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ACKNOWLEDGEMENTS. I thank the members of my laboratory who have participated in the determination of the structures described here: Brian Guenther, Dorothea Kominos, Xiang-Peng Kong, Talleru S. R. Krishna, Jennifer L. Martin, Scott Robertson, Gabriel Waksman and Lim Wong. I also thank my colleagues and collaborators, David Baltimore, Stephen K. Burley, David Cowburn, Hidesaburo Hanafusa, Peter Model, Mike O'Donnell, Rene Onrust, Greg Petsko, Marilyn Resh, Robert M. Sweet and Charles Williams, Jr. This work was supported in part by the National Institutes of Health and the Pew Memorial Trust.

Received 21 December 1992; accepted 22 December 1992.

Recent developments in cell cycle regulation

G. Shanmugam

Cancer Biology Division, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

Cell cycle of normal cells consists of precisely regulated series of events. Check points in each phase of the cycle enable the cells to complete specific functions before they transit to the next phase. Earlier studies emphasized the G0 to G1 transition as a crucial stage in cell proliferation; however, recent findings indicate decision-making points also at G1 and G2/M phases. Growth factors, oncogenes and cyclins are the major players regulating the cell cycle; they regulate the cycle by influencing phosphorylation and transcription processes. Entry into and exit from the cell cycle are determined by several processes such as the synthesis of new proteins, phosphorylation/dephosphorylation of pre-existing proteins and degradation of proteins like cyclins. Deranged regulation of cell cycle is the essence of neoplastic development.

A new-born cell has several options for its future depending on the program it has and on the signals it receives from the environment. When the signals are appropriate, the cell enters into the cycling process called cell cycle; if conditions are not favourable, the cell enters into a state of latency called quiescence. When the cell comes across differentiation factors, it starts to differentiate. Most normal cells have definite life span which is roughly equivalent to their division number *in vivo*; however, some of these cells overcome the rule of definite life span and get immortalized acquiring the capacity to divide infinitely. Programmed cell death (apoptosis) is another option in the life of cells. Since the cell cycle is a remarkably regulated phenomenon of normal cells and this type of regulation is often lost in the proliferation of cancer cells, the salient features of normal cell cycle and the various aspects of regulation of this cycle will be covered in this article. The objective of this review is to expose various aspects of recent developments in cell cycle research to an interdisciplinary readership. References of reviews on specific aspects of cell cycle are presented at appropriate places for those who seek detailed information on one or more aspects of cell cycle. Unless otherwise stated, the studies discussed in this review were from cultured mouse embryo fibroblasts or mouse fibroblastic cell lines (Balb/c 3T3).

The cell cycle of a growing cell is the period between the formation of the cell by the division of its mother cell and the time when the cell itself divides to form two daughters[1]. It is a fundamental unit of time at the cellular level since it defines the life cycle of a cell. A plethora of investigations into the temporal organization of cell division in a variety of cell types revealed that control of cell proliferation is principally determined in G1 phase of the cell cycle[2], and mitosis (M), cytokinesis and DNA synthesis (S) are other landmarks[3,4]. The temporal gaps separating cell division (mitosis) from S phase are designated as G1 from one side and G2 from the other side of the cycle. Modifications to this general concept were introduced to accommodate the G0—a state in which cells may exist for long periods of time in a quiescent stage[5,6].

Recent studies, however, show the presence of several stages which can be termed as sub-phases or check points[7], suggesting that the classical designation of four phases (G1, S, G2, M) may only serve as an organizing

principle. A comprehensive outline of different molecular and regulatory events that occur at different stages of the cell cycle are depicted in Figure 1.

The signal cascade

Normal cells enter into a quiescent state called G0 under unfavourable conditions like nutrient shortage or overgrowth. The growth-arrested cells constantly receive signals from the environment in the form of ion transport, changes in pH and mitogens[5]. These signals are compiled by the resting cells which then decide whether to enter into proliferative phase or not. When G0 cells are triggered to enter into G1/S phase by mitogens a complex series of molecular events occur which culminate in DNA synthesis[6].

The first step in signal transduction is the binding of growth factors with receptors. This binding results in the down regulation and autophosphorylation in the tyrosine moiety of receptor[9]. The secondary response of interaction of growth factors is the recruitment of cytosolic enzymes to the membrane site[10]. For example, in response to platelet-derived growth factor (PDGF) treatment, the PDGF receptor recruits phosphatidyl inositol-3-kinase to the membrane within a minute[10]. The association of cytosolic enzymes with PDGF receptor was shown to be dependent on autophosphorylation of tyrosine in position 751 of PDGF receptor. Receptor type and non-receptor type tyrosine kinases contain certain stretches of sequences called *src* homology (SH-2 and SH-3) domains[8]. Even proteins that are unrelated to tyrosine kinases contain SH-2 and SH-3 domains and these proteins (phospholipase c-γ[11], *ras*-GAP[12] *and crk*[13] *oncoprotein)* are involved in signal transduction. The SH-2 domains bind to tyrosine phosphorylated proteins while the SH-3 domains promote the binding of these molecules to membranes[8].

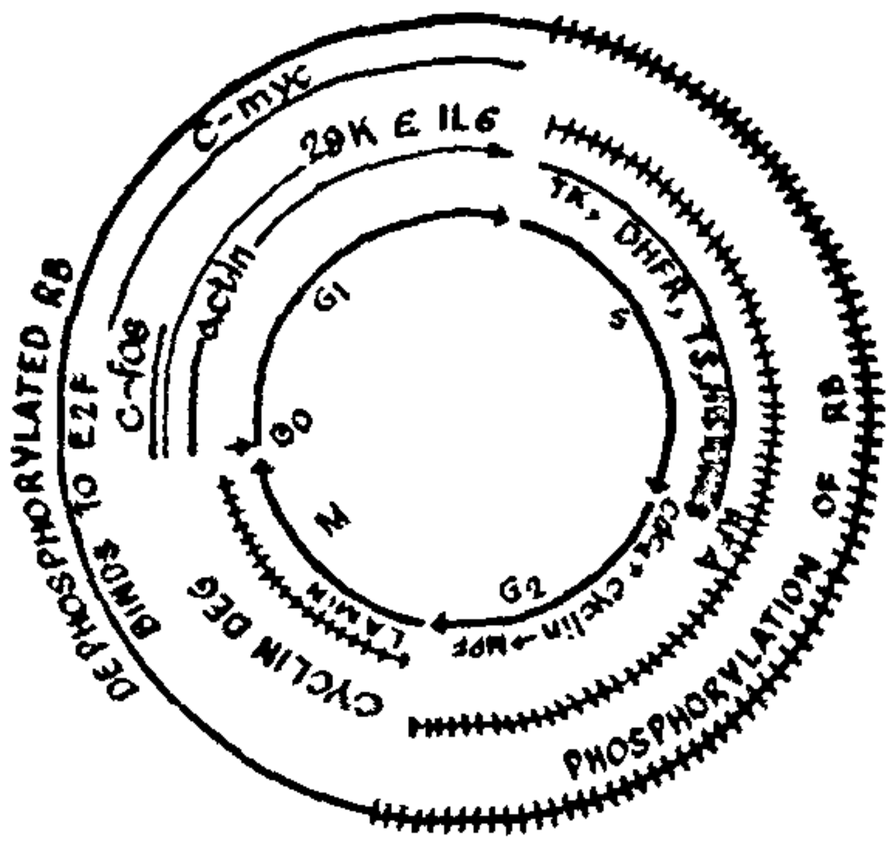

Figure 1. Molecular events of cell-cycle. C-*fos* and c-*myc* respectively refer to the cellular counterparts of feline osteo sarcoma virus and myelocytomatosis virus oncogenes; 29 K, a serum (PDGF) induced early growth response protein; IL-6, interleukin-6; TK, thymidine kinase; DHFR, dihydrofolate reductase, TS, thymidylate synthetase; RF-A, replication factor A; RB, retinoblastoma protein; cdc2, a cell division control gene; MPF, maturation promotion factor; cyclin DEG, degradation of cyclin; E2F, a transcription factor. START is a *decision-making point at which cells become committed to DNA* synthesis.

Among secondary responses of signal transduction, phosphoinositide metabolism plays an important role. For example exposure of quiescent cells to PDGF results in a fifty-fold increase in phospholipase C activity[14] (this enzyme is also activated by tyrosine phosphorylation). This enzyme acts on phosphatidyl inositol-4, 5-biphosphate which results in the production of intracellular second messengers inositol-1, 4, 5-*triphosphate and 1, 2-diacyl glycerol.* These molecules respectively mobilize stored calcium and stimulate protein C-kinase[14]. The kinase cascade continues further with the involvement of cytoplasmic S-6 and casein kinases which are serine–threonine kinases[15].

Some of the earliest responses elicited by the addition of PDGF and other growth factors to quiescent cells are an increase in Na^+ entry into cells by an amiloride sensitive Na^+/K^+ pump activity, increase of intracellular K^+ levels, pH and restoration of the electrochemical gradient for Na^+. An increase in the intracellular pH and K^+ concentration may play a triggering role in mitogenesis[16]. In addition to monovalent fluxes, stimulation of Ca^{2+} efflux from intracellular stores by activation of the plasma membrane Ca^{2+} dependent ATPase has been reported. Enhanced transport rates for various nutrients and ions were observed in the process of activation of quiescent cells[17–19].

Later stages of signal transduction include increased expression of oncogenes and early growth response genes[20–22]. The roles of these genes in cell proliferation are described below. The complex signal transduction process can be simplified into instantaneous membrane events, early cytoplasmic events, early nuclear events, late cytoplasmic events and late nuclear events (Table 1).

G1 events in signalling

The G1 phase of the cell cycle is the functional period during which cells prepare for S phase. Cell biological and biochemical studies revealed that cells require many hours to transit a series of G1 sub-phases starting either from G0 or quiescent state[23–25]. Extracellular factors determine whether a quiescent cell will begin to proliferate and also whether a proliferating cell will continue to proliferate or revert to quiescence. Cell cycle events become largely independent of these extracellular factors at the middle of G1; they become completely independent of exogenous factors from S

Table 1. Summary of signal transduction processes.

1. Membrane events:
   Growth factor—receptor interactions, *ras* activation, phospholipase C activation, conversion of PIP_2 to IP3 and diacyl glycerol, C-kinase activation, pH increase, Ca^{++} mobilization
2. Early cytoplasmic events:
   Activation of S6 kinase, casein kinase, *mos* and *raf* kinases, *raf* and RB phosphorylation during G0 and G1
3. Early nuclear events:
   Expression of early growth response genes like oncogenes *fos* and *myc* and transcription factors
4. Late cytoplasmic events:
   Action of early growth response genes and mitogen response factors
5. Late nuclear events:
   Interaction of various factors with genome and DNA synthesis

phase onwards[26,27]. Control of cell proliferation in cancer cells is lost mainly due to dysregulation of G1 phase events[28,29]. For example, most normal cells require efficient protein synthesis and growth factors to progress through G1 phase while transformed cells do not[28,29]. In addition to proliferation control, cell differentiation also is initiated in G1 phase with an inverse relationship between the two[30].

G1 events require many hours and they appear sequentially, terminating in DNA synthesis. Certain temperature-sensitive mutants are specifically blocked at different stages, thereby providing a means to sequence metabolic events[31]. G1 phase has been divided into sub-phases depending on the effects of limiting growth factors, nutrients and inhibitors as measured by the time to reach S phase after the block is removed. These sub-phases are competence, entry and progression separated by C, V and R points, respectively[32–36].

Competence and progression

Normal mouse fibroblasts do not progress to S phase from G0 phase if they are provided with either PDGF or platelet poor plasma (PPP) alone[32]. The cells progress to S only if they are treated with PDGF first and then with PPP, but not *vice versa*[32]. Quiescent cells exposed to PDGF become competent to replicate their DNA even if PDGF is removed from cell culture medium, suggesting that PDGF-induced competence state reflects a rapidly induced and relatively stable biochemical change within the target cells. Competent cells require several hours (12 h for 3T3 cells) to reach S phase[37]. Other competence-inducing factors include FGF, calcium phosphate, cycloheximide, and the *myc* oncoprotein[5].

Competent 3T3 cells incubated with progression factors like PPP, EGF, or insulin reach S phase in about 12 h. When essential amino acids are depleted in the medium, they reach to a point in G1 phase named V. After the amino acids are provided, the cells reach S phase in another 6 h. Continuous presence of these progression factors are necessary for their transition from competence phase[32].

The point in G1 at which commitment occurs and the cells no longer require serum growth factors to complete the remainder of cell cycle is called the restriction point (R)[32]. The commitment at R appears to coincide with the synthesis of a labile protein of Mr 68,000 which has a half-life of 2.5 h in normal cells and shows enhanced synthesis and stability in transformed cells[38].

Early growth response proteins

Protein synthesis plays a central role and is required throughout G1 phase until the cells enter S phase for the initiation of DNA synthesis. A three-fold increase in protein synthesis was observed within 60 min of serum-stimulation. The changes in gene expression induced by growth factors in quiescent cells are generally considered only as part of the mitogenic response. However, it is possible that many of these gene products are necessary to integrate and co-ordinate complex biological processes in which cell proliferation is a common event[39,40].

Electrophoretic analysis of [^{35}S] methionine labelled cell extracts from mitogen-induced BALB/c 3T3 cells revealed the selective synthesis of five unique proteins designated PI to PV of molecular weights 29,000, 35,000, 45,000, 60,000 and 70,000 respectively[41]. The 29,000 Da PI has growth-related properties consistent with its putative role as the cellular mediator of PDGF[41,42]. It was transiently induced upon mitogenic stimulation and has been identified as a nuclear protein tightly bound to chromatin and superinduced by cycloheximide treatment[43]. Maximum PI synthesis was observed between 2–4 h after addition of PDGF or other competence-inducing agents. Progression factors did not induce the synthesis of this protein. Constitutive synthesis of this protein was observed in transformed fibroblasts. PI was not induced in proliferating cells, indicating that it may function exclusively to facilitate the entry of G0 cells into the proliferation cycle[23].

Cochran *et al.*[44] have identified two clones JE and KC which are abundantly transcribed in PDGF-stimulated quiescent fibroblasts. They were the first PDGF inducible genes to be cloned in mouse fibroblasts. Three growth factor or serum-inducible genes (4F1, 2F1, 2A9) were identified from a cDNA library of 'temperature sensitive' mutant fibroblasts during their transit from G0 to G1 stage[45]. A set of five serum-inducible genes was identified and later, another five mRNA species of this class were cloned by Lau and Nathans[46,47] from mouse fibroblasts. Nearly 82 im-

mediate early mitogen-induced gene sequences were isolated by differential cDNA screening technique (by cross hybridization preferentially with cDNA derived from RNA of stimulated cells rather than with cDNA derived from non-stimulated cells); some of them were found to code for cytoskeletal proteins and zinc finger binding proteins[48].

In order to dissect early growth response genes that are involved in growth control from other mitogen-induced genes, we have successfully used dimethyl sulphoxide (DMSO) treatment[37,49]. Our results indicate that DMSO selectively inhibits the expression of early growth response genes such as *fos*, *myc* and *IL*-6 (ref. 37). Treatment of proliferating cells with 2% DMSO arrests them in G0 stage; the arrested cells transit to S phase synchronously when maintained in serum-containing medium.

Several of the mitogen-induced genes were found to be involved in the regulation of transcription; such genes are: c-*fos*[20], *fra*-1[50], c-*rel*[51], c-*myc*[51], c-*jun*[52], *junB*[53], *egr*-1[22] (krox-24), *zif*/268[54], *NFkB*[51], NGF1-B[55] and SRF[56]. The immediate early mitogen response gene products fall in two categories. The first group consists of leucine zipper proteins including the products of c-*fos*, *fra*-1, *fos* B, c-*jun*, *jun* B and the second group consists of zinc finger DNA binding proteins EGR1, NGF-A, krox24, EGR3 and EGR-4[57]. Presence of leucine zipper or zinc finger motifs is a common feature of transcription factors and induction of the expression of these genes in quiescent cells following exposure to mitogens indicates that the final stages of mitogenic stimuli involve the modulation of transcription process. The role of some of the mitogen-induced proteins (the *fos* protein) in transcription has been studied in detail. The *fos* gene product was found to be a nuclear phosphoprotein[51]. A protein complex containing the *fos* protein and *jun* protein (another mitogen-induced protein) binds to regulatory sequences in DNA which is identical to AP-1 sequence[51]. A few other *fos* related proteins such as *fos*-related antigens (*fra*) and *fos*-B were often found in crude AP-1 preparations[51].

Oncogenes

The first oncogenes to be identified as cell cycle regulated were c-*myc* and c-*fos*[20,21]. The expression of c-*myc* proto-oncogene was induced in serum-stimulated cells[58] and expressed constitutively in all phases of cycling cells[59]. The expression of c-*fos* was found to be rapid, transient and preceeds c-*myc* expression in serum-stimulated fibroblasts[20,21,60]. The c-*myc* oncogene encodes a nuclear protein which plays an important role in normal and abnormal cell proliferation. Prendergast and Cole[61] have identified *mr1* and *mr2* as *myc*-regulated genes. The *mr1* encodes for a plasminogen activator inhibitor-1 (PAI-1), a regulator of extracellular proteolysis[62]. This is the first cellular gene identified to be regulated by c-*myc* protein during cell proliferation.

Fra-1 (*fos*-related antigen) has been identified as an immediate early serum inducible-gene which encodes a protein having regions of extensive aminoacid homology with *fos* protein and can dimerize with *jun* protein in inducing transcription[50]. The proto-oncogene *jun* was induced in quiescent cells upon serum-stimulation during G0/G1 transition. The *jun* oncogene product was found to be a component of transcription factor AP-1 and is the first oncogene identified which codes for a transcription factor[52,63,64]. The murine cellular homolog (c-*rel*) of transforming gene of reticuloendothelial virus was identified as a transiently serum-induced immediate early gene[65] which reaches maximum levels at 1 h and declines to basal level by 3 h after stimulation.

The *ras* oncogene codes for proteins which bind to guanine nucleotides[66] with GTPase activity[67] and are related to G proteins of adenylate cyclase system[68]. The *ras* gene product is a 21 kDa protein associated with plasma membrane[69] which modulates the phosphoinositol turnover[70]. Microinjection of *ras* protein into quiescent fibroblasts induces membrane ruffling and fluid phase pinocytosis within 30 min to 1 h and is accompanied by stimulation of phospholipase A2 activity and DNA synthesis[71,72]. The oncogenic form T24 of the *ras* gene product has a reduced GTPase activity relative to proto-oncogene[73], and the reduction in enzyme activity correlates with the ability to stimulate DNA synthesis. It can induce DNA synthesis at concentrations at which normal p21 protein has no effect[71]. Nuclear microinjection of oncogenic and protooncogenic forms of *ras* DNA induces DNA synthesis in quiescent human cells but not in senescent cells[74]. Microinjected Harvey sarcoma virus genome and its product p21 v-Ha-*ras* protein. K-*ras* p21 protein and Kirsten sarcoma virus genome were shown to induce DNA synthesis in quiescent mammalian cells[75,76]. The *ras* family consists of three proto-oncogenes, H-*ras*, K-*ras* and N-*ras*[77,78] which can acquire oncogenic properties by single missense mutations usually at either codon 12 or codon 61 and prevalent in human and rodent tumor cells[79].

Tumour suppressor genes

A cellular phosphoprotein of molecular weight 53,000 (p53) has recently been found to possess many of the properties of tumor suppressor genes[80,81]. The p53 that was first detected in SV40 transformed cells[82] and later shown to be involved in cell transformation[83] was in fact the mutant form of wild type (wt) p53[84,85]. Current data indicate that the wild type p53 is a gene with

growth-arrest property[81] while various mutated forms of this gene are involved in cell transformation and tumorigenesis[86]. The mutant p53 appears to act in a dominant fashion by oligomerizing and trapping wild type p53[87]. Inactivation of wild type p53 by complexing with mutant p53 may destroy the growth suppressor function of wt p53 and push the cell towards proliferative path. The role of p53 in cell cycle control is not clear. Phosphorylation of p53 has been suggested to be an attractive mechanism for its regulatory role[88]. Over-expression of wt p53 was shown to arrest the growth of osteosarcoma cells and the growth arrest was shown to be due the inability of transfected cells to transit into S phase[89].

Very interesting information is available on the role of another tumour suppressor gene called the retinoblastoma (RB) gene on cell-cycle control and neoplastic development[90]. RB gene encodes a protein of Mr 110,000 which is a nuclear phosphoprotein with presumptive growth suppressor activity. The loss of function by mutation or deletion of the two alleles of RB gene is associated with loss of growth control. It has been suggested that the association of RB with viral protein blocks the growth suppressive activity of RB[91]. In normal cells, RB gene is expressed throughout the cell-cycle. The RB protein exists in multiple phosphorylated forms that are specific for certain phases of the cell-cycle. Highly phosphorylated forms are seen during S and G2/M whereas underphosphorylated forms are seen in G1 and in the growth arrested state. The underphosphorylated form would be the form with growth suppressive activity and is the only form bound by T-antigen, the transforming protein of SV40[92,93]. Recent findings indicate that the transcription factor E2F binds only to unphosphorylated RB protein. This binding inactivates the transcription factor and thereby prevents cells moving out of G0 state[94]. The RB protein interaction studies with transcription factors and transforming proteins have explained the roles of RB and transforming proteins in cell transformation (Table 2). All these studies were done with E2F; however one has to see whether the tumor suppressor genes act in a similar manner with other transcription factors.

Table 2. RB protein and growth control.

1. In quiescence, unphosphorylated RB protein binds to transcription factor E2F and inactivates it.
2. In proliferation, phosphorylation of RB protein results in the release of E2F to promote cell growth.
3. In neoplasia, mutated RB protein does not bind with E2F and as a result transcription is turned on constantly; absence of RB protein also promotes unhindered transcription.
4. In transformation by oncogenic viruses, e.g. adenovirus, the E1a protein of virus complexes with RB protein and thus takes it away from E2F.

Checkpoints

The events of cell cycle are sequentially executed in a definite order. Recently, Hartwell and Weinert[7] proposed and provided evidence for the presence of checkpoints in the cell cycle. These are points of surveillance in which the early events are monitored; if the early events are incomplete or defective, a signal blocks the later event. An S-phase checkpoint prevents nuclear division if the DNA synthesis is blocked[95]. Another checkpoint dictates that chromosome replication should occur before chromosome segregation[95]. A third checkpoint makes sure that the spindle formation is complete and correct for the completion of mitosis[96,97]. When a particular chromosome does not reach the metaphase plate in time, anaphase is delayed till that chromosome reaches the plate[98].

The studies with *RAD9* gene system in yeast indicate the involvement of specific genes in the surveillance of completion of functions in the cell cycle. The *RAD9* gene product was found to regulate the progression of cells in to G2/M phases. Temperature-sensitive mutants defective in DNA replication do not undergo mitosis at the restrictive temperature. If the same mutants have a defective *RAD9* gene, they proceed to mitosis at restrictive temperature suggesting that the normal *RAD9* gene product exerts a check points function[7].

Cyclins

As the name indicates, cyclins are cell cycle specific regulatory proteins that show oscillations in their levels across cell cycle[99-101]. In sea urchin eggs where it was first discovered, the level of this protein rises in interphase and falls in mitosis[99]. Later similar variations of cyclin levels were observed in other systems[100]. There are two major groups of cyclins. The G2-M specific cyclins include cyclins A and B while the G1 cyclins include cyclins C, D and E. The mitotic (G2-M) cyclins regulate mitosis while the G1 cyclins are involved in the signal transduction at G1 phase at a place called 'Start'[99]. The start point is the time at which the cells become committed to enter into S phase[101]. Although it is not proven, cyclins C, D and E were suggested to be involved in G1/S transit of quiescent cells[102,103]. Recent studies with human diploid fibroblasts indicate that the levels of cyclin D mRNAs decreased upon serum starvation and increased upon mitogenic stimulation of serum-starved cells[104].

Mitotic cyclin is a component of maturation promotion factor (MPF)[99]. The M phase induction involves the activation of another component of MPF which is p34 cdc2 kinase[102]. MPF initiates mitosis by breaking the nuclear envelope by phosphorylation of lamin. The consequence of lamin phosphorylation is

dissociation of lamin sub-units leading to break-down of nuclear envelope held together by unphosphorylated lamin. Once mitosis is complete cyclin is degraded by the ubiquitin pathway in order to reset the cycle[105].

Conclusion

In the last decade phenomenal progress has been made in understanding the mechanisms of cell cycle and cell proliferation. Cell cycle research has penetrated into the hottest areas of cancer biology and transcription and it has become a meeting ground of scientists working in interacting areas of cell biology, tumour virology, carcinogenesis and molecular biology. The outcome of the interdisciplinary studies resulted in clearing some of the enigmas surrounding cell proliferation and neoplastic development. For example, now we know how a tumor suppressor gene (RB) can play multiple roles such as in cell cycle regulation, in tumor suppression and in neoplasia. Phosphorylation–dephosphorylation processes at the protein level and mutations at the genome level manifest pleotropic effects in cell function. Literature on the positive and negative regulatory signals of cell cycle has already begun to accumulate. The stage is set to gather more information on the functional aspects of early growth response proteins and their role in normal and neoplastic development. Enormous information is available now on how cell cycle is triggered, mitogenic stimuli are transduced and subsequently on how the cell divides. During deregulation of normal process, oncogene products were found to substitute growth factors or growth factor requirement and thus perturb the signalling mechanism. The future seems to be on tumour suppressor genes. Already evidences are available on the growth suppressive property of these genes. Further studies on tumour suppressor genes are likely to elaborate the roles of these genes in normal cell cycle and in unregulated cell proliferation.

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ACKNOWLEDGEMENTS. I thank Dr S. Mohanam and Mr M. V. V. S. Vara Prasad for reading the manuscript and for suggestions. The studies cited from the author's laboratory are supported by the Department of Science and Technology, Government of India.

Received 14 February 1992; revised accepted 10 August 1992