Reappraisal of serum starvation, the restriction point, G0, and G1 phase arrest points

STEPHEN COOPER[1]

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, USA

ABSTRACT The restriction point in the G1 phase of the mammalian cell cycle is the oldest, best-known, and widely accepted control point regulating division cycle in mammalian cells. Origins of the restriction point and its subsequent history are reanalyzed here. The initial proposal of the restriction point has an alternative explanation, which is that cells arrested with a G1 phase amount of DNA can arise from the inhibition of a process or processes occurring throughout the cell cycle and are not restricted to any particular phase of the cell cycle or specifically related to any event in the G1 phase of the cell cycle. The initial evidence and subsequent analyses require reexamination. It is proposed that the arrest of cells with a particular DNA content equivalent to that in cells in the G1 phase of the division cycle does not mean there is any particular G1 phase control point.—Cooper, S. Reappraisal of serum starvation, the restriction point, G0, and G1 phase arrest points. *FASEB J.* 17, 333–340 (2003)

Key Words: G0 · G1 arrest · G1 phase · restriction point · cell cycle · quiescence

THE RESTRICTION POINT in the G1 phase of the mammalian cell cycle is a well known and widely accepted control element regulating cell growth and the division cycle. More than a quarter of a century ago Pardee (1) defined the "restriction point" as a unique point in the G1 phase of the eukaryotic or mammalian cell cycle at which starved or inhibited cells come to rest. Since that initial proposal, the restriction or R point has grown in importance so that recently it was stated that ". . . the decision to traverse the R point is the central event in normal cellular proliferation control" (2). The impact of the proposal of the restriction point may be seen from the fact that there are more than 764 recorded references to the original proposal (1) and more than 1419 references to a review article on the restriction point proposal (3). The idea of the existence of a restriction point has become so common that many papers refer to the concept of the restriction point without giving it a specific citation.

A recent example of the ubiquity of serum starvation and confluent growth arrest for synchronizing cells to analyze the cell cycle comes from experiments that analyzed the effect of Rho on the timing of expression of cyclin D1 in the G1 phase of the cell cycle (4). This article elicited a positive commentary in the same journal (5). Three different methods are described to analyze the events during the cell cycle. One method is described as follows: "In most experiments, confluent monolayers of NIH-3T3 cells or MEF were G0 synchronized by serum starvation for 1 and 2 days respectively in Dulbecco's modified Eagle's medium (DMEM) with 1 mg ml^{-1} fatty acid free bovine serum albumin (BSA)." After this treatment, the cells were trypsinized and grown in DMEM with 10% fetal calf serum. Other methods used stimulation of quiescent cells by recombinant growth factors. We see in this experimental description the current standard approach to synchronization and arrest of cells in a proposed G0 state.

Why should the proposal of a restriction point, now more than a quarter of a century old, be reexamined and reappraised at this time? This experiment should be reexamined because it has so dominated thinking about the cell cycle. If there are problems or questions regarding such a fundamental result, then it is important to revisit the experiment to see whether our views of the restriction point proposal should be modified. Here I present a detailed reanalysis of the original serum starvation experiments that led to the restriction point proposal.

The analyses presented here will concentrate on the use of serum-starved or growth-arrested cells to produce a synchronized culture. The example quoted above is merely one of thousands of similar papers that use this approach. It is generally believed that the serum-starved cells are "arrested at a point in the G1 phase" or in a "G0 phase" and that upon stimulation to regrow, the stimulated cells move as a synchronized cohort through the cell cycle. I wish to take issue with this interpretation of starvation/release synchronization by looking at the archetypal experiment that defined this method, the postulation of the restriction point. Pardee proposed that during quiescence, irrespective of how quiescence is achieved, cells are arrested at a unique "restriction point" located within the G1 phase of the division cycle (1). Pardee further proposed the existence of a "single switching point, the

[1] Correspondence: Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-0620, USA. E-mail: cooper@umich.edu

restriction point (or R point) in G1, that regulates the reentry of a cell into a new round of the cell cycle."

The restriction point is one of many postulated G1 phase arrest points. The G0 phase (6) is another well-known example. The G0 phase is a quiescent arrest state for mammalian cells where the cells are proposed to be "out of the cycle." G0 phase and the restriction point are operationally indistinguishable. However, a relationship between the G0 phase and the restriction point has been proposed. Zetterberg and Larsson (7) suggested that the ability of the cell to enter the G0 state is determined by a cell's temporal position relative to the restriction point when starvation or inhibition is imposed.

Cells arrested at the restriction point and cells in G0 arrest are examples of growth-arrested cells having a G1 phase amount of DNA. This common situation is usually referred to as arrest in G1 phase or G1 phase arrest.

Here we consider all G1 phase arrest proposals as common phenomena. An analysis of one G1 phase arrest proposal can be directly applied to other G1 phase arrest proposals, irrespective of the arrest conditions or cells studied. An alternative view of the cell cycle presented below suggests that the restriction point does not exist, the G0 phase does not exist, and one should not even use the locution "arrest in G1 phase" to describe cells that are merely "arrested with a G1 phase amount of DNA."

THE ORIGIN OF THE RESTRICTION POINT

Pardee's experiments (1) were simple. Exponentially growing cells were starved for different growth requirements. Cells stopped growth and were arrested with a G1 phase amount of DNA. The growth-arrested cells were then refed the missing supplement to allow regrowth. The time until DNA synthesis resumed was measured using autoradiography. Cells that achieved quiescence by any of three different treatments (64 h with low serum, isoleucine deprivation, or glutamine deprivation) were analyzed. DNA synthesis resumed after 8 h in all three cases. This result was interpreted as indicating that cells arrested by different means were all stopped at a point 8 h before initiation of the S phase. As summarized by Pardee (1), "In each experiment, the quiescent cells required the same length of time to recommence DNA synthesis. These results are consistent with each of the cell populations being blocked at the same point."

Additional experiments with sequential applications of different starvation regimens supported the notion of a unique restriction point. No sequence of different growth arrest conditions allowed cells to escape inhibition and enter S phase. If there were different arrest points for different starvation conditions, then initial arrest at a point later in the cell cycle, followed by relief of this arrest and arrest with a condition causing arrest earlier in the cell cycle, would let cells proceed to the initiation of S phase. No escape from inhibition as indicated by DNA synthesis was observed for any combination of arrest sequences. Pardee concluded that there was a single, unique arrest point for different arrest protocols. Pardee termed this point the "restriction point."

The conclusion from the timing experiment, however, was tempered and restrained by the fact that not all cells initiated DNA synthesis at the same time upon release from starvation. As Pardee pointed out (1) in the original restriction point paper, ". . . different cells begin thymidine incorporation at different times. Thus, measurement of the time of initiation of DNA synthesis by a cell population depends upon the behavior of an early initiating subclass of the population. We can only conclude, therefore, that this subclass is at the same point in different quiescent cultures."

If there were a "restriction point" and if cells were arrested at a unique point in the G1 phase, then the population of arrested and subsequently released cells would be expected to proceed toward S phase in a relatively synchronous manner. This synchronous entry into S phase is not observed. Entry into S phase is asynchronous as indicated by the initial results of Pardee as well as by subsequent experiments on serum starvation and release (see refs 8–10).

Thus, the original experiments of Pardee do not support the proposed restriction point. As cells do not form a synchronized cohort upon release from the arrest conditions, one could conclude that a restriction point does not exist at a particular time during the G1 phase of the eukaryotic cell cycle.

The sequential starvation results are vitiated by the suggestion that the three starvation protocols all lead to a cessation of mass growth. Exchanging one mode of growth inhibition for another mode of growth inhibition should merely lead to the observed inhibition of initiation of DNA synthesis. Thus, if inhibition of protein synthesis, a process that occurs continuously throughout the cell cycle, leads to arrest of initiation of DNA replication, then replacing one method of protein synthesis inhibition with another method of protein synthesis inhibition would merely lead to a continuous inhibition of protein synthesis.

RELATIONSHIP OF THE RESTRICTION POINT AND G0

Analysis of cell division patterns after short serum starvations (i.e., 1 h) led Zetterberg and Larsson (7) to propose that cells are able to enter the G0 phase when the cells are before the restriction point at the instant of starvation. In their experiments, the restriction point was proposed to occur 3.5 h after cell birth. According to Zetterberg and Larsson, cells before the restriction points are able to enter G0 and cells after the restriction point (i.e., closer to the start of S phase) are unable to enter G0. Entry into the G0 phase by cells before the restriction point was implied by the observation of an

8 h delay in the next cell division upon release from a short 1 h starvation period. Cells after the restriction point did not exhibit the 8 h delay in the next cell division in response to a 1 h starvation. Fundamental problems with the Zetterberg and Larsson experiments have been discussed before (11, 12). Not only does the data from the time lapse analyses of Zetterberg and Larsson have an alternative explanation, but internal controls (e.g., cycloheximide inhibition gives the same kinetics of division inhibition as serum starvation, and cycloheximide is a general inhibitor of protein synthesis throughout the cell cycle) argue for a revision of this specific G0 model (11, 12). The difference between the Pardee view of a G1 phase arrest state or restriction point and the G0/G1 state is that the restriction point is within the G1 phase, whereas cells in G0 are proposed to be "out of the cycle." According to Zetterberg and Larsson (7), such out-of-cycle G0 cells require a significant amount of time to reenter the cell cycle.

Illustrations of these two different views of G1 phase arrest are presented in **Fig. 1**. The restriction point and G1 phase arrest points are viewed as points within the G1 phase at which cells come to rest. The restriction point may be imagined as representing some function a cell must perform as it passes through the G1 phase of the division cycle. Upon growth arrest, cells in all phases of the division cycle accumulate "at the restriction point" (Fig. 1a). The idea of pattern in Fig. 1a is that if cells are truly arrested at a point in the division cycle, the aligned cells should be synchronized when starvation ceases and growth resumes.
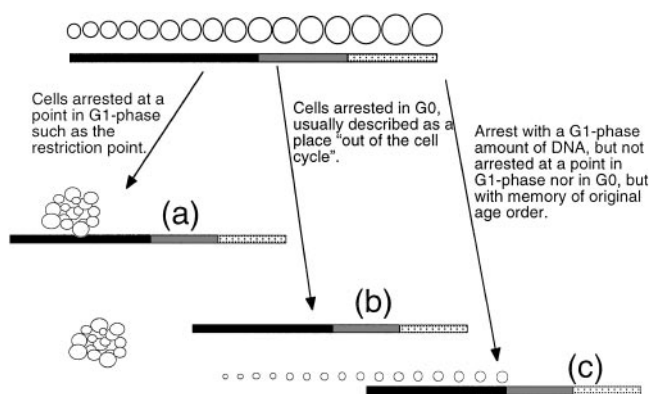

Figure 1. Alternative views of arrest with a G1 phase amount of DNA. At the top is a representation of cells growing through the division cycle. Cells are born at one size (leftmost circle) and move through the division cycle increasing in cell mass (considered here as cytoplasmic mass) throughout the division cycle. Cells in each phase of the cell cycle (G1, S, and G2/M phases indicated by the different shadings on the horizontal bar) are related in size to the particular cell cycle phases. When cells are starved, the resulting population of cells with a G1 phase amount of DNA is proposed to be arrested at a point in the cell cycle (a) such as the restriction point or arrested in some out-of-cycle phase (b) such as G0. An alternative model postulates that arrest is not at any particular cell cycle point but that cells with a G1 phase amount of DNA are representative of cells in different phases of the cell cycle (c).

Figure 1b illustrates arrest with a G1 phase amount of DNA except that the arrested cells are "out of the cycle." It is not possible to operationally distinguish cells at the restriction point from G0 phase cells by merely studying the DNA contents of arrested cells. Both arrested cell types have a G1 phase amount of DNA and are operationally indistinguishable.

Pardee did not explicitly discuss cell synchronization when the restriction point was proposed. However, this idea has spread from researcher to researcher until there are literally hundreds, and possibly thousands, of papers that propose cell synchronization by cell arrest at a particular G1 phase arrest point. The proposal of a restriction point was thus applied willy-nilly to any starvation protocol where the fraction of cells with a G1 phase amount of DNA increased. It was assumed that upon release from G1 phase or restriction point arrest, the cells would be synchronized.

One of the most popular synchronization methods is to starve cells of serum for extended periods, then restore serum to produce a presumably "synchronized" culture. Sometimes the arrest point is formulated as "G1/G0." Other times the cells are explicitly described as arrested at the restriction point. Irrespective of what they are called (G1 phase arrest, G0, quiescence, arrest at a restriction point), all the arrest conditions may be considered similar phenomena.

AN ALTERNATIVE VIEW OF ARREST WITH G1 PHASE AMOUNT OF DNA

An alternative view of "G1 phase arrest" is presented in Fig. 1c. Cells are arrested with a G1 phase amount of DNA but are not arrested at any particular point in the cell cycle. When cells are starved, initiation of S phases ceases, but cells complete the S and G2/M phases in progress and continue until cell division. Cells in G1 phase remain with a G1 phase amount of DNA, as the cells do not initiate DNA synthesis. Cells in S and G2/M proceed through the cycle to divide and produce daughter cells, each with a G1 phase amount of DNA. Cessation of S phase initiations and completion of S/G2/M phases in progress leads to the accumulation of cells all with a G1 phase amount of DNA. In contrast to Fig. 1a, b, there is no arrest at a particular point (Fig. 1c). Cells all have a G1 phase amount of DNA but are arrayed sequentially, reflecting their original order at starvation. Upon resumption of growth, cells do not enter S phase synchronously nor do the cells divide synchronously. The cells at the end of G1 phase enter S phase first, as they were closest to initiation when the starvation or inhibition was imposed. Cells then enter S phase in a sequence reflecting their order in the original, growing cell population. Only after one full doubling time have all the remaining cells initiated DNA synthesis (8, 13). The alternative model has been termed the continuum model.

It is interesting to ask, what is the evidence for the statement, made above, that the cells are arrayed se-

quentially reflecting their original order at starvation? This is the essence of the problem. The continuum model of the cell cycle asks, what is the evidence that they are not so arrayed, and rather are arrested at a particular point? The model given in Fig. 1c is a counter-explanation that has not been taken into account to explain arrest of cells with a G1 phase amount of DNA without the need to invoke G1 phase-specific events. Two pieces of evidence support the proposal that cells are not arrested at a point. The first is the fact of G1 phase arrest. The continuum model predicts this as well as the G1-control model or restriction point model. But when cells are released, the continuum model predicts that cells will not be synchronized. Thus, the original evidence from the Pardee restriction point experiment actually favors the continuum model.

The incorrect interpretation of evidence for the restriction point does not automatically mean that the continuum model is correct. The continuum model is presented as a framework within which to reexamine the restriction point proposal and to see where there are problems.

The restriction point has also been described as a point determining when cells become independent of outside stimuli for division. As recently summarized (2), "After the cell has advanced two-thirds of the way through G1, the cell may decide to commit itself, essentially irrevocably, to continue its advance and complete its cell cycle." Cells past the restriction point are committed to entering S phase whereas cells before the restriction point have not yet made that commitment.

The alternative view of the cell cycle (Fig. 1c) proposes that leakage can account for the differences between cells in the early and late parts of the G1 phase. If starvation is not perfect and absolute, but some leakage occurs, cells slowly accumulate material leading to an initiation of S phase even during the period of incubation in low serum. Cells closer to initiation (i.e., later in S phase) will reach initiation mass sooner than cells earlier in the G1 phase. Cells later in the G1 phase at the time growth arrest is imposed are less likely to have a delayed cell division in the Zetterberg-Larsson experiment. Cells earlier in the cycle will not accrue enough leakage to initiate DNA synthesis and thus will exhibit a delayed cell division.

THE BACTERIAL RESTRICTION POINT

It is ironic that a bacterial restriction point was proposed 5 years before the mammalian restriction point (14). This parallel proposal was never explicitly recognized as a precursor of Pardee's work because the bacterial points were not called restriction points. It was soon shown, experimentally (15) and in theory (16), that the bacterial restriction points were merely the result of leakage during starvation. That is, the bacterial restriction point did not exist. The same reasoning that

applies to the bacterial restriction points applies directly to the mammalian restriction point.

From simplicity, parsimony, or Occam's razor considerations alone, I suggest that pattern c in Fig. 1 is the preferred explanation of arrest with a G1 phase amount of DNA. The support of pattern c by experimental results merely adds to the strength of this conceptual analysis.

RETHINKING G0

The G0 phase proposal has been expanded from its original formulation to include chemical or cellular changes as cells enter G0. The prime example of the G0 state is a differentiated cell. Differentiated cells are proposed to be unable to resume division after differentiation. Differentiated and nondividing cells in a proposed G0 state are recognized as different from dividing and growing cells by their particular biochemical differentiation characteristics as well as their inability to divide.

An alternative view of the G0 phenomenon related to differentiation is illustrated in **Fig. 2**. Growth arrest is not necessary to have cells look as though they are in G0. Chemical changes may occur at slower growth, but these changes are not necessarily related to the cell
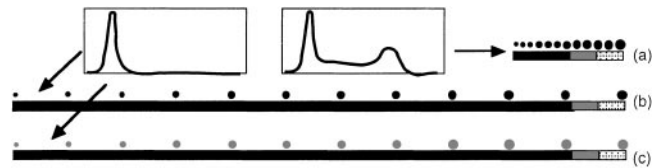

Figure 2. G1 phase arrest and the G0 proposal. Consider cells that are growing with some relatively short interdivision time as illustrated in the top right panel of cells (*a*). Approximately half the cells would have a G1 phase amount of DNA, one-quarter would be in S phase, and one-quarter would be in G1/M phase. All of the cells are now in different parts of the cell cycle and are not representative of any particular phase of the cell cycle. Now imagine that some condition is encountered so that the interdivision time increases enormously but the length of S and G2/M phases does not change. One might imagine with a very slow-growing culture (*b*) that perhaps 95–99% of the cells would have a G1 phase amount of DNA and only 1–5% of the cells would be represented as cells in S/G2/M. A flow cytometric analysis of the fast-growing and the slow-growing cells would show that the cells changed their pattern from the classic three-phase pattern to one where essentially all of the cells have a G1 phase amount of DNA. Whereas all the cells may have a G1 phase amount of DNA, the cells represented in the middle panel are not at any particular point in the cell cycle but are representative of all parts of the cell cycle. That is, each cell represents an equal fraction of the cell cycle, and it is merely a result of the invariant S and G2/M phase lengths that the cells all have a G1 phase amount of DNA. Note that at no time are the cells arrested at a particular point in the cell cycle. If, in addition to slowing growth and having a longer interdivision time, the cells change biochemically (*c*), one can have a population of cells that are both enriched in cells with a G1 phase amount of DNA and biochemically altered.

cycle. If growth slows so cells have a long interdivision time, it will appear that cells have a G1 phase amount of DNA (Fig. 2). But these cells are not arrested at any point in the cell cycle. The increase in G1 phase cells is due to the relative invariance of S and G2/M phases (17, 18). The increased interdivision time is accommodated by a substantially elongated G1 phase (12, 18, 19). Slow-growing cells may have a very long or even an infinite interdivision time. The cells with an infinite interdivision time are cells that do not divide. It should not be assumed that simply because the cells exhibit a preponderance of G1 phase DNA contents that the cells are arrested at a particular point in the cell cycle or that the cells are in an "out of phase" condition such as G0. As cells go from fast growth (Fig. 2a) to slow growth (Fig. 2b, c), it *appears* that cells enter a G1 phase arrest state. But as drawn here, the cells are not necessarily arrested at any particular point, either within or without the cell cycle.

If cells alter their biochemistry due to slow-growth conditions, then one may associate biochemical changes with the increase in G1 phase cells. But this association may just be fortuitous (20). Starvation or arrest conditions may elicit biochemical changes in cells as the cells cope with starvation stress or the cells differentiate. But one must not conflate or confuse these two independent phenomena. Cells may differentiate and may grow slowly and increase the G1 phase cells. However, these two independent phenomena do not prove the existence of any particular G1 phase arrest point or restriction point (20).

The proposal made here accepts that differentiated cells may never divide again. There very likely are such cells (e.g., nerve and brain cells), and such cells may all have a G1 phase amount of DNA. This combination of G1 phase DNA content and different cellular chemistry has been used to argue there is a unique and definable G0 state. What is argued here is that simply because cells accumulate in a uniform state of DNA content and exhibit biochemical changes does not mean that these two results are causally associated.

It is important to understand the verbal implications of the term "G0." G0 arose to define a state "before G1." Cells enter G0 from a point "in G1 phase." Upon release from G0, cells reenter the cell cycle "in G1 phase." These two suggestions support the proposal that there exist G1 phase events or decision points. If cells were described as being in an "X" state, with a name unrelated to the cell cycle, then there would be less argument over whether there is a G0 (or X) state. The proposed existence of G0 and its relationship to the G1 phase—as evidenced by all G0 cells having a G1 phase amount of DNA—is implicit support for the existence of G1 phase events. It is this support for G1 phase events that is dismissed here.

One may attach any label to a particular collection of cells but it is confusing, with unwanted and unnecessary baggage, to call such cells G0 cells, implying that such arrested cells are related in some way to arrest at a point in the cell cycle.

ON THE EXISTENCE OF G1 PHASE EVENTS

Support for G1 phase-specific events rests not only on the kinetic analysis of cells entering arrest states, but on the identification of G1 phase events. There are numerous G1 phase events, syntheses, expressions, and other phenomena that are adduced to support the existence of G1 phase-specific functions. What can one say in the face of this enormous outpouring of support for the existence of G1 phase events?

It is important to realize that essentially all of the proposed G1 phase phenomena are based on experiments using arrest or inhibition methods to produce a population of cells "arrested in G1 phase" or a "synchronized culture" from such cells. The arguments presented here indicate the problem with this approach. If the cells are not arrested at a particular point in G1 phase and are not synchronized, then cell cycle phenomena identified using such cells are not necessarily related to the cell cycle (21).

Until a particular G1 phase phenomenon (for example, expression of a particular cyclin in the G1 phase of mammalian cells) is reproducibly studied and analyzed and rendered a reproducible phenomenon similar to the β-galactosidase of *Escherichia coli*, one does not have to accept the weak evidence in support of such phase-specific phenomena (22). For example, cyclin expression in the G1 phase of mammalian cells rests almost entirely on homology with fungi or lower invertebrates. Direct physiological evidence in mammalian cells that G1 phase cyclins actually vary in expression during the normal, unperturbed division cycle is sorely lacking. There is no mammalian cell system studied in numerous laboratories where the cyclins are analyzed and their rate of synthesis during the division cycle pinned down so that it is irrefutable that cyclin expression is taking place specifically in the G1 phase. Most important, much of the data proposing the existence of G1 phase events are based on methods subject to the introduction of artifacts where artificial periodicities may be introduced by the methodology (8, 21, 23–25).

An instructive example of the introduction of artifacts by the use of growth-arrested cells comes in the study of c-myc expression during the cell cycle. The original proposal that c-myc was expressed specifically in G1 phase (26, 27) was shown to be incorrect when growing cells were studied (28, 29). A reinterpretation of these experiments in terms of the continuum model has been published (21).

ON THE EXISTENCE OF "OVERWHELMING" EVIDENCE FOR G1 PHASE EVENTS

It has been said, "The amount of data supporting G1 events is, indeed, overwhelming" (anonymous reviewer). I agree that the reports of G1 phase events, and even G1 phase-specific synthesis, are numerous and indeed, overwhelming. But I would prefer to characterize these reports as "claims" of evidence for G1 phase

events. If these claims for G1 phase events are all based on the same problematic experimental approach—that of starvation of cells to produce a population of cells with a G1 phase amount of DNA, then release of such cells with analysis of the biochemistry of the cells as they resume growth—all we have is a collection of experiments that are subject to the same criticism as presented here in reanalysis of the restriction point.

EXPERIMENTAL SUPPORTS FOR THE ALTERNATIVE MODEL

An asynchrony of S phase initiation is predicted by the alternative explanation of cell arrest and release from growth arrest. This alternative continuum model predicts that arresting cells with a G1 phase amount of DNA will not produce a synchronized population (13). The evidence from Pardee's experiments (1) does not support a unique restriction point in the division cycle, but rather a collection of cells that are unified with respect to one parameter (i.e., DNA content) but are different by other parameters (e.g., ability to initiate DNA synthesis).

Recent results from my laboratory support the proposed alternative view of the cell cycle and the critique of G1 phase arrest points presented above. For example, I have studied the classical G1 phase phosphorylation of retinoblastoma protein. Retinoblastoma protein phosphorylation in G1 phase is as close as one could come to an accepted cell cycle phenomenon that is widely studied and reproducibly reported to exist. Yet our studies show that in some cells there is no G1 phase phosphorylation event, as cells are phosphorylated at all times during the division cycle (24). Even more to the point, our results explain why many researchers have come to think there is a G1 phase phosphorylation event when the results are merely due to cells not being grown at a low enough cell density (13, 25). To briefly summarize the experimental evidence for an alternative view of G1 phase Rb phosphorylation, if one grows cells to different levels of confluence (from 100% down to 5%), there is a continuous variation in the degree of phosphorylation, with low density cells having 100% Rb phosphorylated. This result is interpreted as indicating that cells at even moderate levels of confluence (e.g., 50% confluence) are a mixed culture of arrested cells (cells in groups are proposed to be arrested by some sort of contact inhibition) and cells that are freely growing. The arrested cells have dephosphorylated Rb and have a G1 phase amount of DNA (by the analysis presented above); the growing cells have phosphorylated Rb and cells in all phases of the cell cycle. Looking only at the G1 phase cells in this mixed population reveals that phosphorylated and dephosphorylated Rb is found. This finding has been interpreted as supporting a mid-G1 phase phosphorylation of Rb protein. The alternative explanation, that growing cells have Rb phosphorylated throughout the cell cycle, and non-growing cells have dephosphorylated Rb protein, ade-

quately explains the data on Rb phosphorylation. An experimental (24) analysis as well as a review (25) should be consulted for the details of this analysis.

In addition, we have reanalyzed a recent paper that proposed numerous cell cycle-specific syntheses (30) and have shown that the data are neither reproducible nor supportive of cell cycle events (31). Finally, time lapse videographic studies of starvation and inhibition synchronization methods indicate that the cells produced by this type of manipulations (e.g., lovastatin inhibition) are not synchronized (32). Together, these results support the alternative view of the cell cycle (13). Other experimental support of the alternative model has been summarized (13).

The conclusion of this analysis of the restriction point is that even this forerunner of all G1 phase arrest points did not lead to a synchronized culture, either in the original work as measured by the initiation of DNA synthesis or in later work looking at cell division. Thus, the restriction point is another example of arrest "with a G1 phase amount of DNA" where the cells are not synchronized.

THE CONTINUUM MODEL

Two decades ago it was proposed (17) that there are no G1-specific events and the G1 phase exists when the interdivision time or mass doubling time of a mammalian cell is greater than the sum of the S+G2+M phases of the division cycle (12, 13, 33). The G1 phase was proposed to be the time when biosynthetic processes begun at the previous S phase are completed. Since that initial proposal, this viewpoint—since codified as the continuum model—has been applied to a large number of experimental observations (12, 13, 33).

Although the analysis presented here has been directed primarily at the phraseology "the restriction point," the results apply to other formulations of G1 arrest such as G1 arrest, entry of cells into G0, and quiescent states where cells accumulate an excess of cells with a G1 phase amount of DNA. Many of these proposals have been addressed in previous publications (8, 13, 23).

APPLICATION OF THESE IDEAS TO THE RHO/CYCLIN D1 KINETICS

We can now return to the original article that began this discussion. It was found that when various mutants were studied, there was a change in the kinetics of expression of cyclin D1. There is no problem with this result. The problem lies in the interpretation. In the original paper, the experimental results are interpreted as changing the expression time of cyclin D1 within the cell cycle. The alternative view would state that whatever kinetics of expression of cyclin D1 occurs after release of starved cells, this expression is different in a mutant cell. There is no need to relate this to timing

during the division cycle until such timing is shown to be altered in exponentially growing, unperturbed cells.

BIOCHEMISTRY OF PROPOSED RESTRICTION POINT ELEMENTS

Transition of the restriction point was proposed to be determined by accumulation of a labile protein (R-protein) (1–4). R-protein was proposed to be a functionally short-lived (labile) regulatory protein whose synthesis is sensitive to growth factors and that must accumulate to a critical amount before a cell can pass the restriction point and proceed toward DNA synthesis 2.

One candidate for the R-protein is cyclin D. I will not review the evidence for or against this proposal. Here I just wish to emphasize that simply because various molecules have been identified as important regulators for passage of the cell through the cell cycle does not imply that the existence of this molecule is support for the existence of the restriction point. One can have cyclin D1 as an important regulator of cell growth throughout the cell cycle. Interfering with cyclin D1 synthesis or activity would lead to the arrest of a cell culture with a G1 phase amount of DNA. But it is not logical to conclude this means that the existence of cyclin D1 supports the restriction point idea. The restriction point proposal must stand on its own. The question raised here is, was the original postulation of a G1 phase restriction point correct or not?

ON THE ATTRACTION OF G1 PHASE EVENTS

If the arguments against the restriction point and related G1 phase arrest points are valid, a legitimate question is, "Why have the concepts of restriction points, G1 phase events, and arrest points gained such a stronghold in the current consensus view of the cell cycle?"

One answer is that the idea of arrest points has been useful to some researchers. The shorthand of arresting cells at a restriction point (rather than describing growth conditions, time of treatment, and so forth in detail and leaving the interpretation of the observations for a separate treatment) allows a field to communicate ideas. Phrases such as "G0" and "restriction point" are simple shorthand for experimental treatments that produce a type of cell collection. *But because something is useful does not mean it is true.* Utility and consensus should not lead to the conclusion that near-universal acceptance ensures that a phenomenon exists. Until the arguments presented in this paper are dealt with, we must be skeptical of the existence of restriction points and other related G1 phase phenomena.

It is important to distinguish the utility in facilitating communication from utility in understanding a biological phenomenon. The use of the terms restriction point and G0 to describe cell cycle controls may actually prevent one from seeing the underlying biological controls. Thus, the shorthand terms for control systems may be anti-utilitarian.

Besides utility, structural aspects of the development of a scientific field make it difficult for workers to discard ideas held by the majority of the field. Because of the repeated use of the terms "restriction point," "G0," and "G1 phase arrest" by investigators of cell cycle studies, it becomes difficult to avoid using these terms even when they are not necessary for a particular analysis.

Furthermore, new researchers entering the field see these terms in textbooks and assume that these words describe a reality that can be used in discussions of the cell cycle. It is rare that a researcher goes back to old work (e.g., 29 years old) and checks to see whether the experiments have an alternative explanation. The historical development of a field leaves certain ideas ingrained in the development of the field and it becomes difficult to discuss ideas outside of the consensus view of the field.

What about the hundreds, if not thousands, of papers that rely on restriction point theory and starvation methodology to study the cell cycle? I can only suggest that whatever experimental results are obtained by these experiments are valid experimental results, but that any interpretation of these results in terms of the cell cycle, and G1 phase in particular, must be reevaluated and, sadly, discarded.

In short, the acceptance of the restriction point and related G1 phase arrest points is due to the acceptance of a general belief system regarding the cell cycle. Many experiments do not actually distinguish between the restriction point belief system and the alternative view of the cell cycle. The arguments presented here demonstrate that it is important to rethink and revisit the restriction point and other arrest points to see whether these points actually exist. FJ

REFERENCES

1. Pardee, A. B. (1974) A restriction point for control of normal animal cell proliferation. *Proc. Natl. Acad. Sci. USA* **71,** 1286–1290
2. Lundberg, A. S., and Weinberg, R. A. (1999) Control of the cell cycle and apoptosis. *Eur. J. Cancer* **35,** 1886–1894
3. Pardee, A. B. (1989) G1 events and regulation of cell proliferation. *Science* **246,** 603–608
4. Welsh, C. F., Roovers, K., Villanueva, J., Liu, Y., Schwartz, M. A., and Assoian, R. K. (2001) Timing of cyclin D1 expression within G1 phase is controlled by Rho. *Nat. Cell Biol.* **3,** 950–957
5. Coleman, M. L., and Marshall, C. J. (2001) A family outing: small GTPases cyclin' through G1. *Nat. Cell Biol.* **3,** E250–E251
6. Lajtha, L. G. (1963) On the concept of the cell cycle. *J. Cell. Comp. Physiol.* **60,** 143
7. Zetterberg, A., and Larsson, O. (1985) Kinetic analysis of regulatory events in G1 leading to proliferation or quiescence of Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA* **82,** 5365–5369

8. Cooper, S. (1998) Mammalian cells are not synchronized in G1-phase by starvation or inhibition: considerations of the fundamental concept of G1-phase synchronization. *Cell Prolif.* **31,** 9–16
9. Di Matteo, G., Fuschi, P., Zerfass, K., Moretti, S., Ricordy, R., Cenciarelli, C., Tripodi, M., Jansen-Durr, P., and Lavia, P. (1995) Transcriptional control of the Htf9-A/RanBP-1 gene during the cell cycle. *Cell Growth Differ.* **6,** 1213–1224
10. Kim, T. Y., and Kaelin, W. G., Jr. (2001) Differential control of transcription by DNA-bound cyclins. *Mol. Biol. Cell* **12,** 2207–2217
11. Cooper, S. (1987) On G0 and cell cycle controls. *Bioessays* **7,** 220–223
12. Cooper, S. (1998) On the proposal of a G0 phase and the restriction point. *FASEB J.* **12,** 367–373
13. Cooper, S. (2000) The continuum model and G1-control of the mammalian cell cycle. *Prog. Cell Cycle Res.* **4,** 27–39
14. Lark, K. G., and Renger, H. (1969) Initiation of DNA replication in *Escherichia coli* 15T-: chronological dissection of three physiological processes required for initiation. *J. Mol. Biol.* **42,** 221–235
15. Cooper, S., and Weusthoff, G. (1971) Comment on the use of chloramphenicol to study the initiation of deoxyribonucleic acid synthesis. *J. Bacteriol.* **106,** 709–711
16. Cooper, S. (1974) A criterion for using chloramphenicol to define different processes in the initiation of DNA synthesis in bacteria. *J. Theor. Biol.* **46,** 117–127
17. Cooper, S. (1979)) A unifying model for the G1 period in prokaryotes and eukaryotes. *Nature (London)* **280,** 17–19
18. Cooper, S. (1998) On the interpretation of the shortening of the G1-phase by overexpression of cyclins in mammalian cells. *Exp. Cell Res.* **238,** 110–115
19. Cooper, S. (1981) The continuum model: application to G1-arrest and G0. In *Cell Growth* (Nicolini, C., ed), pp. 315-336, Plenum Press, New York
20. Cooper, S. (2001) Revisiting the relationship of the mammalian G1 phase to cell differentiation. *J. Theor. Biol.* **208,** 399–402
21. Cooper, S. (1988) The continuum model and c-myc synthesis during the division cycle. *J. Theor. Biol.* **135,** 393–400
22. Cooper, S. (2000) Toward a standard system for the mammalian cell cycle. *ASM News* **66,** 71–75
23. Cooper, S. (1997) G1 and S phase gene expression cannot be analyzed in mammalian cells synchronized by inhibition. *Microb. Comp. Genomics* **2,** 269–273
24. Cooper, S., Yu, C., and Shayman, J. A. (1999) Phosphorylation-dephosphorylation of retinoblastoma protein not necessary for passage through the mammalian cell division cycle. *IUBMB Life* **48,** 225–230
25. Cooper, S., and Shayman, J. A. (2001) Revisiting retinoblastoma protein phosphorylation during the mammalian cell cycle. *Cell. Mol. Life Sci.* **58,** 580–595
26. Kelly, K., Cochran, B., Stiles, C., and Leder, P. (1984) The regulation of c-myc by growth signals. *Curr. Top. Microbiol. Immunol.* **113,** 117–126
27. Kelly, K., Cochran, B. H., Stiles, C. D., and Leder, P. (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. *Cell* **35,** 603–610
28. Hann, S. R., Thompson, C. B., and Eisenman, R. N. (1985) c-myc oncogene protein synthesis is independent of the cell cycle in human and avian cells. *Nature (London)* **314,** 366–369
29. Thompson, C. B., Challoner, P. B., Neiman, P. E., and Groudine, M. (1985) Levels of c-myc oncogene mRNA are invariant throughout the cell cycle. *Nature (London)* **314,** 363–366
30. Cho, R. J., Huang, M., Campbell, M. J., Dong, H., Steinmetz, L., Sapinoso, L., Hampton, G., Elledge, S. J., Davis, R. W., and Lockhart, D. J. (2001) Transcriptional regulation and function during the human cell cycle. *Nat. Genet.* **27,** 48–54
31. Shedden, K., and Cooper, S. (2002) Analysis of cell-cycle-specific gene expression in human cells as determined by microarrays and double-thymidine block synchronization. *Proc. Natl. Acad. Sci. USA* **99,** 4379–4384
32. Cooper, S. (2002) Reappraisal of G1-phase arrest and synchronization by lovastatin. *Cell Biol. Int.* **26,** 715–727
33. Cooper, S. (1991) *Bacterial Growth and Division,* Academic Press, San Diego