cell types have shown that the G1 period is the most variable while S + G2 + M tend to remain constant (Prescott, 1982). This same pattern has been observed in lower eukaryotes.

Several lines of evidence suggest that cells begin DNA synthesis after attaining a critical cell mass. Smaller, post-fission cells have a longer G1 than do larger cells (Killander & Zetterberg, 1965a; Yen et al., 1975; Shields et al., 1978). When growth is restricted by nutritional limitation, cell mass is reduced, and the length of G1 increases (Kimball et al., 1971; Yen et al., 1975; Shields et al., 1978). Starvation characteristically causes cells to arrest in G1 (Kimball et al., 1971). Microphotometric measurements of cell mass demonstrate that variation in cell mass is lowest just after the start of DNA synthesis (Killander & Zetterberg, 1965b; Kimball et al., 1971). This is consistent with the predicted effect of a mass-related control over entry into S phase.
Effect of cell mass on cell cycle progression

While the observations described above suggest that increase in mass is responsible for initiation of DNA synthesis, increase in mass is not an absolute requirement for progress through the cell cycle. Once DNA synthesis has been initiated, cell cycle progression is moderately insensitive to the action of growth inhibitors (Prescott, 1982). It has also been demonstrated that cells can be induced to enter S phase and will complete mitosis and cytokinesis, in the absence of any increase in mass (Zetterberg & Engstrom, 1983; Ronning & Lindmo, 1983; Mercer et al., 1984).

Cell growth cannot be entirely excluded as a contributing factor in cell cycle regulation. The rate of cellular proliferation has been correlated with the rate of protein accumulation (Baxter & Stanner, 1978), and it has been suggested that some aspect of cell growth is required for initiation of DNA synthesis (Liskay et al., 1980). Cells which complete this requirement by the end of mitosis start DNA synthesis almost immediately, while cells which have not, have an intervening G1 period. V79-8 Chinese Hamster cells have no measurable G1 under optimal growth conditions (Liskay, 1977). However, when the rate of protein synthesis is decreased by treatment with cycloheximide, a G1 period is observed and the length of S + G2 + M is unchanged (Liskay et al., 1980). This is consistent with the suggestion that G1 is not a part of the DNA-division cycle, and exists only to satisfy a growth-related requirement for initiation of DNA synthesis (Stancel et al., 1981).

The rate of DNA synthesis can also be reduced without affecting cell growth, by treating cells with hydroxyurea. Thus, S phase can be
extended without an increase in mass doubling time. The length of the cell cycle is unchanged, but G1 is decreased by an amount equal to the extra time required for replication (Stancel et al., 1981). Thus, it appears that the G1 phase of the mammalian cell cycle exists only when the cell growth cycle is longer than the time required to complete the DNA-division cycle. These observations are consistent with a mass-related control over entry into S phase.

Cell cycle regulation models

The evidence so far suggests that cell mass, initiation of DNA synthesis and the timing of cell division are under the control of homeostatic mechanisms. Three classes of theoretical models for the regulation of cell division have been proposed recently (Fantes & Nurse, 1981).

1) Cell division mass. Division could be triggered by the attainment of a critical cell mass. Cells would all divide at a predetermined size. Thus, large cells would have short cell cycles, while small cells would have long cell cycles.

2) Increment in mass between divisions. Regulation could be by incremental growth during the cell cycle. All cells would grow the same amount, thus reducing variation by one-half by partitioning to daughter cells.
3) Cell cycle duration. Cells might grow for a fixed length of time. However, since cells grow exponentially, cell mass will double and there will be no reduction in the amount of variation.

Two more specific classes of models, concerned with the mechanisms of regulation have also been proposed (Fantes et al., 1975). These models, and most models devised, are based on three assumptions.

1) Cells tend to maintain a constant ratio of mass to genome, alternately referred to as the nucleocytoplasmic (n/c) ratio, or gene dosage. The timing of cell cycle events is assumed to be a response to the constraints imposed by this ratio becoming either too high (excess mass) or too low (deficient mass). All models are an attempt to describe the mechanisms that cells utilize to maintain a constant gene dosage.

2) Evidence indicates that the factor(s) regulating cell cycle events are cytoplasmic. Thus, cell mass should be strongly correlated with the control exerted by the regulatory substances.

3) Nuclei in a common cytoplasm initiate DNA synthesis and divide synchronously. Sister cells show less variation in the time of DNA synthesis onset and cell division than do unrelated cells.

Regulatory models can be roughly divided into two classes, concentration and structural. Concentration models describe the effect of regulating substances as a function of their cellular concentration. Structural models postulate that accumulation of subunits to form a completed structure mediates the occurrence of cell cycle events.
Concentration models

Concentration models postulate that cell cycle events are triggered when the cytoplasmic concentration of an effector molecule reaches a critical threshold concentration. There are two variations of this model, simple concentration, or active decay of an inhibitor. The simple concentration model predicts that events occur when 1) the concentration of an activator rises above a critical threshold (activator accumulation) or 2) the concentration of an inhibitor falls below a critical threshold (inhibitor dilution). These models are mathematically equivalent (Fantes et al., 1975).

In the activator accumulation model, the rate of activator production will be correlated with cell mass, since the rate of activator synthesis should be a function of cell growth. This prediction is consistent with the observation of growth requirements for initiation of DNA synthesis (Liskay, 1977). This model is also consistent with the size-related thresholds observed at the start of S phase in several eukaryotes (Nurse, 1975; Johnston et al., 1977; Nasmyth, 1979).

In the inhibitor dilution model, the rate of synthesis is nil for most of the cell cycle, and decrease in inhibitor concentration occurs by passive dilution as cytoplasmic volume increases. The observations regarding size thresholds are also consistent with this model. There is evidence suggesting an inhibitor regulation of DNA synthesis onset at least in quiescent cells (Yanishevsky & Stein, 1981; Burmer et al., 1983; Polunovsky et al., 1983). However, both concentration models are contradicted by studies which show that DNA synthesis can be initiated in the absence of net growth (Zetterberg & Engstrom, 1983; Ronning &
The unstable inhibitor model was first proposed by Ycas et al. (1965). The model suggests that cell cycle regulation is under the control of an inhibitor substance whose absolute amount is proportional to gene dosage. Rather than the amount being solely a function of dilution, the inhibitor is proposed to decay at a constant rate, independent of its concentration. Cell cycle events are triggered when the amount of inhibitor falls below a critical threshold. Thus, the regulatory effects exerted by the inhibitor will be dependent on both growth and gene dosage. This model is consistent with the growth-related requirement for initiation of DNA synthesis (Liskay, 1977), and the existence of size-thresholds for cell cycle events (Nurse, 1975; Johnston et al., 1977; Nurse & Thuriaux, 1977; Nasmyth, 1979).

Positive vs. Negative control mechanisms

Most of the controversy about cell cycle regulation concerns the nature of the control mechanism. Are cell cycle events triggered by activating substances or modulated by inhibitors? (i.e. positive or negative control). There is evidence supporting both views.

Most of the evidence supporting positive control mechanisms is derived from cell fusion studies. Mammalian culture cells, when treated with polyethylene glycol and Sendai virus, fuse to form a heterokaryon. When cells in S phase were fused with G1 cells, the G1 nucleus was induced to begin DNA synthesis (Rao & Johnson, 1970). G2 cells fused with G1 cells do not have this effect. This suggests that
a substance, present only in S phase nuclei, is responsible for initiating DNA synthesis. It is important to note, however, that this does not exclude the possibility that initiation of DNA synthesis may still be under negative control even though the maintenance of DNA synthesis under positive control.

Other cell fusion experiments have given different results. Quiescent human diploid cells (HDC) in G1 fused to cells in S phase are not induced to synthesize DNA (Yanishevsky & Stein, 1980). In addition, several other cell types when fused to HDC, not only fail to induce DNA synthesis in the HDC, but are themselves inhibited from entering S phase (Yanishevsky & Stein, 1980). Finally, inhibition of protein synthesis in quiescent NIH-3T3 and human diploid fibroblast-like cells, prevents these cells from inhibiting DNA synthesis in proliferating cells (Burmer et al., 1982; Polunovsky et al., 1983). Thus, it appears that, at least in quiescent cells, initiation of DNA synthesis is under the negative control of a protein or a group of proteins.

Structural Models

Structural models propose that cell cycle events are triggered by the completion of a particular structure. The structure is composed of subunits, so that the rate of assembly depends on the rate of subunit synthesis. In turn this will be proportional to cell growth. At least two cases have suggested a structural model for regulation of the cell cycle. In *Tetrahymena*, observations are consistent with cytoplasmic subunits controlling cell division (Zeuthen & Williams, 1969). In *Saccharomyces*, the spindle pole body has been suggested as the structure
regulating completion of the start function, since the SPB is completed just prior to start and its number is proportional to cell mass (Pringle & Hartwell, 1982).

The cell cycle in Paramecium tetraurelia: The subject of this study

The cell cycle in Paramecium is similar to that of most eukaryotes. There is a substantial G1 phase which extends from fission to 0.25 of the cell cycle. Macronuclear DNA synthesis starts abruptly at this point, and continues until nuclear division at 0.87 (Berger, 1971) (Figure 1-2). There is no macronuclear G2.

In Paramecium the coefficient of variation in cell mass is typically 15-20 % (Kimbball, 1959; Berger, 1982). Until recently, very little was known about cell cycle regulation in Paramecium. Early work by Kimball (1959, 1967) demonstrated a strong correlation between macronuclear DNA content and cell mass over a wide range of values. The only other study of cell mass regulation showed that small clonal differences in cell mass were maintained over several cell cycles (Kimball, 1967). However, this slow regulation of mass does not explain the maintenance of a constant variance in cell mass from one cell cycle to the next. Some other mechanism must be functioning to keep the variation which is introduced at each fission within limit.

In contrast to the lack of understanding of cell mass regulation, the regulation of DNA content is much better understood. Several
studies indicate that cells make a standard amount of DNA during the cell cycle regardless of initial DNA content (Berger & Schmidt, 1978; Berger, 1978, 1979). The amount made is equal to the mean DNA content of the population (Berger, 1978, 1979). Thus, regulation of DNA content operates by an incremental mechanism (Fantes & Nurse, 1981), so that half the variation in DNA content is removed in each cell cycle.

The $cc_1cc_1$ and $cc_2$ mutations of Paramecium have proven to be very useful in examining the coordination between DNA synthesis and cell growth in this organism. Both mutants are temperature-sensitive and block cell division. In addition, $cc_2$ partially inhibits DNA synthesis while $cc_1cc_1$ totally inhibits DNA synthesis (Peterson & Berger, 1982).

Aims of this Study

This study has five objectives.

1) To further characterize the cell-cycle mutant $cc_1cc_1$.

2) To examine the consequences of cell cycle blockage on cell mass, DNA content and cell division.

3) To examine the regulation of cell mass, after cells have been experimentally enlarged, and the effects of excess mass on cell cycle duration, macronuclear DNA content and cell mass in subsequent cell cycles.
4) To determine the effect of initial cell mass and parental DNA content on initiation of DNA synthesis.

5) To develop a simulation model of the *Paramecium* cell cycle, that can account for the experimental observations.

The availability of the ts cell cycle mutants *cc1cc1* and *cc2*, which interfere with cell cycle progression and DNA synthesis, and the recessive mutation *tamG*, which alters the distribution of the macronucleus to daughter cells, allow these problems to be studied.
Figure 1-1. Cell cycle organization in several eukaryotes. 0 = start of cell cycle; 1 = end of cell cycle. ND = nuclear division, S = period of DNA synthesis. 1-1A. Cell cycle organization in Saccharomyces cerevisiae. 1-1B. Cell cycle organization in Schizosaccharomyces pombe. 1-1C. Cell cycle organization in a typical mammalian tissue culture line (Balb-c/3T3 cells).
Figure 1-2. Cell cycle organization in *Paramecium tetraurelia*.  
0 = start of cell cycle; 1 = end of cell cycle; 0.25 = normal time of DNA synthesis initiation; 0.87 = end of S and start of macronuclear division (MND).
CHAPTER II.

A Fast-acting Temperature-sensitive Cell Cycle Mutation of *Paramecium tetraurelia* Affecting DNA Synthesis and Cell Cycle Progression, During both G1 and S: Implications for Cell Cycle Regulation.
Introduction

The cell cycle in eukaryotes is composed of two interdependent pathways; the cell growth and DNA division cycles (Mitchison, 1971). Normally these two pathways are coordinated such that one cycle does not outstrip the other. Many temperature-sensitive cell cycle mutations, which interfere with a wide variety of functions have been isolated in eukaryotes (review; Simchen, 1978). The use of these mutations has revealed much about the relationships between the cell growth and DNA division cycles (Hartwell, 1978; Fantes, 1982).

In particular, mutations which block DNA synthesis while allowing nearly normal patterns of cell growth to continue are useful in examining the coordination of DNA synthesis with cell growth and cell cycle progression. One such mutation, cci, has been isolated in Paramecium (Peterson & Berger, 1976). This mutation blocks macronuclear DNA synthesis and cell cycle progression at 34.4 C. This study examines three additional aspects of the cci defect: namely a) kinetics of inactivation and reactivation of macronuclear DNA synthesis; b) blockage of cell cycle progression in G1 and; c) separation of the cell division and DNA synthesis pathways late in the cell cycle.

The results indicate that cci causes rapid inhibition of both macronuclear DNA synthesis and cell cycle progression at the restrictive temperature. This inhibition is reversible, and cells resume normal
rates of DNA synthesis after being returned to permissive conditions. Cell cycle progression is blocked even when the heat treatment lies totally within the GI period. There is neither progression through the cell cycle at the restrictive temperature, nor excess delay of cell division when cells are returned to permissive conditions. Macronuclear DNA synthesis is not required for the completion of cell cycle past 0.72 of the cell cycle. This point of commitment to cell division coincides with the micronuclear mitosis and the onset of oral morphogenesis in Paramecium.
Experimental Design

Protocol for Temperature Shift Experiments

Shift from 27 C to 34.4 C

Synchronous samples of *ccl* cells were incubated at 27 C for three hours after fission to allow the cells to reach mid S phase. Cells were then placed in a water bath at 34.4 C. Ten minutes prior to fixation, medium with [3H]-thymidine labelled bacteria, pre-warmed to 34.4 C was added to the sample. At the scheduled time, samples were removed and cells killed in a solution of ethanol/Cerophyl (3% v/v) to prevent further incorporation of label into the macronuclei. The rate of DNA synthesis was determined by direct grain counts as described earlier (Berger, 1971), and compared to an untreated (27 C) control group.

Shift from 34.4 C to 27 C

DNA synthesis was first completely blocked by incubating *ccl* cells in mid S phase at 34.4 C for one hour. Cells were then returned to 27 C, the permissive temperature, and transferred to medium with [3H]-thymidine labelled *E. coli*. Samples were fixed at 10 minute intervals. The rate of DNA synthesis was determined and compared to an untreated control group.
Results

Effect of temperature shift on macronuclear DNA synthesis

Following a rapid shift from 27 C to 34.4 C the rate of macronuclear DNA synthesis declined to 50% of initial activity within 6 minutes and was completely inhibited within 30 minutes (Figure 2-1; open circles). The rapid inhibition of DNA synthesis at 34.4 C was found to be reversed with equal rapidity when cells were transferred from restrictive to permissive conditions. The rate of macronuclear DNA synthesis in heat-treated ccl cells rose to 50% of the control level within 5 minutes, and was normal within 20 minutes after return to permissive conditions (Figure 2-1; filled circles).

Delay of initiation by heat treatment in G1

When ccl cells were exposed to heat treatment at 34.4 C and subsequently returned to permissive conditions the cell cycle was extended (Figure 2-2, solid line). The duration of this extended cell cycle, less the length of heat treatment was always nearly equal to the length of the control cell cycle (96.8±2.5%; n=13)(Figure 2-3). In these experiments, heat treatment always began 30 minutes after fission (early in G1), suggesting that the ccl defect, in addition to blocking DNA synthesis, also blocked progression through the cell cycle in G1. If ccl
blocked only DNA synthesis then a cell cycle delay would be expected only when heat treatment extended past the normal point of DNA synthesis initiation (Figure 2-2, dashed line). The observation that cell cycle duration is increased when heat treatment is wholly contained within the G1 period (Figure 2-2) supports the first alternative.

If \textit{ccl} blocked progression through G1, then heat treatment would delay initiation of DNA synthesis. On the other hand, if heat treatment in G1 causes cell cycle elongation only through interference with DNA synthesis, then cells heat treated in G1 should initiate DNA synthesis at the normal time when heat treatment is contained completely within the G1 period. Such cells would also be expected to initiate DNA synthesis very soon after return to permissive conditions if the period of heat treatment extended past the normal time of initiation, since during heat treatment cells would have accumulated at the G1/S interface.

To distinguish between these two alternatives, the timing of macronuclear DNA synthesis onset was determined in synchronous samples of \textit{ccl} cells under different conditions. A control sample was grown continuously at 27 C, while, starting 30 minutes after fission, other samples were subjected to either a 1.0 hour, a 3.0 hour or a 5.0 hour heat treatment. Comparison of the time elapsed between the end of heat treatment and initiation of DNA synthesis in the experimental cells, with the duration of the portion of the G1 period remaining at the start of the heat treatment shows that there was no progression through G1 during heat treatment (Table 2-1). This conclusion is supported by the observation that the difference between the duration of the remaining portion of the G1 period and the time from the end of heat treatment to
initiation of DNA synthesis did not increase when the duration of the heat treatment was increased.

Completion of DNA synthesis is not a precondition for cell division

Studies on *P. tetraurelia* suggest that cells synthesize a standard amount of DNA during the cell cycle (Berger, 1979). This is true even in cells which begin the cell cycle with abnormally low or high macronuclear DNA contents. Consequently it is important to know whether completion of the full schedule of DNA synthesis is a precondition for cell division in this organism. The complete and rapid blockage of macronuclear DNA synthesis at restrictive conditions by the *ccl* defect makes it possible to directly test whether blockage of DNA synthesis during the latter part of the macronuclear S period is associated with blockage of cell division.

The transition point (execution point) for cell division in *ccl* was estimated to occur at 0.73 of the normal 27°C cell cycle by Peterson & Berger (1976). This study, using the residual cell division method of Howell et al. (1975), estimates the transition point at 0.72±0.03 of the normal cell cycle. After the transition point, incubation of *ccl* cells at 34.4°C does not prevent cell division in the current cell cycle. To test whether DNA synthesis was inhibited after the transition point, synchronous samples of *ccl* cells were shifted to the restrictive temperature at four points (0.64, 0.72, 0.80, 0.88) in the cell cycle, and incorporation of tritiated thymidine into macronuclear DNA was examined after transfer of cells to the restrictive temperature. Earlier samples contained a significant number of cells which had not yet
reached the transition point, and were blocked, while in the later samples most of the cells had passed the transition point and completed the cell cycle on schedule. There was no incorporation of label into macronuclei of either group of cells, while controls at 27 C showed normal labelling. Thus, macronuclear DNA synthesis is not precondition for cell division after 0.72 of the cell cycle.
Discussion

The ccl phenotype appears to be the result of a mutation in a single gene mutation that is pleiotropic (Peterson & Berger, 1976). At the restrictive temperature, DNA synthesis is rapidly inhibited, and progression through the cell cycle is arrested provided that the cells are shifted to restrictive conditions prior to 0.72 of the cell cycle. This effect occurs if cells are in either the G1 or the S phase of the cell cycle. Cell growth is not affected and cell mass increases normally while cells are at the restrictive temperature (Rasmussen & Berger, 1982).

Effect on DNA Synthesis

Several mutations with temperature-sensitive defects in DNA synthesis have been described in Saccharomyces (review; Pringle & Hartwell, 1982) and Schizosaccharomyces (review; Mitchison, 1971); ciliates (Peterson & Berger, 1976; review: Berger, 1984) and mammalian cell cultures (e.g. Basilico, 1977; Sheinin & Lewis, 1980; Hyodo & Suzuki, 1982; McCracken, 1982). Many ts-mutants have defects in metabolite uptake or production. These characteristically produce a slow inactivation of DNA synthesis (McCracken, 1982). The rapid inactivation and reactivation of DNA synthesis in the ccl mutant is
unusual, and suggests that the mutation does not interfere with metabolite uptake or production but may represent a defect in the replication mechanism itself.

A similar defect is observed in the cdc8 mutant of *Saccharomyces cerevisiae* (Arendes et al., 1983). The cdc8 gene is required for DNA synthesis and in vitro replication of yeast 2 μm plasmid. The cdc8 gene product has recently been isolated and has been shown to be a single-stranded DNA binding protein. After 20 min. incubation at the restrictive temperature, more than 80% of the DNA binding and 90% of the complementation activity were lost (Arendes et al., 1983). However, unlike ccl, the effect of heat treatment is not reversible. Also, ccl blocks progression through G1 while cdc8 blocks only after the initiation of DNA synthesis.

**Effect on Cell Cycle Progression**

Cell cycle progression can be blocked by incubating cells in the presence of metabolic inhibitors such as cycloheximide (Polunovsky et al., 1983) or hydroxyurea (Worthington et al., 1976; Stancel et al., 1981; Singer & Johnston, 1983), or through the action of ts-mutations (Hartwell, 1970; Nurse et al., 1976; Peterson & Berger, 1976; Hyodo & Suzuki, 1982). However, cell cycle blockage is commonly either incomplete, and allows some progression through the cell cycle under restrictive conditions, or imposes excess delay (Prescott, 1982). The ccl mutation is unusual in that it blocks progression through the cell cycle completely, with neither residual progression nor excess delay. This study indicates that an indispensible cell function, altered by the
action of ccl at the restrictive temperature, is required for progression through the G1 portion of the cell cycle, as well as for maintenance of DNA synthesis.

Cell growth has been suggested as a major determinant of cell cycle progression (Liskay et al., 1980; Stancel et al., 1981). Attainment of a critical minimum cell mass is correlated with initiation of DNA synthesis in many eukaryotes (Prescott, 1956; Killander & Zetterberg, 1965; Nurse, 1975; Johnston et al., 1977; Nasmyth, 1979; Nasmyth et al., 1979; Rasmussen & Berger, 1982). However, the mechanisms coordinating cell growth with cell cycle events are not fully understood and the available evidence is conflicting. The effect of critical cell mass on control of initiation of DNA synthesis has been rationalized as a consequence of either accumulation of an activator (Rao & Johnson, 1970; Prescott, 1982) or dilution of an inhibitor (Stein & Yanishevsky, 1981; Burmer et al., 1982; Polunovsky et al., 1983). In each case, initiation of DNA synthesis is a function of cell growth. Cell growth also plays a role in determining the timing of other cell cycle events, e.g., nuclear division (Hartwell, 1974; Nurse, 1975; Nurse & Thuriaux, 1977; Craigie & Cavalier-Smith, 1982).

The present experiments indicate that cell growth by itself is not sufficient to bring about DNA synthesis in Paramecium. Since ccl cells continue to grow normally at the restrictive temperature, there should be no delay in the onset of DNA synthesis if initiation of DNA synthesis is dependent solely on attainment of a critical cell mass. Other observations have demonstrated that initiation of DNA synthesis and progression through the entire mitotic cell cycle can occur in the
absence of a significant increase in cell mass (Zetterberg & Engstrom, 1983; Ronning & Lindmo, 1983).

Effect of DNA Synthesis Inhibition on Cell Division

Earlier studies suggested that completion of the full schedule of DNA synthesis may be required to initiate cell division in Paramecium (Berger & Schmidt, 1978; Berger, 1978; 1979). All cells, regardless of initial DNA content, synthesize a standard amount of DNA. Cells with very low initial DNA contents have extremely long cell cycles with greatly prolonged S periods. Our results show that the full schedule of DNA synthesis is not required for the initiation and completion of cell division or nuclear division. When ccl cells were shifted to restrictive conditions after the cell division transition point, they completed the cell cycle in the absence of any measurable macronuclear DNA synthesis, and post-fission DNA content was reduced by the expected amount (Ching, unpublished results). Similar divergence of the DNA division cycle from the cell division cycle (Mitchison, 1971) has been observed in other organisms (Hartwell, 1978; Mitchison, 1974). In most of these cases the point at which cell division becomes independent of the DNA division cycle occurs after the start of nuclear division. In Paramecium, however, the point at which blockage of DNA synthesis no longer blocks cell division (0.72 of the cell cycle) occurs at the start of stage 1 of oral morphogenesis, as defined by Kaneda & Hanson (1974). This point also coincides with the beginning of micronuclear mitosis, but occurs substantially prior to the start of macronuclear amitosis (0.87). Similar, though less precise observations have been made with
Tetrahymena (Anderson, 1972; Jeffery, 1972; Worthington et al., 1976). Thus the coordinated initiation of oral morphogenesis and micronuclear mitosis appears to define a commitment point for cell division in both *Paramecium* and *Tetrahymena* (review; Berger, 1984).
Table 2-1. Effect of heat treatment on cell cycle progression during G1.

<table>
<thead>
<tr>
<th>Length of HT (hr)</th>
<th>Control G1 (hr)</th>
<th>Remainder of G1 at SHT (hr)</th>
<th>Time from EHT to S (hr)</th>
<th>DIFF (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 hr</td>
<td>1.66 hr</td>
<td>1.16 hr</td>
<td>1.06 hr</td>
<td>0.10 hr</td>
</tr>
<tr>
<td>3.0 hr</td>
<td>1.38 hr</td>
<td>0.88 hr</td>
<td>0.85 hr</td>
<td>0.03 hr</td>
</tr>
<tr>
<td>5.0 hr</td>
<td>1.47 hr</td>
<td>0.97 hr</td>
<td>0.90 hr</td>
<td>0.07 hr</td>
</tr>
</tbody>
</table>

HT = Heat Treatment  
SHT = Start of Heat Treatment  
EHT = End of Heat Treatment  
DIFF = (Remainder of G1 at SHT) - (EHT to S phase)
Figure 2-1. Rate of macronuclear DNA synthesis after temperature shifts. Open circles = cells shifted from 27°C to 34.4°C; Filled circles = cells shifted from 34.4°C to 27°C.
Figure 2-2. Cell cycle length vs. length of heat treatment. Dashed line = expected result if heat treatment extends cell cycle by interfering with cell cycle progression in S phase; Solid line = expected result if heat treatment blocks cell cycle progression in G1; Open circles = existing data. Length of heat treatment is expressed as a percentage of the control cell cycle duration.
Figure 2-3. Portion of the cell cycle remaining after the length of heat treatment is accounted for vs. the length of heat treatment. Both variables are expressed as a percentage of the control cell cycle duration.
Extended cycle - Length of HT

Length of Heat Treatment

0 25 50 75 100 100 200
CHAPTER III.

Downward regulation of Cell Size
in *Paramecium tetraurelia*:
Effects of Increased Cell Size,
With or Without Increased DNA Content, on the Cell Cycle.
Introduction

In a typical eukaryotic cell the nuclear division mechanism ensures that daughter cells receive quantitatively and qualitatively identical chromosomal complements. The partitioning of cell mass (cytokinesis) is not as precise, and sister cells typically differ in size (e.g. Killander & Zetterberg, 1965a). Rates of growth and generation times also vary slightly. As a result, variation in cell mass is introduced during each cell cycle. If no regulatory mechanisms existed, the variance in cell mass within a cell population would increase without limit. Obviously this does not happen and cell mass shows only a limited variation (Killander & Zetterberg, 1965a; Killander, 1965; Kimball, 1967; Kimball et al., 1971; Yen et al. 1975; Fantes, 1977). Thus, one may conclude that regulation of cell mass occurs in all organisms.

Regulation of cell mass has been demonstrated in several organisms including mammalian cell cultures (Prescott, 1956; Kimball et al., 1971), and the yeasts *Schizosaccharomyces pombe* (Fantes, 1977; Fantes & Nurse, 1977) and *Saccharomyces cerevisiae*, (Johnston et al. 1977). These studies show that cells tend to maintain a constant cell mass under stable growth conditions. However, when growth conditions are altered, cell mass is modulated. Studies in *S. pombe* (Fantes & Nurse, 1977), *Saccharomyces* (Johnston et al., 1980), mammalian cell cultures (Kimball et al., 1971) and *Tetrahymena* (Zalkinder 1979a, 1979b) show that
cell mass decreases as growth conditions decline and increases when conditions improve.

Cell mass, or some cell mass related factor, appears to play an important role in regulating the cell cycle. Several studies have shown a correlation between the attainment of a critical cell size and the onset of DNA synthesis (Killander & Zetterberg, 1965b; Nurse, 1975; Yen et al., 1975; Fantas, 1977; Johnston et al., 1979; Nasmyth, 1979; Nasmyth et al., 1979). A similar relationship exists between cell size and the triggering of cell division (Frazier, 1973; Fantas & Nurse, 1977, 1978; Johnston et al., 1977; Zalkinder, 1979a, 1979b; Craigie & Cavalier-Smith, 1982; Soll et al., 1982). The mechanism that translates cell mass into a regulatory signal for the cell is, as yet, unknown.

Variability in the generation times of sister cells is universal. Much of the work in cell cycle kinetics has been undertaken to explain how genetically identical cells can vary in their rates of growth, and generation times. The result of such studies suggests that in proliferating cells, the length of S + G2 + M tends to remain constant over a wide range of growth rates. (Kimball et al., 1971; Johnston et al., 1979; Nasmyth, 1979; Nasmyth et al., 1979). Most, if not all, of the variation in cell-cycle length is a consequence of variation in the G1 phase of the cell cycle (Prescott, 1976). There are contrasting explanations for the observed variability in the length of G1.

Paramecium is no exception to the rule. The generation time of exponentially growing cells in Cerophyl medium (Kimball et al., 1959) ranges from 5.5 - 6.5 hours at 27 C, and sister cells typically differ in their generation times, cell mass and DNA contents. Paramecium is
particularly useful for examining the cell cycle and cell mass regulation as it is possible to isolate and study individual cell lines. Further, the use of fluorochrome staining procedures in conjunction with a computerized system for quantifying staining intensity, makes it possible to do rapid and accurate measurements of both cell mass (protein) and macronuclear DNA content (Berger, 1979). In addition, the use of tritiated thymidine labelled \textit{E. coli} (Berger, 1964) is a sensitive assay for DNA synthesis and allows direct determination of the length of G1 (Berger, 1971; Rasmussen & Berger, 1982).

\textit{Paramecium tetraurelia} is a relatively large protist with a volume 50 - 100 times that of a typical diploid eukaryotic cell. It has a large polygenomic macronucleus, which can tolerate extreme variation in DNA content without the occurrence of genic imbalance. Cell mass also varies considerably, with a coefficient of variation on the order of 15 - 20\% (Kimball et al., 1959; Rasmussen, unpublished data). In spite of the normal variation in both macronuclear DNA content and cell mass, there is usually a high correlation between mean cell mass and mean DNA content in \textit{Paramecium} populations (Kimball, 1967; Berger, 1982a). These observations suggest that \textit{Paramecium} can regulate cell mass, and that regulation of cell mass and DNA content are interrelated processes (Berger, 1982a).

This chapter is concerned with the effect of heat treatment on two temperature-sensitive (ts) cell-cycle mutants, and examines the kinetics of cell mass regulation in \textit{Paramecium} and its relation to the cell cycle. The ts mutation \textit{ccl} blocks macronuclear DNA synthesis and cell division at the restrictive temperature (34.4°C). However, protein synthesis and accumulation continue, so that cells with excess cell mass
and normal DNA content can be produced. A second ts mutation, cc2, also blocks cell division, but allows macronuclear DNA synthesis to continue at the restrictive temperature (34.25°C), although at a reduced rate. These cells show an increase in both cell mass and DNA content at the restrictive temperature, although DNA content increases at a lower rate than cell mass under these conditions.

These mutations make it possible to experimentally generate cells of increased size, with or without a concomitant increase in macronuclear DNA content. The kinetics of downward regulation of cell mass were examined, and the effects of increased DNA content and cell mass on the rates of growth and DNA synthesis, cell-cycle duration and the changes in DNA content and cell mass over the course of a recovery cycle after the return of experimental cells to permissive conditions were studied. In addition experimentally enlarged cc1 cells were followed over several recovery cell cycles to examine long term downward regulation of cell mass.
Experimental Design

Two groups of approximately 20 dividing cells were hand-selected prior to fission. One group, the control sample, was incubated at the permissive temperature (27°C). This group was used to determine the length of the normal cell cycle, and the protein and DNA contents of untreated cells. The other sample, the experimental group, was incubated at 27°C for 0.5 h before the start of heat treatment to ensure that all cells had completed division prior to incubation at the restrictive temperature. Heat treatments were carried out in a water-bath with an electronic temperature controller, which maintained water temperature to within 0.02°C of the desired temperature. At the end of the heat treatment cells were returned to the permissive temperature and allowed to reach division. After the first division, one of the daughter cells was fixed, and the other was allowed to progress through the next cell cycle. After the second cell cycle, both daughter cells were fixed. The cell cycle that included the heat treatment was designated the first, or experimental, cell cycle and the recovery cell cycle was designated the second. The permissive temperature for both ts mutants was defined as 27°C. The restrictive temperature for ccl was 34.4°C, and for cc2, 34.25°C.
**Results**

**Analysis of ccl: Increase in cell size alone**

**Growth and DNA synthesis during heat treatment**

At 34.4°C, ccl cells were unable to synthesize macronuclear DNA. However, the rate of growth was only slightly less than that of wild-type cells at 34.4°C (Peterson & Berger, 1976). Cell mass, as estimated by protein content, increased linearly during heat treatment while DNA content remained at the G1 level. The rate of growth (protein accumulation) was nearly normal as compared to cells grown at the permissive temperature (27°C).

**Cell cycle durations**

Heat treatment caused an extension of the cell cycle proportional to the length of the heat treatment (open circles, Figure 3-1). The duration of the experimental cell cycle less the duration of the heat treatment was consistently 90-100% of the control cycle length (n = 13; mean = 96.8±2.53%). This suggests that ccl cells did not progress through the cell cycle while at the restrictive temperature when heat treatment began in early G1. It is not known if the same effect occurs when heat treatment is started at other points in the cell cycle. Presumably the same effect on cell cycle progression should occur up to the transition point at 0.72.
The second cycle was quite different from the first (filled circles, Figure 3-1). There was a linear decrease in the length of the second cell cycle in cells heat treated for up to 30% of the control cell cycle length. For cells treated longer than this, a consistent second cell cycle duration of 75% was obtained.

DNA content and DNA synthesis

Heat treatment of ccl cells during the first cell cycle caused no change in macronuclear DNA content (open circles, Figure 3-2). The post-fission DNA content of heat treated cells was never significantly different than the control sample. Therefore, ccl cells synthesized the normal amount of macronuclear DNA once returned to the permissive temperature even though their cell mass was up to twice the normal amount. The DNA content was still at the normal level following the second cell cycle (filled circles, Figure 3-2). Thus, the normal amount of DNA was made, even though this cell cycle was up to 25% shorter than normal, and despite increased cell mass.

Cell mass and growth rate.

Since growth continued during heat treatment, ccl cells were larger than normal, the increase in cell mass was directly proportional to the length of the heat treatment (open circles, Figure 3-3). Cell mass was reduced during the second cell cycle. (filled circles, Figure 3-3). Cells heat treated for less than 0.3 of a cell cycle returned to normal mass, while those heat treated longer than 0.3 of a cell cycle retained greater than normal mass. The amount of cell mass reduction increased with increasing initial cell mass. The amount of protein
accumulation during the second cell cycle closely parallels the pattern of decreased cell cycle length as initial cell mass increased (Figure 3-4) (compare filled circles in Figure 3-2 and Figure 3-4). Thus although the amount of protein synthesized in cells with different initial masses varied, this was a consequence of differences in the length of the second cell cycle and not due to the rate of protein accumulation which was constant and independent of cell mass.

Analysis of cc2: Increase in cell mass and DNA content

Growth and DNA synthesis during heat treatment

At 34.25 C, cc2 cells did not undergo cell division. However, unlike cc1, the cc2 cells were able to synthesize macronuclear DNA at the restrictive temperature. Cell mass increased linearly throughout heat treatment at 77% of the normal rate. Macronuclear DNA content also increased linearly, but only when heat treatment extended past 0.25 of the cell cycle. This point coincides with initiation of DNA synthesis in normal cells (Berger, 1971).

Cell cycle durations

The first cell cycle was extended by heat treatment (open circles, Figure 3-5). However, unlike cc1 cells, the increase in cell cycle duration was less than the length of the heat treatment. The time between the end of heat treatment and cell division decreased as the duration of the heat treatment increased (Figure 3-6). This suggests that cc2 cells progress through the cell cycle while at the restrictive
temperature. The rate of progression was estimated to be about 40% of the normal rate. The duration of heat treatment also affected the length of the second cell cycle (Figure 3-5). The pattern was nearly identical to that of cc1 cells. There was a linear decrease in the duration of the second cell cycle with heat treatments up to 30% of normal cell cycle length. Longer heat treatments produced a second cell cycle 75% of normal length.

**DNA content and DNA synthesis**

When heat treatment was restricted to the G1 portion of the cell cycle, there was no significant change in DNA content. However, when heat treatment extended into macronuclear S phase, DNA content increased (Figure 3-7). The rate of accumulation was about 40% of normal. Thus, although DNA synthesis occurred in cc2 cells at the restrictive temperature, the rate was reduced. During the second cell cycle there was a strong correlation between the initial DNA content and the rate of macronuclear DNA synthesis, so that the increased DNA content was maintained (Figure 3-7) \( r = 0.97 \).

**Cell mass and growth rate**

Like cc1, cc2 cells had an extended cell cycle at the restrictive temperature. The cells continued to grow normally, and so were larger than normal at the end of the first cell cycle (open circles, Figure 3-8). The increase in cell mass was directly proportional to the duration of the heat treatment.

Reduction in cell mass occurred during the second cell cycle
(filled circles, Figure 3-8). Cells heat treated entirely within the G1 period of the first cell cycle returned to normal size, while those given heat treatments longer than 0.3 of a cell cycle were still larger than normal. The amount of cell mass reduction during the second cell cycle increased with increasing initial cell mass.

Unlike cc1 cells, in which the rate of growth during the second cell cycle was constant, the rate of growth in cc2 cells showed a strong positive correlation between G1 DNA content and growth rate (r = 0.92). This observation, and the fact that the macronuclear DNA content was always relatively less than the cell mass, suggests that macronuclear gene dosage is the rate-limiting variable for cell growth and DNA synthesis when cell mass is increased relative to macronuclear DNA content.

Long-term downward regulation of cell size

Synchronous samples of cc1 cells were heat treated for several hours in order to produce cells with increased cell mass. Cell mass at the start of the second cell cycle was about 150% of normal. Cell mass returned to the control level within two cell cycles while DNA content remained at the control level throughout (Figure 3-9).
Discussion

Downward regulation of cell mass in *Paramecium* involves the combined action of two processes which control both the rates of cell growth and DNA synthesis, and the length of the cell cycle. Each process is linked to different aspects of the macronuclear replication cycle. The rates of cell growth and DNA synthesis appear to be controlled by macronuclear gene dosage and cell mass, while the length of the cell cycle appears to be regulated by a cell-mass-dependent control over the initiation of macronuclear DNA synthesis as shown in the subsequent chapter. This two-part homeostatic mechanism allows cells with a mass of up to 1.18 times normal to regulate back to normal mass within one cell cycle. Since normal variation in cell mass is less than 20%, most, if not all variation in cell mass can be removed by the next fission.

Control of growth and DNA synthesis

The results of this study are consistent with the hypothesis that the rates of cell growth and DNA synthesis are jointly determined by cell mass and macronuclear DNA content. When one variable is increased relative to the other, the smaller of the two becomes rate-limiting (Berger, 1978, 1982a). In the present study, cell mass was increased
relative to DNA content in both heat treated ccl and cc2 cells. In ccl, where DNA content remains at normal levels, the rates of cell growth and DNA synthesis were also normal. By comparison, in cc2, where DNA content was also increased but to a lesser extent than cell mass, the rates of cell growth and DNA synthesis were increased at a rate proportional to initial macronuclear DNA content. The inverse situation has been previously studied. Cells with experimentally reduced DNA content showed reduced cell growth and DNA synthesis (Berger, 1979).

It appears that regulation of growth and DNA synthesis involves a coordinated interaction between cell mass and DNA content. The coordination is probably fairly stringent, in order to account for the strong correlation between mean DNA content and mean cell mass (Kimball, 1967; Morton & Berger, 1978; Berger, 1982a), and the rapid downward regulation of cell mass in ccl, reported in this study.

The relationship between protein synthesis rate and gene dosage in Paramecium has been more thoroughly examined by Berger (1982a). At low DNA content, the rate of protein synthesis is proportional to DNA content. The rate of protein synthesis increases with increasing gene dosage up to a critical level. The critical gene dosage is on average 8% higher than the mean DNA content (Berger, 1982a). Further increase in gene dosage does not increase the rate of protein synthesis and the rate of protein synthesis reaches a plateau. There is a strong correlation between the plateau level of protein synthesis and cell mass in Paramecium (Berger, 1982a).

The means by which Paramecium regulates growth and DNA synthesis can thus be explained in terms of a saturation hypothesis. When gene dosage is below the critical level, as in cells with normal or reduced
DNA content, the rate of cell growth will be limited by the availability of DNA template. Above the critical level, the rate of cell growth might be limited by the size of the protein-synthesizing system of the cell, or by a concentration-dependent feedback system as has been suggested as the basis for cell mass regulation in the fission yeast, *Schizosaccharomyces* (Fraser & Nurse, 1978, 1979; Barnes et al., 1979). The present data are insufficient to exclude one hypothesis in favour of the other, but the implications from this study are clear. In experimentally enlarged cc1 and cc2 cells, cell mass is always relatively greater than DNA content. Thus, the rate of cell growth is expected to be limited by the gene dosage of the cell. The finding that growth rate is correlated with initial DNA content is consistent with this expectation.

The involvement of cell mass in the regulatory system is not fully understood. It may be that a feedback mechanism exists which prevents cell mass from going above or below a certain level without influencing the DNA content of the cell. *Paramecium* must have some means of coordinating DNA content and cell mass in order to explain the simultaneous reduction of both variables in chemostat cultures at low growth rates (Ching & Berger, unpublished results). It is likely that the level of protein synthesis activity is a major component in the regulation of DNA content and cell mass.

**Control of cell cycle length**

The second aspect of cell mass regulation in *Paramecium* involves the length of the cell cycle. As initial cell mass increases there is a
corresponding decrease in cell cycle duration, to a minimum of 0.75 of its normal length. Further increase in cell mass has no effect on cell cycle duration. Macronuclear S phase is known to start at 0.25 of the cell cycle in normal cells (Berger, 1971). Therefore, S + M (there is no macronuclear G2 in Paramecium) equals 0.75 of the normal cell cycle. The minimum cell cycle length observed in cells with increased mass suggests that G1 is reduced as initial cell mass increases. If the initiation of DNA synthesis requires the attainment of a critical cell mass, then the mass just required to produce a cell cycle of minimum duration should be equal to the cell mass at the time of initiation in normal cells. Cell mass in normal cells at the onset of S phase is about 120% of initial cell mass (Berger, 1982a). This is consistent with the observation that protein content increases exponentially (Kimball et al., 1959).

These observations suggest that DNA synthesis in Paramecium begins when cell mass exceeds 120% of the mean initial value. When the initial cell mass is equal to or greater than this threshold, the G1 phase of the cell cycle is eliminated. Labelling experiments reported later in this study (Chapter IV) show this to be the case.

Elimination of G1 following the production of abnormally large cells through blockage of DNA synthesis has been observed in Tetrahymena (Worthington et al., 1976) and in some studies on mammalian cells (Gerner et al., 1976; Cress & Gerner, 1977). In addition, two recent studies have shown that treatment of Chinese hamster cells (Stancel et al., 1981) or Saccharomyces (Singer & Johnston, 1981) with hydroxyurea can cause a lengthening of S phase at the expense of G1. The length of the cell cycle remained unchanged at low inhibitor levels, so
that cells are of normal size.

Prescott (1982) suggests that G1 is not a part of the chromosome cycle, but rather a component of the growth cycle. Cells will only have a G1 phase when the cell mass doubling time is greater than the time required to complete S phase. Early mouse embryos in cleavage stage lack a G1 phase (Gamow & Prescott, 1970). Only later, when the rate of cell division decreases, is a G1 period introduced. There are also several cultured cell lines which lack a G1 (these are termed G1-) (Robbins & Scharf, 1967; Liskay & Prescott, 1978; Prescott, 1982). One of these, Chinese hamster line V79-8, has been studied extensively. The results indicate that G1 is dispensible and the G1- condition is phenotypically dominant (Prescott, 1981). The fact that Paramecium appears to delete G1 when initial cell mass is increased agrees with the interpretation that G1 is only required to prevent the chromosome cycle from out-stripping the time required to double cell mass. It is not known whether initiation of DNA synthesis in eukaryotes is strictly determined by cell mass, or is a consequence of some other aspect of cell growth (Liskay et al., 1980).

There is, however strong evidence that some cell types have rigid cell mass requirements for initiation of DNA synthesis, the length of G1 being negatively correlated with cell mass (Johnston et al., 1977; Nurse & Thuriaux, 1977; Killander & Zetterberg, 1965a, 1965b; Kimball et al. 1971; Yen et el. 1975; Schafer & Cleffman, 1982). This section has only addressed the question of downward regulation of cell mass in experimentally enlarged cells. It is not known whether the observed correlation between cell mass and initiation of DNA synthesis is absolute. Evidence suggests that cells which are smaller than normal
are able to traverse the cell cycle. The ts mutation sm2 interferes with cell surface proliferation and causes a reduction in cell mass and DNA content (Jones & Berger, 1982; Berger, 1979). At the restrictive temperature cells continue to traverse the cell cycle, but become progressively smaller at each successive fission. It appears that reduction of cell surface by the sm2 defect, causes a shortening of subsequent cell cycles, thus limiting the amount of protein and DNA accumulation (Rasmussen, unpublished results).

The two regulative processes revealed by this study - regulation of growth rate and shortening of the cell cycle in enlarged cells - act to quickly attenuate experimental increases in cell mass. The limitation of growth rate to the level of the initial DNA content when cell mass is increased results in the loss of 50% of the excess mass per cell cycle. This occurs by partitioning of the excess mass between the two daughter cells produced at fission. The shortening of the cell cycle accounts for a further reduction in cell mass by restricting the length of time available for growth. A computer-simulation model of the combined regulatory processes produces a good fit to the observed consequences of increasing cell mass (Berger, 1982a). The model predicts that increases in cell mass of up to 20% are accommodated within one cell cycle. Since the coefficient of variation in cell mass is normally about 20% (Kimball et al. 1959; Berger, 1978), most, if not all of the normally introduced variation in cell mass is removed within one cell cycle of its occurrence.

This study suggests that cell-mass-dependent initiation of DNA synthesis is an important regulatory component of the cell cycle in Paramecium. Recent data indicate that there may also be a regulatory
point for cell division at or near 0.72 of the cell cycle (Chapter II). It is apparent that the effect of increased cell mass on the timing of DNA synthesis is not just a function of absolute cell mass. Doublet cells have twice the normal cell mass yet begin DNA synthesis at 0.25 of the cell cycle (Morton & Berger, 1978). Incomplete doublets are smaller, but still maintain cell and nuclear sizes that are greater than those in normal singlet cells (Sonneborn, 1963). This suggests that the cell cortex may play an important role in the regulation of cell mass and DNA content. The observation that reduction of the cell surface in sm2 cells causes a shortening of the subsequent cell cycle at the expense of S phase suggests that the cell cortex may mediate its effect quite late in the cell cycle (Rasmussen, unpublished data).

A control mechanism is also required for termination of the cell cycle. In Paramecium this system is not time-dependent. Cells with highly decreased DNA content require three to five times the normal cell cycle duration to reach fission (Berger, 1979), even though the timing of DNA synthesis is normal (Berger, 1982b). The observations that these cells synthesize the normal amount of DNA (Berger, 1979) and reach at least normal cell mass at division suggest that cell division depends on growth and DNA synthesis and may also be dependent on cell mass as in fission yeast (Fantes & Nurse, 1977). The uncoupling of DNA synthesis and cell division after the transition point in ccl may be a consequence of the completion of a DNA synthesis-related event required to initiate the cell division pathway.
Figure 3-1. Effect of heat treatment on the length of the 1st and 2nd cell cycles in \textit{cc1/cc1} cells. Open circles = 1st cell cycle; Filled circles = 2nd cell cycle. Cell mass is expressed as a percentage of the initial cell mass of control cells.
Initial Cell Mass

Length of Heat Treatment

Cell Cycle Length
Figure 3-2. G1 macronuclear DNA content in heat-treated cc1/cc1 cells. Open circles = start of 2nd cell cycle; Filled circles = start of 3rd cell cycle. Cell mass is expressed as a percentage of the initial mass of control cells. DNA content is expressed as a percentage of the G1 DNA content of control cells.
Figure 3-3. Post-fission cell mass in heat-treated ccl/ccl cells. Open circles = start of 2nd cell cycle; Filled circles = start of 3rd cell cycle. Mass is expressed as a percentage of initial cell mass of control cells. Length of heat treatment is expressed as a percentage of the control cell cycle duration.
Figure 3-4. Protein accumulation during the 2nd cell cycle in heat-treated ccl/ccl cells. Cell mass is expressed as a percentage of the initial mass of control cells.
Figure 3-5. Effect of heat treatment on the length of the 1st and 2nd cell cycles in \textit{cc2/cc2} cells. Open circles = 1st cell cycle; Filled circles = 2nd cell cycle. Cell mass is expressed as a percentage of the initial mass of control cells.
Figure 3-6. Time required for \textit{cc2/cc2} cells to complete the cell cycle after the end of heat treatment.
Time from EHT to Division

Length of Heat Treatment

0
25
50
75
100
125
40  60  80  100
Figure 3-7. G1 macronuclear DNA content in heat-treated cc2/cc2 cells. Open circles = start of 2nd cell cycle; Filled circles = start of 3rd cell cycle. Cell mass is expressed as a percentage of the initial mass of control cells.
Figure 3-8. Post-fission protein content in heat-treated \textit{cc2/cc2} cells. Open circles = start of 2nd cell cycle; Filled circles = start of 3rd cell cycle.
Figure 3-9. Downward regulation of cell mass in experimentally enlarged ccl/ccl cells. Cell mass is expressed as a percentage of initial cell mass of control cells.
CHAPTER IV.

Initiation of DNA synthesis in

*Paramecium tetraurelia*:

The effect of Cell Mass and Parental DNA content

on the Timing of DNA synthesis Initiation.
**Introduction**

For most cells the time needed to double cell mass is greater than the time required for DNA replication. Mechanisms must exist to ensure that successive rounds of DNA synthesis are not initiated before the cell has completed sufficient growth, otherwise cell mass would quickly decrease in subsequent generations. Thus, the central problem for cycling cells is coordination of cell growth with DNA synthesis.

A large body of evidence suggests that attainment of a critical cell mass prior to initiation of DNA synthesis is a major mechanism through which this coordination is achieved (Killander & Zetterberg, 1965; Nurse, 1975; Yen et al., 1975; Johnston et al., 1977; Nasmyth, 1979; Rasmussen & Berger, 1982). If initiation of DNA synthesis is solely dependent on attainment of a critical cell mass, then the length of the G1 period should represent the time required to reach the size threshold, and should vary with cell size. The G1 phase is the most variable portion of the cell cycle while the remainder (S + G2 + M) is of relatively constant duration (Precott, 1976), suggesting that the G1/S boundary is the major coordination point for the cell cycle. However, the mechanisms responsible for this coordination are not understood.

There is evidence that G1 can be eliminated in *Paramecium* when cells begin the cell cycle with increased cell mass (Rasmussen & Berger,
Further, in cleavage stage embryos G1 is absent but appears as cell size is reduced, and the rate of cellular proliferation decreases (Gamow & Prescott, 1970). In mammalian tissue culture cells, mutants have been obtained which lack a G1 period. However, a G1 period can be introduced by reduction of growth rate (Liskay et al., 1980). These studies raise the question as to whether there are any G1-specific events, or whether the presence of a G1 period merely reflects a requirement for cell growth before DNA synthesis initiation.

*Paramecium* is a useful organism for studying coordination of growth and DNA synthesis since both cell mass and gene dosage can be varied over a wide range. The availability of gene mutations affecting 1) cell division and DNA synthesis, and 2) distribution of macronuclear DNA at fission, make it possible to independently vary either cell mass or gene dosage, in order to study the effects of change in either variable on initiation of DNA synthesis.

Cell mass has been correlated with initiation of DNA synthesis in many eukaryotes, including *Paramecium* (e.g. Nurse, 1975; Rasmussen & Berger, 1982). While gene dosage in G1 has no effect on the timing of DNA synthesis onset, increase in the variance of gene dosage is accompanied by increased variability in the timing of DNA synthesis initiation (Berger, 1982b). This suggests that parental gene dosage may affect the timing of DNA synthesis in the subsequent cell cycle. This study examines the effects of initial cell mass and parental gene dosage on initiation of DNA synthesis in *Paramecium*. 

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Experimental Design

Effect of cell mass on initiation

Experimentally enlarged cells were produced by heat treating synchronous samples of cecl cells for 5% to 35% of a normal cell cycle. This produced groups of cells with mean initial cell masses from 5% to 33% above normal. From these groups, several sub-samples of dividing cells were hand selected at fission and these synchronous cell samples were then placed into medium containing [3H]-thymidine labelled bacteria. At 15 minute intervals, samples of 20-30 cells were removed, washed in unlabelled Cerophyl medium, and cells fixed individually onto a slide. One group was fixed immediately after fission as a cell mass control. The length of G1 was determined as described previously (Rasmussen & Berger, 1982), and compared to the mean initial cell mass.

Effect of parental DNA content on initiation

Individual tamG cells were selected at division and both daughters isolated in medium containing [3H]-thymidine labelled bacteria for times ranging from 30 minutes to 3 hours before fixation. The time between collection and fixation for each pair of daughter cells was recorded and the stage in the cell cycle determined. DNA content was determined as described previously (Rasmussen & Berger, 1982), and the parental DNA content derived by summing the G1 DNA contents of the two daughter cells. The results were sorted according to cell cycle stage, parental DNA content and whether or not the cell had initiated DNA
synthesis, as determined by autoradiography (Berger, 1971).
**Results**

**Effect of cell mass on initiation of DNA synthesis**

Synchronous samples of *ccl* cells were heat treated for varying lengths of time to produce abnormally large cells. At the end of the heat-treated cell cycle two groups of dividing cells were collected. One group was fixed immediately after fission as a measure of initial cell mass, while the second group was allowed to complete the subsequent recovery cell cycle to determine the cell cycle duration after experimentally increasing cell mass. Both the initial cell mass and duration of the recovery cell cycle were scaled relative to control values from a parallel experiment involving non-heat-treated cells.

It was found that the duration of the subsequent cell cycle decreased as initial cell mass increased (Figure 4-1). There was a strong negative correlation between the two variables ($r=-0.87$). Cell cycle duration approached a minimum of 75% of the control value when initial cell mass exceeded 120% of the normal post-fission value. G1 normally encompasses 25% of the cell cycle, while S phase and nuclear division (there is no G2) require 75% of the cell cycle for completion. Thus, the data indicate that increasing initial cell mass shortens the cell cycle by reducing the G1 period. This idea was tentatively examined in an earlier paper (Rasmussen & Berger, 1982).

This hypothesis was tested in detail by directly comparing the
length of G1 (as opposed to cell cycle duration), with initial cell mass in heat treated groups of ccl cells. The point of DNA synthesis initiation was taken as the time at which 50% of the nuclei in a group showed significant incorporation of label. Typically, the median 50% of the population initiate DNA synthesis within a 7 minute interval.

The results are clear. Increase in initial cell mass leads to a shortening in the length of the G1 phase of the cell cycle (Figure 4-2). There was a high negative correlation between initial cell mass and length of G1 up to cell masses of 118% of normal (r = -0.92). The G1 phase of the cell cycle was completely eliminated when initial cell mass exceeded 118.5% of normal. Cells which start the cell cycle with an initial mass greater than this amount initiate macronuclear DNA synthesis within 5 minutes after fission.

**Effect of parental DNA content on initiation of DNA synthesis**

To test the effect of parental DNA content on initiation of DNA synthesis, tamG cells were collected at fission and each pair of daughter cells was individually isolated in medium containing [3H]-thymidine labelled E. coli. At various times between 0.20 and 0.40 of the cell cycle, each pair of cells was fixed onto a slide. The parental DNA content was determined by summing the individual DNA contents of each daughter cell. Autoradiographs were made, and the state of DNA synthesis (labelled vs. non-labelled) was determined for each pair of daughters. The data from several experiments were pooled and sorted according to cell cycle stage and the state of DNA synthesis. The cumulative distributions of DNA content in labelled and
non-labelled nuclei were compared at two different cell cycle stages; 0.20-0.30 and 0.30-0.40 (Figure 4-3).

The results indicate that, at the mean time of initiation (0.25 of the cell cycle), the average macronuclear DNA content of cells which have initiated DNA synthesis is 0.79 times that of cells which have not yet started macronuclear S. Thus, it appears that cells with smaller nuclei initiate DNA synthesis slightly earlier than do cells with larger nuclei. Later in the cell cycle (at 0.35), there is no significant difference between the distributions of labelled and unlabelled macronuclei.
Discussion

Effect of cell mass on the timing of DNA synthesis

The results demonstrate a strong correlation between cell mass and DNA synthesis initiation in *Paramecium*. Cells initiate DNA synthesis when cell mass exceeds 118.5% of the normal post-fission mass. This is the cell mass that would be expected in at cells at 0.25 of the cell cycle if growth occurs exponentially as has been observed (Kimball et al., 1959). Correlations between cell mass and timing of DNA synthesis have been observed in several other eukaryotes (Prescott, 1956; Killander & Zetterberg, 1965; Kimball et al., 1971; Nurse, 1975; Yen et al., 1975; Nurse & Thuriaux, 1977; Johnston et al., 1977; Nasmyth, 1979). The popular interpretation suggests that cells must attain a critical cell mass or volume, before DNA synthesis can begin.

Other studies show that cell mass alone is insufficient to trigger DNA synthesis. In cases where growth is inhibited by serum deprivation, cells can enter S phase and traverse the cell cycle in the absence of cell growth (Zetterberg & Engstrom, 1983; Ronning & Lindmo, 1983). Also, *ccl* cells blocked early in G1 do not begin DNA synthesis immediately upon return to permissive conditions, but complete the remainder of G1, even though cell mass at the end of heat treatment is greater than the size at which cells normally begin DNA synthesis (Chapter II). Thus, while initiation of DNA synthesis appears to be related to some aspect of cell growth, as has been previously suggested, (Liskay et al., 1980; Singer & Johnston, 1981; Stancel et al., 1981), cell mass alone is not sufficient for entry into S phase.
Effect of parental DNA content on the timing of DNA synthesis initiation

*Paramecium* is unusual compared with most eukaryotes in that it has a large polygenomic macronucleus which carries out the somatic functions of the cell. Since the macronucleus divides amitotically, DNA content, and consequently, gene dosage varies. The action of the *tamG* gene mutation causes this variation to increase considerably (Berger & Schmidt, 1978).

Variation in G1 gene dosage has no effect on the timing of DNA synthesis initiation (Berger, 1982b; this study). However, this study shows that DNA synthesis initiation is influenced by parental gene dosage. This effect is not due to cell mass since cell mass was not significantly different in labelled vs. non-labelled cells. This observation is consistent with the interpretation that initiation of DNA synthesis is under the control of an inhibitory factor, produced in the previous cell cycle, and whose amount is proportional to the rate of growth. The effect of gene dosage on protein synthesis has been previously discussed (Berger, 1982a). Protein synthesis rate in cells with normal cell mass, but greater than normal gene dosage is limited by cell mass, so that the amount of inhibitor produced will be normal. Protein synthesis in cells with with normal mass, but low parental gene dosage is limited by gene dosage and thus, the amount of inhibitor produced will be proportional to gene dosage. Thus, the effect of gene dosage on initiation of DNA synthesis should only be observed in cells where gene dosage is rate-limiting for growth, i.e. in cells with low
Recent observations in human tissue culture cells also suggest inhibitor control over DNA synthesis initiation (Yanishevsky & Stein, 1980; Burmer et al., 1982; Polunovsky et al., 1983). Blockage of protein synthesis in quiescent fibroblasts using cycloheximide makes these cells unable to inhibit DNA synthesis in proliferating fibroblasts (Polunovsky et al., 1983). Thus, it appears that entry into S in quiescent cells is prevented by a protein inhibitor of DNA synthesis initiation. However, functions controlling entry of cells from quiescent to cycling state are not necessarily the same functions which control initiation of DNA synthesis in cycling cells. The recent isolation of a ts-mutant, specific for entry into the cell cycle from G0, suggests very strongly that these are indeed separate mechanisms (Ide et al., 1984).

**Nature of the G1 period in Paramecium**

Normally, the cell mass doubling time is longer than the time required to complete DNA replication. Therefore, a mechanism must exist to coordinate growth and DNA synthesis. Otherwise succeeding rounds of DNA synthesis would outpace the cell's ability to increase in size, and cell mass would decrease. In most, but not all, cycling cells each round of DNA synthesis is preceded by a G1 period. It has been suggested that G1 reflects a requirement by the cell to attain a critical cell size before DNA synthesis can begin (Liskay et al., 1979). Cells which have completed this requirement by the end of mitosis initiate DNA synthesis with minimum or no G1 phase (Prescott,
Cells which have not completed the size requirement for initiation by the end of mitosis, necessarily have an intervening G1 period. DNA synthesis begins once the critical cell mass has been reached. Studies in which the rate of DNA synthesis was reduced, so that the time required for replication more nearly equaled the cell mass doubling time, showed that as the length of S phase increased, there was a corresponding decrease in the length of G1. Cell cycle duration was unaffected (Singer & Johnston, 1981; Stancel et al., 1981). In Paramecium, the G1 period can be reduced or eliminated by increasing initial cell mass. This is consistent with the model that G1 is a general period of growth required for cell mass to keep pace with succeeding rounds of DNA synthesis, and is not an intrinsic part of the DNA division cycle (Prescott, 1982).
Table 4-1. Comparison of pre-fission DNA content in \textit{tamG} cells with labelled or non-labelled nuclei, at 0.25 of the cell cycle. DNA content is expressed as percentages of the G1 DNA content of control cells.

<table>
<thead>
<tr>
<th>DNA Synthesis state</th>
<th>Mean Cell Mass</th>
<th>Mean DNA Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labelled</td>
<td>114.5%</td>
<td>169.3%</td>
</tr>
<tr>
<td>Non-labelled</td>
<td>117.9%</td>
<td>214.3%</td>
</tr>
</tbody>
</table>
Figure 4-1. The effect of initial cell mass on cell cycle duration. Initial cell mass is expressed as a percentage of the initial mass of control cells. Cell cycle duration is expressed as a percentage of control cell cycle duration.
 Initial Cell Mass vs. Cell Cycle Length
Figure 4-2. Effect of initial cell mass on the length of the G1 period. Initial cell mass is expressed as a percentage of the initial mass of control cells. G1 duration is expressed as a percentage of control cell cycle duration. Filled circles = length of G1 determined directly by autoradiography; Open circle = Onset of DNA synthesis estimated cytofluorimetrically (see Chapter III).
Figure 4-3. Cumulative percentage plot of parental macronuclear DNA content vs. DNA synthesis state at 0.25 of the cell cycle. Open circles = cells not synthesizing DNA; Closed circles = cells which are synthesizing DNA. Parental DNA content is expressed as a percentage of the initial DNA content of a control sample.
Figure 4-4. Cumulative percentage plot of parental macronuclear DNA content vs. DNA synthesis state at 0.35 of the cell cycle. Open circles = cells not synthesizing DNA; Closed circles = cells which are synthesizing DNA. Parental DNA content is expressed as a percentage of the initial DNA content of a control sample.
CHAPTER V.

Summary: A Cell Cycle Model for *Paramecium*
This study has revealed three major points about cell cycle regulation in *Paramecium*. First, the present data show that there is a mass-related control over DNA synthesis initiation (Chapter IV). Second, DNA synthesis is not required for completion of cell division past 0.72 of the cell cycle (Chapter II). Finally, there is evidence which suggests that the timing of DNA synthesis initiation is determined in the previous cell cycle (Berger, 1982b; Chapter IV).

Recent observations indicate that the onset of DNA synthesis does not depend strictly on the attainment of a critical cell mass through cell growth (Ronning & Lindmo, 1983; Zetterberg & Engstrom, 1983; Chapter II). We have shown in this study that varying gene dosage between cells influences the kinetics of entry into S phase, such that cells with lower than average gene dosage initiate DNA synthesis earlier than cells with higher than average gene dosage. However, sister cells initiate DNA synthesis at the same time despite larger differences in gene dosage (Berger, 1982a). These observations suggest that the timing of DNA synthesis initiation is determined in the preceding cell cycle. The point of determination must be prior to the cci transition point (0.72) since inhibition of DNA synthesis in cci cells after this point reduces gene dosage, but does not affect the timing of DNA synthesis initiation in the subsequent cell cycle (Ching, unpublished results).

There are four major observations concerning the cell cycle in *Paramecium*. 1) The cci defect blocks cell cycle progression completely
during the G1 period. Thus some function uncovered by this defect is required for cell cycle progression. 2) G1 gene dosage has no effect on the timing of DNA synthesis initiation. Sister cells with extreme differences in DNA content, caused by the action of the tamG mutation, initiate DNA synthesis at the same time. 3) There is a cell mass related threshold for initiation of DNA synthesis in *Paramecium*. 4) The timing of DNA synthesis initiation appears to be determined in the previous cell cycle, and is affected by parental gene dosage. Table 5-1 summarizes these observations, and lists the cell cycle regulation models consistent with each observation. Only one model, the unstable inhibitor model, is consistent with all observations.

A simple computer model describing the cell cycle in *Paramecium* has been discussed previously (Berger, 1982a). This model makes four assumptions; (1) DNA synthesis begins when a critical cell mass is reached; (2) Cell division is triggered once sufficient macronuclear DNA has been synthesized; (3) Gene dosage and cell interact to control the rate of protein synthesis; (4) The rate of protein synthesis is determined by the relatively smaller of gene dosage or cell mass. This model has been further refined in order to account for the present observations. The revised model closely parallels the unstable inhibitor model of Fantes et al. (1975). The BASIC code for the model is shown in Appendix II.

The criteria for initiation of DNA synthesis and start of the cell division pathway have been altered. DNA synthesis begins when the level of inhibitor falls below 0.85. Inhibitor concentration at the start of the cell cycle is set at 1.00. The inhibitor is pulse synthesized at the point of commitment to cell division. In normal
cells this occurs at 0.72 of the cell cycle. The amount of inhibitor produced is proportional to a rate-limiting-variable, whose value is equal to either gene dosage or cell mass, whichever is relatively smaller. The inhibitor decays at a constant rate such that the amount will be reduced by 1/2 during the course of a normal duration cell cycle. One assumption remains. The cell division pathway is triggered when DNA content passes 168% of the initial amount. This is an a priori assumption based on the estimated DNA content in cells at 0.72 of the cell cycle. For cells with increased cell mass, or increased DNA content, the values predicted by the simulation model closely parallel the experimental observations (Table 5-2).

In conclusion, this study has demonstrated four components of cell cycle regulation in Paramecium tetraurelia. (1) There is a cell mass-related control over initiation of DNA synthesis. Cells enter S phase when cell mass exceeds 118.5% of normal mass. (2) There is a commitment point for cell division at 0.72 of the cell cycle. After this point cell division can be completed in the absence of macronuclear DNA synthesis. (3) There is an indispensable function in G1 required for progression through the cell cycle. (4) There is evidence that the timing of DNA synthesis initiation is determined in the previous cell cycle.

There are several questions which remain unanswered. What is the mechanism which controls initiation of DNA synthesis? What is the event which causes the cell to become committed to cell division, before replication is complete in the macronucleus? How does upward regulation of cell mass occur? What are the effects of nutritional limitation on the organization of the cell cycle? These are all
questions which need to be examined in order to develop a fuller understanding of how cell cycle events are determined in *Paramecium* and other organisms.
Table 5-1. Summary of Observations on Cell Cycle Regulation in *Paramecium* and Models Consistent with each Observation.

1. Blockage of cell cycle progression by ccl.
   
   (a) Activator accumulation
   - assumes that ccl prevents activator production.

   (b) Unstable inhibitor
   - assumes that ccl prevents inhibitor decay.

   (c) Structural model
   - assumes that ccl prevents subunit production.

2. G1 gene dosage has no effect on initiation of DNA synthesis.
   
   (a) Inhibitor dilution
   - assumes near normal growth in cells with small nuclei.

   (b) Unstable inhibitor
   - decay constant is not affected by rate of growth in current cell cycle.

3. Cell mass threshold at start of S phase.
   
   (a) Activator accumulation
   - activator synthesis proportional to growth.

   (b) Inhibitor dilution
   - inhibitor is diluted as cell volume increases.

   (c) Unstable inhibitor
   - production of inhibitor proportional to mass in previous cell cycle.

   (d) Structural model
   - subunit production is a function of growth.

4. Parental effect on initiation of DNA synthesis.
   
   (a) Inhibitor dilution
   - inhibitor produced late in previous cell cycle, amount depends on growth rate before fission.

   (b) Unstable inhibitor
   - same as inhibitor dilution.
Table 5-2. Comparison of observed consequences of changes in DNA content and cell mass with results predicted by the cell cycle model (Appendix II). D = macronuclear DNA content; P = size of protein synthesis system; M = cell mass. Means are shown with standard errors in parentheses. Both DNA content and cell mass are expressed as percentages of initial control values.

a) Increased DNA Content

<table>
<thead>
<tr>
<th></th>
<th>D</th>
<th>P</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Observation</td>
<td>193 (8.6)</td>
<td>-</td>
</tr>
<tr>
<td>Values</td>
<td>Simulation</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>Final</td>
<td>Observation</td>
<td>165 (15.5)</td>
<td>-</td>
</tr>
<tr>
<td>Values</td>
<td>Simulation</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>Cycle Length</td>
<td>Observation</td>
<td>91.4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simulation</td>
<td>93%</td>
<td></td>
</tr>
</tbody>
</table>

b) Increased Cell Mass

<table>
<thead>
<tr>
<th></th>
<th>D</th>
<th>P</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Observation</td>
<td>102.5 (6)</td>
<td>-</td>
</tr>
<tr>
<td>Values</td>
<td>Simulation</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Final</td>
<td>Observation</td>
<td>100.0 (5)</td>
<td>-</td>
</tr>
<tr>
<td>Values</td>
<td>Simulation</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cycle Length</td>
<td>Observation</td>
<td>75%</td>
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</tr>
<tr>
<td></td>
<td>Simulation</td>
<td>76%</td>
<td></td>
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</table>
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APPENDIX I.

Materials and Methods
Stocks & Culture

*Paramecium tetraurelia* (Sonneborn, 1975), was grown in phosphate-buffered Cerophyl medium (Sonneborn, 1970) with *Enterobacter aerogenes* as the food organism. Culture medium was grown up overnight and the pH adjusted to 6.8-6.9 prior to use. Heat treatments were carried out in a water bath equipped with an electronic thermostat which maintained the water bath within 0.02 C of the desired temperature.

Three mutant stocks, all derived from the wild-type stock 51-S were used. Stock d4-1001 carries the ts mutation *cc1*, which completely blocks macronuclear DNA synthesis and cell division at 34.4 C (Peterson & Berger, 1976). Stock d4-1003 carries the ts mutation *cc2*, which completely blocks cell division but only partially blocks macronuclear DNA synthesis at 34.25 C (Peterson & Berger, 1976; Rasmussen & Berger, 1982). Stock d4-1031 carries the recessive mutation *tamG*, which causes missegregation and unequal division of the macronucleus at amitosis.

Synchronization of cells

Synchronous samples of cells were obtained by hand-selection of dividing cells from exponential growth-phase cultures. For each sample, cells were collected for a period of 5 minutes or less and 40-50 dividing cells collected per sample.
Cytological Procedures

Cells were placed by micropipette on albumin-coated slides, and most of the culture medium removed to ensure maximal flattening of cells as they dried. Cells were fixed in ethanol/glacial acetic acid (3:1) for 30 minutes, and stained by the two-color method described by Cornelisse & Ploem (1976) using the following procedure:

1) RNA was removed by hydrolysis in 5N HCl at room temperature for 45 minutes.

2) Cells were stained in an Acriflavine solution for 30 minutes. The solution is of the following composition:

\[
\begin{align*}
0.05 \text{ gm Acriflavine-HCl} \\
2.0 \text{ gm Sodium metabisulfite} \\
30 \text{ ml IN HCl} \\
170 \text{ ml distilled water}
\end{align*}
\]

3) Unbound stain was removed by rinsing cells for 1 minute in an acid-ethanol rinse of the following composition.

\[
\begin{align*}
70 \text{ ml 95\% Ethanol} \\
62 \text{ ml Distilled water} \\
68 \text{ ml IN HCl}
\end{align*}
\]

4) Cells were then stained in a 0.10 mM solution of Primulin for 20 minutes.

Microfluorimetry

Microfluorimetry was used to estimate DNA and protein content in single cells. The microscope was equipped with an incident light
illuminator. For measurement of fluorescence of Acriflavine-stained DNA, a 490 nm low-pass excitation filter, 500 nm dichroic reflector and 520 nm barrier filter were used. For measurement of Primulin-stained protein, a UG3 excitation filter, 400 nm dichroic reflector and 430 nm barrier filter were used.

Each fluorescence measurement was obtained by taking the difference between the intensity of the field with the specimen and one over an adjacent area without the specimen. The output from the photomultiplier was monitored by a computer (Berger, 1979). The measurement sequence was initiated at a fixed interval (0.1 sec) after the exciting radiation was switched on, and consisted of a series of 32 digitizations of the output from the photomultiplier, averaged to give an intensity reading.

Labelling & Autoradiography

Labelling with tritiated thymidine-labelled bacteria, and autoradiography have been described previously (Berger, 1971).

Statistical Procedures

Statistical procedures were performed as described in Sokal & Rohlf (1969).
APPENDIX II.

*Paramecium* Cell Cycle Model
A simple BASIC computer simulation of the *Paramecium* cell cycle was developed to incorporate the experimental observations to date. In this model, both the rate of growth and DNA synthesis are a function of the protein synthesis rate. Protein synthesis is in turn limited by either cell mass or gene dosage, depending on the relative amount of these two variables.

DNA synthesis initiation is controlled by an unstable inhibitor (DECF), which decays at a rate independent of its concentration. The initial level of inhibitor is set at 1.00. DNA synthesis begins when the inhibitor decays to 0.85 of the starting amount. The rate of decay (DECR), is set so that the inhibitor decays by one-half during the course of a normal cell cycle.

Nuclear division is triggered when the scheduled amount of DNA is made. This is an assumption, and does not account for the observation that DNA synthesis is not required after 0.72 of the cell cycle. In the absence of a demonstrable correlation between mass or DNA content and the triggering of the cell division sequence, it is difficult to use either variable as a component in causing cell division to begin.

The value for the decay rate of protein synthesis activity (PDEC) was determined by Berger (1982a). Each pass through the program loop equals 1% of a normal cell cycle. The rate of increase in DNA content (DRATE) is set to produce a doubling in amount during the course of a normal S phase (from 0.25 to 0.87). The rates of increase in protein synthesis activity (PRATE) and cell mass (MRATE) were set so that each exactly doubles during a normal duration cell cycle. The input values
are expressed as a percentage of normal initial values. The output values are expressed as a percentage of initial values at the start of the subsequent cell cycle.

The effect of changes in initial DNA content or cell mass were tested by altering initial values. There were no other changes made during the simulations.
1000 REM *** Constants & control variables ***
X=0:ZZ=0:Z=0
1001 PDEC=.89663:PRATE=.1125:DRATE=.01145:MRATE=.007345
DECF=1.:DECR=.9930925
REM *** Starting conditions ***
2100 PRINT CHR$(12)
PRINT "Paramecium Cell Cycle Model"
PRINT "----------------------":PRINT
2000 INPUT "Initial DNA content ";D
INPUT "Initial synthesis activity ";P
INPUT "Initial cell mass ";M
PRINT
INPUT "# of cell cycles ";ZZ
DECF=DECF/(M/100)
REM *** The cell cycle begins ***
INPUT "For continual output ENTER 1 ";X:GOTO 2001
X=0
2001 T=1:N=0
PRINT CHR$(12)
DI=D
PI=P
DSTOP=D+P
REM *** Final DNA content is set ***

3000 N=N+1

IF M>D THEN RLV=D ELSE RLV=M

REM *** Rate limiting variable determined ***
P=P*PDEC+(RLV*PRATE)

REM *** Protein synthesizing system grows ***

IF DECF<.85 THEN D=D+(P*DRATE)

REM *** DNA synthesis if critical size is reached ***

DECF=DECF*DECR

REM *** Unstable Inhibitor Decays

1500 M=M+(P*MRATE)

REM *** Cell Growth ***

IF X<>0 THEN PRINT N,D,M,DECF

IF D<DSTOP THEN GOTO 3000

ND FOR I=1 TO 13

M=M+(P*MRATE)

P=P*PDEC+(RLV*PRATE)

N=N+1

DECF=DECF*DECR

IF M>D THEN RLV=D

IF D>M THEN RLV=M

IF X<>0 THEN PRINT N,D,M,DECF

NEXT I

PRINT

Z=Z+1

REM *** The cell divides ***

DECF=DECF*RLV/100

M=M/2:D=D/2:P=P/2
PRINT CHR$(12)
PRINT :PRINT
PRINT "Cell cycle # ";Z
PRINT "---------------":PRINT
PRINT "Cell Cycle length ";N
PRINT "DNA Content ";D
PRINT "Cell Mass ";M
PRINT "Synthesis System ";P
PRINT :PRINT
IF Z<ZZ THEN INPUT "Press <RETURN> to continue...";A
IF Z<ZZ THEN GOTO 2001
PRINT "DONE"
9999 END