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Control of the Cell Cycle and Apoptosis

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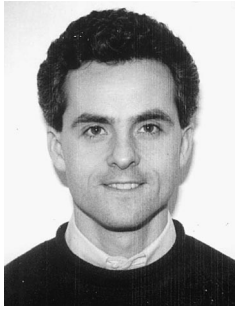

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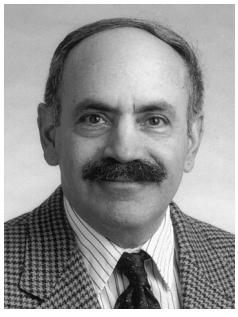

Dr Robert A. Weinberg is a founding member of the Whitehead Institute for Biomedical Research and the Daniel K. Ludwig Professor for Cancer Research at the Massachusetts Institute of Technology. He has been an American Cancer Society Research Professor at Whitehead and MIT since 1985. Dr Weinberg and his colleagues discovered the first human cancer-causing gene, the *ras* oncogene, and the first known tumour suppressor gene, *Rb*, the retinoblastoma gene. The principal goal of his research programme is to determine how oncogenes, their normal counterparts (proto-oncogenes), and tumour suppressor genes fit together in the complex circuitry that controls cell growth. He is particularly interested in applying this knowledge to improve the diagnosis and treatment of breast cancer. He is an elected member of the US National Academy of Sciences and Fellow of the American Academy of Arts and Sciences.

OUR UNDERSTANDING of the molecular mechanisms controlling cell proliferation underwent a revolution over the past decade with the discovery and description of the cell cycle clock apparatus. This molecular machinery, operating in the cell nucleus, functions as the master controller governing the decision of the cell to proliferate, to enter into reversible quiescence, or to enter into a postmitotic differentiation state. Before this machinery was uncovered, many believed that oncogene proteins and the products of tumour suppressor genes would provide us with a reasonably complete explanation of proliferation control. Now we know that these proteins, aberrant versions of which are often found in cancer cells, are nothing more than peripheral players that function by funneling their signals to the central decision-making machinery—the cell cycle clock—the ultimate governor of the cell's fate.

Any scheme depicting such a master control circuitry must address and resolve two major conceptual problems. First, how does this machinery gather a multitude of diverse, incoming growth-controlling signals, integrate and process them, and ultimately decide on the cell's fate? Second, once a decision is made, how is it executed? How does this central controller succeed in orchestrating the complex series of events that we call the cell cycle or the alternative courses that lead to quiescence or endstage differentiation? In fact, answers to these questions have begun to come from detailed study of the components of the clock apparatus itself.

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A BRIEF DESCRIPTION OF THE CLOCK APPARATUS

The clock machinery is composed of two core components, cyclin-dependent kinases (cdks) and cyclins (Figure 1). Like the great bulk of the cell's protein kinases, the cdks phosphorylate target proteins on critical serine and threonine sites. Protein kinases offer great opportunity for amplifying and broadcasting regulatory signals, in that a single kinase molecule can modify a number of distinct target proteins, phosphorylating hundreds or thousands of each species of protein in a short period of time.

Without their cyclin partners, the cdks are blind and inactive. Once physically associated with the cdks, cyclins are able to guide the cdks to appropriate substrates and activate their catalytic activity. A variety of cyclin:cdk complexes are formed during distinct phases of the cell cycle, each dedicated to the phosphorylation of a distinct set of target proteins. In general, the levels of cdks are relatively constant throughout the cell cycle, whilst the levels of the cyclins vary substantially. Indeed, the first cyclins were discovered and so named because of the dramatic periodic variations in their levels during each cell cycle [1]. This behaviour indicates that the activity of cyclin:cdk complexes is determined in part by the levels of available cyclins. But, as indicated below, additional levels of control may be superimposed on these cyclin:cdk complexes.

An assemblage of cyclin:cdk complexes orchestrates the advance of the cell through the phases of its growth cycle. As cells emerge from quiescence in response to mitogenic stimuli, the synthesis of D-type cyclins is induced. The continued presence of mitogens ensures that the levels of these cyclins remain high throughout the remainder of the cell cycle. Once synthesised, the D-type cyclins associate with cdk4 and cdk6, two alternative partner cdks whose functional distinctions have yet to be elucidated. In mid/late G1, several hours before the onset of S phase, cyclin E is induced and forms complexes with cdk2. The activity of this complex appears to be essential for entrance into S phase, when cyclin A appears in concert with the onset of DNA synthesis. Cyclin A associates initially with cdk2 and later with cdc2. This association continues until the late G2 phase B-type cyclins appear, forming complexes with cdc2 and triggering the complex series of events associated with mitosis [2].

G1 PROGRESSION AND THE RESTRICTION POINT

The above description alone provides little insight into the physiology of cell growth control. The guiding concept here is that external mitogenic and antimitogenic signals are received by the cell; they are processed and funneled to the clock apparatus; and this apparatus responds by programming the advance of the cell through the various phases of its growth cycle. How does the clock machinery make this regulation possible and how is this regulation disturbed in tumour cells?

In fact, the solutions to these questions were greatly simplified by the work of Arthur Pardee, who discovered that the dependence of a normal cell on exogenous mitogenic stimulation operates only during a limited phase of its growth cycle [3]. He found that cells require growth factor stimulation only during the first two-thirds of their G1 phase. Having experienced continuous mitogenic stimulation during this time window, cells can then complete the remainder of their growth cycle through mitosis in the absence of further exposure to mitogens. This behaviour suggests the existence of a decision point at the end of this G1 time window in early/mid G1 which Pardee termed the 'restriction point' (R point). At this juncture, after the cell has advanced two-thirds of the

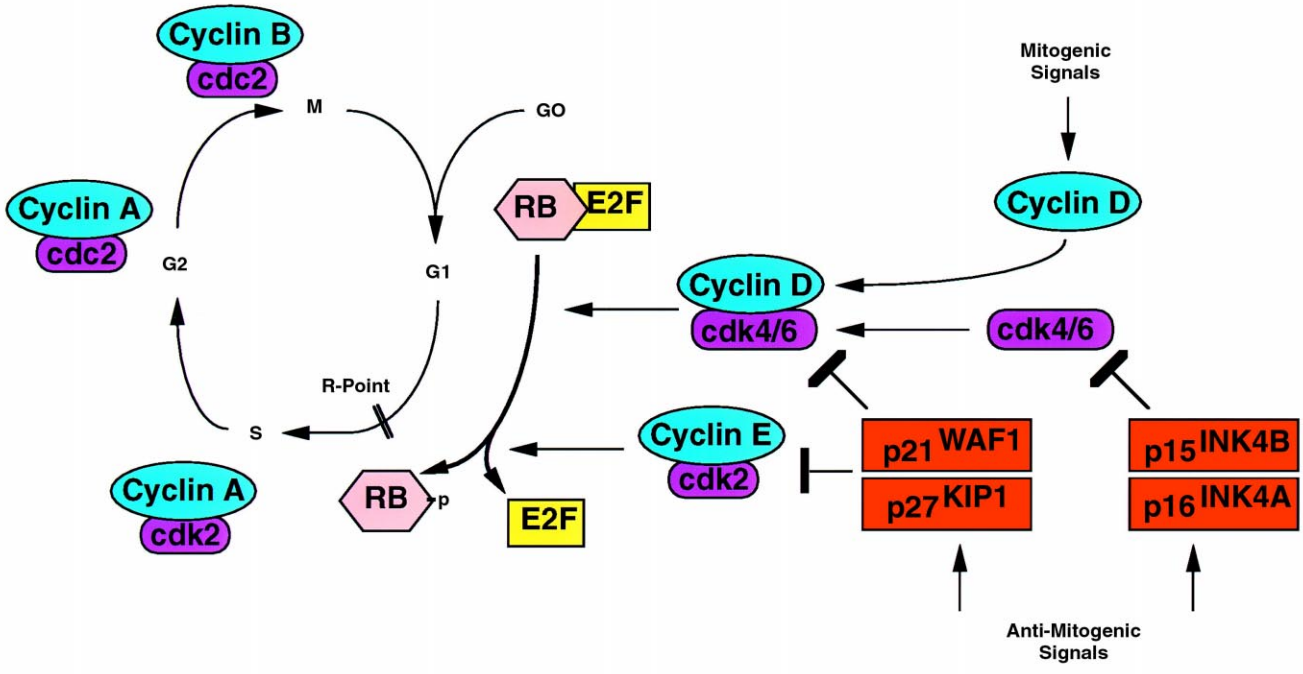

Figure 1. The cell cycle clock machinery. G0, M, G1, S, and G2 refer to the quiescence, mitosis, first gap, DNA synthesis, and second gap phases of the cell cycle, respectively. The restriction point (R-Point) is shown. RB and RB-p represent the unphosphorylated and hyperphosphorylated forms of the retinoblastoma protein.

way through G1, the cell may decide to commit itself, essentially irrevocably, to continue its advance and complete its cell cycle. Alternatively, if the signals received during this period have not been propitious for growth, the cell may turn back and re-enter into the G_0, quiescent phase from which it emerged previously, or may commit itself to enter into a postmitotic, differentiated state.

When depicted in this way, it becomes apparent that the decision to traverse the R point decision is the central event in normal cellular proliferation control. As a consequence, the regulatory processes occurring during the remainder of the cell cycle assume secondary importance for those intent on understanding neoplastic growth deregulation. Once a cell has committed itself to leave the G1 phase and enter into S phase, the rest of cell cycle progression is effectively automatic and fully predictable. To be sure, a series of checkpoint controls operate during the remainder of the cell cycle to ensure that the complex programme of growth and division through mitosis (M phase) is completed properly. The discrete steps executed during this remaining period in the cell cycle (from the onset of S phase to the end of mitosis) are rigidly programmed and proceed on a pre-arranged timetable unless some unanticipated disaster such as metabolic disruption, DNA damage, or disrupted spindles intervenes and threatens the normal routine. For example, checkpoint controls ensure that the DNA replication of S phase is completed before preparations for M phase entrance are undertaken; that damage to the cell's genome causes a halt in cell cycle progression until the genome is made whole once again; and that advance through M phase is stopped until disrupted spindles become properly assembled and aligned (reviewed in Refs [4, 5]). Still, these controls are essentially housekeeping functions, ensuring that a complex choreography is carried out efficiently and flawlessly and that it follows a detailed, pre-arranged script.

pRB AND THE R POINT TRANSITION

How is the R point decision executed, and what afferent signals converge on this decision? A diverse body of evidence accumulated over the past decade has shown that the retinoblastoma protein, pRB, is the molecular device that serves as the R point switch. When unphosphorylated or hypophosphorylated, pRB blocks the R point transition. Once phosphorylated, pRB loses much, if not all, of its growth-inhibitory powers and permits advance into late G1 and thence into the remainder of the cell cycle (reviewed in [6]). This central role implies that pRB function must be compromised in all tumours in which R point control is disrupted. Indeed, the R point transition may well be deregulated in all malignant human tumours.

We now have a reasonable understanding of how pRB phosphorylation is regulated and how pRB, for its part, is empowered to control cell cycle advance. The core components of the cell cycle clock, in particular the G1 cyclins D and E and their associated cdks, are responsible for pRB phosphorylation. Specifically, D-type cyclins (D1, D2 or D3) acting with associated cdks (CDK4 or CDK6) initiate pRB phosphorylation. This partial phosphorylation prepares pRB for subsequent hyperphosphorylation at the hands of cyclin E:CDK2 complexes [7]. Only after this latter modification occurs is pRB rendered inactive and thus unable to continue to block cell cycle advance. This inactivation opens a regulatory door, permitting the cell to advance into late G1.

pRB controls this door and thus cell cycle advance through its ability to regulate the activities of transcription factors, most importantly members of the E2F family such as E2F1, E2F2 and E2F3 [8]. When in its actively growth-suppressing, hypophosphorylated state, pRB physically associates with these factors and blocks their ability to activate expression of a constituency of responder genes; these genes, in turn, are known or surmised to encode products necessary for S phase progression. In fact, the inhibitory effects of the hypophosphorylated form of pRB extend beyond its ability to occlude the transcription-activating domains on the E2F transcription factor molecules: hypophosphorylated pRB can bind the E2F factors while they sit on specific DNA sequence binding sites located in the promoters of the downstream responder genes. Once associated with a DNA-bound E2F, the pRB molecule can actively repress the activity of an adjacent promoter, perhaps through its ability to recruit proteins like histone deactylases that create functionally inactive chromatin [9–11].

EXOGENOUS SIGNALS AFFECTING pRB PHOSPHORYLATION

As stated above, extracellular physiological signals affect the decision to transit the R point and this decision is executed through pRB phosphorylation. This must mean that extracellular signals directly control pRB phosphorylation, a speculation borne out by much evidence accumulated over the past 5 years.

Most dramatic is the evidence that mitogens directly and rapidly induce expression of cyclin D1, the first of the D-type cyclins to be discovered [12]. Once formed, cyclin D1, like other D-type cyclins, begins the job of inactivating pRB through its ability to associate with its previously cited CDK4 and CDK6 partners. Mitogen-dependent induction of cyclin D1 synthesis is often mediated by the centrally important Ras–Raf–MAP kinase signal transduction cascade [13, 14]. Strikingly, in certain cells lacking pRB, blockage of the Ras–Raf–MAPK pathway fails to stop cell G1 progression [15–17], implying that the essential purpose of this pathway in these cells is pRB phosphorylation and attendant inactivation.

The downstream effects of the other D-type cyclins (D2 and D3) would appear to be identical to those of cyclin D1; they too initiate pRB phosphorylation. However, the upstream signals controlling the levels of these other D-type cyclins are distinct from those that regulate cyclin D1. For example, in certain cell types, such as the granulosa cells of the ovary, cAMP levels are positive regulators of cyclin D2 levels; the cAMP levels in turn are controlled by a seven-membrane-spanning cell surface receptor, quite distinct from the tyrosine kinase mitogen receptors that control cyclin D1 [18]. This suggests that each of the three D-type signals responds to a distinct set of afferent signals impinging on the cell surface; and that control of cell proliferation in various cell types, known to be exercised by a diverse array of mitogens, is achieved in part through the use of three highly divergent, functionally distinct transcriptional promoters, each of which regulates expression of a distinct D-type cyclin gene. Once expressed, these three D-type cyclins then funnel their signals in a convergent manner to a common target—pRB.

There is no evidence to date that extracellular signals directly affect the levels or activity of cyclin E or cyclin E:CDK2 complexes. Rather, it appears that after cyclin D levels reach a certain critical threshold, they associate with

their CDK partners and initiate pRB phosphorylation. Cyclin E expression and the activity of cyclin E:CDK2 complexes is then activated as one of the events occurring subsequent to the initial creation of active cyclin D:CDK4 or cyclin D:CDK6 complexes. The detailed mechanisms that activate the cyclin E:CDK2 are complex and remain to be elucidated.

Extracellular signals can affect this cyclin:cdk phosphorylation machinery negatively as well. Serum starvation leads to a collapse of cyclin D levels and activity [12, 19]. In addition, serum starvation and the resulting withdrawal of cells from the active growth cycle is accompanied by increased levels and activity of the cdk inhibitor p27 [20]. Once synthesised, it can associate with and block the actions of a variety of cyclin:cdk complexes including notably those of cyclin E:cdk2.

The levels of another cdk inhibitor, $p15^{INK4B}$, are strongly induced by treating certain types of cells with the growth-inhibitory factor TGF-β [21]. Once formed, $p15^{INK4B}$ blocks the activity of cyclin D:CDK4/6 complexes, thereby preventing advance through the R point in mid/late G1. In consonance with this, TGF-β loses its growth-inhibitory powers once cells have passed through the R point, after which the cyclin D:CDK4/6 complexes appear to no longer play a role in cell cycle advance.

In many cell types trapped in G_0, high levels of the p21 and p27 cdk inhibitors ensure that any cyclin:cdk complexes that may be present in the cell remain inactive. As cells emerge from G_0, increasing levels of cyclin D1 form complexes with CDK4/6. Moreover, ongoing mitogenic stimulation often leads to a decrease in the levels of p27 [20]. Both mechanisms favour an increase in the molar ratio of the cyclin D:CDK4/6 complexes to cdk inhibitors, eventually leading to a reduction in the inhibitors. This results in the liberation of some cyclin:cdk complexes from inhibition by p27 and appears to play a key role in the activation of cyclin E:CDK2 activity in mid/late G1 [20, 22].

Curiously, other signalling pathways connecting mitogen receptors and the cell cycle clock apparatus remain poorly described. Mitogens induce synthesis of Myc protein, and this in turn has been reported to increase synthesis of the cdc25A phosphatase, which in turn is able to activate cdks by removing phosphate groups that inhibit their activity [23]. However, the physiological role of this phosphatase in actual cell cycles remains unresolved. The Myc protein clearly has a variety of other downstream targets that strongly affect cell cycle progression; the identities of these targets remain elusive.

DERANGEMENT OF R POINT CONTROL IN CANCER CELLS

R point control is deranged in many and possibly all types of tumour cells. How precisely do the growth-deregulating lesions found in cancer cells affect this decision, or more specifically, how do they affect the known components of the cell cycle clock? A most obvious point of deregulation comes from the well-documented aberrations of the mitogen-activated pathway in many human tumours, perhaps in all of them. Once activated constitutively, this pathway can drive the expression of D-type cyclins, notably cyclin D1, which in turn can prepare pRB for inactivation by phosphorylation.

This mitogen-activated pathway may be deranged in a number of ways. Many tumour cells release mitogens constitutively that bind and activate their cognate receptors displayed by these same cells. The resulting autocrine signalling liberates tumour cells from exogenous mitogenic stimulation [24] and ensures the continuous induction of cyclin D1. Overexpression and mutation of tyrosine kinase-containing receptors, notably the EGF receptor and HER2/erbB2/neu, appears to allow for ligand-independent firing of the receptor molecules with identical consequences [25]. And defects in the downstream signal-transducing components have equally devastating effects on normal regulation: the Ras protein is mutated and constitutively active in more than a quarter of human tumours [26]. As with the other mechanisms, the mutant Ras liberates the cell cycle clock from dependence on exogenous mitogens, perhaps largely through its ability to drive expression of cyclin D1.

Overexpression of D-type cyclins is also encountered in a variety of human tumours. In some cancers this may be achieved through amplification or rearrangement of the cyclin genes (reviewed in [27]). In others, deregulation of the upstream signalling pathways, as suggested above, may be responsible. In most cases, however, it remains unclear whether the overexpressed D cyclins derive from deregulation of these upstream cytoplasmic signalling cascades or, alternatively, from aberrations in the transcription factors directly responsible for modulating the activities of the cyclin D transcriptional promoters.

pRB, the central target of control by these various factors, is also frequently perturbed in tumour cells. In some, pRB is absent due to mutational inactivation of the *Rb* gene copies. This is true for virtually all retinoblastomas and small cell lung carcinomas and for a substantial proportion of sarcomas, non-small cell lung, bladder, and breast carcinomas (reviewed in [28]). In the vast majority of cervical carcinomas, pRB is inactivated through sequestration, being bound by the human papilloma virus-encoded E7 protein [29].

As described earlier, the TGF-β signalling cascade also converges on pRB phosphorylation through its ability to increase levels of the $p15^{INK4B}$ cdk inhibitor [21]. This cascade is also disrupted in a variety of tumours. Some may not express TGF-β receptors [30], others may express mutant receptors, and yet others may express defective or absent signal Smad proteins that operate downstream of the receptors and serve as intermediaries between the receptors and target genes [31, 32]. The disabling of this pathway liberates the R point transition from control by a widely acting extracellular growth-inhibitory signal.

CONTROL BY INTRACELLULAR SIGNALS

The cell cycle clock must respond to other signals beyond those conveyed by extracellular factors such as mitogens and antimitogens. Equally important are the signals that reveal the status of the cell's own internal metabolism and its genome. A well-organised cell physiology dictates that cell cycle advance is only appropriate and permissible when the intracellular household is in order.

The effects of genomic integrity on cell cycle advance have been intensively studied, if only because the cell's genome is easily perturbed experimentally by a variety of chemical and physical mutagens. Damage to the genome occurring during the G1 phase of the cell cycle causes rapid cell cycle arrest (Figure 2). Much of this is effected by the p53 protein whose levels increase within 30 min of damage to the cell's DNA [33]. The triggering of this response clearly requires a surveillance apparatus that rapidly and efficiently detects minor amounts of damage occurring anywhere in the genome. Indeed, even one double-strand DNA break appears

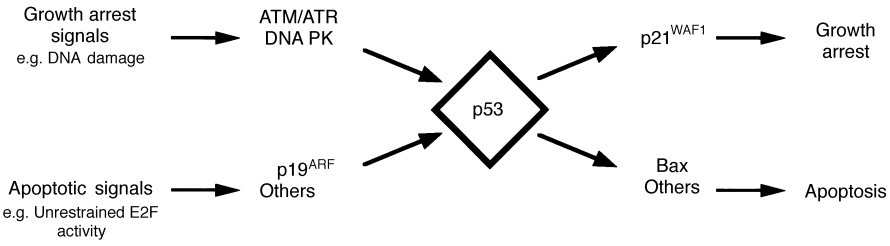

Figure 2. Signal transduction pathways controlling arrest and apoptosis. Intermediate steps have been omitted for simplicity.

sufficient to alert this apparatus. DNA damage activates the ATM/ATR and DNA PK family of protein kinases which leads to the post-translational stabilisation of a normally labile p53 protein [34].

The effect of p53 accumulation on the cell cycle clock machinery is very clear: p53 acts as a transcription factor that causes expression of several responder genes, among them the p21 cdk inhibitor. Because p21 acts so widely on a variety of cyclin:cdk complexes, a p53-initiated block can apparently occur in both the G1 and G2/M phases of the cell cycle [35, 36]. In the G1 phase of the cell cycle, a block imposed by p21 affords great advantage to the cell. By holding the cell temporarily in G1, DNA replication is prevented, thereby preventing the inadvertent replication of damaged DNA [33]. Such replication would result in the fixation of heritable mutations. The DNA repair apparatus is thereby given the opportunity to repair damage to its genome, and should this damage be reparable, p53 will, in some instances, revert to its basal level of expression, and the cell will now be permitted to re-enter into the active growth cycle and copy its now-restored genome [33].

Clearly many types of physiological perturbation are so severe that they warrant a response far more drastic than a temporary cessation of cell cycle advance. For example, substantial damage to the cell's genome may overwhelm the restorative powers of the DNA repair apparatus. Similarly, metabolic perturbations including anoxia may provoke imbalances that may exceed the abilities of the cell's homeostatic mechanisms. In situations like these, elimination of a damaged cell represents the most desirable response. Once again, p53 acts as an important governor, in this case of the apoptotic response. The connections between the p53 transcription factor and the apoptotic circuitry are not yet mapped out in detail. One way by which p53 may activate apoptosis seems to depend on its ability to induce expression of the pro-apoptotic Bax protein [37, 38], but other connections appear to exist as well.

This scheme implies that p53 sits as a master governor of two distinct downstream machineries: it may inhibit the cell cycle clock or may activate the apoptotic apparatus. The ability of p53 to evoke either stasis (i.e. a block of cell cycle advance) or apoptosis creates a dilemma that remains unresolved: Which signals collaborate with p53 to divert the cell in one direction or the other?

APOPTOSIS WITHIN THE CELL CYCLE CLOCK

The above scenario suggests that two distinct machines operate within the cell, both under the control of p53. However, this implied separation between the cell cycle clock and the apoptotic machinery represents an over-simplification. The cell cycle clock apparatus itself can directly participate in triggering the apoptotic response.

This connection was first suggested by investigation of the E2F1 transcription factor. Genetically altered mice lacking the *E2F1* gene develop lymphoid hyperplasias and lymphomas at an unusually high rate [39, 40]. This result was at first counter-intuitive because E2F1 was known to promote cell cycle advance late in the G1 phase of the cell cycle. Accordingly, the absence of the E2F1 factor seemed likely to lead to hypoplasia in various tissues throughout the body including components of the haematopoietic compartment.

Soon ectopic expression of E2F1 was found to cause cells initially in G_0 to progress all the way through G1 into S phase [41], but the continued expression of E2F1 into and through S phase was discovered to trigger apoptosis [42]. From this it could be concluded that during the normal cell cycle, the activity of E2F1 is confined to a narrow window of time beginning at the R point (when E2F1 is freed from pRB-imposed control) to the onset of S phase (when cyclin A:cdk2 complexes inactivate E2F1 complexes through phosphorylation) [43–46]. Hence, a deviation from this schedule involving continued activity of E2F1 into and through S phase may be a means of inducing the apoptotic response. And cells intent on triggering apoptosis may resort to this expedient as a way of physiologically activating the apoptotic death programme. Consequently, cells deprived of E2F1, including the lymphoid cells of E2F1-negative mice, may lack the ability to enter apoptosis through their normal access route (which may depend directly on prolonging E2F1 expression into S phase), leading, in turn, to excess lymphoid populations in these animals.

This interconnection between the cell cycle clock and apoptosis can be interpreted in two ways. According to one scenario, a cell intent on entering apoptosis will upregulate E2F1 as a means of directly activating specific components of the apoptotic machinery. However, an alternative model has recently become more likely. This one would suggest that E2F1 levels are carefully monitored in the cell, and that cells having inappropriately high levels of E2F1 activity, which might occur as a pathological consequence of damage to the pRB control pathway, may trigger apoptosis by signalling the universal alarm, p53; the latter then proceeds to initiate apoptosis directly as it does in response to a diversity of other afferent signals [47–50].

Although several members of the E2F family of transcription factors can transactivate E2F-dependent genes, the ability to induce apoptosis appears to be unique to E2F1 [51]. Evidence of specialisation among these factors is beginning to emerge. For example, E2F1 may regulate entry of a quiescent cell into the cell cycle, whereas E2F3 may regulate continued cell growth [52], providing a teleological explanation why the activation of E2F1 needs to be more tightly constrained than other E2F family members. Both E2F1-dependent transcription and apoptosis can be suppressed by pRB, the prime

antagonist of E2F1 [53]. Indeed, there is accumulating evidence that pRB acts as a general suppressor of apoptosis, as it is capable of suppressing apoptosis induced by IFN, TGFβ, and p53 [54–57]. Furthermore, cleavage of pRB by caspases, the proteolytic signalling molecules in apoptosis, appears to be required for TNF-induced apoptosis [58–62].

Most recently another, very distinct connection has been forged between the cell cycle clock apparatus and apoptosis. The paclitaxel-induced apoptosis of human breast cancer cells has been found to be dependent upon the induction of the cdc2 kinase at the G2/M phase transition of the cell cycle. This apoptosis can be blocked through dominant-negative mutants of this cdk or through the induction of the p21 cdk inhibitor [63].

ESCAPE FROM CELL SENESCENCE AND APOPTOSIS

The model of multistep tumour progression implies that clones of tumour cells must address and solve a succession of biochemical and cell regulatory problems en route to becoming fully malignant. Among these problems are an acquired independence from exogenous mitogenic stimulation, an acquired resistance to exogenous growth-inhibitory signals, and an acquired ability to resist apoptosis.

The first two of these problems can largely be solved by deregulating the cell cycle clock apparatus, specifically the R point control mechanism described above. This deregulation is often effected through the mutational activation of oncogenes or the inactivation of tumour suppressor genes, but such deregulation is achieved at a price: these genetic changes often evoke compensatory defensive responses from the cell that thwart further clonal expansion. For example, activation of a *ras* oncogene may elicit cell senescence [64, 65]; activation of a *myc* oncogene has been shown to elicit apoptosis [66, 67]. These secondary responses are seemingly designed to eliminate rare cells that carry growth-deregulating mutations, or at least block their further multiplication. Hence, subsequent expansion of premalignant cell clones and their progression toward malignancy depend importantly on disabling these important antineoplastic cellular defences.

The senescence response mechanism appears closely tied to the actions of cdk inhibitors, notably $p16^{INK4A}$ and p21. This may explain why many and perhaps all tumour cells seem to have inactivated these genes or at least their upstream regulators. Recent work has demonstrated that the two cdk inhibitors are tied together in a common circuitry because of the two reading frames inherent in a single gene (Figure 3). In particular, the $p16^{INK4A}$ reading frame specifies a cdk inhibitor which, like $p15^{INK4B}$, blocks CDK4 and CDK6 action. An alternative reading frame of the same gene encodes the 19^{ARF} protein [68] which antagonises the MDM2 protein [69–71]; MDM2 in turn antagonises p53 action by driving p53 degradation [72, 73]. In the absence of $p19^{ARF}$, MDM2 is given free rein to degrade the cell's complement of p53, effectively eliminating p53 from the regulatory circuitry. Consequently, a deletion of this single p16/p19 genetic locus, often seen in human tumours [74], causes the simultaneous loss of $p16^{INK4A}$ activity (thereby deregulating the R point transition) and p53 activity. Loss of p53 cripples the cell's ability to mobilise p21 for inducing senescence and to mobilise proteins like Bax that are important for triggering apoptosis.

This logic makes it clear that premalignant cells often shed the p16/p19 locus as one means of escaping senescence or apoptosis. Direct inactivation of the p53, seen in as many as half of all human tumours [75], may achieve the same end result. Through either means, cells have inactivated a prime defence against the unbridled proliferation that occurs following oncogene inactivation or tumour suppressor gene inactivation.

It can be concluded that inactivation of $p19^{ARF}$ or p53 allows cells to escape the apoptosis normally provoked by genotoxic stress or oncogene activation. As a consequence, additional mutations in survival (anti-apoptotic) genes such as *Bcl-2* may be rendered unnecessary. Indeed, activation of the *Bcl-2* oncogene is relatively uncommon in human tumours, perhaps because it exerts its effects only on the apoptotic machinery without achieving concomitant deregulation of the cell cycle clock [76–78].

The inactivation of antineoplasia defences yields other benefits for the evolving premalignant cell. The anoxia experienced by nests of premalignant cell clones early in tumour progression (prior to the acquisition of angiogenic abilities) appears to evoke apoptosis in a high proportion of these cells. Inactivation of p53 may provide some protection against this apoptosis [79], thereby shifting the balance of apoptosis versus growth and yielding a net gain in cell number in these nests. Moreover, as discussed earlier, p53 plays a role in shutting down proliferation in response to genomic damage, resulting in its being termed the ''guardian of the genome'' [75]. This implies that cells lacking p53 function may continue to grow and replicate their genomes in the face of unrepaired genomic damage, resulting in heritable mutations being accumulated at abnormally high rates. One demonstrated consequence is that genes in p53-negative cells may undergo amplification at rates 1000-fold higher than normal [80, 81]. Increased mutability may greatly amplify the rate with which mutations strike growth-regulating genes, thereby accelerating the entire process of tumour progression. To summarise, because of the pleiotropic activities of the p16/p19 and p53 genes, their inactivation may yield a series of distinct cellular changes that are highly advantageous to evolving populations of premalignant cells.

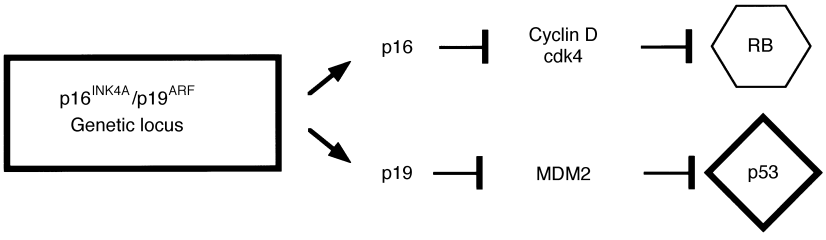

Figure 3. The $p16^{INK4A}/p19^{ARF}$ locus. A deletion of this single p16/p19 genetic locus causes the simultaneous loss of $p16^{INK4A}$ activity, deregulating the R point transition, and of p53 activity, disabling the response to genotoxic stress or oncogene activation.

PRECIS AND PERSPECTIVE

The deregulation of the cell cycle clock and the apoptotic apparatus as described here represents only a portion of the changes that are required to make a malignant cell. A cell clone on the way to becoming malignant gains substantial benefit from the cited alterations in its growth-regulating genes, including among other functional advantages: (1) mitogen independence and an ability to resist antimitogens; (2) an ability to tolerate anoxia and other apoptosis-inducing conditions; (3) an ability to tolerate oncogene activation and tumour suppressor gene inactivation; (4) a mutable genome that hastens subsequent rate-limiting steps in the progression to fully-fledged malignancy.

Still, these changes do not explain the entirety of malignant progression. Yet other problems must be solved by premalignant cell clones in order for them to succeed ultimately in developing into aggressively growing tumours. Each one of these problems suggests the operations of a defence mechanism that is placed in the path of these cell clones by the host organism in order to prevent completion of tumour progression.

Cell mortality is one hurdle that must be overcome by these evolving cell clones. Recent work strongly suggests that in some cells this problem can be solved by activation of expression of a previously repressed telomerase gene [82, 83]. This activation implies the operations of a 'generational clock' that is embodied in the telomeres at the ends of chromosomes and is quite distinct mechanistically from the cell cycle clock discussed here.

Yet other phenotypic shifts of premalignant cell clones involve the acquisition of angiogenic ability and ultimately invasiveness and metastatic ability. These latter steps in tumour progression are apparently not directly connected with the regulatory machinery that is described here in some detail. Thus, an elucidation of their mechanistic bases will require an entire new body of molecular and genetic phenomenology.

None the less, the information already in hand provides some indication about early 21st century directions in cancer research. The overall outlines of the control mechanisms governing cell cycle advance and apoptosis are in clear view, although certain critical details involving the connections between component parts are still obscure. These details will come to light during the early years of the next decade. Their discovery will be enormously facilitated by technology already in hand, including gene mapping, genetic strategies for detecting protein–protein interactions such as the yeast two-hybrid procedure [84] and large-scale analyses of gene expression [85].

The existing information, limited as it may be, holds rich promise for clinical oncology. Therapies developed over the past quarter of a century have been largely empirical and thus not inspired by insights into the mechanistic bases of tumorigenesis. The discovery of the genetic determinants of tumour cell growth, some of which have been described above, has changed this situation dramatically. The availability of large-scale genome and gene expression analyses may soon make it possible to tailor chemotherapy specifically to the characteristics of each tumour encountered in the oncology clinic. Apoptosis will attract the attentions of those intent on developing new types of anticancer therapy. In particular, workers designing novel therapies will focus increasingly on pharmacological strategies that succeed in inducing apoptosis in cancer cells.

The cell cycle clock apparatus will also figure largely in future thinking about anticancer therapy. Initially, it was portrayed only as a means of programming the cell's advance through its normal mitotic growth cycle. However, the recently uncovered interconnections between the clock machinery and the apoptotic apparatus will mean, inescapably, that cyclins and cdks will become more than laboratory curiosities and instead assume central roles among the molecules targeted by new generations of anticancer therapeutics.

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