The Molecular Basis of Cell Cycle & Growth Control

THE MOLECULAR BASIS OF CELL CYCLE AND GROWTH CONTROL

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PREFACE

During the past several years, significant advances have been made in understanding control of proliferation at the cellular, biochemical, and molecular levels. As the complexity and interdependency of parameters mediating growth control become increasingly apparent, the demarcations between regulated and regulatory components of mechanisms that promote or inhibit proliferation are eroding. There is recognition of both cause and effect relationships between the activities of factors that modulate the cell division cycle reflecting multidirectional signaling between segments of regulatory cascades that are selectively operative in specific cells and tissues. The necessity of accommodating the integration of positive and negative growth regulatory signals is now appreciated in a broad spectrum of biological contexts. These include but are not restricted to (1) repeated traverse of the cell cycle for cleavage divisions during the initial stages of embryogenesis and continued renewal of stem cell populations, (2) stimulation of quiescent cells to proliferate for tissue remodeling and wound healing, and (3) exit from the cell cycle with the option to subsequently proliferate or terminally differentiate. Equally important is an appreciation of the cell cycle regulatory mechanisms that have been compromised in transformed and tumor cells and in nonmalignant disorders where abnormalities in cell cycle and/or growth control are operative. These insights into regulatory mechanisms operative in cell cycle and growth control are rapidly being translated to clinical diagnosis and therapeutics.

Proliferation is explored in this volume from the perspectives of conceptual and experimental approaches to fundamental principles as well as biomedical applications. The biological problem of cell cycle control is introduced within the historical context of contributions from a broad spectrum of model systems that have provided the foundation for our current understanding of growth control in mammalian cells. The identification of checkpoints and restriction points as well as the factors responsible for proliferation competency and cell cycle progression are presented. The chapters focus on DNA replication and S phase; mitosis and meiosis; regulation of gene expression; growth factors and growth factor receptor–mediated pathways; signal transduction and integration of regulatory information; differentiation; development and programmed cell death; and cellular senescence and immortalization. The concluding chapter addresses cancer and other disorders that are linked to perturbations in control of proliferation. Each chapter was developed with a balance between presentation of biological parameters and gene regulatory mechanisms that mediate growth control and cell cycle progression. A concerted effort has been made to provide a comprehensive bibliography and illustrations that assimilate components of the increasing complexity associated with proliferation.

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CHAPTER 1

INTRODUCTION TO THE CELL CYCLE

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HISTORY OF THE CELL CYCLE

The First Days of Creation

Like Sleeping Beauty, the study of cell division slept for almost a hundred years after mitosis was first described in 1875. Despite countless cytological descriptions of mitosis in its classic four stages (prophase, metaphase, anaphase, telophase), very little progress was made on its biochemical basis, and interphase (the status of cells when not in mitosis) simply remained terra incognita. The measure of our ignorance is best illustrated by an example. It is now well established that colchicine (or Colcemid) blocks cells in mitosis; it is, in fact, used to synchronize cells in mitosis so that we can collect almost pure populations of mitotic cells. But back in the 1930s, scientists, having observed that the number of mitoses increased sharply in tissues of animals given injections of colchicine, came to the conclusion that colchicine was . . . mitogenic, a not unreasonable conclusion given the circumstances. So, let us follow, briefly, how we have come from such a naive view of cell division to the sophisticated cell cycle of the molecular biologist.

The first breakthrough, indeed, the very concept of a cell cycle, came from Alma Howard and Steve Pelc in 1951. Using ^{32}P to label the roots of *Vicia faba* seedlings and autoradiography, Howard and Pelc (1951) concluded that DNA was synthesized during a discrete period of the interphase, that the interval between mitosis and DNA synthesis was a long one, and that the interval between DNA synthesis and mitosis was a short one. Their paper succinctly layed the basis for the four phases of the cell cycle that we all know: G_1, between mitosis and S phase (DNA synthesis); S phase; G_2, between S phase and mitosis; and mitosis itself. The almost contemporary demonstration

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that DNA is the genetic material of cells made their observation even more important: The cell, before dividing, replicated its genetic material. The method used by Howard and Pelc to label cells was, so to speak, primitive, requiring digestion with RNase to distinguish DNA from RNA synthesis, and made even more difficult by the use of ^{32}P, a high energy emitter that gave only an approximate intracellular localization of the radioactivity.

The second breakthrough came with the introduction of high-resolution autoradiography with tritiated thymidine (^{3}H-Tdr). The advantage of ^{3}H-Tdr was twofold: Tritium is a weak emitter and its label can be localized precisely not only in cells but also in cell compartments, and Tdr is a specific precursor of DNA. The original paper by Hughes et al. (1958) merits some discussion. For many years, histologists had observed that, in the lining epithelium of the small intestine of rodents, all mitoses occurred at the bottom, in an area called the *crypts,* while no mitoses were found in the lining epithelium of the villi, intestinal folds that project into the intestinal lumen. Imagine yourself again in the place of an investigator of the 1930s. The number of epithelial cells in the crypts of the adult rodent is constant, so what happens to the siblings of a mitotic cell? Does one of the siblings die in the crypt? Or does it migrate along the villi? The use of autoradiography with ^{3}H-Tdr solved the problem because DNA, once synthesized, remains stable until the cell dies and because Tdr in the living animal, if not incorporated into DNA, is broken down to nonutilizable products in 45 minutes. Only crypt cells were labeled within 30 minutes after an injection of ^{3}H-Tdr, indicating that the epithelial cells of the villi did not synthesize DNA. But, when the animals were sacrificed 24 hours after the single injection of ^{3}H-Tdr, labeled epithelial cells were now visible along the villi, where, with time, they progressed slowly to the tips of the villi before being sloughed off into the intestinal lumen, roughly 48 hours after the initial labeling. It showed that the dividing cells originally labeled in the crypts moved out of the crypts and replaced the lining epithelium of the villi, pushing the older cells upward toward the lumen of the intestine. The similarity of cellular progression in the mucosa of the small intestine to academic or corporate careers is striking: One slowly glides to the top, against much resistance, and, when the summit is finally reached, one is simply discarded into oblivion. Armed with ^{3}H-Tdr, it was not difficult for subsequent investigators to define the phases of the cell cycle in a variety of cells.

The third breakthrough came when Stanley Cohen (1962) purified from the salivary glands of mice a polypeptide that caused early eruption of the incisor teeth and precocious opening of the eyelids in newborn mice. These activities do not strike us as particularly exciting, but what Stanley Cohen had done was to purify the first growth factor, which he called *epidermal growth factor* (EGF). So, by 1962, we had the cell cycle, we had cells that moved from one phase of the cell cycle to another or even out of the cell cycle, and now Stanley Cohen had found the force that moved the cell cycle: growth factors.

The Growth of the Cell Cycle: Kinetics

It would be impossible to give credit to the many scientists who slowly built upon the concepts outlined above: more refined cell kinetics, populations of cells, mathematical models, more growth factors, biochemistry of the cell cycle, genetics of the cell cycle, molecular biology of the cell cycle. But some highlights should be mentioned. Baserga et al. (1960) were the first to apply cell kinetics to the study of tumor growth, and, surprisingly, they found that the cell cycle of tumor cells was not necessarily shorter than that of normal cells. This implied that other parameters, besides cell cycle length, were at work, parameters that allowed tumors to grow while normal adult tissues did not grow. Mendelsohn (1962) then introduced the concept of growth fraction: Not all cells are cycling; in fact, some are fated to die without dividing again, and others are in a state of inactivity from which they can re-enter the cell cycle if appropriately stimulated. We call the former ones *terminally differentiated cells,* the latter ones G_0 cells. It is now generally accepted that G_0 cells are biochemically different from G_1 cells and are therefore entitled to be separately classified. But a vigorous discussion of whether G_0 was simply a long G_1 or not, enlivened the cell cycle circles for many years, a discussion that produced countless papers but little information.

If you now look at Figure 1.1, it is easy to see that, when it comes to cell division, there are three populations of cells: the ones actively dividing (cycling cells), the ones optionally out of the cell cycle (G_0 cells), and the ones that are destined to die without further divisions. The third group was all but neglected until recently, when apoptosis suddenly became the fashionable way for cells to die. But Bresciani et al. (1974) had shown a long time ago that even tumor cells die in large amounts, unfortunately not large enough to cause the tumors to regress. From Figure 1.1, we can draw a generalized rule for any increase in cell number. The three mechanisms that produce an increase in cell number of any given cell population are (1) a shortening of the cell cycle, i.e., the cells divide more frequently; (2) more cells participate in the cell cycle, i.e., a decrease in the G_0 fraction; and (3) a decrease in the rate of cell death.

The next question is whether an increase in cell number is the only mechanism for tissue and organismic growth. A tissue can grow by (1) increasing the number of cells, (2) increasing the size of the cells; or (3) increasing the amount of intercellular substance. Because intercellular substance of a tissue, for example, collagen or bone, is usually a secreted product of the cell, it can be considered as an extracellular extension of the cytoplasm. We can therefore take an increase in intercellular substance as a variation of an increase in cell size and reduce tissue growth to two mechanisms, growth in size and growth in the number of cells. This is true regardless of whether we are dealing with normal or abnormal growth, with the intact animal or with cells in culture. However, although both mechanisms may be operative, an increase in cell number is (with very few exceptions) by far the most important component in either

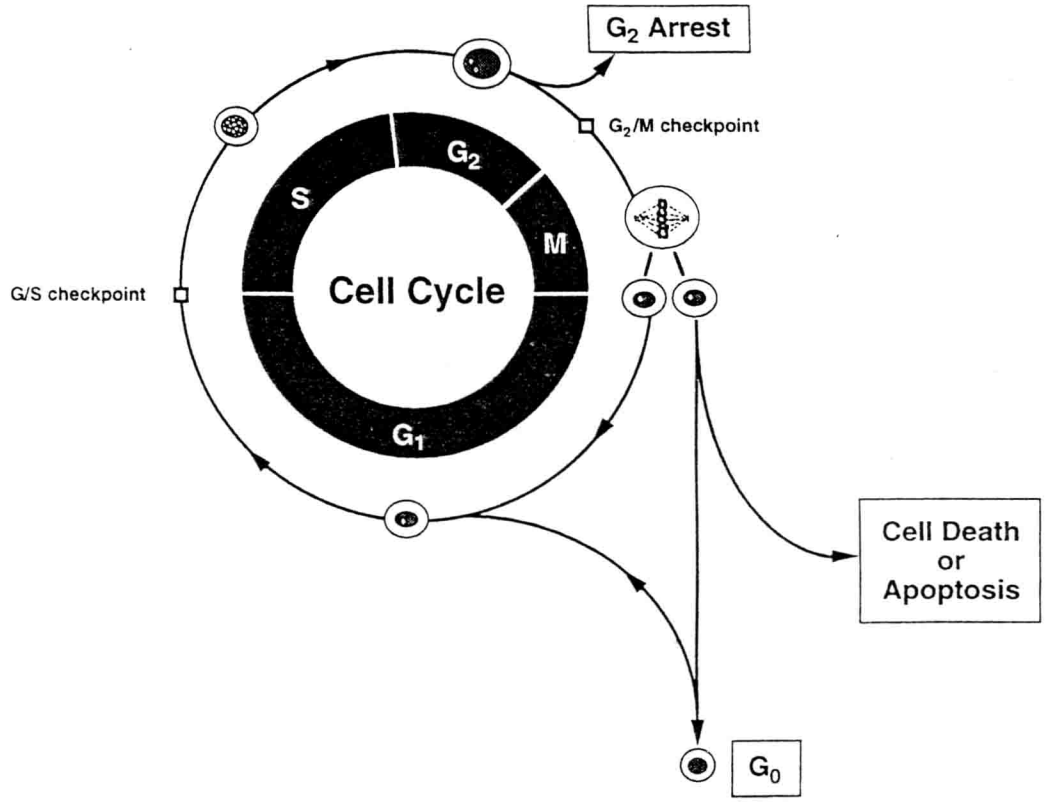

Figure 1.1. Cell Cycle. Note that, as the cell progresses through the cell cycle, there is also an increase in size until, just before mitosis, the size of the cell is twice that of an immediately postmitotic cell. G_2 arrest results in cells with tetraploid amounts of DNA.

normal or abnormal growth (Table 1.1). In abnormal growth, all three mechanisms are operating, but the bottom line is always the same: A tissue will grow only when the number of cells that are produced per unit time exceeds the number of cells that are lost in the same time.

There is another population of cells that deserves to be set apart, a typical example of the fact that some cells are more equal than others. The concept of stem cells originated with the pioneer work of McCulloch and coworkers. Their work identified in the bone marrow a category of pluripotent cells, capable of self-renewal, that could generate from a single cell all the lineages of hemopoietic cells (Becker et al. 1963). It is now accepted that stem cells are present in many other tissues, a reservoir of cells endowed with the ability to regenerate a cell population with several options for differentiation.

From Cell Kinetics to Molecular Biology

Until 1963, the cell cycle was essentially a study in cell kinetics, generating a great amount of useful information on a variety of cells, how long

TABLE 1.1. Postnatal Growth of Rat Liver

Age (Days)	Liver Weight (g)	Protein per Cell (pg)	DNA per Cell (pg)	Cell No. $\times 10^{-6}$
10	0.30	29.3	5.9	168
41	5.7	103.0	11.1	1,060
80	8.1	154	11.4	1,270
182	12.0	155	11.4	1,790

All values are averages of several animals. The protein content is a good measure of cell size. The DNA values at 41 days or after indicate that liver cells undergo polyploidization. Note that whereas cell size stops increasing at day 80 (or before), the number of cells keeps increasing.

Source: Adapted from Baserga (1985).

their cell cycles were, the different phases, the growth fraction, the whole properly spiced with mathematical formulations that looked very clever, but, in reality, were largely descriptive and had little predictive value. It was Irving Lieberman with his collaborators who first suggested that beneath the G_0, G_1, and G_2 phases were a series of biochemical events (Lieberman et al., 1963). A review by Baserga (1968) codified these first experiments and clearly showed that beneath the cell cycle was a whole textbook of biochemistry.

The obvious next steps would be to move to genetics and molecular biology. The genetics of the cell cycle were first studied in the seminal work of Hartwell and collaborators with the yeast *Saccharomyces cerevisiae.* Hartwell (1971), using temperature-sensitive (ts) mutants, showed that cell cycle progression was regulated by the timely expression of specific gene products. Similarly enlightening were the studies of Paul Nurse and coworkers (Beach et al., 1982) with another yeast, *Schizosaccharomyces pombe.* It is regrettable that while the use of ts mutants of the yeast cell cycle generated exceedingly valuable information, ts mutants of mammalian cells were much less informative, with very few exceptions (for a review, see Baserga, 1985). While in yeasts gene expression is cell cycle regulated, it quickly became apparent that in mammalian cells gene products were not only cell cycle regulated but also growth regulated, i.e., they were expressed only in growing cells (for the distinction, see below). Although Cochran et al. (1983) were the first to identify gene products whose expressions was regulated by growth factors, Calabretta et al. (1986) were the first ones to molecularly clone and publish a complete sequence of a mammalian growth-regulated gene (a calcium-binding protein, subsequently called *calcyclin*), i.e., of a gene whose expression was regulated by growth. This first report was followed by many, many others, until now there must be more than 500 genes whose expressions can be said to be growth regulated (see, for instance, the review by Hofbauer and Denhardt, 1991).

The latest developments, regarding cyclins, cyclin-associated proteins, oncogenes, tumor suppressor genes, signal transduction from growth

factor receptors, are part and parcel of the present book, and, as such, they do not belong to a historical survey, but to the present.

RULES AND REGULATIONS OF THE CELL CYCLE

The progress of science depends, at least in part, on the fact that scientists define their terms, which makes for economy of language and for understanding across language barriers. Spirits can be heavenly or alcoholic, a musical pitch varies, the soprano prefers it low, the violinist likes it high, but when we say *cell cycle*, all biologists understand what we mean by it, even though the expression may not be perfectly accurate (years ago, a change was proposed to *nuclear cycle;* it really does not matter, as long as we define the term). So, it is helpful if we define terms related to the cell cycle, i.e., if we establish a few rules and regulations, some of which have been consecrated by use, others by common sense.

Mitogenicity

Cells synthesize DNA (i.e., enter S phase) before dividing (mitosis), but cells can synthesize DNA and not divide, something that occurs not too infrequently in both normal and abnormal conditions. Most investigators will say that, for instance, a growth factor is mitogenic because it increases the incorporation of ^{3}H-Tdr into an acid-insoluble fraction of the cell. What the incorporation of ^{3}H-Tdr into an acid-insoluble fraction measures is the incorporation of ^{3}H-Tdr into an acid-insoluble fraction, period. It is very distantly related to mitogenicity and is not even an accurate measurement of how many cells are actually entering S phase. Growth factors increase the permeability of cell membranes (Pardee et al., 1978) and stimulate kinases (e.g., thymidine kinase), which, in turn, change the deoxyribonucleoside triphosphate pool (Walters et al., 1973) so that a measurement of thymidine incorporation into an acid-insoluble fraction can tell us as much about membrane function as about DNA synthesis.

Determining the number of cells entering S phase (by autoradiography, or by FACS analysis, or by bromodeoxyuridine [BUdR] incorporation) is much better, but it still tells us only how many cells enter S phase, not how many cells divide. If we wish to classify a growth factor, or its receptor, as mitogenic, we have to show that it induces mitosis, and by far the best way to show this is by the very old-fashioned method of counting cell numbers. One has to be careful, though, because we are used to a six- to eightfold increase in thymidine incorporation or a 20-fold increase in the number of cells labeled by either ^{3}H-Tdr or BUdR, whereas the increase in cell number may only be one- or twofold. The point is that, when the number of cells labeled, for instance, by BUdR in 24 hours, goes from 1% in unstimulated cells to 95% in stimulated cells, the increase in cell number will only be a doubling. The choice depends on the investigator's taste, whether he or she prefers a large

increase in a less accurate value or a modest increase in the correct measurement.

In theory, the number of mitoses ought to be the best indicator of mitogenicity. However, mitoses are fleeting; in most cells they last only 45 minutes, and, unless one looks at precisely the right moment, one can miss them. Furthermore, the duration of mitosis can increase in certain cells, especially in transformed cells (Sisken et al., 1982). Everything else being equal, if the duration of mitosis in cell line A is twice that of cell line B, the number of mitoses in A will also be twice that of B, although the two cell lines may grow at the same rates. In tissues, the number of mitoses per 1,000 cells (the mitotic index) is a reasonable measure of the *proliferating activity* of a cell population, but not of its growth. For instance, in the crypts of the lining epithelium of the small intestine, there are many mitoses. Fortunately for us, the small intestine in the adult individual does not grow, because, for every new cell produced in the crypts, one dies at the tips of the villi. Again, the most reliable method of assessing an increase in cell number is the counting of cells.

The Wind and the Leaves

One of the most charming suggestions I ever read was James Thurber's poetic insistence that it is the leaves of the trees that generate the wind, because every time the leaves move you can feel the breeze coming. Some scientists are like James Thurber: They identify a growth-regulated gene, and they present it as a growth regulatory gene. The two things, of course, are not mutually exclusive, but they are not the same thing, either (for a discussion, see Baserga, 1990). The best illustration is the regulation of expression of the thymidine kinase gene. In growth-regulated cells (like 3T3 cells and human diploid fibroblasts) the expressions of thymidine kinase RNA and protein are not only growth-regulated, but are exquisitely cell cycle regulated (Coppock and Pardee, 1987): They both increase at the G_1/S boundary and decrease when the cell reaches mitosis. Yet thymidine kinase is no growth regulatory gene product: It is not even necessary for cell proliferation; many cells and even some animals grow perfectly normally in the complete absence of thymidine kinase (discussed in Baserga, 1985). There are many genes like that; in fact, one ought to say that if the expression of a gene changes during the cell cycle, or even under different growth conditions, all it tells us is that the expression of that gene is growth regulated or cell cycle regulated. To be growth regulatory, a gene must be capable of altering the growth rate of a cell population.

Distinction Between Cell Cycle–Regulated and Growth-Regulated Genes

This brings us to another important distinction if we wish to understand each other, especially in studies dealing with mammalian cells, and this

is the distinction between cell cycle–regulated and growth-regulated genes (Hofbauer and Denhardt, 1991). There are certain genes that are cell cycle regulated (i.e., the proteins are expressed in a cell cycle–dependent manner). For instance, in most mammalian cells, thymidine kinase (Coppock and Pardee, 1987) and histones (Plumb et al., 1983) are expressed only during the S phase of the cell cycle. However, there are many other genes that are simply not expressed in nongrowing cells (G/0 cells) but are differentially expressed when the cells are stimulated to grow. However, once activated, these genes are not cell cycle regulated; in other words, they continue to be expressed as long as the cells are proliferating, without any particular localization to one or the other phase of the cell cycle. Typical of these genes are, for instance, c-myc, vimentin, ornithine decarboxylase, and many others (see review by Hofbauer and Denhardt, 1991). The distinction between these two types of regulated genes is important because it implies diverse regulatory mechanisms.

When Is a Gene a Regulatory Gene of the Cell Cycle?

Thymidine kinase is superfluous: You can delete the gene, and the cells will still grow in a perfectly normal way (see above). But suppose you find another gene that, deleted or inhibited, causes growth arrest; can you call it growth regulatory? This is an important question because there are many gene products in this category, notably the DNA synthesis genes, the cyclins, the cyclin-associated proteins, protooncogenes, and, indeed, many other gene products. Literally, we can say that these genes are growth regulatory because when they are inhibited or suppressed the cells are arrested in their cell cycle progression. But the cells will also stop cycling if you decrease the supply of adenosine triphosphate (ATP), and there are a great number of gene products that behave similarly: When they are inhibited (e.g., by antibodies, or dominant negatives, or antisense oligodeoxynucleotides, or in other ways), the cells stop their progression in the cell cycle. If anything that is required for cell cycle progression is growth regulatory, then we should include ATP and at least a thousand other molecules in this group. Such a broad definition would have scarce informational content (Baserga, 1990).

Perhaps a more rigorous definition of a growth regulatory gene is in order. I have a suggestion. Let us call the genes that are required for cell cycle progression exactly that: *genes required for cell cycle progression.* And let us reserve the term *growth regulatory* only for those genes (a handful) that actually decide whether a cell will enter the cell cycle or not. Let me illustrate this suggestion with an analogy: (1) You can drive a car without windows. It may not be comfortable in winter, but it can be done. That is thymidine kinase. (2) You cannot drive a car without the steering wheel, the four wheels, the engine, the differential, or gasoline. These items are the equivalent of the genes required for cell cycle progression. (3) But you can have all this, and more, and the car, shining in all its splendour, will sit in G_0 in the showroom of the car

dealer. Somebody has to turn the ignition key and press the gas pedal to move that car, and that is, for me, a growth regulatory gene. The fact that, under physiological conditions, I think that growth factor receptors activated by their ligands qualify as growth regulatory may reflect my prejudice, or it may not. Inside the cell, the best candidate for growth control is probably the inhibitor of cyclin-dependent kinases, $p27^{kip1}$ (Sherr and Roberts, 1995; Russo et al., 1996).

UNIVERSALITY OF THE CELL CYCLE

The Cell Cycle in Different Cells

I do not know of any *proliferating* eukaryotic cell that does not have a cell cycle. In fact, prokaryotic cells also have a cell cycle, which becomes similar to that of eukaryotic cells when the growth of bacterial cells is slowed down by nutritional manipulations. In this case, bacteria show the presence of a G_2 phase, although the S phase still occupies a preponderant fraction of the cycle (Helmstetter, 1971). Furthermore, from bacteria comes the discovery of thymineless death, which is now used extensively both at the basic science level and for therapeutic interventions. The yeast cells have been a gold mine for our understanding of the cell cycle (see Section 1.1.3.), and the amount of information and insight provided by the use of yeast cells cannot be overemphasized. Their greatest contribution has been the discovery and the elucidation of the role of cyclins and cyclin-associated proteins in cell cycle progression. Because these are the subject of another chapter, I will not discuss them here, but this should not be taken as a disregard for them. On the contrary, I will venture to say that the yeast cell cycle has provided the frame for our understanding of the mammalian cell cycle at the molecular biological level.

Returning to mammalian cells, there are cells that do not have a G_1 phase and others that do not have a G_2 phase, but all dividing cells have three features that are universal: They must grow in size, they must replicate the genetic material, and they must undergo mitosis. Growth in size is self-evident: If the cells were not to double in size before dividing, they would get smaller and smaller at every division and eventually vanish. In yeast, the evidence is substantial that cell size is a determinant for entry into S phase and mitosis (Hartwell, 1978). In mammalian cells, one can occasionally have division without concomitant growth in size, but it is exceptional and hardly normal. The S phase, during which the genetic material is replicated, is obligatory, although the duration may vary from a few minutes in embryo cells (Graham and Morgan, 1966) to several hours in tumor cells (see references in Baserga, 1985). Needless to say, the whole cell cycle in early embryo cells is also very short, something like 14 minutes.

Some cells may grow in size, double their DNA content, and stop in G_2. This happens not unfrequently during normal development, especially in