Cell Cycle Control and Cancer Chemotherapy

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Abstract   As detailed information accumulates about how cell cycle events are regulated, we can expect new opportunities for application to cancer therapy. The altered expression of oncogenes and tumor suppressor genes that commonly occurs in human cancers may impair the ability of the cells to respond to metabolic perturbations or stress. Impaired cell cycle regulation would make cells vulnerable to pharmacologic intervention by drug regimens tailored to the defects existing in particular tumors. Recent findings that may become applicable to therapy are reviewed, and the possible form of new therapeutic stratagems is considered.   © 1994 Wiley-Liss, Inc.*

Since an essential characteristic of malignant tumors is abnormal regulation of cell proliferation, the question arises whether such abnormalities could be utilized to advantage for therapy. It may become possible to design chemotherapeutic stratagems on the basis of detailed knowledge of the regulatory mechanisms and the nature of their defects in particular tumors. Early efforts to explore this avenue were limited by a lack of detailed knowledge of the regulatory mechanisms and how they might be defective in tumors [Bradley et al., 1977; Studzinski and Gierthy, 1973]. Now that these molecular details are becoming accessible and there are excellent prospects of their further elucidation, one can think more cogently about the outlook for therapeutic applications. The genetic changes commonly observed in human malignant tumors involving oncogenes and tumor suppressor genes present a broad scope for potential applications. Defective control of cell cycle events may enhance the sensitivity of cancer cells to cytotoxic drugs, and may in large part be responsible for the antitumor responses that have been attained with standard cytotoxic chemotherapy.

Current anticancer drugs, with few exceptions, have one or more of the following actions: (1) damage DNA (e.g., nitrogen mustards, cis-platin, bleomycin), (2) block a DNA topoisomerase (e.g., doxorubicin, etoposide, camptothecin), (3) inhibit DNA synthesis and/or become incorporated into newly replicated DNA (e.g., methotrexate, fluorouracil, thioguanine), or (4) block the mitotic spindle (e.g., vincristine, taxol). DNA damage such as interstrand crosslinks, if not adequately repaired, can lethally perturb the orderly progress of DNA replication and cell division. The topoisomerase-targeted drugs stabilize covalent complexes of topoisomerase with DNA; these complexes seem to affect cells in a manner resembling the effects of DNA damaging drugs. Lethal lesions may be generated when blocked topoisomerase complexes are encountered by a moving replication fork or by a transcription process [D'Arpa et al., 1990; Holm et al., 1989; Hsiang et al., 1989; Kaufmann, 1991]. The chemical damage to DNA produced by alkylating agents can be removed only by slow and uncertain DNA repair processes; on the other hand, the topoisomerase–DNA complexes are able to reverse spontaneously. The antitumor actions of these diverse drugs may be based in large part on an impaired ability of malignant cells to control critical events in DNA replication and cell division. The presently available cytotoxic therapies may work by taking advantage of metabolic impairments in malignant cells; however, they are overly destructive to critical normal cells. It may become possible to devise optimized therapies that would be more civilized and less destructive. To this end, it will be

necessary to identify the relevant alterations in gene expression existing in individual tumors and to tailor the therapy accordingly.

It has often been stressed that cancer is a multitude of diseases, and yet, because of limited knowledge, tumors have been classified primarily according to tissue of origin and histologic appearance. A better focus would be on the altered expression patterns of regulatory genes. One way in which this development may take place is through comparisons between tumor cells that differ in sensitivity to particular types of drugs. Differential drug sensitivity could be related to differences in gene expression. A complementary path will be to work out the control networks and to devise drug stratagems to act selectively against cells having particular kinds of control defects.

Although the cell cycle is usually described in terms of a linear sequence of G1, S, G2, and M phases, the inadequacies of this description have been pointed out [Hartwell and Weinert, 1989; Murray, 1992; Nurse, 1990]. This sequence of phases is not always obeyed in biologically specialized cells or in cells perturbed by drugs. A more generally useful description utilizes the concept of checkpoint controls on the initiation of key cell cycle events pending the completion of critical preceding events [Hartwell, 1992; Hartwell and Weinert, 1989; Murray, 1992] (Fig. 1). If a checkpoint control is defective, cell cycle events can occur in an abnormal order, leading to major cell injury or death. Weak checkpoint controls in malignant cells would provide opportunities for new therapeutic stratagems.

With this framework in mind, we now discuss some of the possibly useful information currently at hand relating to events in the regulation of cell division.

Preparations for DNA Replication ("G1-S Transition")

In preparation for DNA replication, the metabolism of the cell must be geared for the synthesis of nucleic acid precursors and of the replication machinery itself. The decision to prepare for genome replication appears to be determined by switch-like mechanisms involving the SWI and CLN gene families in yeast [reviewed by Andrews and Mason, 1993] and an at least partially homologous system in mammalian cells involving some of the cyclin subfamilies, their associated kinases, and regulators [reviewed by Farnham et al., 1993]. The control system in mammalian cells probably corresponds to the "restriction point" (R) described by Pardee and his colleagues [Dou et al., 1993; Pardee, 1974; Rossow et al., 1979]. An identified output of the system is the E2F transcription factor(s), which activates many genes involved in DNA replication and cell proliferation; the active form appears to be a heterodimer consisting of an E2F species bound to another protein, such as DP1 [Helin et al., 1993b]. E2F1 has been shown to induce quiescent mammalian cells to initiate DNA replication [Johnson et al., 1993].

In its most primitive form, one might imagine that the decision to prepare for replication would simply require turning on a master gene that makes an E2F-like product. Eukaryotic cells
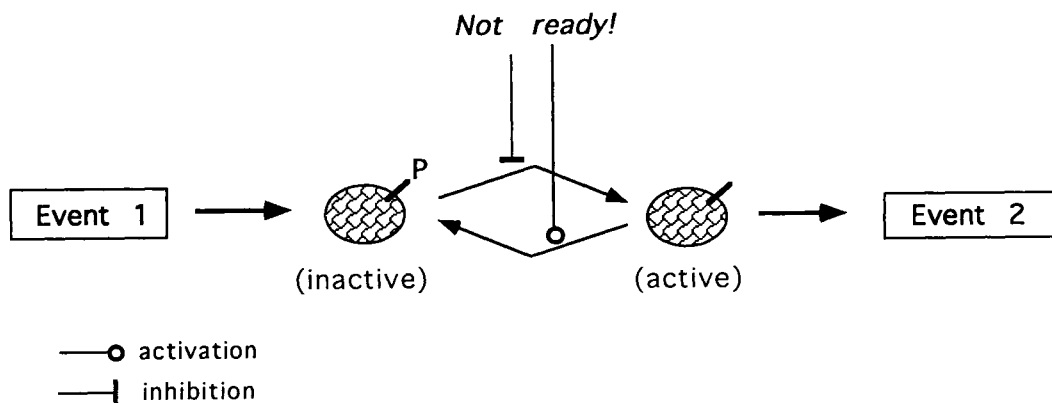
Fig. 1. Checkpoint control logic. Event 1 sets up the conditions for the initiation of a subsequent event 2 by preparing an initiating protein in an inactive form. In this illustration, the protein is shown as being inactivated by a specific phosphorylation (as is the case for cdc2, for example). Activation of the protein and initiation of event 2 is controlled by the removal of this phosphate. The checkpoint signal is essentially a "not ready" signal that inhibits the dephosphorylation and/or stimulates the reverse reaction.

have developed a further level of control which inhibits the action of this product and allows for further control inputs. The E2Fs are kept in check by binding to proteins of the Rb/p107 family (Rb = retinoblastoma tumor suppressor gene product) [Helin et al., 1992a; Nevins, 1992b; Schwarz et al., 1993; Zhu et al., 1993]. (The control by binding to Rb also affects other transcription factors, such as c-myc [reviewed by Kouzarides, 1993].) No abnormalities of p107 have so far been found in cells, perhaps because it operates at a fundamental level such that deficiency would be lethal. Rb, on the other hand, may operate at a higher level of control, and cells may survive despite deficiencies in Rb function. Many embryonic tissues develop normally in the absence of Rb up to the stage of neural tube development [reviewed by Jacks et al., 1992, and by Nevins, 1992b]. Rb is often defective in a variety of human tumor types [see review by Wiman, 1993].

Activation of E2F may occur by enhanced phosphorylation of p107 or Rb which then releases the E2F in free and active form [Cobrinik et al., 1992]. The phosphorylation is brought about by the action of cyclin–kinase pairs [Nevins, 1992a,b]. The cyclin subfamilies D, E, and A appear to be activated sequentially in a finely choreographed process leading towards the onset of DNA replication. Human cancer cells often have abnormally high levels of expression of some of these cyclins [Keyomarsi and Pardee, 1993]. The increased expression of cyclins may enhance the stimulus for initiation of cell cycle events.

Another way in which active E2F may be released is by displacement by a competitor for the binding site on p107 or Rb. The best known of such competitors are proteins such as E1A of adenovirus which is necessary (although not sufficient) to make the infected cell grow and divide in a relatively unchecked fashion [reviewed by Nevins, 1992a].

Although the function of Rb has been studied primarily with respect to its inhibitory role in the initiation of S phase events, it has recently been reported that overproduction after the initiation of S phase can cause G2 arrest [Karantza et al., 1993]. This is another example of the complexity of overlapping functions that is emerging as a common feature in biological control systems.

Superimposed over these levels of control is the p53 tumor suppressor gene product, a pro-

tein that appears to have a multiplicity of actions. The superimposed nature of p53 function is suggested by its relatively restricted phylogenetic distribution—so far, it has been reported only in vertebrates [Soussi et al., 1990]—and by its apparent lack of essential function in the normal development of the mammalian embryo [Donehower et al., 1992]. p53 may be designed to act upon regulatory proteins of more fundamental and more widely conserved function. This is suggested by the finding that overexpression of p53 in yeast can exert at least some of the controls operative in vertebrate cells [Bischoff et al., 1992; Nigro et al., 1992]. A recent study of Xenopus embryos notes that, although the gene is not transcribed, p53 protein (presumably derived from maternal genes) persists in a stable cytoplasmic form up to the hatching of the tadpole [Tchang et al., 1993]. It is not clear however whether this protein has any essential function in the embryo; perhaps it is merely readied for action in the event of DNA damage to the egg developing in an exposed environment, such as a sunlit pond. We do not know whether p53 protein persists in the mammalian embryo.

A major role of p53 seems to be to monitor against inappropriate uncontrolled proliferation of damaged or mutant cells. The growth control exerted by transfected p53 in yeast suggests that the targets of control are widely conserved, but does not imply that a p53-like protein actually functions in these unicellular organisms. This is therefore not inconsistent with the major role attributed to p53 as a tumor suppressor. The incidence of mutation or functional impairment of p53 varies among different types of human tumors, with an overall incidence that may be as high as 70% of cases [Harris and Hollstein, 1993; Levine, 1992]. Thus p53 harbors the most common gene alteration found in human cancer.

Control defects in cancer obviously would have to be such that the altered function is compatible with the life and reproductive capability of the cell. Abnormalities in the control of the preparations for genome replication (G1–S transition) that are commonly defective in cancer include (1) the Rb tumor suppressor gene, (2) viral oncoproteins that compete at binding sites for E2F, (3) types D and E cyclins, and (4) the p53 tumor suppressor gene. Other oncogenes, such as c-myc, probably influence this control, but their manner of action is not yet clear.

These alterations are points of potential vulnerability that might be utilized to advantage in new drug strategies.

The inputs to the regulator of preparations for genome replication (termed START in yeast [Wittenberg and Reed, 1991] and restriction point (R) [Pardee, 1974, 1989; Pardee and Keyomarsi, 1992] in mammalian cells) probably include cell size, nutritional or metabolic state, growth factors, and DNA damage. Cells can enter a quiescent state, which must require additional metabolic controls to assure that nothing gets seriously out of balance. Quiescent tumor cells may at times be pushed out into a replicative state by an occasional imbalance arising because of defective metabolic controls which may allow periods of chaos in the system. Thus efforts to push quiescent tumor cells into cycle might become more promising when these controls are elucidated.

p53 Function, G1 Arrest, and Sensitivity to DNA Damage

Much attention has recently been given to the role of p53 as monitor of cell proliferation and as a determinant of response to DNA damage [Brachman et al., 1993; Kastan, 1993; Lee et al., in press; Lee and Bernstein, 1993; Lowe et al., 1993a,b; Murnane and Schwartz, 1993; Zambetti and Levine, 1993]. The extraordinary range of diverse targets of action of p53 that has recently been disclosed [see reviews by Frebourg and Friend, 1993; Kastan, 1993; Vogelstein and Kinzler, 1992; Zambetti and Levine, 1993] presents a challenge to coherent interpretation. (1) p53 functions as a transcriptional activator for genes, including *mdm2, gadd45, wafl/cip1* [El-Deiry et al., 1993; Harper et al., 1993], and perhaps muscle creatine kinase, which contain a recognition element—RRRC(A/T)(T/A)GYYY (R = purine; Y = pyrimidine)—to which it binds. (2) It may suppress transcription of a wide variety of genes by binding to transcription factors such as TATA-binding protein (TBP), CCAAT-binding factor (CBF), and the GGGCGG-binding factor Sp1. (3) It binds to replication protein A (RPA) which functions at replication origins. (4) It binds nonspecifically to regions of single-stranded DNA, such as may exist in regions of replication forks or DNA repair, and favors the annealing of base-paired regions. (5) It binds to the MDM2 protein to yield an inactive complex, and activates the *mdm2* gene, thereby seeming to form a negative feedback control loop which

would tend to buffer changes in p53 concentration. (6) The p53 protein is subject to conformational changes between an active and inactive form, and is degraded rapidly, probably while in the inactive conformation. (7) It binds to the heat-shock protein Hsp70 which perhaps is involved in the conformational transition. It is hard to imagine how such a complex of functional components could evolve in a largely nonessential protein—unless, of course, p53 is not as highly integrated a system as it may seem, but rather represents a kind of jury-rigged arrangement that works but does not have ready access to evolutionary paths towards increased efficiency. Thus not every binding interaction of p53 need be functionally important.

Cells that have been exposed to X-rays or DNA damaging drugs normally do not progress to DNA replication or to mitosis. They become arrested either in G1 at (or near) the R point, or in G2 at the point where preparations for mitosis are initiated, whichever occurs first [O'Connor and Kohn, 1992]. The delays in entry into S phase or into mitosis while DNA damage is present allow more time for DNA repair and presumably protects cells against major genome damage. Defects in either of these control points would increase the likelihood of chromosome alterations and could be responsible for the genomic instability which is a hallmark of most cancers [Hartwell, 1992]. The arrest in G1 requires normal p53 function, suggesting that p53 may function as a "guardian of the genome" [Kastan et al., 1991; Kuerbitz et al., 1992; Lane, 1992].

We recently studied the X-ray response of 17 Burkitt's lymphoma cell lines, some having normal and some having mutated p53 genes [O'Connor et al., 1993b]. Five of the lines responded with a strong G1 arrest, and all of these had two normal p53 alleles. Ten of the lines exhibited little or no G1 arrest, and all of these had at least one mutant p53 gene. The remaining two lines had normal p53 genes, but showed reduced responses; these cells, however, failed to show the normal increase in p53 protein (p53 protein levels normally increase because of reduced lability of the protein). Thus the G1 arrest responses of these cells correlate perfectly with p53 function.

From the point of view of the "guardian of the genome" hypothesis [Lane, 1992], p53 function might be expected to help cells survive DNA damage, since more time would be given for

DNA repair before the onset of S phase. Contrary to this expectation, however, normal p53 function is often associated with death rather than survival after DNA damage [Lee and Bernstein, 1993; also see letter by Murnane and Schwartz, 1993, and reviews by Kastan, 1993, and by Lee et al., in press]. In our study of the effects of X-ray on Burkitt's lymphoma cells, most of the p53 mutant lines were less sensitive than the p53 normal lines [O'Connor et al., 1993b]. The basis for this unexpected relationship may be that p53 has an essential role in programmed cell death or apoptosis [reviewed by Lane, 1993]. The p53-mediated response to DNA damage may depend on the cell type: apoptosis may be the usual response of hematopoietic cells, whereas fibroblasts perhaps are not programmed as delicately for apoptosis and therefore may exhibit G1 arrest [reviewed by Kastan, 1993]. Lymphoid cells are especially prone to apoptosis, a logical function in the life history of these cells. However, the association between p53 mutation and reduced sensitivity to radiation and drugs appears to hold in a variety of cell types, suggesting that p53-dependent apoptosis of tumor cells may be an important mechanism of chemotherapeutic response.

The complex role of p53 in respect to the G1–S transition is in part suggested in Figure 2. Commitment to DNA replication is made at the restriction point (R), which is activated by growth signals and dependent on protein synthesis [Pardee, 1974, 1989]. p53 is activated by DNA damage, particularly DNA strand breaks, by an as yet unelucidated mechanism which leads to the stabilization of active p53 protein. p53 inhibits the process which commits cells to S phase, probably by acting at the R point through inhibition of cyclin E/cdk2 activity [O'Connor et al., in preparation]. p53 also provides a necessary function for apoptosis. The role of p53 in apoptosis hypothesized in Figure 2 is that it monitors whether a growth signal received at the R point has a valid growth factor origin; if the R point is activated in the absence of a valid signal, the cell would be forced to apoptose. For example, E1A or c-myc may act at R to initiate S, and can stimulate apoptosis, especially in the presence of DNA damage [discussed by Lowe et al., 1993a]. Some Burkitt's lymphoma cell lines, for example, appear to be driven by simultaneous overexpression of c-myc and mutation of p53 [O'Connor et al., 1993b].
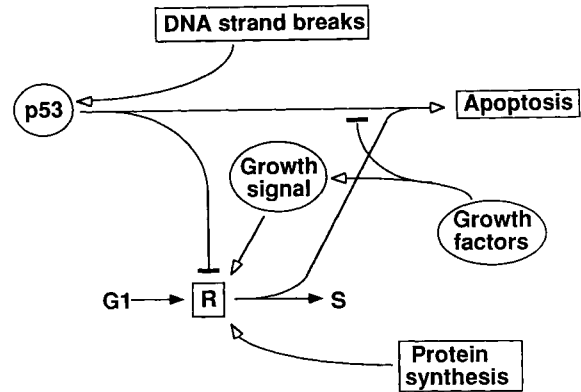

Fig. 2. General scheme for p53 function in G1 arrest and apoptosis. p53 tends to limit commitment for S phase, especially when overexpressed in response to DNA damage. p53 stimulates apoptosis when (we propose) cells are initiating S phase, unless there is a confirmatory signal from an appropriate growth factor receptor.

According to the model in Figure 2, the p53 monitor system would detect an invalid signal in the absence of a validating signal, for example from a growth factor receptor, and would then stimulate apoptosis. The model predicts that apoptosis under these circumstances could be inhibited by adding the right growth factor.

Orderly Replication of the Genome (S Phase)

DNA synthesis can be blocked by hydroxyurea, an inhibitor of ribonucleotide reductase, or by aphidicolin, an inhibitor of DNA polymerase. RNA and protein synthesis, however, continue and produce an imbalance which stresses the ability of the cell to restore a viable metabolic state. Accordingly, the ability of cells to survive a period of DNA synthesis inhibition can sometimes be improved by cotreatment with a protein synthesis inhibitor [Kung et al., 1990b]. Perhaps one of the functions of the p53 tumor suppressor gene product is to downregulate transcription of many genes, for example through its interaction with the TATA-binding protein [Seto et al., 1992], thereby tending to restore balance between cell growth and cell division when the latter is inhibited.

Kung et al. demonstrated in a line of Chinese hamster ovary cells that cotreatment with the protein synthesis inhibitor, cycloheximide, could protect against loss of clonogenic survival otherwise engendered by an 18 hr inhibition of DNA synthesis by hydroxyurea or aphidicolin. The physical death of cells (i.e., loss of plasma membrane integrity) required not only ongoing pro-

tein synthesis but also active DNA synthesis. As long as the DNA synthesis inhibition was maintained, the cells did not die physically. Death, as measured by loss of plasma membrane integrity, occurred only after removal of the inhibitor and resumption of DNA synthesis. Again, cycloheximide prevented the cell death. Time-lapse photomicroscopy showed that some cells, perhaps depending upon their cell cycle state at the time of drug addition, seem to undergo abortive steps toward division, as indicated by their transient assumption of a refractile morphology and evidence of chromosome condensation. This seemed to occur at irregular intervals, sometimes repeatedly, as if a chaotic process were at work. Some cells died physically while others, even sister cells, reverted to a normal morphology. Normal morphology was sometimes restored after several irregularly spaced episodes of refractile appearance. One can envision these disturbed cells undergoing an irregular oscillation of metabolic state that may trigger death or that may find its way back to a stable and viable condition, the final outcome perhaps depending unpredictably upon which of these ultimate fates occurs first.

Although of considerable interest with respect to drug therapy, it is not clear whether the protective actions of protein synthesis inhibition against the lethality of pure DNA synthesis inhibitors would apply generally to human cells, normal or malignant. It is interesting to note, however, that DNA synthesis inhibitors such as methotrexate and 5-fluorouracil, which also inhibit RNA synthesis, are much more useful as anticancer drugs than is hydroxyurea, a pure DNA synthesis inhibitor. The therapeutically effective metabolic stress induced by methotrexate or 5-fluorouracil may be DNA synthesis inhibition with simultaneous restraint on RNA and protein synthesis, and this may be the condition of stress that distinguishes malignant from normal cells with respect to their ability to adapt.

Some cell types, when treated with DNA synthesis inhibitors, tend to initiate new rounds of replication of local chromosome regions, leading to the phenomenon of gene amplification. Gene amplification seems to occur in neoplastic or transformed cells, but not in normal human cells [Tlsty, 1990; Tlsty et al., 1992], suggesting that neoplastic cells sometimes are defective with respect to the stringency of prevention of DNA re-replication in normal cells. Pure DNA synthesis inhibitors, such as hydroxyurea, however, have not been very effective in therapy.

Preparations for Mitosis (G2 Phase)

Eukaryotic cells normally do not begin mitotic events, such as chromosome condensation, while DNA replication remains incomplete. The inhibitory effect of unreplicated DNA on mitosis promoting factor (cdc2–cyclin B complex) has been demonstrated in cell-free extracts of Xenopus eggs [Dasso and Newport, 1990]. The system that recognizes the presence of incompletely replicated DNA in eukaryotic cells involves the abundant DNA-bound protein, RCC1 ("regulator of chromosome condensation"), and its even more abundant partner, Ran, a ras-like GTP-binding protein [recently reviewed by Dasso, 1993]. Temperature-sensitive RCC1 mutants, at restrictive temperature, undergo premature and catastrophic chromosome condensation. The same monitoring system may also detect the presence of damaged DNA and delay the onset of mitosis while substantial DNA damage remains unrepaired. We have recently found that the G2 block that normally follows radiation does not occur in some human cancer cell lines [O'Connor et al., in preparation]. By progressing to mitosis without allowing extra time for DNA repair, such cells could be selectively vulnerable to DNA damaging drugs.

Delayed onset of mitosis (G2 block) is commonly observed in cells that have been exposed to DNA damaging agents and topoisomerase blockers [reviewed by O'Connor and Kohn, 1992]. The cells are able to complete a full or nearly full round of DNA replication (assuming that the cells were not arrested at the R point), but do not proceed to condense chromosomes. The G2 block is prevented by methylxanthines such as caffeine and pentoxifylline; these compounds enhance the cytotoxicity of alkylating agents, presumably by reducing the time available for the repair of DNA damage prior to the start of mitosis [Fingert et al., 1986, 1988; Lau and Pardee, 1982; O'Connor et al., 1993a; Teicher et al., 1991].

The control pathways for initiation of mitotic events are only partially understood. The proximal step is thought to be the activation of kinase cdc2 which catalyzes extensive phosphorylations of histones and nuclear lamins. In cells that have become arrested in G2 due to DNA damage or topoisomerase blockade, cdc2 remains in an inactive tyrosine-phosphorylated state [Lock, 1992; O'Connor et al., 1992, 1993a] although in some cases cyclin B synthesis may

also be inhibited [Muschel et al., 1991, 1993; Tsao et al., 1992]. The challenge next is to trace the control steps from cdc2 back to the DNA damage regulation point which may involve RCC1. In general outline, the links between cytotoxic drugs and S/G2 cell cycle controls are represented in Figure 3 [see also the review by Li and Deshaies, 1993].

The activation of cdc2 appears to take place by a switch-like mechanism illustrated in Figure 4. Cdc2 is maintained in an inactive form by phosphorylation of tyrosine-15 by Wee1-like kinases. The switch may operate through a positive feedback loop in which the inhibitory phosphates are removed by phosphatase cdc25, thus activating cdc2 which then phosphorylates and thereby increases the activity of cdc25. The next outer layer of control would include kinases and phosphatases that, respectively, inactivate and activate wee1. Also included here would be the phosphatase that inactivates cdc25. A further aspect of control is the location of these species in the nucleus or cytoplasm, and movement from one compartment to the other. cdc25, lo-

cated in the cytoplasm, is required for the premature condensation of chromosomes caused by loss of RCC1 function, whereupon cdc25 appears to move into the nucleus [Seki et al., 1992]. The details of such translocations between cytoplasm and nucleus, and their generality for different cell types, are not yet clear.

Cyclin-dependent kinases (cdks), including cdc2 (which is also known as cdk1), as their name implies, must be bound to a cyclin to be active. The major active form of cdc2 during mitosis is cdc2–cyclin B. To a lesser extent, and slightly earlier in time, cdc2 is also active as cdc2–cyclin A. Cyclin B is synthesized and accumulates as cells progress from S phase to mitosis. Cyclin A accumulates earlier, at about the time that cells begin to replicate DNA. The kinase cdk2–cyclin A is the major form present during S phase. These events were monitored in cells treated with the DNA crosslinking drug, nitrogen mustard [O'Connor et al., 1993a]. The cells were synchronized at the beginning of S phase using the DNA polymerase inhibitor, aphidicolin, and exposed to nitrogen mustard
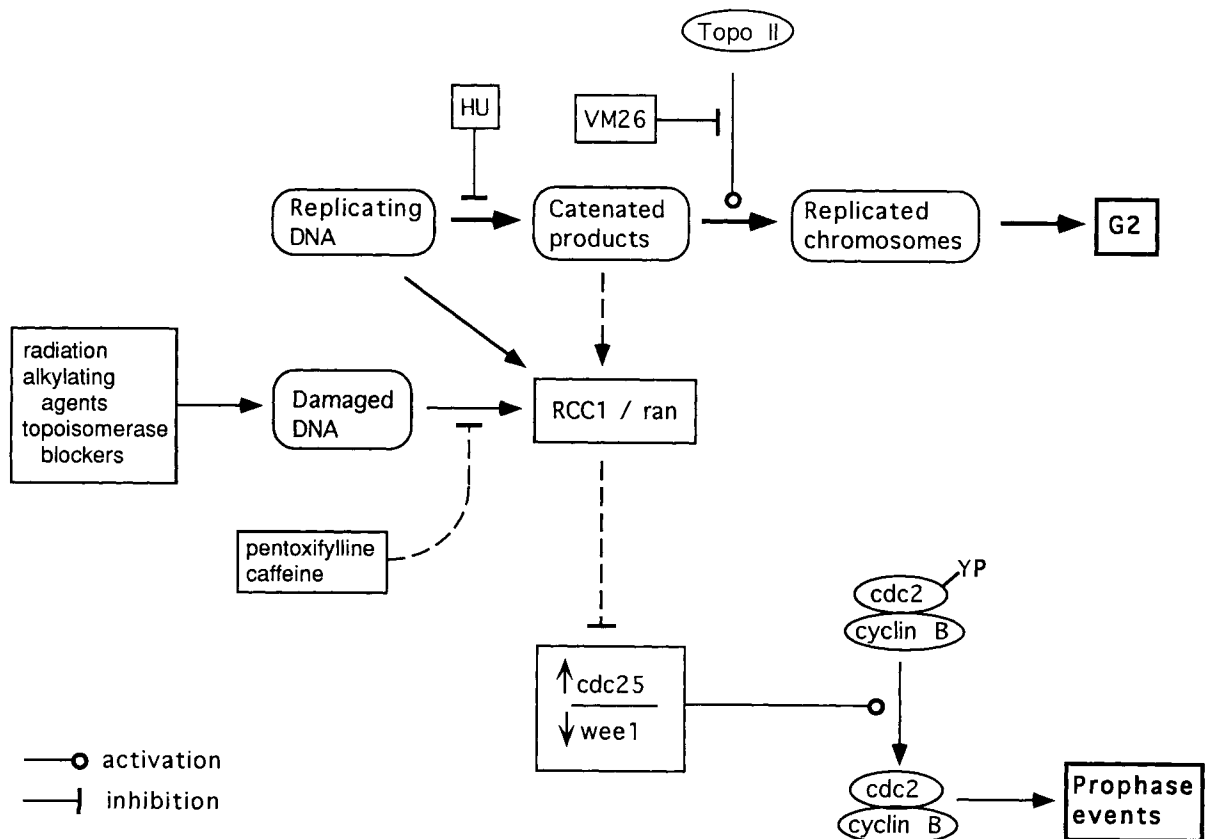

Fig. 3. General scheme for how various types of drugs could affect mitotic events by signaling via RCC1/ran.
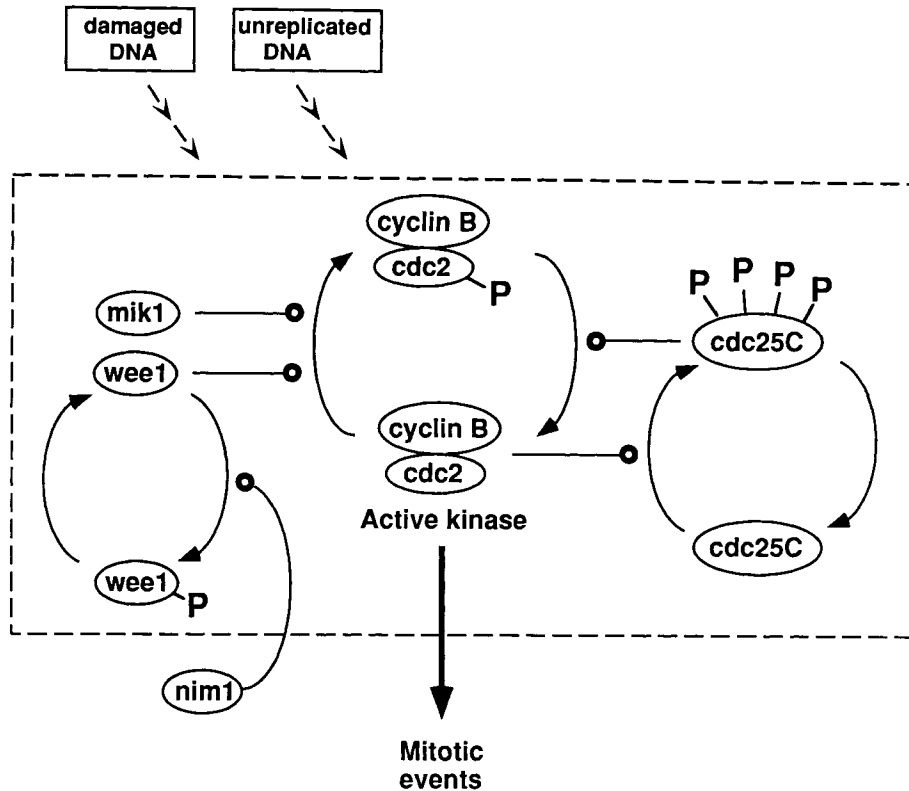
Fig. 4. Model of the cyclin B switch.

for 30 min after the removal of aphidicolin. S phase, although somewhat slowed, proceeded to a complete, or nearly complete, duplication of DNA. Most of the cells, however, failed to initiate mitosis. This is typical behavior for cells exposed to a DNA damaging agent (although, as previously mentioned, we have found that the ability to arrest in G2 is deficient in some human cancer cell types). The cyclins and cyclin-dependent kinases in the nitrogen mustard–treated cells exhibited the following behavior as the cells completed DNA duplication and waited for a mitosis that did not come. The kinase activity of cdc2–cyclin B failed to rise as it normally does in control cells entering mitosis. The phosphorylations of cdc2 at tyrosine 15 and threonine 14 failed to be removed. The kinase activity associated with cdk2–cyclin A normally rises during S phase and then falls prior to mitosis; in treated cells the kinase activity rose during S phase and continued to rise to abnormally high levels. Therefore the control point of the block in treated cells precedes both the rise in cdc2–cyclin B activity and the fall in cdk2–cyclin A activity. Although drug treatment did not greatly alter the amount of cyclin B present

in the cells, the treatment did have a major effect on the amount of cyclin A protein. Normally, cyclin A protein decreases markedly as cells enter mitosis; but in treated cells, the amount of cyclin A remains high. Hence the control point of the block also precedes the fall in cyclin A protein.

At the next outer level of control, we have looked at the behavior of phosphatase cdc25C [O'Connor et al., in preparation]. The system of synchronized and treated cells was the same as above. When cells go into mitosis, cdc25C normally becomes highly phosphorylated, and thereby activated. In nitrogen mustard–treated cells, there was neither phosphorylation nor activation of cdc25C. This result is consistent with cdc25C being part of an integrated switch that activates the output of the control system, kinase cdc2. Therefore the control point sensed by DNA damage must be at or before the input to this switch.

Controls During Mitosis

Cells can be blocked in metaphase by microtubule inhibitors such as colchemid, nocodazole, vincristine, and taxol. In order to retain viability

during a period of metaphase arrest, cells must (1) inhibit exit from mitosis (e.g., chromosome decondensation) and (2) remain able to restore a functional metaphase spindle when drug is removed. The inhibition of exit from mitosis varies among mammalian cells, human cells generally being better able to accomplish this than are rodent cells [Kung et al., 1990a].

In experiments with the mitotic inhibitor, vincristine, Kung et al. [1990b] obtained results that were in most respects similar to their findings with DNA synthesis inhibitors. Conducted using Chinese hamster ovary cells, these experiments again showed that inhibition of protein synthesis by cotreatment with cycloheximide prevented the loss of clonogenic survival that otherwise followed an 18 hr exposure to vincristine. Again, the onset of physical cell death (loss of plasma membrane integrity) was delayed until the inhibitors were removed and the cells presumably attempted to consummate mitosis.

An Integrated Processor?

Immunofluorescence studies sometimes show cyclins and associated kinases localized in granular or punctate structures [Girard et al., 1991; Pines and Hunter, 1991; Riabowol et al., 1989]. Are these proteins integrated in some kind of superstructure which functions efficiently to choreograph cell division events? A recent crystallographic analysis of CksHs2, a human version of the Suc1/Cks proteins, has suggested the form of a possible superstructure [Parge et al., 1993]. The Cks proteins bind strongly to cyclin-dependent kinases, are essential for the in vivo function of the kinases, and are functionally conserved from yeast to human. The structural analysis of CksHs2 showed that two of these molecules can bind tail-to-tail, and three of these homodimers can join to form a ring-like hexamer having a 12-Å-diameter central tunnel. This hexamer structure may function as a core to which cdks (cyclin-dependent kinases) could bind; molecular modeling showed that a cdc2 (cdk1) molecule could be bound to each Cks unit in the hexamer simultaneously without steric interference.

Parge et al. go on to suggest that the cdks could be bound to cyclins which would add a third layer to the structure (Fig. 5). This structure would deserve a name, such as "cyclosome." The cyclosome would interact with kinases such as wee1 and mik1, and with phosphatases such as cdc25. Phosphates, viewed as negative charges

that can be added or removed from specific sites on a cyclosome, evoke an image of a kind of integrated computing circuit. If cdc25 could interact with a cyclosome cluster containing several phosphorylated cdc2 molecules, its phosphatase function would be highly efficient, as demanded for a switch. The mutual activation of cdc25 and cdc2 could occur by the shuttling of a single cdc25 molecule from one cdc2 molecule to the next in the cluster. This would be consistent with the much lower amount of cdc25 than cdc2 present in the cell, and with the finding that cdc25 is bound to cdc2 during a specific time shortly before mitosis [O'Connor et al., in preparation].

The decision network operating at the restriction point (R) may involve cyclins E and A [Dou et al., 1993], suggesting that these cyclins, together with their associated cdks, would be constituents of a "G1/S cyclosome." The "G2 cyclosome," on the other hand, would contain cdc2 and cyclins B and A (since cyclin A, as well as cyclin B, has been found associated with cdc2 in nitrogen mustard–treated cells arresting in G2) [O'Connor et al., 1993a].

The cyclosome may also interact with (or even be built up further to include) other regulators, such as the Rb/p107 proteins and their associated E2F transcription factors. Since a variety of proteins are known to bind to cyclin/cdk pairs and to become phosphorylated by them, one might anticipate the existence of other proteins that would bind but inhibit function at the cyclosome level. This could explain the puzzling findings recently that phosphorylation of cdc2 is not essential for G2 control in budding yeast [Amon et al., 1992; Sorger and Murray, 1992]. In nuclear extracts, Rb and a cdk have been detected in a high molecular weight complex, designated Yi1, which is converted to a lower molecular weight form, Yi2, at or about the time of the G1/S transition [Dou et al., 1992]. A particularly interesting recent finding is that cyclins and cdks are found in complexes with PCNA (proliferating cell nuclear antigen) and a p21 protein; these complexes are disrupted in cells that have been transformed by SV40 T antigen or that have a p53 deficiency (cells from Li-Fraumeni patients) [Xiong et al., 1993]. It seems that all of the key participants in cell cycle control logic may function in integrated superstructures, and it is here that regulatory deficits of cancer cells might be found and exploited.
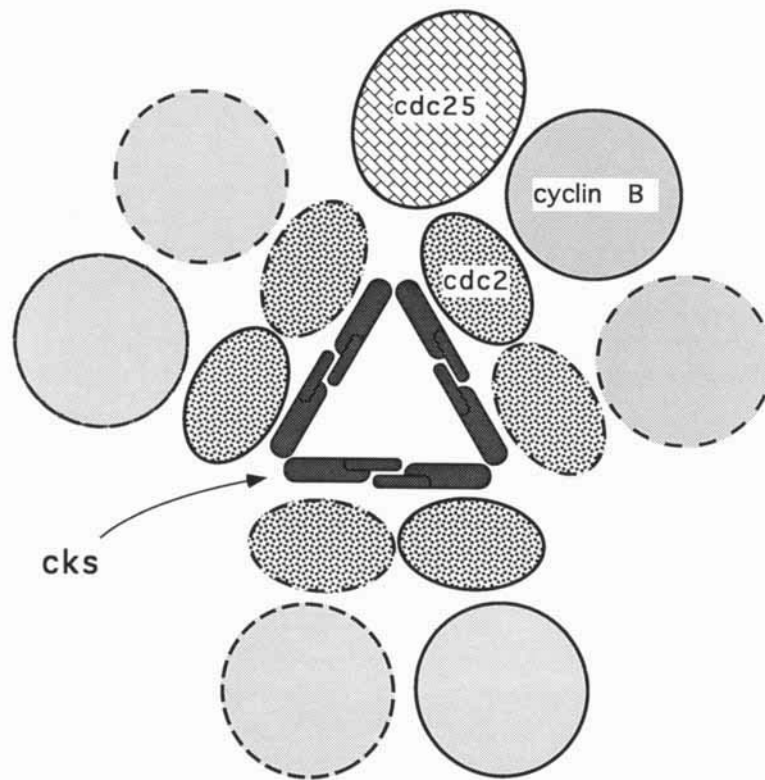
Fig. 5. The "cyclosome" model, elaborated from the model proposed by Parge et al. [1993]. The core of the structure is made up of three Cks homodimers which form a somewhat triangular ring with a 12-Å-diameter solvent tube within which divalent cations necessary for stabilization of the structure may be located. Cdk molecules, such as cdc2, would bind to the Cks proteins to form an outer layer. According to the model of Parge et al., three would be centered above and three below the plane of the page. Cyclins could then be imagined bound in a next outer layer to the cdks. To illustrate the functional possibilities of this structure, a phosphatase cdc25 is shown bound to a cdc2–cyclin B pair. Other control molecules, such Rb, p107, and E2F, that bind to cdk–cyclin pairs could also interact forming integrated logic units.

Prospects for Chemotherapy

Much has been learned recently about the constituent molecules and component reactions that control cell proliferation. This information is accumulating at an extraordinary rate. A coherent picture of how these components work together as a system still seems a long way off, but some tentative possibilities are beginning to emerge. We have reviewed some aspects that seem potentially applicable to cancer chemotherapy and finally consider the general forms that such applications might take.

Cell proliferation and cell cycle events evidently are controlled by complex reaction networks. The state of a complex reaction system fluctuates in response to physiologic variations in conditions, such as concentrations of nutrients, metabolites, and regulator substances; physiologic stress is especially severe when cells move from one cell cycle phase to another and

when cells respond to DNA damage. Stress here refers to perturbations in the concentrations, as well as in the rates of synthesis and degradation, of regulatory components. Normal control is so tight that a cell almost never enters a condition (i.e., a point in "state space") from which it could not fully recover. A hallmark of neoplasia is a weakening or disturbance of these controls. As a result, neoplastic cells have an increased probability of undergoing irreversible changes leading to progressive abnormalities or death. The main point here is that this same weakening of control could increase vulnerability to some kinds of pharmacologically induced stress. The challenge, therefore, is to identify the regulatory weaknesses in individual cancers and to use this information to tailor therapies (preferably not involving actual DNA damage) to push the cells into a region of state space from which they cannot recover.

One general stratagem would be to use a pharmacologic agent (for example, an inhibitor of certain kinases or phosphatases or of the expression of certain genes) to activate a regulatory factor that normally prevents cells from entering a vulnerable phase, such as S phase or mitosis. The inhibitor selected would be such that the factor it activates is defective in the targeted cancer cells. The cancer cells that enter the vulnerable phase, while critical normal cells remain arrested in a safe state, would be vulnerable to selective attack with available drugs that specifically kill cells in S phase or mitosis. This general type of stratagem has been suggested using puromycin aminonucleoside [Bradley et al., 1977; Studzinski and Gierthy, 1973] or staurosporin [Crissman et al., 1991; Gadbois et al., 1992; Kraemer and Bradbury, 1993] to selectively arrest normal cells in a drug-insensitive state of the cell cycle.

Another type of binary drug stratagem that has been proposed would combine a cell cycle arresting drug, such as hydroxyurea, etoposide, or taxol, with an inhibitor, such as 2-aminopurine, that would override the arrest; 2-aminopurine, a protein kinase inhibitor, was found to induce premature entry into mitosis in hamster cells arrested in G1, S, or G2, and premature exit from mitosis in cells arrested in M [Andreassen and Margolis, 1992]. Premature entry into mitosis, however, would occur only if adequate levels of cyclin B and cdc2 are present during S phase, as is the case in hamster cells, and may not occur in some human cells in which cyclin B levels remain low during much of S phase [Steinmann et al., 1991]. The earlier expression of cyclin B found in human breast cancer cells suggests a basis for selectivity against some tumors [Keyomarsi and Pardee, 1993].

Much still needs to be done to develop specific inhibitors of key kinases, phosphatases, or genes. Another means of pharmacologic intervention would be through inhibitors of proteases that limit the ambient concentrations, for example, of cyclins or p53; the cell cycle perturbations caused by exposure of cells to a peptide that blocks the degradation of cyclins have recently been described [Sherwood et al., 1993].

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