Overview

All definitions of cancer stress the defective regulation of cell growth and differentiation because such physiological aberrations underlie the gross derangements of the disease. An important goal of basic cancer biology is to provide molecular explanations for these defective cellular processes. With recent developments in molecular and cellular biology, investigators have obtained penetrating insights into cell processes and their respective derangements. These are being investigated at four experimental levels: genes and their mRNAs; growth factors and their receptors; biochemical regulation; and cell biology in culture. The results are interrelated and integrated within the framework of the classical cell proliferation cycle.

Cell proliferation, the creation of two cells from one, is the culmination of a vast number of biochemical events which are needed to duplicate all of a cell's components. Among these processes in the cell only a few are regulating pacemakers; the rest are controlled although they may be essential for growth. It is extremely important to our further understanding of growth control to discover the regulating events and the molecules involved in them. Current studies demonstrate that there are a limited number of such events, located at definite points in the cell cycle. These regulatory processes are deranged in cancer cells; they are the ones on which our research focuses.

Within the context of these general thoughts, research from our laboratory and others has led to several conclusions. A major regulatory control occurs prior to the entry of cells into S phase. This event appears to regulate not only the onset of DNA synthesis but other events which take place simultaneously, such as syntheses of histones and specific enzymes involved in DNA synthesis (e.g., thymidine kinase). Cells are committed to complete their DNA duplication about 2 h before the onset of S phase. At this point (the restriction point) they can go on to make DNA in the absence of external growth factors. Proliferation is thus controlled by events during G\textsubscript{1} rather than by processes occurring during the S, G\textsubscript{2}, or M portions of the cell cycle. These latter cycle stages are terminal and necessary for production of a new cell but they appear much less dependent upon regulatory molecular processes than are G\textsubscript{0} and G\textsubscript{1} events.

Some regulation-related events occurring prior to the restriction point have been identified. IGF-1\textsuperscript{1} is the only growth factor required by BALB/c 3T3 fibroblasts in the interval from 2 to 6 h prior to S phase. During this same time the ras oncogene is activated to produce its message. Studies with inhibitors show that cells cannot initiate S phase without rapid protein synthesis during this prerestriction point interval. The data strongly suggest that normal cells need to make a critical amount of a particular protein that is quite unstable, having a half-life of about 2.5 h, in order to pass the restriction point. Various tumorigenic cells behave as if this protein is stabilized or made in excess, suggesting that greater availability of this protein may permit the escape of cells from growth control.

The proposal that a labile protein is necessary for proliferation of normal cells and that its levels are altered in tumorigenic cells led us to examine proteins on two-dimensional gels. A candidate protein which was unstable in normal cells but relatively stabilized in tumorigenic cells was identified. It has been shown to possess a molecular weight of 68,000. This protein is one of a very small number closely linked to cell proliferation during G\textsubscript{1}. Our work in progress is designed to further identify this protein, clone its gene, and study its properties in normal cells that have been transfected with this gene.

We are also investigating events following the restriction point. The rather long time interval (about 2 h) between the restriction point and the onset of S phase suggests that some complex process must occur, one that appears not to require de novo RNA synthesis. During this time some enzymes needed for DNA synthesis become activated. We have proposed that these and other proteins are assembled into a multiprotein complex which is the actual machinery for synthesis of DNA. This assembly process may be triggered at the restriction point after an ample supply of protein p68 has been made in the cell. p68 possibly could thus catalyze production of a component that "glues" the multienzyme complex together.

A Restriction Point Protein

Information regarding the regulation of cell growth has been summarized frequently. An excellent recent review is in a book by Baserga (1). The principal guides to my thinking on the problem of growth control in normal cells and its derangement in cancer cells are briefly stated at the beginning of this article, and they have been discussed more extensively in several recent reviews (2, 3). This information and these ideas will not be presented extensively here. Instead, I will discuss what I consider to be the central component of this control system, namely the agent responsible for activation of the onset of DNA synthesis, a prime growth-controlled event in the cell cycle. Discussion of our discovery of a possible central regulatory protein will be followed by brief comments on the events that lead to its production and the events that it can subsequently trigger.

Numerous experiments using inhibitors have demonstrated that protein synthesis is essential for cell cycle transit. The cells must be able not only to synthesize proteins but to make them rapidly. This requirement is specific for the G\textsubscript{1} period of the cell cycle, the part during which cells prepare for the synthesis of DNA and the other events in S phase. This particular sensitivity to protein synthesis inhibitors during G\textsubscript{1} led to the idea that an unstable protein with a short half-life (2.5 h) is essential in order to initiate S phase (4-6).

During a sabbatical year (1972-1973) in the laboratory of Dr. Michael Stoker I investigated conditions that differently affect the ability of untransformed versus transformed cells to transit the cell cycle (7). Prominent among the conditions that preferentially inhibited the growth of nontransformed cells were those that limit protein synthesis, such as shortages of essential amino acids or low concentration of cycloheximide-related pro-

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2 The abbreviations used are: IGF-1, insulin-like growth factor 1; p68, a protein with a molecular weight of 68,000; TK, thymidine kinase.
tein synthesis inhibitors. These results suggested that an unstable regulatory protein may be more stable in the transformed cell. Further work in my laboratory by Drs. Peter Rossow, Veronica Riddle, Judith Campisi, and Estela Medrano extended these findings and provided kinetic data consistent with the increased stability of a regulatory protein in tumor cells (4, 8, 9). At about the same time we demonstrated a similar phenomenon in yeast, attesting to the generality of labile regulatory proteins in the cancer biology of G₁ transition and S phase entry (10). We found that the labile protein of yeast has a half-life of only about 10 min in contrast to the 2 to 3 h half-life reported with mammalian fibroblasts. Popolo and Alberghina (11) and Popolo et al. (12) using two-dimensional gels have identified such a labile protein in yeast which seems to satisfy the criteria that we had proposed for the regulator of the start event.

These studies have all pointed to the central role of a labile protein acting specifically at the onset of DNA synthesis to regulate the progression of cells through the cycle. Such a protein, however, had not actually been identified. The aforementioned kinetic and inhibitor studies had yielded the following three major properties which were used to seek for and identify such a protein: (a) the regulatory protein should be produced in G₁ of the cell cycle; (b) in normal cells this protein should be unstable with a half-life of 2–3 h; and (c) the protein should be altered in transformed cells so that its apparent stability is increased.

Dr. Robert Croy, working in my laboratory, then set out to find a protein that satisfied all of these criteria (13). Using the technique of two-dimensional gel electrophoresis he observed at least 1000 spots. Among these about a dozen satisfied the criterion of being made during G₁, but not by resting cells. Pulse-chase experiments revealed that most of these proteins in normal cells had half-lives of longer than a few h; only one of the dozen G₁-related proteins was unstable. Furthermore, this protein alone was made in large amounts in transformed cells, where it was stable for at least 8 h. This protein has been called p68 because of its molecular weight of 68,000. It has an isoelectric point of 6.3.

We consider p68 to be of special interest because its properties coincide so well with the conditions we defined, and because it is synthesized so differently by normal cells as compared to a variety of transformed cells. Few other proteins have been identified that satisfy even one of these criteria. Enzyme activities and messenger RNAs have been reported that differ in normal versus transformed cells or that are made specifically in the pre-S phase part of the cell cycle. However, since none of these proteins satisfy the three criteria outlined above, they are probably not regulatory proteins. It is more likely that they are the consequences of regulatory events (e.g., housekeeping enzymes needed for processes such as nucleotide synthesis).

Our current work is aimed at testing the role of p68 in growth regulation. In order to accomplish this goal we have purified this very rare protein and commenced production of monoclonal antibodies. Our ultimate goal is to clone the gene for p68 and make antibodies to the purified protein. These tools will enable us to examine its role in cell cycle progression, its location in the cell, and its properties.

Events Leading to the Synthesis of p68 and the Subsequent Onset of S Phase

Cell proliferation is primarily regulated by extracellular factors. Prominent among these are growth factors such as platelet-derived growth factor, epidermal growth factor, and insulin-like factors (IGF-1) which appear to act sequentially to bring the cells out of the quiescent state and into S phase of DNA synthesis. This emergence of cells from quiescence involves the activation of transcription and protein synthesis (1). This is interesting from our point of view since rapid protein synthesis is essential for the later G₁ events, as discussed above. In the early stages of the G₀ to S-phase transition, the cell is put back into working order after a period of quiescence which has caused the gradual disappearance of labile components of the cell’s metabolic apparatus. It is well known that a longer time is required for cells to traverse the G₀ to S-phase process than is required for cycling cells to pass from mitosis to S phase. In mouse 3T3 cells the G₀ to S-phase time is 12 h, whereas the time from M to S phase is only 6 h. Very likely some of the events that transpire during the 6 extra h in the former situation occur in cycling cells during the prior cycle.

Events leading more directly to DNA synthesis occur during the latter part of the G₀ to S-phase transition approximately 6 h prior to DNA synthesis. This period corresponds to the M to S-phase part of the cycle in proliferating cells. The production of adequate amounts of labile protein(s) including p68 occurs during the first 4 h of this G₁ period. Synthesis of these protein(s) requires the presence of growth factors (14, 15) and is blocked by mild inhibition of protein synthesis. Experiments are currently in progress to determine which growth factors are most responsible for progression through G₁. It has been found that epidermal growth factor seems to be essential for early G₁ events. However, IGF-1 is the principal growth factor required by mouse cells during late G₁, since within about 6 h of S phase these cells can progress when only IGF-1 is supplied (9, 16).

Relatively little else is known about specific events during this 6-h pre-DNA synthetic period except that during this time the ras oncogene is activated (17) and appears to be essential for cell progression, as shown with microinjected antibody (18). Also, except for the production of the p68 protein, differences during this period between normal and cancer cells remain largely unknown.

Events That Follow the Restriction Point

The cell’s metabolic requirement(s) change dramatically at about 2 h before the onset of S phase (8, 15, 19). After this time, serum and growth factors are not required to complete the cycle, and neither is rapid protein synthesis (8). We have designated this point of commitment to begin S phase as the restriction point (7). We consider it as a key regulating event in the cell cycle, because after cells come within about 2 h of the onset of S phase, their progression through the remainder of the cycle and into the next G₁ becomes essentially automatic and uncontrolled by extracellular factors. Although it is possible to stop cells in this latter part of the cycle (e.g., with inhibitors of DNA synthesis such as hydroxyurea), no major physiological controls have yet been reported for cell lines in culture such as we have been studying.

It is remarkable that approximately 2 h transpire between the loss of serum dependence and the onset of S phase. What happens during this time? We have been investigating this question using two different approaches. One is to study the production of enzymes that are known to be needed for DNA synthesis, and the other is to investigate the assembly of these enzymes into a multienzyme complex which functions to synthesize DNA.

For many years it has been known that histones and certain
DNA-related enzymes are synthesized at the onset of S phase. Therefore, importantly, S phase is not synonymous simply with DNA synthesis. Rather a set of more or less tightly coupled events occur at the same time (20, 21). For example, histone synthesis commences with and is tightly coupled to DNA synthesis, primarily through changes in the stability of histone mRNAs (22): if DNA synthesis is arrested by an inhibitor, histone mRNA decays very rapidly and histone synthesis then comes to a halt, providing another example of control through lability.

A different mechanism is involved in turning on the production of certain enzymes that are involved in DNA synthesis. Several of these enzymes increase dramatically at the onset of DNA synthesis. TK is one such enzyme which has been studied in our laboratory (20). The activity of this enzyme increases at least 40-fold during S phase. The enzyme is not obligatory for DNA synthesis (as shown with thymidine kinase-negative mutants), nor is DNA synthesis essential for its appearance since DNA synthesis inhibitors (such as hydroxyurea) do not block increases in TK activity.

Our most recent studies have centered on regulation of the mRNA for thymidine kinase. This increase in TK mRNA, which occurs at the beginning of S phase, is partly due to an enhanced transcriptional rate. We have reported enhanced transcription as a mechanism for increasing levels of TK mRNA. However, there also appears to be a strong component of stabilization of this mRNA or its precursor, so that the amount of TK mRNA is dependent on both its increased rate of production and a decreased rate of degradation. Other mechanisms such as more efficient nuclear processing or increased mRNA transport may also contribute to the accumulation of this message during S phase.

An important consequence of studies on DNA synthetic enzymes is the opportunity to investigate cell cycle regulation by working backwards from a measurable event turned on at the beginning of S phase. In particular, the appearance of TK mRNA provides a tool for studying growth control. It will evidently be much easier to study the mRNA regulation of a single gene, which is dependent upon transcriptional activation, stabilization, and processing, than to study the yet incompletely understood and multicomponent process of DNA synthesis. By investigating the molecular events which control the production of an enzyme such as TK, we will learn about molecules produced during G1 that regulate various coordinated events of S phase. This knowledge will thereby permit us to understand more completely molecular biology of growth control.

Our second approach to post-restriction point events is to investigate the multienzyme complex that is required for DNA synthesis. Many data over the past decade, starting with work by Seki and Muller (23) and Baril’s laboratory (see Ref. 24), have suggested that DNA is made in eukaryotic cells by a multienzyme complex rather than by soluble enzymes. Dr. G. P. V. Reddy and I have extended this concept to include not only the enzymes directly involved in DNA synthesis, such as DNA polymerase, topoisomerases, primase, etc., but also enzymes of DNA precursor synthesis including ribonucleotide reductase, thymidine kinase, thymidylate synthase, and others (25). Various replisome enzymes may or may not be present during G1; also, those found then are principally located in the cytoplasm. We propose therefore that assembly of the replisome complex occurs during the 2-h period between the restriction point and the onset of S phase and that the complex, once assembled, permits DNA synthesis. It is of great interest to learn what mechanism is responsible for the assembly of such a complex. Is it the final synthesis of some glue molecule which provides nucleation for replisome assembly? Is it controlled by spontaneous intermolecular forces (much as the classical work of Dr. Nomura has shown for ribosome assembly)? And, finally, what relation does this multienzyme complex have to the nuclear matrix, to which several enzymes, including DNA polymerase-α, are at least in part bound?

Some of our recent work to determine answers to the above queries has shown that the activation of thymidylate synthase, a component of this replisome, occurs an hour or so before the onset of S phase and does not require protein synthesis (21). This result is consistent with activation of this enzyme being related to its assembly into the complex. Furthermore, studies by us and others have shown that the activity of thymidylate synthase in intact cells is strongly inhibited by drugs that directly inhibit ribonucleotide reductase, DNA polymerase-α, or DNA topoisomerase II (27). Our explanation for this indirect inhibition, which does not occur in extracts, is that it is caused by allosteric interactions between thymidylate synthase and the other, inhibited enzymes within the replisome complex. All of these data suggest an important role in DNA synthesis for multienzyme complexes and therefore, between assembly of the complex and regulation of the cell cycle at onset of DNA synthesis.

On the Dynamic State of Regulatory Processes

One generalization that arises from these studies is the importance of dynamic states in regulation. This dynamic state is a reflection of the age-old principle of Ying and Yang, in which all the world’s processes are considered to be balanced between active and passive components. Again and again, at every level of cell biology and biochemistry, one observes that molecules such as mRNAs, proteins, and phosphorylated compounds are synthesized and broken down, or phosphorylated and dephosphorylated, etc. (28). At first glance these processes appear to be wasteful of metabolic energy. However, they can be justified upon realizing that they permit rapid adjustments of a system to altered conditions and therefore are vital for the maintenance of tight control of important processes.

According to this reasoning, a substance subject to both rapid synthesis and degradation exists in a steady state, which in this dynamic system is highly dependent upon both inflow and outflow rates, and so its amount changes dramatically owing to small changes in either of those rates. An analogy that comes close to home is the dynamic state of one’s checking account, which can fluctuate widely depending upon the amount of input and output. Similarly in cell cycle regulation, the dynamic state of the p68 protein in untreated cells could allow rapid responses to growth-controlling substances depending upon
events which govern its production, such as rates of protein synthesis and availability of growth factors, and also on other events that determine its stability. Stabilization of this protein in tumor cells would therefore increase the steady state level of p68 and result in relaxation of growth control. Given this apparent power to modulate concentrations of regulatory constituents, it seems apparent that the dynamic state will prove to be a major principle for biology of regulation at every level.

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References

Molecules Involved in Proliferation of Normal and Cancer Cells: Presidential Address

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