

On the Interpretation of the Shortening of the G1-Phase by Overexpression of Cyclins in Mammalian Cells

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Increasing the concentration of cyclins in mammalian cells leads to a shortening of the G1-phase of the division cycle. This observation has been interpreted as indicating that these cyclins act during, and are rate limiting for, passage through the G1-phase. Here it is argued that it is not possible to interpret experiments involving cyclin overexpression-induced changes in the lengths of individual cell cycle phases without considering changes in the overall cellular growth rate. A rigorous reanalysis of these experiments demonstrates that the results are consistent with the proposal that the shortening of the G1-phase is merely due to an increase in the rate of mass synthesis in all phases of the cell cycle. Increased cyclin concentrations leads to a faster rate of mass synthesis and a concomitant shortening of the G1-phase. Cyclins can also affect the length of the S- and G2-phases, which leads to the observed shortening of the G1-phase. Thus, the experiments on cyclin overexpression and their effect on G1-phase length cannot be used to support the proposal that cyclins act specifically during the G1-phase of the division cycle. © 1998 Academic Press

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INTRODUCTION

A number of experiments have demonstrated that increasing the expression of certain “G1-cyclins” can lead to a shortening of the G1-phase of mammalian cells. These experiments have been interpreted as indicating that these cyclins are specifically associated with, and are rate-limiting for, passage through the G1-phase.

It is improper to interpret the results of such overexpression experiments without considering the consequences of cyclin overexpression on the overall cellular growth rate or on the other phases of the cell cycle. An alternative interpretation of these experiments presented here indicates that it is just as valid to conclude that the shortening of the G1-phase is due simply to

an increase in the rate of mass synthesis in all phases of the division cycle. Since the S- and G2/M-phases of mammalian cells are relatively invariant in length with changing interdivision time [1, 2], a decrease in the interdivision time due to the increased rate of mass synthesis necessarily leads to a shortening of the G1-phase.

OVEREXPRESSION OF CYCLINS AND THE CELL CYCLE

The current paradigm of the cell cycle proposes that there are various proteins, the cyclins, that regulate other molecules, specifically the cyclin-dependent kinases. Progression through the G1-phase of the cell cycle is believed to be stimulated or regulated by some of these cyclins [3, 4]. Cyclins are proposed to vary or “cycle” within the cell cycle in either amount or activity. An increase in a cyclin leads to an increase in cyclin-dependent-kinase activity, and this activity leads, in some way, to the triggering of various cell-cycle-specific events. One major event in the mammalian cell cycle is the initiation of DNA replication or the start of S-phase. A subset of cyclins, the G1-cyclins, are believed to act in the G1-phase and to perform functions leading to DNA replication.

A number of experiments using cyclin overexpression support this notion. These experiments overexpress a cyclin (for example, by adding an expression vector coding for that cyclin and turning on production of the cyclin) and the change in the length of the G1-phase is measured. Overexpression of G1-cyclins leads to a shortening of the G1-phase. The general conclusion of these experiments is that G1-cyclins act during the G1-phase and are rate-limiting for passage through the G1-phase. The increase in cyclin concentration is presumed to speed up progression through the G1-phase of the cycle.

The leading experiment was performed by Ohtsubo and Roberts [5]. They tested the idea that cyclins control G1-progression in mammalian cells by analyzing fibroblasts that constitutively overexpress human cyclin E. When the cycle phases were measured by three different methods (by measurement of the pattern of thymidine incorporation into cells synchronized

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by mitotic release; by flow cytometry; and by the method of frequency of labeled mitoses), it was found that the G1-phase was shortened when a cell had an abundance of cyclin E. They concluded that this cyclin could be the rate-limiting substance for G1-progression in mammalian cells. As the doubling time of the culture did not change there were, perforce, changes in other phases of the division cycle. In these experiments the S-phase lengthened. The possibility that the extension of the S-phase could "cause" the shortening of the G1-phase was discounted as the cyclin overexpressing cells were slightly smaller than the parental cells. The argument was made that if only the S-phase was extended the cells would be expected to be larger. It was concluded that the G1-phase was regulated by a rate-limiting step related to the action of cyclin E. (This anomalous change in the S/G2/M-phases and cell size will be analyzed in more detail below.) More recently, it was found that retinoblastoma gene deficient fibroblasts, which have an elevated cyclin E content, exhibit a shortened G1-phase [6]. This result supports the observations of Ohtsubo and Roberts.

Following Ohtsubo and Roberts, a number of experiments have given similar results. For example, using a combination of video-time-lapse cinematography, along with Budr labeling, it was shown that overexpression of human cyclin E in HeLa cells leads to a shortening of the G1-phase by 1.5 h [7]. Overexpression of mouse cyclin D1 in serum-stimulated mouse NIH-3T3 and rat-2 fibroblasts decreased the time for serum-stimulated cells to leave their quiescent state and start the synthesis of DNA (that is, the G(0)- to S-, or the G1-phase of the cell cycle, was decreased when a cyclin was overexpressed). A similar result with cyclin D2 in rodent fibroblasts also indicated that the G(0)- to S-phase interval was shortened [8]. In this last example the shortening of the G1-phase did not occur with an increase in the length of the S/G2-phase.

In another experiment, cyclin D1 expression was coupled to a metallothionein promoter so that addition of zinc could lead to induction of cyclin D1. In cycling cells, the induction of cyclin D1 resulted in a shortening of the G1-phase, leading to the conclusion that cyclin D1 is rate limiting for progress through the G1-phase [9]. Similarly, overexpression of cyclin D1 in Rat6 embryo fibroblasts using retrovirus-mediated transduction decreased the duration of the G1-phase [10]. In another experiment with results similar to that of Ohtsubo and Roberts, it was observed that induction of either of the human cyclins D1 or E (using a tetracycline regulated expression vector) led to a decrease in the length of the G1-phase interval [11]. There was a compensatory lengthening of the S- and G2-phases, so that the mean cell cycle length in the population was unaltered. When cells were studied for their entry into S-phase from quiescence, only cyclin D1 had a strong

effect (cyclin E having a much smaller effect). The conclusion from this experimental result, as in the others as well, was that the cyclins studied were "rate-limiting activators of the G1-to-S-phase transition."

The overall conclusion of all of these experiments is that the cyclins act during the G1-phase of the cell cycle, and thus there are G1-phase-specific events that regulate the mammalian division cycle.

AN ALTERNATIVE EXPLANATION FOR THE EFFECT OF CYCLINS

Two types of results have to be explained. First, there are experiments where the shortening of the G1-phase is associated with a shortened interdivision time. This is relatively easy. Second, and somewhat more complicated, there are experiments that are associated with an invariant interdivision time. When the interdivision time decreases with a shortening of the G1-phase, there is an invariance of the S- and G2-phases. With no change in the interdivision time and a shortened G1-phase, the S- and/or G2-phases must necessarily increase in length.

The essence of the problem stems from the difficulty of interpreting changes in the lengths of individual cell cycle phases without considering changes in either the total interdivision time or the lengths of the other phases of the cell cycle. Cell cycle lengths are not independent entities, for the sum of the cell cycle phases must be equal to the interdivision time. A particular phase of the cell cycle cannot change length unless there is a change in some other entity of the division cycle, whether it be some other phase, or the total interdivision time of the cell.

Consider cells where the shortening of the G1-phase due to overexpression of a cyclin associated with a decreased interdivision time and where the S- and G2-phases do not change when the cyclin is overexpressed. The decrease in G1-length must equal the decrease in interdivision time. For any cell component, the ribosomes, for example, the doubling time during steady-state growth equals the doubling time of the cell. A cell with a shortened G1-phase would double mass in a shorter interval. Over one doubling time it is necessary that the rate of mass synthesis increase when the interdivision time is shortened. For steady-state growth we can immediately say that in addition to the observed change in G1-phase length, the overexpressed cyclin must also change the rate of mass increase for that cell. If the interdivision time were not equal to the mass doubling time then over time cells will change size and no steady-state size will be obtained. The overexpressed cyclin must therefore have a pleiotropic effect. The cyclin must affect the rate of synthesis of everything in that cell. When the cyclin concentration is increased, the cell increased its rate of mass synthesis so

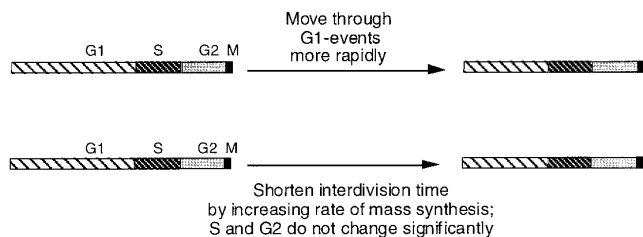


FIG. 1. Comparison of the G1-event model and the mass synthesis model with regard to predictions of G1-length. The upper diagram illustrates the shortening of the G1-phase (without a concomitant change in the S- and G2/M-phases) due to the proposed passage of cells through the G1-phase events more rapidly with overexpression of a cyclin. The lower diagram is the expected change in cycle phases if cyclin overexpression leads to an increase in the rate of mass synthesis and a shorter interdivision time. Note that the two predictions are identical. A shorter G1-phase is observed.

that the mass doubling time decreased. This leads to a completely different way of looking at the effect of the cyclin. The cyclins can be general and cell-cycle-phase-independent promoters of mass increase. Whatever kinases are induced or activated, or whatever cell elements are altered by the cyclin, all we have to do is propose that the cyclin affects any aspect of the cell that is limiting for growth over the entire cycle. The simplest explanation is that the cyclins produce an increase in the rate of cell growth throughout the division cycle. From this viewpoint, the cyclins are important molecules because they regulate the synthesis of all of the mass and cytoplasm of the cell, in all phases of the division cycle, and not because they have cell-cycle-specific effects or functions.

The rate of mass increase does not refer to the absolute increase in mass during an interval, but to the relative rate of mass increase—that is, the rate relative to the extant mass. Thus, if the time for the mass of a cell to double is 24 h, the rate of mass increase is not changed for cells with a newborn cell size of 1 or 2 or 4 or whatever. Absolute cell size should not be confused with the rate of mass increase. In steady-state growth, an increase in the rate of mass increase necessarily means a shortening of the interdivision time, and if S- and G2-phases are unaltered, then the G1-phase must necessarily shorten.

To put the conclusion in its most concise form, one cannot distinguish, looking merely at the shortening of the G1-phase and an associated decrease in the interdivision or doubling time, between cyclins affecting G1-passage specifically, and a general effect of cyclins on the rate of mass synthesis.

A comparison of the G1-cyclin rate-limiting model and the alternative mass synthesis model (for shortened interdivision times) is presented in Fig. 1. Both models make exactly the same prediction for changes in cell-cycle phases with overexpression of cyclins. In

the rate-limiting model (top panel, Fig. 1) an overexpression of cyclins leads to a more rapid passage through the G1-phase of the division cycle. As the S- and G2-phases do not vary, there must be a concomitant shortening of the interdivision time. In this viewpoint, the shortening of the G1-phase by increasing passage through the G1-phase induces an increase in the rate of mass increase. The alternative “mass synthesis model” presents the same predictions (bottom panel, Fig. 1) as the G1-cyclin-rate-limiting model. The main difference is that in the bottom panel it is proposed that the overexpressed cyclin directly increases the rate of mass synthesis in all phases of the cycle. The shortened interdivision time leads (due to the relatively invariant S- and G2-phases) to an ineluctable shortening of the G1-phase.

A more general view of the effect of a change in the rate of mass synthesis on the interdivision time and on the length of the G1-phase is presented in Fig. 2. Here it is shown that a change in the rate of mass synthesis produces a change in the length of the G1-phase. Since S- and G2-phases are relatively constant [1, 2], changing the rate of mass synthesis leads to large changes in the G1-phase. In this viewpoint, the G1-phase is merely what is left over when the S/G2/M-phases are less than the interdivision time or mass doubling time. At this time there is no way to distinguish between the effect of the cyclins on a specific action in the G1-phase, and the effect of the cyclins on the total rate of mass synthesis.

This alternative explanation is independent of whether or not cyclins vary during the division cycle, whether or not cyclins activate various kinases at specific times during the division cycle, or whether or not cyclins are associated with any particular cell-cycle event (such as the start of S-phase). This analysis deals only with the interpretation of experiments on the overexpression of cyclins and whether or not it is valid to use these experiments to support the proposal that there are G1-specific cyclins associated with a rate-limiting step in the G1-phase of the cell cycle. Whether or not the cyclin story is correct should not depend on experiments using overexpression of cyclins. A rigorous examination of these overexpression experiments suggests that they cannot be used to support the G1-cyclin regulatory model of the cell cycle.

EFFECT OF G1-CYCLINS ON THE S-PHASE

We have noted that cyclins also affect S-phase length in the experiments of Ohtsubo and Roberts [5] and Resnitzky *et al.* [11]. A similar effect was noted in cells deleted for the retinoblastoma gene, which induces increased synthesis of cyclin E [6]. In these experiments the interdivision time did not change, G1-phase was shortened, and the S-phase lengthened. In all of these

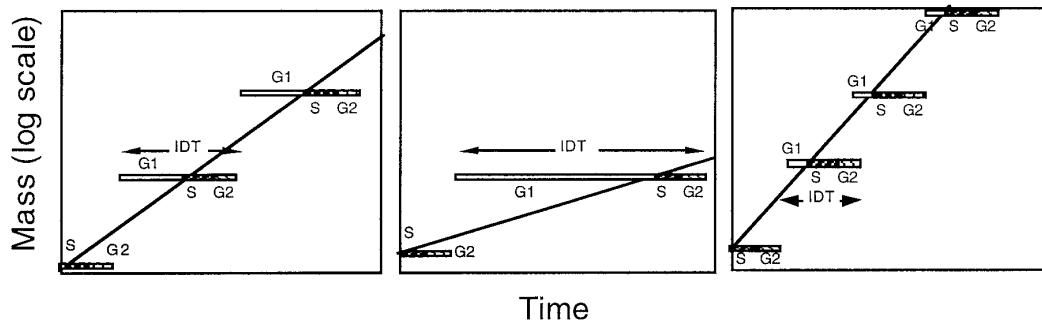


FIG. 2. Illustration of the effect of change in rate of mass synthesis on G1-phase. In the three panels intermediate, a long, and a short interdivision times are illustrated for cells where the S- and G2-phases do not vary with growth rate. The angled line indicates the rate of mass synthesis; a steep slope is a rapid synthesis of mass with a short doubling time, and a shallow slope indicates a long doubling time. The slower the rate of mass synthesis, the longer the interdivision time (IDT) and the longer the observed G1-phase.

experiments, as the doubling time of the culture did not change when the G1-phase was shortened, there was an inevitable extension of the S/G2/M-phases. The possibility that the extension of these later phases could “cause” the shortening of the G1-phase was discounted as the cyclin-overexpressing cells were slightly smaller than the parental cells [5, 6]. If only the S/G2/M-phases were extended, it was argued, the cells would be expected to be larger. This prediction is based on the assumption that the S-phase is initiated at a specific cell size in cells independent of the interdivision time and that the elevation of the cyclin concentration does not change the cell size at initiation. Therefore, Ohtsubo and Roberts concluded that the G1-phase was regulated by a rate-limiting step related to the action of cyclin E.

An alternative view is presented in Fig. 3. Here the interdivision time does not vary for cells with different lengths of G1-phase. If cyclins work by extending the S/G2-phases, and the interdivision time does not change, then the G1-phase will shorten. Thus we see that the conclusion that the cyclins work in the G1-phase of the

division cycle is not inevitably drawn from the experimental observations. Rather it is equally valid to propose that cyclins work by slowing down passage through the S-phase; that is, the cyclins may decrease the rate of DNA replication.

What about the arguments related to cell size? All other things being equal, it is expected that extension of the S-phase would increase cell size. In fact, the cells were smaller after cyclin induction. Consider cells in culture growing with a 24-h doubling time with the S/G2/M-phases totaling 14 h; this means that G1-phase is 10 h. Keeping the cell-cycle interdivision time constant, and merely extending the S/G2/M-phases, there is a decrease the G1-phase in the subsequent cycle (Fig. 3). This is because the birth of the daughter cells at the end of the G2-phase is delayed. In this analysis there is no invocation of any cell-cycle event in the G1-phase. The G1-phase appears because division occurs prior to the start of an S-phase. The shortened G1-phase is due to lengthened S/G2-phases. If the S-G2-M-phase was long enough to cause division to occur at the same time as S-phase started, then there would be no G1-phase at all (bottom panel, Fig. 3). All that has to be proposed to explain the observed results on cell cycle phases is that the cyclins lead to a slowing of passage through the S/G2/M-phases of the cell cycle.

Sometimes one cannot propose that the cyclins are involved as limiting elements in the rate of mass synthesis, as when the rate of mass synthesis does not change upon cyclin induction. How can we explain the observation of Ohtsubo and Roberts [5] and Herrera *et al.* [6] that cells are smaller when cyclins are induced? Extension of the S/G2-phases, with a constant interdivision time, would presumably lead to an increase in average cell size (Fig. 3). This is because cells divide later (when they are larger) and the newborn cells resulting from that division are also larger. This would only be true, of course, if the size of the cell at initiation was unchanged. If, however, increasing initiator con-

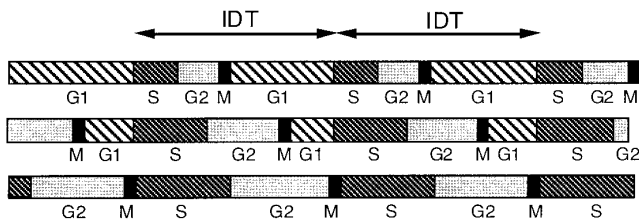


FIG. 3. Analysis of shortening of G1-phase with no change in interdivision time. As indicated in this figure, if the interdivision time does not vary and the G1-phase shortens, there must be some increase in the S/G2/M-phases. In the middle and bottom panels the S- and G2-phases are shown extended, yielding a shortened G1 phase (middle) and no G1-phase (bottom). In all cases, the interdivision time (IDT) does not vary. Observe that as the S- and G-phases are lengthened, the G1-phase decreases.

centration leads to a slightly smaller cell size at the start of S-phase, one can have a smaller size distribution, have cells with a longer S/G2-phases and a shorter G1-phase, and keep the interdivision time constant. Let us assume that initiation of S-phase occurs when a particular "initiator" of S-phase, synthesized in all phases of the cell cycle, reaches a particular value per extant DNA origins. For simplicity, assume that the initiator is either the cyclin or a complex of the cyclin with other molecules. If cyclin overexpression leads to a small increase in the cyclin concentration per cell mass, then S-phase initiation will occur when the cell size is smaller; the "initiation mass" will decrease with an increase in cyclin concentration. At the smaller cell size the cell will reach the required number of cyclin molecules compared to the parental cell that does not overexpress cyclin. The smaller cell size at initiation would produce a smaller average cell size in the population. One can have an unchanged interdivision time, an enlarged S/G2/M-phase, a shortened G1-phase, and a smaller cell size according to this analysis. According to this analysis, experiments with an invariant interdivision time do not distinguish between the current G1-event model and a model of the division cycle where there are no G1-specific events rate limiting for passage through the G1-phase of the division cycle.

It could be argued that this is an *ad hoc* solution to the problem of cell size. This critique can be made for the Ohtsubo and Roberts interpretation as well. Their results require that the cyclins work to both shorten the G1-phase *and* increase the length the S/G2-phases. The alternative analysis presented here requires that the added cyclin decrease the initiation mass (by having the cyclin or initiator be a larger fraction of total mass), *and* that the cyclin slow down replication and passage through the S/G2-phase of the cell cycle. This lengthening of the S- and G2-phases when associated with an unchanged interdivision time leads to a shortening of the G1-phase. Regarding the parsimony of hypotheses there is no reason to choose the G1-event model over the alternative model. Both models contain *ad hoc* solutions (i.e., two independent postulates) to explain changes in cell kinetics. The Ohtsubo and Roberts model requires both a shortening of G1-phase *and* a compensatory lengthening of the G2/S/M-phases. The alternative model, similarly, requires two changes to occur due to the increased cyclin. There is a slowing down of passage through the S/G2/M-phases (as in the Ohtsubo and Roberts model), *and* an increase in the concentration of the molecules involved in the initiation of DNA synthesis. There is no change in the rate of mass synthesis, and the cell size would be expected to decrease.

A slowing of cell growth due to overexpression of cyclin D1 has been reported [12]. This slowing of growth is associated with a prolonged S-phase, indicat-

ing that the increase in cyclin D1 affects not only DNA synthesis, but also affects the entire metabolism of the cell as indicated by the increased doubling time of the cells in culture. This is experimental support for the idea, proposed here, that an overexpression of a particular cyclin can lead to the observed increase in the S-phase, which can lead to a shortening of the G1-phase. In this particular case the increase in the S/G2/M-phases overcompensates for the increased interdivision time, leading to a shortened G1-phase.

One could question this analysis because two different explanations are presented to explain the results of the overexpression of cyclins. But that is because two different results have been obtained in these experiments. At this time it is not known why two different types of results are obtained. All that is proposed here is that the two different types of results (no change in interdivision time and a shortening of the interdivision time) can be accommodated by a general model whereby other changes caused by the cyclins (in one case an increase in the growth rate, in the other case the lengthening of the S-phase) can lead to the observed G1-shortening. No G1-specific activity needs to be postulated.

The ultimate verdict of this reanalysis is that the observed changes in the G1-phase of mammalian cells when cyclins are induced or overexpressed is that these experiments need not support the invocation of G1-specific syntheses, events, or activities. If the cyclins increased the general rate of growth or mass synthesis, or perhaps adversely affected passage through the S-phase, the G1-phase would be shortened without the need to invoke cell-cycle-specific events.

There is no argument with the published experiments or their general interpretation with regard to the specific phenomena measured. Cyclins can and do affect the lengths of the G1-phase and upon occasion affect the total interdivision time of cells. It is proposed that there is an additional and equally valid interpretation of the data. We must distinguish experiments that actually demonstrate a difference between cell-cycle models from experiments that merely show a consistency with one or another model. The experimental results analyzed here are consistent both with the G1-event model and with the alternative model. To put the conclusion more strongly, the experiments analyzed here are explained quite easily by the a model that invokes no G1-specific syntheses.

THE CONTINUUM MODEL

The analysis presented here has only one small and limited purpose. That is to show that a fashionable and influential experiment supporting the notion of G1-specific controls or events may have another interpretation. This alternative interpretation, sometimes

referred to as the continuum model, has been applied to a number of other experimental situations [1, 2, 13–19].

The analysis presented here suggests that some experiments used to support the G1-model of the division cycle are equally consistent with the continuum model proposing that there are no G1-specific events during the animal cell division cycle. It is not possible to use experiments on changes in G1-length following overexpression of cyclins as an unequivocal support of the G1-cyclin model of the division cycle. If one takes into account the overall cellular growth rate, or variations in other phases of the cycle, one can interpret the results without invoking any cell-cycle-specific properties of cyclins.

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