Histol Histopathol (1998) 13: 1197-1214

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Invited Review

Cell proliferation and cancer*

J.F. López-Sáez[1], C. de la Torre[2], J. Pincheira[3] and G. Giménez-Martín[2]

[1]Department of Biology, Autonome University of Madrid, Madrid, [2]Centre of Biological Researches, CSIC, Madrid, Spain and [3]Department of Cell Biology and Genetic, School of Medicine, University of Chile, Santiago, Chile

Summary. The discovery that phosphorylation of selected proteins by cyclin-dependent kinases is the engine which makes the cycle run provides a new image of the control of proliferation and of its deregulation. The high conservation of this machinery in the different eukaryotic organisms emphasizes its early origin and its importance for life. It also makes the extrapolation of findings between different species feasible. The control of proliferation relies basically on accelerating and braking mechanisms which act on the engine driving the cycle. This review particularly stresses the importance of checkpoint or tumor suppressor pathways as transduction systems of negative signals which may induce a cycle braking operation. They prevent any important cycle transition, as the initiation of proliferation, that of replication, mitosis, etc., until the DNA and other cellular conditions make such a progression safe. These checkpoint pathways are able to recognize and transduce signals about the adequacy of initiating or continuing proliferation for a cell at a particular time, under a particular set of external and internal conditions. Crucial components of these pathways are proteins encoded by some of the checkpoint genes that evaluate the final balance of mitogenic and antimitogenic pathways reaching them and, if the balance is negative, they prevent temporarily cycle inititation or its progression by inhibiting the corresponding cyclin-dependent kinases. On the other hand, when the balance becomes positive, they allow the activation of the cyclin-dependent kinases. Uncontrolled cell proliferation associated with cancer always depends on the functional abrogation of at least one of the checkpoint pathways. The checkpoint or tumor suppressor protein p53 is one of the proteins in them, and mutations in the gene encoding it are present in more than half of all human tumours. The review touches new pharmacological strategies which have been opened by the discovery of portions of some of the signal transduction cascades involved in the transient brake of cell proliferation. Restoration of checkpoint pathways either prevents further proliferation of cells with damaged genome until repair is over or, alternatively, the dismantling of these checkpoints induce those cells to commit suicide (apoptosis). The fact that both restoration and dismantling of checkpoint pathways sensitive to DNA damage have not disturbing effects on any other proliferating cell with undamaged DNA makes these selective strategies promissing.

Key words: Cell proliferation, Cyclin-dependent kinases, Checkpoints, Tumor-suppressor genes, Cancer

Offprint requests to: Prof. Dr. Jorge F. López-Sáez, Departamento de Biología, Universidad Autónoma de Madrid, Canto Blanco, E-28049 Madrid, Spain

*This review is dedicated to Jesús Vázquez, a great teacher, a great histopathologist and, overall, a great man who left us prematurely.

Introduction

Cell proliferation should be considered as the most fundamental property of living beings. The maintenance of life directly depends on cell proliferation: the survival of a species relies on the reproduction of its germ cells while the survival of the individual depends on the reproduction of its somatic cells. Only a very few specialized cells are able to survive for a long time without reproduction, such as gametes under extreme environmental conditions (germ cells) or neurons (somatic cells). Most cells, however, are faced with the reproduction-death dilemma.

Cell reproduction accounts for the formidable increase in cell numbers which go from the single cell of the zygote up to the 10^{13} cells which form an adult man. Moreover, cell renewal is estimated to be supported by 20×10^6 cell divisions per second throughout the whole life span of a man!

How is cell proliferation controlled and how can its deregulation be cured? The control of the cell cycle depends on the availability of nutrients in the unicellular organisms. On the other hand, the problem of nutrients is usually solved in the multicellular organisms while other mechanisms account for the control of cell growth in them.

A simple model of a cell cycle takes into account that proliferation is mainly controlled by two opposing mechanisms -accelerator and brake- which act on the

cyclin-dependent kinases, the engine which drives the cycle. Failures in the accelerator tend mostly to permanently stop the engine, while those in the brake often allow its unrestrained progression.

1. Proliferation and cell cycle

As a way to study real cell proliferation, the use of its ideal model, the cell cycle, is a useful tool. Proliferation is the biological fact, while the cycle is only a model which tries to explain the behaviour of the different cells responsible for such a proliferation in a particular tissue. Whereas duplication of the cell components and their segregation between the daughter cells is exact in the ideal cell cycle, they certainly change to some extent among the different cells proliferating in a tissue.

Cycle studies were initiated by Howard and Pelc (1953). They partially unveiled the function of a proliferating cell during interphase. They demonstrated that the interphase preceding the chromosomal segregation possesses three different parts (Fig. 1). The actual period where DNA is replicated was named the S period or period of DNA synthesis, while two gaps between the replication and segregation of DNA were named G1 and G2 (gaps 1 and 2) for the pre- and post-replication periods, respectively.

The sixties were the years when the kinetics of proliferation and the S period were preferentially analyzed. The seventies were mostly devoted to the genetics of the cell cycle, a task which has been specially successful in yeasts. As a consequence of these studies, the cycle was defined as a cellular process dependent on the ordered expression of a relatively small number of genes, the cell division cycle genes (cdc genes). Moreover, Mitchison (1971) proposed a model in which the cell cycle resulted from the integration of two cycles: that of growth in the cell mass which is a continuous one and that of replication and segregation of DNA which is formed by the two discrete S and M (mitosis) phases (Fig. 2). The integration of both cycles obviously depends on a certain coupling between them. The requirement to reach a certain mass for initiation of replication (m_i in Fig. 2) to be triggered was first shown in animal cells (Killander and Zetterberg, 1965), then in prokaryotes (Donachie, 1968) and later in higher plant cells (Navarrete et al., 1983). Analogous mass requirements were demonstrated for cells to initiate division (m_d in Fig. 2). As a consequence, G_1 and G_2 were considered to represent virtual cycle stages where the cell should reach a minimum size compatible with replication and mitosis, respectively.

However, it was later apparent that some requirements should be fulfilled by a cell before reaching both the S and mitotic periods. The stringency level of both G_1 and G_2 requirements need not be similar, as shown when looking at the response to the inhibition of protein synthesis in discrete portions of both cycle phases in plant proliferating cells (Fig. 3) (De la Torre et al., 1989). This different level of stringency, if large, can make one of these controls cryptic under physiological conditions. In general, the controls in G_1 are also more strict than those in G_2 in mammalian cells.

A new paradigma on the control of proliferation was established in studies by Rao and Johnson (1970) because of their elegant experiments based on fusing cells at different stages of the cycle. They showed that the progression of the cycle was mostly conditioned by

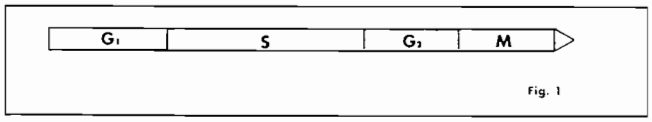

Fig. 1. Howard and Pelc's model for cell cycle. Replication in the S period of interphase is preceded and followed by the two short gaps G_1 and G_2 where the interphasic cells are not replicating their DNA. M: mitosis.

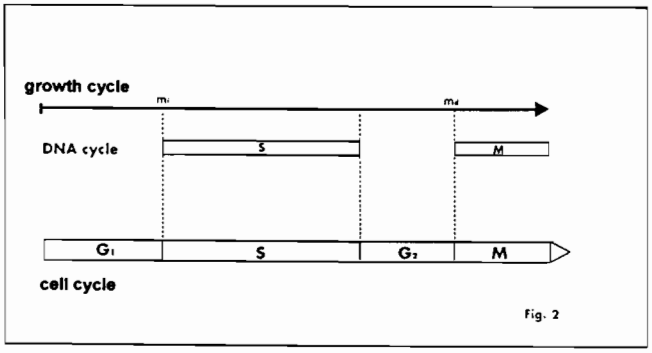

Fig. 2. Mitchison's cycle model. It integrates the fact that growth in mass is a continuous process and that replication and division only initiate when the cell reaches a minimum initiation and division masses, respectively. G_1 and G_2 are phases where cells wait until a proper size is achieved to progress into the subsequent phases of the DNA-division cycle. M: mitosis.

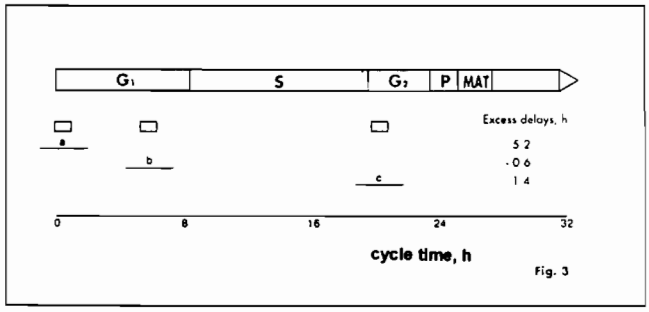

Fig. 3. The detection of interphase regions outside the S period where the transient inhibition of protein synthesis produces excess delays. This means that the synthesis of some specific proteins in discrete times of the cycle regulates its progression. The relative stringencies of the three interphasic regions where this phenomenon is produced are the following: a>c>b (De la Torre et al., 1989). P: prophase. MAT: metaphase + anaphase + telophase.

the cytoplasm stage. Cytoplasm dominated the nucleus for the control of cycle progression (Fig. 4, upper line A). Quantitative features reinforced this finding, since the rate of induction of any late cycle state was a direct function of the ratio of advanced to early state cytoplasms (see dosage effect at the intermediate line B of Fig. 4). Moreover, the cytoplasm of mitotic cells always induced chromosome condensation in the interphase nuclei, independent from their cycle stage (bottom line C in Fig. 4). At the same time, some nuclear conditions were critical to allow specific cycle transitions. Thus, G_2 nuclei are unable to reinitiate replication again when located in a cytoplasm which immediately induces it in G_1 nuclei. In fact, under physiological conditions, the replicated chromatin of the nuclei where replication is in progress does not reinitiate replication again in that cycle.

2. Molecular biology of the cell cycle

It took about 20 years to get an explanation for the results obtained when fusing heterophasic cells. The molecular biology of the cycle discovered how progression through it relied on the cyclic activation and desactivation of a family of kinases: the cyclin-dependent kinases or cdks. The kinases are enzymes which transfer phosphate groups from ATP to the hydroxyl groups of serine and threonine side chains of proteins. Changes in the activity of the cyclin-dependent kinases were in turn brought about by their association with cyclin, by their phosphorylation and dephosphorylation and by their interaction with inhibitors, as will be shown below. Molecular biology of the cycle started in 1988 when independent studies in budding and fission yeast on both Western and Eastern Atlantic sides, respectively, converged with studies in *Xenopus* and in mammalian cells. The cyclin-dependent kinase involved in the induction of mitosis in yeast was the same protein initially isolated and known as the maturation promoting factor (MPF) in animal cells. It had finally been purified (Lohka et al., 1988) and cross-reacted with antibodies raised against both the yeast cyclin-dependent kinase and the cyclin B partner protein. It was an indispensable component of the cytoplasm of maturing oocytes, i.e. initiating meiosis after progesterone treatment. MPF not only triggers meiosis in a recipient oocyte, but also induces mitosis in somatic cells. For this reason, MPF is indistinctively used to mean maturation, meiosis and mitosis promoting factor, given the identity of the initial letters. MPF is the alternative name for the universal cyclin-dependent kinase which specifically induces the G_2 to mitosis transition, i.e. for the mitotic cyclin-dependent kinase. Only two years after this confluence of discoveries, the conservation of the tools and the engine functions for cycle regulation was obvious (Nurse, 1990). The enormous amount of information accumulated on cycle transitions, dependencies and correlations among different cycle processes, as well as the detailed knowledge of many cell division cycle genes in two yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) permitted that extraordinary fast leap in the knowledge of the molecular control of cycle progression. How the balance between opposing mitogenic and antimitogenic signals is achieved by the operation of multiple checkpoint pathways is the field where the study of the cycle control is today.

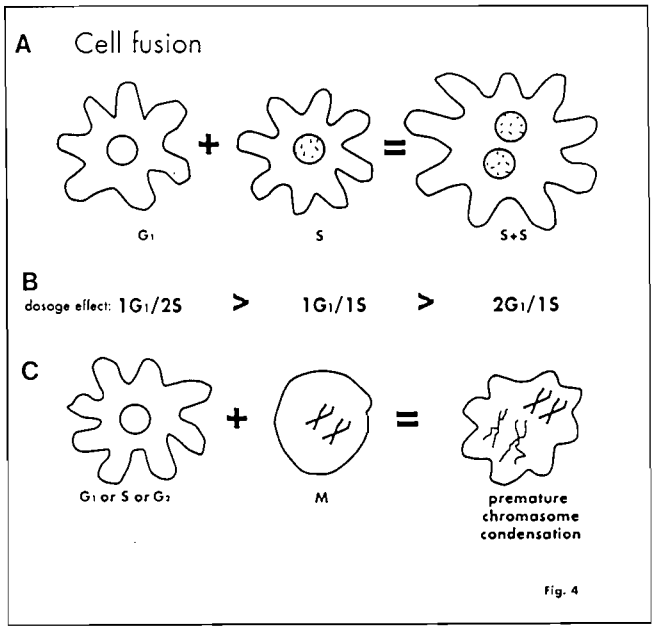

Fig. 4. Cell fusion experiments by Rao and Johnson (1971). They showed that the nucleus of a cell in G_1 when fused with a cell in S started replication immediately (upper line A). Dosage effects are schematized in mid line B: If two cells in S were fused with a single cell in G_1, the initiation of replication in this latter nucleus occurred much earlier than when fusing 1 or 2 G_1 cells with a single cell whose nucleus is replicating (line B). Another set of experiments (line C) demonstrated that the cytoplasm of a metaphase (M) always induced the condensation of chromosomes in any nucleus, independent from the stage of the interphase where it was located. When the induced nucleus was in G_1, chromosomes with a single chromatid appeared.

3. The cyclin-dependent kinases, the machinery which drives the cycle

By looking at a model which integrates the known features of the cyclin-dependent kinases, the multiple ways to regulate them are immediately apparent. Thus, the mammalian kinases are formed by different cdk heterotrimers (Fig. 5):

i) the catalytic subunit, sometimes called p34 (for its mass in kilodaltons) or $p34^{cdc2}$ if the gene encoding it in fission yeast is considered. On the other hand, there is a family of cyclin-dependent kinase subunits in the mammalian cells (cdks 1-7), each of them functional at a different cycle stage (Fig. 6). This component is the actual cyclin-dependent kinase or cdk. It is a serine-threonine kinase that is inactive as a monomer. This

subunit binds one molecule of ATP in a pocket, at the bottom of a cleft between its two lobes (see bottom left part of Fig. 5). All cdks contain, in their small lobe, a tyrosine residue in position 15 (Y15) and, in higher eukaryotes, also a threonine in position 14 (T14). Their phosphorylation by a tyrosine kinase prevents their activation (negative regulation), until the corresponding adequacy signals dephosphorylate them. The small lobe also conserves a region, the PSTAIRE (one single letter code for aminoacids) consensus sequence in cdk 1, 2 and 3, which is slightly modified in the other cdks. This sequence is apparently located in the cdk interface which binds to cyclin (Fisher, 1997). Mutations in that region can inactivate the kinase (Ducommun et al., 1991).

On the other hand, the threonine located at position 161 in the human cdk in the large lobe, the so-called T-loop, of the cyclin-dependent kinase, should be phosphorylated by the CAK (cdk-activating kinase) to become active (positive regulation).

ii) the cyclins or regulatory subunits of the kinase (p62 in mammalian cells). Cyclins from A to H have been described up to now in human cells. Cyclins contain the so-called "cyclin box" or region which binds the catalytic subunit. Its binding activates the cdk and allows its entrance into the nucleus. Phosphorylation of cyclins apparently potentiates the kinase activity of the multimeric complex (Li et al., 1995).

The pattern of synthesis and function of the main cyclins (A, B, D and E) is shown in Fig. 6, where the different catalytic subunits (cdks 1, 2, 4 or 6) they bind to are also displayed. They are rate limiting for the cdk activation. They integrate the transcriptional control into the cycle, as they are also labile due to a specific sequence, "the destruction box", which binds ubiquitin (a molecule targeting them to proteases). Cyclin D is the only one which is regulated by extracellular signals.

iii) there is a third small subunit, the cyclin-dependent kinase subunit (cks) (bottom right of Fig. 5). It is also named p9 in humans (because of its mass in kilodaltons). This cks exhibits such a high affinity for the catalytic subunit that it is used to isolate it. On the other hand, its function, though essential, has not yet been completely worked out in mammalian cells. In *Xenopus*, it controls the interaction of both positive and negative regulators with the mitotic cdk (Patra and Dunphy, 1996).

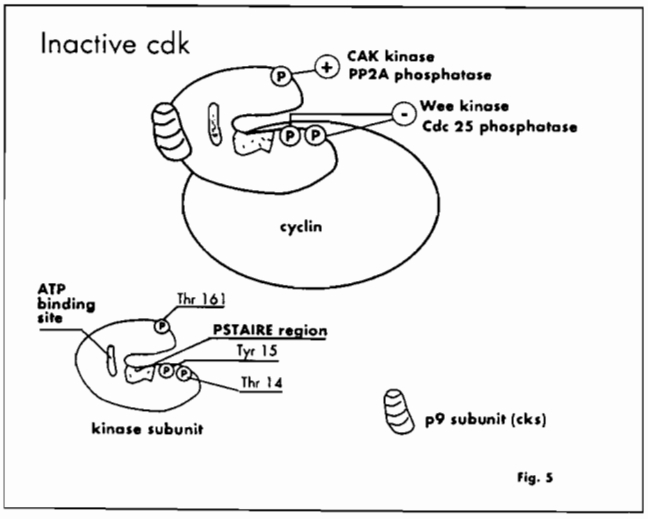

Fig. 5. The universal cyclin-dependent kinase which controls cycle progression. It is a trimer formed by the smallest subunit p9 (cks, analogue of $p13^{suc1}$) + the intermediate sized subunit which is the catalytic one (analogue of $p34^{cdc2}$) + the largest regulatory subunit or cyclin. The catalytic subunit possesses a threonine in position 161 which should be phosphorylated to be active as a kinase (see left bottom part), while it has a threonine in position 14 and a tyrosine in position 15 whose phosphorylation in mammalian cells prevents the final activation of the kinase (left bottom part of this figure). The ATP binding site and the consensus PSTAIRE region which interacts with the cyclin are also schematically displayed. Notice that the kinase has two lobes: the largest one corresponds to the amino-terminal region and it is the one which interacts with the cyclin. The smallest lobe, called the T-loop, correponds to the carboxy-terminal region and is located in all these figures in the upper position. In the upper right part of this figure, the kinases and phosphatases which are active in the phosphorylation sites are also shown. Notice that the CAK kinase is an activating kinase, while the Wee kinase is a negative regulatory kinase, because of the opposite roles both phosphorylations play.

4. Transduction of mitogenic signals

There are two types of control on cycle progression: one positive which drives the cycle and another negative which brakes it. The positive control is brought about by proteins encoded by the cell division cycle (cdc) genes. The proto-oncogenes are responsible for the positive control in mammalian cells. About 50 cdc genes have been described in budding yeast. The cdc genes and the proto-oncogenes when mutated stop cycle progression at some discrete stages. Since cell proliferation is essential for life, only cdc conditional mutants which become unfunctional under non-permissive conditions can be isolated.

Various categories of proto-oncogenes can be thought of. First of all, those which encode for

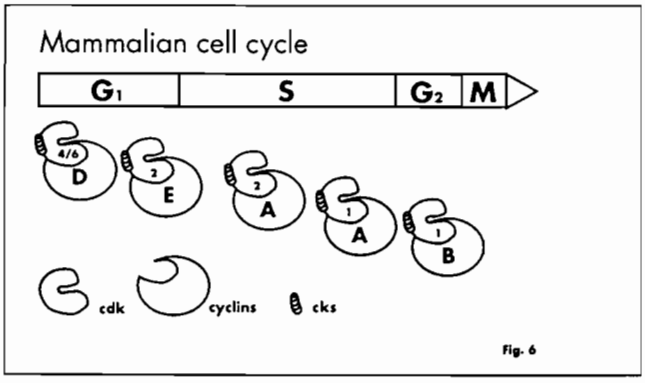

Fig. 6. Cycle-stage specificity of the different catalytic (cdks 1, 2, 4, 6) and cyclin (A, B, D and E) subunits (as specified in Fig. 5) which operate throughout the cell cycle in mammalian cells.

components of the cycle machinery, such as those forming the different cdk heterotrimers, the true positive regulators of cell proliferation. In mammalian cells, not only the cyclins but the different catalytic subunits are produced at different cycle stages (Fig. 6). On the other hand, the gene for the catalytic subunit of the kinase itself is constitutively expressed in yeast, where the introduction of additional copies of the gene had no effect on the cycle (Russell and Nurse, 1987). This stresses that it is the functional state of the kinase and not its quantity which regulates the proliferation rate.

The genes which encode for cyclin D are typical proto-oncogenes and some oncogenic viruses actually encode cyclin D homologues (Jung et al., 1994). In fact, cyclin D had been earlier described as a protein encoded by an oncogene or transformed version of a proto-oncogene (Hinds et al., 1994). The knowledge of cdks and the pathways which result in their activation makes terms as oncogenes and proto-oncogenes no more informative than mitogenic genes.

The overexpression of any mitogenic genes can be achieved in different ways. For example, increases in the rate of synthesis of the protein they encode, anticipation in the time of their expression, increase of their efficiency or prevention of its degradation. The over-expression can be achieved as a result of the translocation of the gene to another genome site (position effect) or by gene amplification (*c-myc*).

There are many other proteins needed for cycle progression to occur which, however, should not be regarded as cycle regulatory proteins. This is evident if we think of the enzymes and precursors needed for DNA replication, and the synthesis and assembly of tubulin for DNA segregation in mitosis, etc.

5. Physiological inhibitors of the cyclin-dependent kinases

The discovery of a group of proteins which are direct inhibitors of the cyclin-dependent kinases (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Serrano et al., 1993; Xiong et al., 1993; Harper and Elledge, 1996) will certainly have a large impact in the field of cancer prevention or cure, as they can control proli-feration. There are at least two families of these cyclin-dependent kinases inhibitors (cki). The p16 family is formed by p15, p16 itself, p18 and p19, while the p21 family is formed by p21 itself, p27 and p57. They associate with the catalytic subunit of the cyclin-dependent kinases in a non-covalent way, preventing the kinase activity. As can be seen in Fig. 7, members of the p16 and p21 families (i.e p15, and p21 itself and p27) have affinities for different cdks.

The discovery that p53 (the product of a tumor suppressor or checkpoint gene) actually activates the transcription of the inhibitor p21, which prevents cdk activation and cycle progression, is the clearest example of the link between tumorigenesis and the cycle machinery.

It should be noticed that p16 is the only inhibitor which can bind monomers of the catalytic subunit of cdks, while the rest only bind the subunit when in the trimer. The antimitogenic signal produced by the transforming growth factor ß (TGF-ß) induces a blockade in various cdks. It activates the synthesis of proteins which leads to the production of p15, a member of the p16 family (left part of Fig. 7). This p15 is also able to inactivate the phosphatase Cdc25 by binding to it (Iavarone and Massagué, 1997).

6. Transduction of antimitogenic signals: the checkpoint pathways

The eukaryotic cells also contain other important controls which are able to brake cycle progression. They can be considered feedback safety mechanisms and they are more sophisticated than the positive ones. These negative controls are integrated in checkpoint pathways and depend on genes known as checkpoint genes (chk) or tumor suppressor genes, as they are called in mammalian cells. As a matter of fact, all human tumours happen to have mutations in some of the tumor suppresor genes (Taylor and Shalloway, 1996a). Moreover, proteins codified by most oncogenic DNA viruses have affinity for, and are inhibitors of, some of these checkpoint genes (see Moran, 1993).

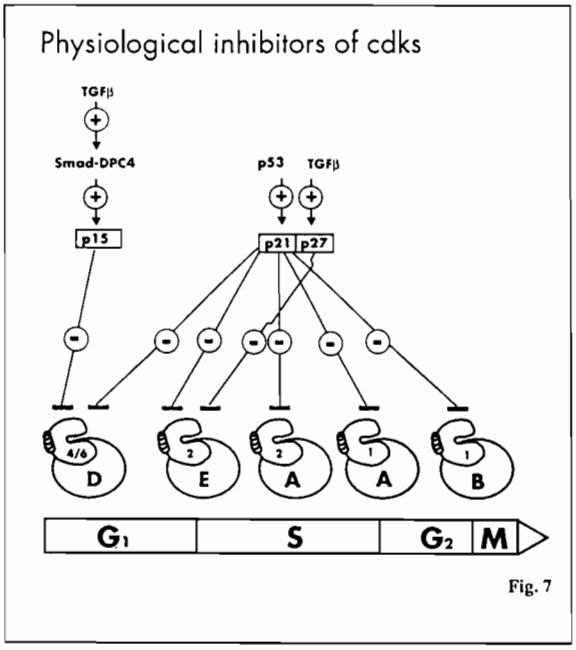

Fig. 7. Effect of different members of the two families p16 (i.e. p15) and p21 (i.e. 21 itself and p27) of physiological inhibitors of cdks. The main routes for specific members of each family are marked. The protein p21 is the most universal inhibitor of cdks.

There are also four classes of tumor suppressor or checkpoint genes: 1) those which encode inhibitors of the cyclin-dependent kinases; 2) those which prevent the repair of the genes encoding for either cdks or for their inhibitors; 3) those which allow the persistence of transformed cells by preventing terminal differentiation; and 4) those which also allow that persistence by preventing programmed cell death (apoptosis) (Harper and Elledge, 1996). As in the case of the proto-oncogenes, we should restrict the use of the terms checkpoint or tumor suppressor genes and use instead terms referring to their mechanism of action, as soon as it is known.

Checkpoint pathways of cycle progression are the most evolved mechanisms we can think of in a cell. They are security mechanisms which ensure that the aim of the cycle will be faithfully accomplished: to produce two cells similar to the one they derive from, including crucial control of the accuracy of the DNA information. For this, the external and internal conditions related to the adequacy for initiating proliferation or for a particular cell transition at a particular time are surveilled. Thus, the negative control integrates hormonal signals, information on the position in the tissue, and on cell size, DNA precursors, the protein synthesis capacity, stress environmental signals, on the integrity and state of the DNA, on the energy available, and on the different pools of molecules required, etc. Controls integrated in the checkpoint pathways which evaluate both mitogenic and antimitogenic signals are multifunctional: they are not only able to recognize a failure but also to weigh contradictory cycle progression signals, to temporarily stop cycle progression if such balance suggests that it will compromise the viability, to induce repair of the detected failure, and to allow cycle progression when it has been repaired. The signals produced by the operation of a checkpoint should be amplified so that it should be efficient to quickly induce a reversible cycle block or to free cycle progression. This is achieved because these signals affect one or more cascades of the antimitogenic signal transduction. Moreover, some of the checkpoint proteins are involved in more than one single checkpoint pathway. It represents the main source for complexity of the proliferation control.

The final effect of the checkpoint functions on cycle progression is produced by either preventing the activation or inactivating the corresponding cyclin-dependent kinases, i.e. by modifying the components of the machinery which actually drives the cycle (Walworth et al., 1993; Sánchez et al., 1997).

7. Discernement of positive and negative cycle controls

Both positive and negative controls are clearly distinguished by the opposite effects they produce when cancelled. Cancellation of a positive control leads to a block in the progression of the proliferating cell at a specific point in the cycle, an effect which is clearly opposite to the induction of uncontrolled proliferation. On the other hand, cancellation of a negative control leads to a precocious progression towards later phases in the cycle, when either the cell is not prepared at all for such a progression, or the tissue signals are against such proliferation. When the checkpoint pathways are functional, cells will not enter mitosis until replication or repair of DNA is completed, or until the minimum cell size for division which characterizes the tissue has been reached, or until the entrance into mitosis is not going against the social signals which try to maintain the specific growth fraction of the tissue.

Such tissue-specific growth fractions usually cover, but do not exceed, the renewal of worn out cells. The cells having precociously and unduly overcome a cycle checkpoint can be considered to be transformed cells indeed. In this way, deficiencies in the DNA cycle when DNA lacks the necessary integrity leads a cell to acquire a mutator phenotype, i.e. to suffer from genomic instability, the main hallmark of cancer. Occasionally, the activity of a checkpoint can lead a cell with damage high enough to be considered irreparable to initiate the suicide pathway (apoptosis). Isolation of checkpoint genes is based on their high sensitivity to irradiation and drugs and on their ability to overcome the cycle arrest induced by the cdc mutants (Murray, 1995).

8. Commitment to proliferate and the retinoblastoma protein pathway

Shortly after completion of telophase, in response to an efficient stimulus, the cell can be committed to proliferate by initiating a cycle. This is the G_0 to G_1 transition. In the presence of signals to proliferate, cells first have to leave G_0 and to commit them to cycle by reaching G_1. This decision is said to be taken at the "Start" or restriction point, as it is named in yeast and mammalian cells, respectively. The discernement between the G_0 to G_1 and the G_1 to S transitions has been supported from the early days by the observation of yeast mutants whose cells arrested in G1 after "Start", but before the S period (Hartwell et al., 1974). Partly because the induction of proliferation in a tissue is more easily discerned by the G_1 to S transition than by the G_0 to G_1 transition there is still some confusion about these two sequential transitions.

Cells in G_0 and G_1 are metabolically different. This difference can be used to estimate the proliferative potential of a tissue which may be expressed by its G_1 to G_0 ratio. This ratio is useful to evaluate cancer remission or release after a specific antitumoral treatment (Hittelman and Rao, 1978). Thus, it changes well before labelling and mitotic indeces modify (Sans and De la Torre, 1979).

When the universal inhibitor of cycle progression p21 is present, two G_1 cyclin-dependent kinases and the PCNA or auxiliary factor of the DNA polymerase δ are inactive (Waga et al., 1994) because of p21 binding to

the N-terminal region of the cdk.

On the other hand, when the p21 pool becomes negligible, three pathways are activated. The first pathway regulates the G_0 to G_1 transition (left part of Fig. 8). It is the retinoblastoma protein pathway and constitutes the most relevant target for oncogenesis. It is implicated in cell committment to proliferate (Bartek et al., 1996). Recently, the role of this pathway has been reviewed (De Luca et al., 1996). First of all, the cyclin D-dependent kinase 4/6 complex phosphorylates the checkpoint retinoblastoma protein (pRb). As can be seen in the left part of Fig. 8, the phosphorylation of pRb causes its inactivation and, concommitantly the release of its associated or bound E2F transcription factor needed to activate the genes whose expression commit a cell to proliferate. Mitogenic signal transduction systems from three different classes of receptors are actually upstream regulators of this checkpoint pathway (Lukas et al., 1996).

9. Integration of the social control in a cell throughout the retinoblastoma pathway

There are two types of signals which convey the adequacy to initiate the cycle. One of them involves the social control in the cell cycle. This control is formed by hormonal signals coming from different tissues and by signals produced in the tissue itself. These signals ensure the size of the specific tissue in which the cell is included, and the number of cells forming it, without blocking the normal process of cell replacement. When a cell overcomes these controls it is said to have been transformed.

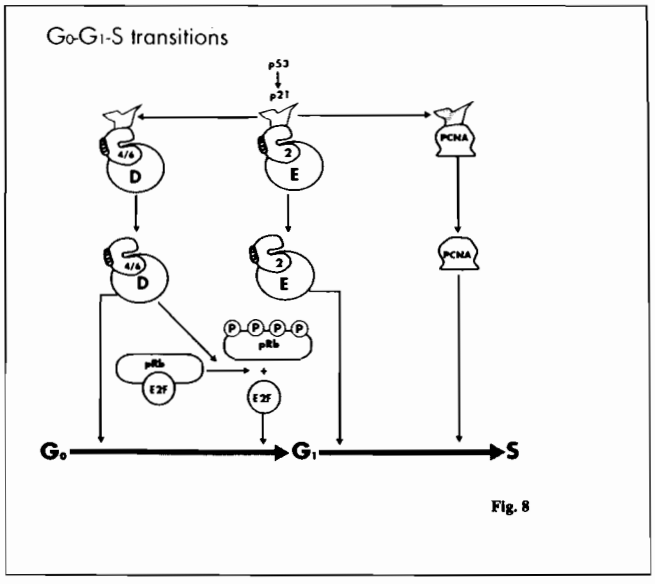

Fig. 8. The role the cdk-inhibitor p21 plays in the activation of the different cyclin-dependent kinases, and the role these kinases play in the G_0 to G_1 and G_1 to S transitions. The protein p21 is continuously synthesized because p53 activates the transcription of the p21 coding gene. When the p21 level decreases, three different routes are activated. First of all, at the left, p21 detaches and activates in this manner the catalytic subunit 4 or 6 already bound to cyclin D. This cdk phosphorylates the pRb (the retinoblastoma protein) which only then frees the transcription factor E2F. This factor induces the transcription of genes related to cycle commitment (G_0 to G_1 transition). In continuation, when p21 disappears, the cdk2-cyclin E complex is also activated (mid line) and, finally, the removal of p21 of the PCNA or auxiliary factor of the polymerase b is also activated (right line), so that replication can now start freely (G_1 to S transition). Encircled P: phosphate.

Extracellular signalling proteins which stimulate cell proliferation are known as growth factors. Downstream, there is always a specific receptor in the membrane which interiorizes each signal. A part of these mitogenic signals are transduced by a set of protein kinases, starting by Ras protein, then Raf and then a set of MAP-kinases (Mitogen-Activated Protein kinases), in a chain of protein to protein interactions. In fact, the expression of the Ras protein also increases the cyclin D level to make the inititation of proliferation possible (Liu et al., 1995). But this particular chain of transmission of mitogenic signals, in the end, activates transcription factors of those genes which are the so-called early response genes when proliferation is induced: *c-fos*, *c-jun* and *c-myc* (Taylor and Shalloway, 1996b). *Fos/jun* heterodimers constitute the AP-1 transcription factor while *c-myc* is mitogenic because it stimulates the transcription of the phosphatase Cdc25, which in turn activates cdks by dephosphorylating their threonine and tyrosine residues located at position 14 and 15 (Zörnig and Evan, 1996).

The response of the hepatocyte to the human recombinant hepatocyte growth factor illustrates the timing of the mitogenic response (Gómez-Lechón et al., 1996), as well as the kinetics of the different intermediate steps in this process (Fig. 9).

The antimitogenic signal produced by the transforming growth factor ß reaches a similar endpoint (Herrera et al., 1996). It activates the synthesis of a different transducer, Smad-DPC4 (left part of Fig. 7), where DPC4 is the product of a tumor suppressor gene

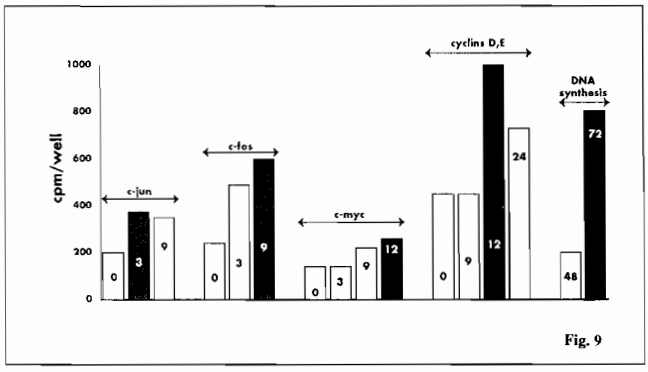

Fig. 9. The kinetics of the appearance of the different molecules related to the G_0 to G_1 and the G_1 to S transitions in the cycle of the hepatocyte, after stimulation by the hepatocyte growth factor. The time when the values were recorded are in the central part of each bar. The highest value for each cycle-related protein is labelled in black.

or checkpoint (Hahn et al., 1996). This complex is a transcription factor which induces the production of p15, one inhibitor of the G1 cyclin D-dependent kinases (Lagna et al., 1996).

It should be recalled that any inhibitor of a checkpoint will also work as an inducer of proliferation. Thus, the protein MDM2 induces proliferation because it binds and inactivates both p53 and pRb (Xiao et al., 1995).

10. Functions of the main tumor suppressor or checkpoint gene encoding p53

More than half of all human cancers present deletions, mutations or changes in the sequence of this checkpoint protein. When operative, p53 prevents the proliferation of a particular cell mostly by inducing the transcription of the gene encoding an inhibitor of cdks, the repressor protein p21 (Fig. 7). The checkpoint or tumor suppressor protein p53 evaluates the adequacy of proliferation by taking into account both external and internal signals, either mitogenic or antimitogenic ones. The regulatory inputs p53 receives can be conveyed through one of the many phosphoaminoacids it contains (Fig. 10), either the various serine residues in the amino-terminal region, or the one which is a target for the cdks and is located towards the carboxy-terminal region of the p53 molecule. Multiple enzymatic cascades converge in p53. They are the multiple upstream regulators of p53 activity.

The behaviour of this checkpoint protein is explained by the different p53 protein domains (Fig. 10). Its amino-terminal domain binds the transcription factor TFIIH subunit p62, the left part of p53 being directly implicated in the activation of transcription. It contains an intercalated region (closer to the carboxy than to the amino terminal extremes) which allows its movement into the nucleus (NLS, nuclear localization signal). On the other hand, the carboxy-terminal third of the molecule is an optional switch: it either activates DNA repair by binding the helicases XPB and D (xeroderma pigmentosum B and D) when damaged DNA is present or, alternatively, induces the transcription of the genes of the apoptotic pathway if the damage is too great for repair to take place. In this latter case, the protein Bax is induced. It dimerizes and inactivates the apoptosis inhibitor Bcl-2 (White, 1996). The expression of other caspases, the ICE-like family of cytoplasmic proteases which operates during apoptosis, is also triggered (Porter et al., 1997). It is interesting to notice that the helicases XPB and XPD are also involved in the apoptotic

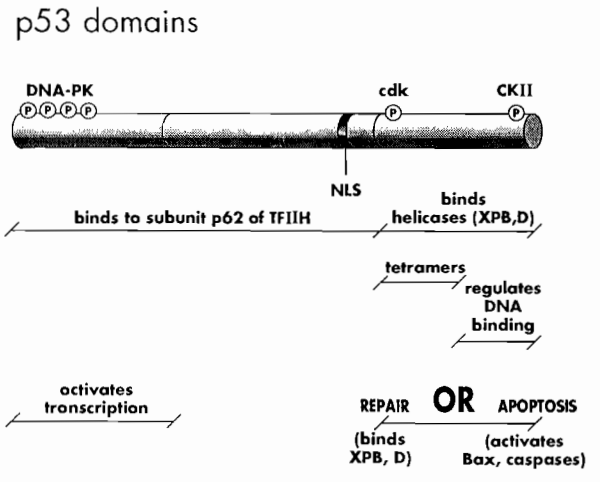

Fig. 10

Fig. 10. The different domains of the universal checkpoint protein p53. It contains six residues which can receive signals by phosphorylation (see upper part). It is a substrate for cdks, and also for DNA-PK (related to the recognition of DNA damage) and by CKII. The entrance into the nucleus of the p53 molecule is ensured by the nuclear localization signal it contains (NLS). The role of p53 in activating transcription of some negative regulatory genes is explained by its binding to the largest subunit of the TFIIH (second line). A subdomain has been positively involved in the activation of transcription (fifth line). But p53 also binds to helicases (as the Xeroderma Pigmentosum B and D) which are needed for DNA repair to take place. In the third line, the relative position of the domain responsible for tetramerization is also shown, as well as that responsible for regulating its binding to DNA (fourth line). Finally, the carboxy-terminal region of p53 behaves as a switch: either binding to helicases to induce repair or, if the damage is sensed as large enough to overcome the repair capacity of the cell, to induce cell suicide (apoptosis).

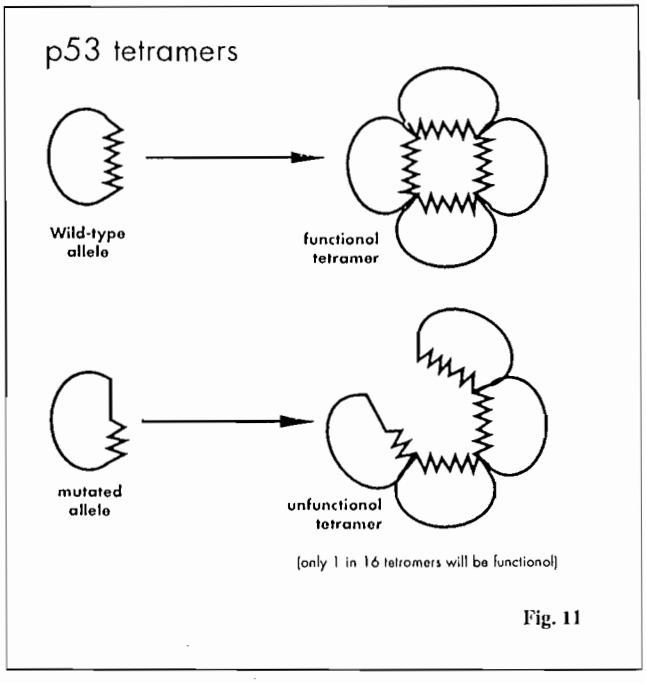

Fig. 11

Fig. 11. The formation of p53 tetramers explains the formation of dominant negative mutants from a recessive mutant. Thus, if a single one of the two alleles is mutated and its trancription is not modified, half of the p53 polypeptides of the cell will be wild type, while the other half will be mutated. This will assure that only 1/16 of the tetramers (the frequency of correct homotetramers) will be functional. The phenotype, under these specific conditions, behaves as mutated.

pathway (Wang et al., 1996).

Finally, since one previous step in the activation of p53 is the formation of tetramers, modifications in one single alelle of the DNA sequences which encode the aminoacids involved in tetramerization may dramatically affect the activity of p53 (Fig. 11). Only those scarce homotetramers (1 in 16), whose four molecules are encoded by the normal allele, are functional. The introduction of a single copy of a mutant gene inactivates the corresponding normal genes, a strategy followed to produce dominant negative mutations (Alberts et al., 1994).

In relation to its operation as a sensor of DNA damage (left part of Fig. 12), p53 is not only able to recognize single strand (Nelson and Kastan, 1994; Jayaraman and Prives, 1995) and double strand breaks in DNA, but also mismatches because of the presence of extra bases on one strand (Lee et al., 1995). This p53 checkpoint protein stabilizes in the cell when DNA damage is present.

The tumor suppressor protein p53 integrates the signal from DNA damage into the cycle by inducing the transcription of genes coding for direct inhibitors of the cyclin-dependent kinases such as p21. Moreover, it also stimulates the transcription of the growth-arrest and DNA-damage-inducible or Gadd genes (Hartwell and Kastan, 1994), which are also overexpressed in cells resting in G_0. It also interacts with and inactivates the auxiliary factor of the DNA polymerase δ which participates both in replication and DNA repair (Smith et al., 1994).

The p53 protein is also able to induce DNA repair by different mechanisms (Fig. 12). One is activation of the transcription of genes related to the repair machinery of the cells, the so-called transcription-repair complex (Wang et al., 1995). It attracts and activates helicases (products of some of the genes which are mutated in xeroderma pigmentosum). In fact, the XPB helicase is the largest subunit of the human RNA polymerase II basal transcription factor (TFIIH). Since this factor is necessary for the start of transcription, this molecule is at

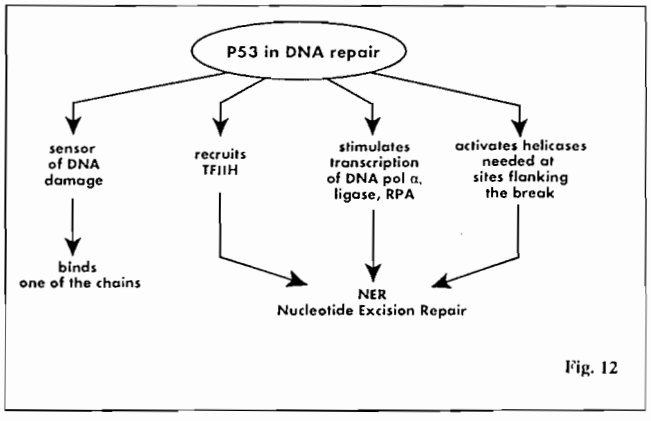

Fig. 12. The multiple roles of p53 in DNA repair.

the cross-roads of many crucial processes in the cell (Nigg, 1996). Helicases unwind DNA, take the energy by hydrolysing ATP, and separate both DNA strands. The replication protein A (RPA) binds to the single strand regions of DNA to prevent them from rewinding. The function of helicases is essential for nucleotide excision repair to take place in the damaged DNA. Then, non-semiconservative or repair replication takes place with the DNA polymerase, the replication factor C (RF-C) and PCNA (the auxiliary factor of the DNA polymerase δ). The repaired and new DNA stretch is finally ligated by a DNA ligase (Griffin, 1996).

In short, the presence of functional p53 tetramers ensures nucleotide excision repair. The p53 tetramers lower the frequency of mutations in the DNA, decrease gene instability, decrease gene amplification and prevent the aneuploidy secondary to a mitosis with damaged chromosomes. In this way, the p53 protein avoids the accumulation of mutations in the proliferating cells; the most obvious hallmark of transformation.

11. Checkpoint pathways related to competence for cycle progression

The control of genome integrity is obviously crucial for the continuation of the DNA duplication-segregation cycle. Signals related to the integrity of the DNA are processed by the operation of some checkpoint pathways. Only pieces of these other enzymatic cascades are known. On the other hand, some proteins like p53 are involved in more than a single checkpoint pathway. As a consequence, many efforts are made to discern them and their interactions.

DNA damage normally occurs due to replication errors or to the presence of genotoxic agents. It is integrated in the life of the cell which has mechanisms to repair it. In its positive aspect, the damage allows DNA

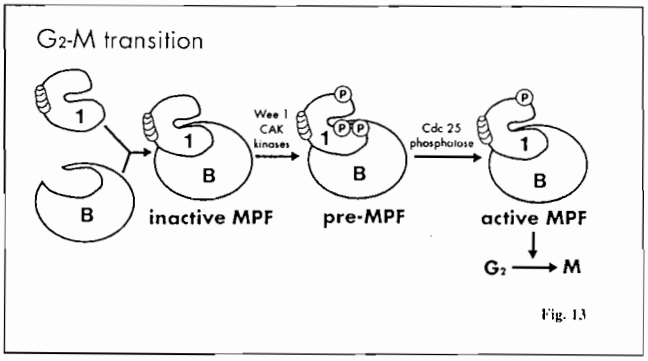

Fig. 13. Activation of the mitotic cdk or maturation promoting factor MPF. In principle, phosphorylation of both the activating and inactivating phosphorylation sites of the catalytic subunits takes place. These phosphorylations transform the inactive MPF trimer into a pre-MPF. The final dephosphorylation of tyrosine 15 and threonine 14 by the Cdc25 phosphatase in response to mitogenic signals fully activates the kinase, which is now called the active MPF. Active MPF induces the G_2 to M transition, by phosphorylation of multiple specific substrates.

evolution to take place. Meiotic recombination and that which takes place during the formation of antibodies are physiological processes which help the organism to survive in stressful environments.

The cell deals with DNA damage through different overlapping pathways which allow its efficient repair. The reversible brake of cycle progression due to the operation of the checkpoint mechanisms, and which depends on the production and transmission of antimitogenic signals, is the process which allows the time needed for repair to take place. Thus, cancer cells mostly result from failures in the surveillance of processes related to safe chromosome transmission (either replication or mitosis), failures in the repair itself and/or from failures in stopping the cycle until DNA integrity is restored. Only if there is cancellation of a checkpoint, can the DNA damage result in genomic instability, characterized by deletions, amplifications and, as a response to this serious stress, to gene translocations and genome rearrangements (McClinctock, 1984). Each transformed cell is a unique experiment in cell evolution. These defects are perpetuated, though they evolve and often increase in subsequent cell generations. Unfortunately, selection among the many cells with different genetic compositions occurs for the most active proliferating cells.

But p53 is only one of the molecules involved in checkpoint pathways sensitive to DNA damage. Thus, members of a family of kinases which phosphorylate lipids, the phosphatidylinositol 3-kinase (PI 3-kinase) family, are importantly involved in the recognition and transduction of signals related to the presence of DNA damage. Apparently, these enzymes do not retain any lipid kinase activity. Instead, they are efficient protein kinases. There are three prominent members: the product of the gene mutated in ataxia telangiectasia patients (ATM); the DNA-dependent Protein Kinase (DNA-PK); and the FRAP protein, another inhibitor of the mammalian G_1 cdks (Brown et al., 1994; Cimprich et al., 1996).

The DNA-PK recognizes and binds DNA double strand breaks. Only then does it become active as a kinase. It is a dimer formed by the catalytic subunit (Hartley et al., 1995) and the Ku subunit, which is a DNA helicase that binds to double strand breaks (Gottlieb and Jackson, 1994; Jackson, 1996). This helicase also controls telomere length (Porter et al., 1996). One prominent member of the DNA-PK, the human gene for ataxia telangiectasia, is also a homologue of a gene controlling telomere length in budding yeast (Greenwell et al., 1995; Morrow et al., 1995).

The DNA-PK is a multifunctional enzyme, also involved in both replication and nucleotide excision repair. Thus, it acts as a transducer for the DNA damage signals. It phosphorylates one essential replication factor (the replication protein A or RPA), inhibits the progression of transcription in the close proximity to the double strand break and also inhibits cycle progression (Gottlieb and Jackson, 1994) because it phosphorylates the amino-terminal region of the checkpoint protein p53. This is one of the most direct examples of how DNA damage generates the transient stop produced in the cycle by the operation of checkpoint pathways. In other words, how DNA damage is converted into an antimitogenic signal.

There is another set of regulatory signals transduced by alternative checkpoint pathways which are related to the state of the cell and its components to endure the different processes of the cycle such as replication and segregation of chromosomes and cell growth. They preferentially control whether the cells are large enough to initiate replication (Fig. 2), whether DNA is unreplicated (for starting replication) and whether it is replicated (for entering into mitosis). The Wee1 kinase selectively phosphorylates the tyrosine in position 15 (Y15) and also the threonine 14 (T14) of the mammalian cyclin-dependent kinases. It was the first enzyme involved in any checkpoint pathway (Russell and Nurse, 1987). This kinase controls whether the cell has reached a minimum size (md biomass) for division to take place.

12. The G1 to S transition

The lack of p21 also has the consequence of activating the cdk2-cyclin E complex, which is needed for the triggering of replication or G_1 to S transition (central part of Fig. 8). In fact, this complex phosphorylates and therefore activates the phosphatase Cdc25 (Hoffman et al., 1994), which will activate cdk by dephosphorylating its inhibitory pY15. Another consequence of the lack of p21, PCNA -which is one auxiliary factor needed for DNA polymerase δ to initiate replication- is also activated and the G_1 to S transition is made feasible (right part of Fig. 8).

13. The regulation of the rate of replication

The rate of replication in a nucleus is a function of both the number of simultaneous active origins as well as the mean rate of DNA elongation. Both factors are indeed modified in the eukaryotic cells during proliferation (Van't Hof, 1976; Painter and Young, 1980; Moreno and De la Torre, 1985). Though replication seems to be a continuous process, activation of the different families of replicons occurs at different times of the S period.

There is a positive control of DNA replication. This accounts for the observation that cells entering into the S period with a biomass larger than the minimum mass required for the initiation of replication (mi) replicate their genome faster than the normal population (Cuadrado et al., 1985; Johnston and Singer, 1985; Cánovas et al., 1990).

The rate of replication is also positively controlled by the rate of protein synthesis, probably due to the fact that shortly after synthesis of the complementary DNA strands, they are packaged with histones which are

synthesized simultaneously with replication (Weintraub, 1972). This positive remains cryptic when there is a large pool of histones in the cell. In addition, the high availability of DNA precursors enhances the rate of DNA replication.

But the replication rate is also under negative control. This control, in principle, should not be necessary under physiological conditions where inititation of the S triggering ensures its completion. A checkpoint which depresses the rate of replication in response to DNA damage exists in mammalian cells (Painter and Young, 1980) as well as in budding yeast (Paulovich and Hartwell, 1995). There are two components which downregulate the replication rate in unfavourable conditions: one with a low radiosensitivity slows down the rate of chain elongation (Watanabe, 1974), while the most radiosensitive one decreases the number of active origins (Makino and Okada, 1975; see Bernhard et al., 1995).

14. The G_2 to mitosis transition

The role of the cyclin-dependent kinases on this other crucial transition was the first to be established in fission yeast. As seen in Fig. 6, the mitotic cyclin-dependent kinase (cdk1-cyclin B-cks), known as the mitosis promoting factor, or MPF, is involved in this transition in all eukaryotic cells. The initial step in MPF activation is the assembly of its subunits (Fig. 13). When they are bound, the trimeric structure constitutes the precursor of the MPF or pre-MPF. Only the binding of cyclin makes the trimer enter the nucleus. In second place, the serine-threonine kinase CAK (cyclin-dependent activating kinase) has previously phosphorylated the threonine in position 161. This is also required for the MPF to be active. Finally, in mammalian cells, a kinase (the analogue of the Wee kinase of fission yeast) phosphorylates not only the tyrosine in position 15 (Y15) but the threonine in position 14 (T14) of the catalytic subunit of the kinase (bottom left of Fig. 5). This inhibitory phosphorylation keeps the nuclear kinase inactive until specific signals reach the nucleus. These signals activate the phosphatase Cdc25. When these two aminoacids are dephosphorylated, the cdk activates. Thus, the inactive intranuclear mitosis-promoting factor is finally activated by the phosphatase Cdc25. It should be noticed how the two kinases CAK and Wee1 have opposite effects on MPF in the nucleus, because one phosphorylation activates it while the other inactivates it.

As commented earlier, the negative regulation of this transition usually becomes cryptic in animal cells under physiological conditions. The product of the tumor suppressor gene p53, throughout the universal cdk inhibitor p21 whose synthesis it induces, also prevents the activation of MPF and, as a consequence, avoids the G_2 to M transition (Agarwal et al., 1995; Fan et al., 1995).

15. The spindle checkpoint

The formation of the bipolar spindle, immediately after prometaphase is over, is a crucial initial part of the microtubular cycle responsible for chromosome segregation. Before it is triggered, another checkpoint pathway exists which ensures all the kinetochores are attached by microtubules to poles (Li and Murray, 1991). This checkpoint is responsible for the arrest in mitosis when antimicrotubular agents are used (see Gorbsky, 1997). This control is evolutionarily conserved in the eukaryotic cells. The product of the tumor suppressor gene p53 also participates in this pathway (Cross et al., 1995).

16. The development of late mitosis

Chromosome segregation in mitosis coincides with the inactivation of the mitotic cyclin-dependent kinase. It takes place by degradation of cyclin B (Fig. 14). Mitotic cyclins are degraded by ubiquitin-dependent proteolysis, favoured by the presence of the cyclin "destruction box", a nine aminoacid motif which links ubiquitin, so that the cyclins become targets for proteases (King et al., 1996). This is an essential step for complete mitosis.

Controls involved in mitosis progression include the checkpoint which verifies that all kinetochores are bound to the spindle poles, and also the one which responds to different mitotic forces and is responsible for chromosomal segregation. Readers can follow them in some excellent papers (Gallant and Nigg, 1992; Holloway et al., 1993; Irniger et al., 1995; Li and Nicklas, 1995; Murray, 1995; Yu et al., 1996). These controls could not be considered minor ones, since this transition is as important as the G_1 to S and G_2 to M transitions. In fact, mitosis producing cells with extra chromosomes or with fewer chromosomes than the diploid number are indeed a very important source of genome instability which in some cases accelerates the progression of cancer.

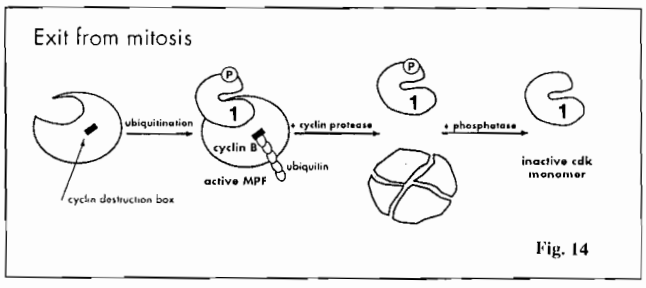

Fig. 14. The exit from mitosis depends on the inactivation of the MPF or active mitotic cyclin-dependent kinase. This inactivation depends on the enzymatic binding of ubiquitin to the cyclin destruction box. When cyclin is ubiquinated, a cyclin protease digests this regulatory component of the cdk. The cdk is immediately inactivated. Phosphatases in the absence of this kinase trigger a cascade of dephosphorylation in different substrates reversing the processess which characterize the initiation of mitosis, leading the cell to an interphase situation.

17. Substrates of the cyclin-dependent kinases

There are many structural and regulatory proteins of the cell which are known substrates for phosphorylation by the active cyclin-dependent kinases and which are responsible for cell progression throughout the different cycle phases. Even checkpoint proteins such as p53 are substrates for phosphorylation for cdks, as well as for DNA-PK and for CKII (Fig. 10), though this loop has not been well studied yet. Moreover, phosphorylation of pRb by the G_1 cdk (cdk4/6-cyclin D) is one important factor to activate the transcription factor E2F (Fig. 8). Another substrate for cdks is the DNA polymerase α involved in replication. Transcription factors are also phosphorylated by cdks, as well as other protein kinases of the cell.

On the other hand, the mitotic cyclin-dependent kinases or MPF display pleiotropic effects (Fig. 15). First of all, both mitotic cyclins (A and B) are targets for the cyclin-dependent kinases. However, it is not yet certain whether their phosphorylation is essential for activating cyclin-binding to the cdk component and/or to make the kinase active. Since both (the phosphatase Cdc25, and the kinase CAK which activates the cdk) are activated by the MPF (mitotic cdk), it is accepted that the activation of the MPF fastly activates other molecules of pre-MPF in the cell. Once all molecules of MPF are activated in the cell and some requirements, as attachment to poles of all chromosomes are fulfilled, MPF itself activates the cyclin degradation machinery. As a consequence, the cell exits from mitosis (Murray, 1993).

In relation to chromosome condensation, the mitotic cyclin-dependent kinase phosphorylates the histone H1 located in the internucleosomal linker. This phosphorylation correlates with condensation of chromosomes (Gurley et al., 1974). Phosphorylation takes place in the serine and threonine residues of the sequences SPKK and TPKK (one letter code aminoacids) located in the amino-terminal region of the histone H1 molecule. In fact, the active form of this cyclin-dependent kinase is frequently evaluated by its histone H1 phosphorylating capacity. Cyclin-dependent kinases are also involved in the phosphorylation of the high mobility group protein 1 (HMG1), one of the non-histone proteins which binds DNA and regulates its expression. It also phosphorylates nucleolin, the nucleolar protein which is involved in the selective transcription of some of the ribosomal DNA genes. RNA polymerase is another target of these cdks.

Finally, in relation to mitosis, proteins of the kinetochore are also targets for cdk phosphorylation, and their sudden dephosphorylation correlates with the separation of the half chromosomes in anaphase, the instant when chromosomes initiate segregation. But cdks seem to be involved in mitosis progression in other ways, since they also phosphorylate and induce changes in microtubules and microtubular proteins, and increase both microtubule turnover and the capacity of the mitotic spindle assembly.

Phosphorylation of the nuclear lamins by cyclin-dependent kinases has been related to the breakage and disassembly of the nuclear envelope, a process which takes place during prometaphase in higher eukaryotic cells.

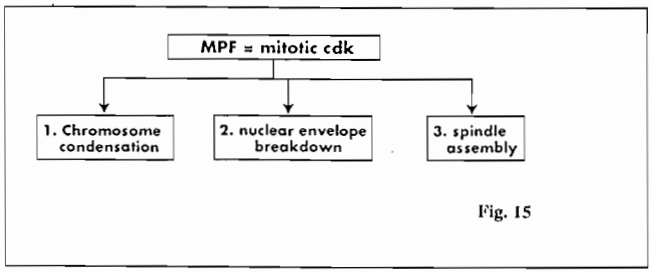

Fig. 15. The three main processes controlled by the phosphorylation of different substrates by the mitotic cdks or MPF. These multiple targets for cdks do not exhaust the range of them, some of them being involved in important signal transduction pathways.

18. Other nuclear conditions integrated into the cell cycle by checkpoint functions

Since early experiments by Rao and Johnson (1970) it has been clear that there are also some intranuclear requirements for a nucleus to respond properly to cytoplasmic signals about cycle progression. The first of these requirements was the stage of the chromatin: one G_2 nucleus was unable to reinitiate replication when properly stimulated (Hervás et al., 1982). In other words: G_2 nuclei are unable to re-replicate, or they are not competent for the initiation of an S period or there are nuclear conditions incompatible with the G_1 to S transition. The disappearance of the nuclear envelope in mitosis and its reconstitution at completion overrides this particular requirement (Blow and Laskey, 1988).

Other requisites for a nucleus to respond to the adequate stimulus have been discovered in plant cells, which share similar control mechanisms with the mammalian ones. To dissect the nuclear requirements, the cells were treated with an agent producing multipolarity in mitosis. After cytokinesis was prevented, the whole chromosome complement was distributed in multiple nuclei (i.e. more than two) in a common cytoplasm. Thus, the whole tetraploid complement remains distributed in different nuclei which share the same cytoplasm. In these cells, both the initiation of replication and that of mitosis depend on the intranuclear presence of some particular chromosomes. This requirement is obviously cryptic when the whole chromosome complement is a nucleus. These experiments point out that specific sequences of the DNA of certain chromosomes constitute *cis*-acting regulatory domains which are required for cycle transitions (Hervás et al., 1982; Giménez-Martín et al., 1992; Panzera et al., 1997).

19. General properties of checkpoint pathways

First of all, the function of checkpoints or tumor suppressor pathways is crucial when something goes wrong or when the cell is growing under stress conditions. These functions are not yet developed in the simplified cycles of the first cell divisions of the embryo (Edgar et al., 1994), but as there is always a basal level of DNA damage, the lack of a checkpoint function in any cell can lead to a rise in that level.

The main function of a checkpoint is the integration of signals of opposite signs about the adequacy to continue proliferation and to activate or inactivate the corresponding cdks (Table 1).

The tumor suppressor genes should, in principle, be recessive. Only the loss of both copies will result in the loss of function in the diploid somatic cells. However, some of them may produce a dominant negative phenotype, as we have seen for p53 (Fig. 11).

Some common features of the different cycle checkpoints are shown in Table 2. The multiple inputs for the regulation of cdks and the fact that these inputs are only steps in parallel regulatory pathways often make checkpoints redundant. Thus, DNA damage produced by UV exposure relies on the redundant inactivation of cyclin-dependent kinases by both p21 induction and by phosphorylation of the cdk inhibitory T14-Y15 residues (Kharbanda et al., 1994; Poon et al., 1996). Another example is provided by the checkpoint which controls whether all the chromosomes are attached to the spindle before the division of centromeres starts. It usually depends on the CAK-phosphatase which controls dephosphorylation of the threonine in position 161 of the cdk. When this control does not work, such degradation starts to depend on the inhibitory phosphorylation of tyrosine 15 and threonine 14 instead (Minshull et al., 1996). Due to redundant checkpoint pathways, genetic analysis of these negative regulators depends on double and triple mutants, even in the haploid cells of yeast.

Lastly, the repression that a checkpoint exerts on cycle progression is transient, so if the damaged DNA is not repaired for a period of time, the cell, depending on the tissue, either leaves the cycle to rest with 2C of 4C DNA contents, triggers a programme of suicide (apoptosis) or fatally jumps into S or into mitosis (Del Campo et al., 1997). The premature progression of unprepared cells towards more advanced phases in the cycle also has fatal consequences, either the initiation of apoptosis itself or the entrance into a deleterious mitosis (mitotic catastrophe). Either cell adaptation to a continuous signal or frailty of one of the components of the transduction signal cascade would account for this effect.

Partially similar checkpoint functions take place for analogous molecular events at different stages of the cycle. For example, evaluation of DNA damage occurs both in G_1 and G_2. Moreover, portions of a single negative regulatory pathway can be involved in the control of processes which are analogous but not identical, such as the presence of DNA damage and the completion of replication.

Finally, purine analogs like pentoxyfilline, caffeine, 2-aminopurine, etc. can specifically cancel checkpoint functions in the cell (Andreassen and Margolis, 1992; Yao et al., 1996) and, as a consequence, allow proliferation of unprepared cells. This effect apparently depends on the activation of the phosphatase Cdc25 which removes the inhibitory phosphate on the T14 and Y15 aminoacids in the catalytic subunit of the cdk. However, their efficiency may depend on the cdk state, since these purines can have the opposite effect, as they can also inactivate cdks by competing for the ATP pocket in the kinase (Vesely et al., 1994).

20. Approaches to the pharmacology of cancer based on the checkpoint pathways

Molecular biology of the cycle has been extremely important in understanding proliferation and cancer.

Table 1. Functions of checkpoints.

1) They work as devices which evaluate the adequacy for a cell to proliferate.
2) They integrate signals of opposite signs and produce a final balance.
3) If the balance is negative, they inactivate cdks and reversibly block cycle progression. If the balance is positive for proliferation, they allow the activation of the corresponding cyclin-dependent kinases.
4) They can induce the process needed for proper cycle progression to occur (i.e., the induction of repair when DNA is damaged).

Table 2. Properties of checkpoints

1) The checkpoint or tumor suppressor genes are recessive. However, some of them may constitute dominant negative mutants when the proteins they codify function as multimers and the mutation prevents the assembly or the function of the multimer.
2) Checkpoint functions are mediated by enzymatic cascades. This property makes both the amplification of their signals and their pleiotropic effects possible.
3) Checkpoints are often redundant. Alternative enzymatic cascades converge in some steps.
4) Checkpoints are frail. The repression that a checkpoint exerts on cycle progression wears out with time if the lesion remains unrepaired for a long time.
5) Different checkpoint pathways can share some segments of their enzymatic cascades. For this, evaluation of DNA damage in G_1, S and G_2 need not to be radically different.
6) One single checkpoint can control processes which are analogous but not identical, such as the completion of replication and the presence of DNA damage.
7) Two alternative outcomes are the consequence of the braking effect of the checkpoint operation when the situation is not adequate for proliferation: either the initiation of a resting state (either G_0 or differentiation) or the induction of a suicide programme (either apoptosis or mitotic catastrophe).
8) Some purine analogues can cancel checkpoint functions, under certain conditions, probably by inducing the dephosphorylation of threonine and tyrosine residues of the cyclin-dependent kinase, and activating it. As a consequence, there will be a premature cycle progression of unprepared cells.

Moreover, it provides new rational strategies to deal with cancer. New targets have been unveiled for antitumoral agents. All have advantages, in principle, over the conventional chemotherapy and radiotherapy treatments which are based on the genotoxic effects they produce on proliferating cells, i.e. on cells which must undergo either replication or mitosis. The efficiency of these treatments are decreased by the repair mechanisms, if some checkpoint functions still remain.

Among the novel approaches, the cascades of reactions involved in the transduction of mitogenic and antimitogenic signals are new targets to be considered. Thus, inhibitors of the enzyme farnesyl-protein transferase to silence the transduction of signals throughout Ras, or the use of antisense technology have been used.

Another new pharmacological approach to cancer is the supply of natural polypeptides involved in checkpoint functions or the use of chemical inhibitors specific for the cyclin-dependent kinases. In this line, a set of new inhibitors is being developed (Meijer, 1996), though a second generation efficient at nanomolar concentrations has to be found before being used in cancer therapy (see Fig. 7). Some of them, as VCN-01 and flavopiridol are already in phase I trials.

Another antitumoral strategy is the re-establishment of the deranged functions of negative regulators of the cycle or checkpoints (Fig. 16). It would be good to know the genetic lesions which are present in a specific tumor to restore the activity of the unfunctional molecules. But in any case, the induction of over-expression or the increase in molecules involved in the checkpoint transduction is very promising. Thus, cell proliferation will only be selectively blocked in the transformed cells possessing damaged DNA, but not in those cells with no specific damage. Not only the enzymes which incide on the inhibition of the cyclin-dependent kinases are targets

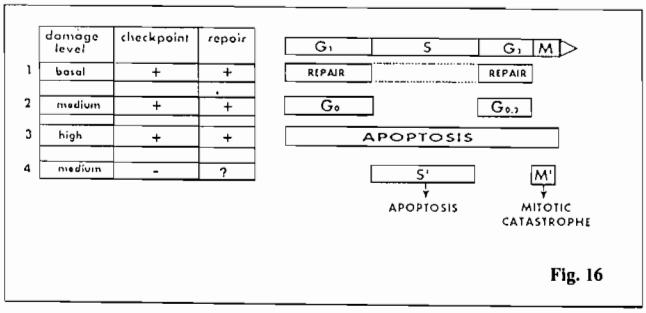

Fig. 16

Fig. 16. The consequences of the presence of damage and the functioning of the corresponding checkpoints in cycle progression. Four alternative situations are outlined. The presence of repair throughout the cycle (situation 1), the arrest in G_0 and G_0, 2 to allow repair as a consequence of the cycle brake induced by checkpoints when damage is present (situation 2), the induction of apoptosis when damage is too high to be repaired by the cell (situation 3) and the induction of apoptosis when unduly entering into a subsequent phase by failure of the checkpoints (situation 4). The restoration of checkpoints in the latter situation will allow the cell to arrest in either G_0 or $G_{0,2}$ to repair.

for this therapy, but also those which activate specific negative regulators of cycle progression: for instance, the cascade of enzymes (kinases and phosphatases) which are upstream of the cyclin-dependent kinases, in the pathways which keep tyrosine 15-threonine 14 phosphorylation or which prevent threonine 161 dephosphorylation (see the model of Fig. 5). The enzymes which activate the functioning of negative regulatory proteins like p53, and also the DNA-dependent protein kinases are also potential antitumoral drugs.

Another very promising approach for antitumoral drugs is the most radical of all, and this way is just the opposite of the second one: to derange any remnant checkpoint function in the cell. It will end in the selective removal of the cells with damaged DNA by favouring the induction of apoptosis. The cancellation of the block produced by the negative regulators preventing the completion of repair after chemo- or radiotherapies is a perfect strategy to get rid of all the cells which have damage in their DNA (Powell et al., 1995). As earlier commented, checkpoints spontaneously cease when the damage overtakes the repair capacity. This approach is very favoured at present given the common properties of all tumors: the increased number of control genes which become deranged with time. This occurs because of the characteristics of the transformed cell. Thus, as soon as a DNA lesion appears and the corresponding checkpoint fails to stop cycle progression, the stress produced by the intranuclear presence of DNA damage leads to increased genome instability. As McClintock (1984) showed, the presence of broken DNA ends triggers the reorganization of parts of the genome, mediated by the movement of transposable elements, accelerating DNA evolution in that particular nucleus, i.e., inducing its transformation.

The fact that purine analogs can selectively annul negative controls in the cell cycle (Cremer et al., 1980; González-Fernández et al., 1985; Andreassen and Margolis, 1992; Vesely et al., 1994; Yao et al., 1996) make them potential drugs for such alternative approach to cancer therapy. They are proving to be especially useful when p53 failure is involved in cell transformation, as in breast cancer (Fan et al., 1995). Then, both the G_1 and the G_2 checkpoint pathways can be eliminated by pharmacological intervention leading to the triggering of apoptosis in the transformed cell population which was unable to be stopped in spite of the presence of DNA damage. It is fortunate that the p53-deficient tumors which are resistant to genotoxic agents are, however, prone to apoptosis induction.

Abbreviations and their short definitions

A: alanine; AP-1: nuclear transcription factor formed by *c-fos*/c-jun heterodimers; ATM: checkpoint gene mutated in ataxia telangiectasia; Bax: gene whose product dimerizes and inactivates the inducer of apoptosis bcl-2; Bcl-2: mammaliam gene which suppresses cell death by apoptosis; CAK: cyclin-

dependent kinase activating kinase; caspases: cysteinyl-aspartate-specific protein-ases, new nomenclature for the ICE-like family of proteases, the main natural mediators for the induction of apoptosis; cdc: cell division cycle; Cdc25: dual phosphatase codified by the gene cdc25 of *S. pombe* which estimulates cdk by dephosphorylating its Y15 and T14 residues; cdk: cyclin-dependent kinase; cki: cyclin-dependent kinase inhibitor; cks: cyclin-dependent kinase subunit; *c-fos*: gene which early responds to mitogenic signals transduced by MAP-kinases by inducing the synthesis of a transcription factor; chk: checkpoint pathway transducing antimitogenic signals in a cascade of products of tumor suppressor genes; c-jun: gene which early responds to mitogenic signals transduced by MAP-kinases by inducing the synthesis of a transcription factor; c-myc: gene which early responds to mitogenic signals transduced by MAP-kinases by inducing the synthesis of a transcription factor which induces the synthesis of the phosphatase Cdc25; DNA-PK: DNA-dependent protein kinase; DPC4: a human checkpoint or tumor suppressor gene integrated in checkpoint pathways; E: glutamic acid; E2F: trancription factor which is activated by early response genes; ERCC: enzymes which cut DNA and are involved in nucleotide excision repair; ERK1 and ERK2: two MAP-kinases of 44 and 42 kD, respectively, which are known as Extracellular Signals-Regulated Kinases; FRAP: inhibitor of G_1 cdks which associates to the rapamycin-receptor; G_0: resting stage for cells with the 2C pre-replicative content of DNA; G_1: interphasic pre-replicative stage of the cycle; G_2: interphasic post-replicative stage of the cycle; HMG1: highly mobility group protein 1. It binds DNA sequences and regulates their expression; I: isoleucine; ICE: family of proteases activated in the apoptosis programme (see caspases) ; K: lysine; Ku: helicase which binds to DNA strand breaks. It accompanies the DNA-PK catalytic subunit; M: mitosis or period of nuclear division in the cell cycle; MAP-kinase: mitogenic-activated protein kinase; MAP 2 kinase: the kinase which phosphorylates MAP-kinase; MAP 3 kinase: the kinase which phosphorylates MAP 2 kinase; MDM2: a protein which binds p53 and inhibits its checkpoint function, i.e. it acts as a mitogen; MPF: mitosis promoting factor = maturation promoting factor = meiosis promoting factor. Equivalent to mitotic cdk = cdk1 + cyclin B; m_d: minimum division mass; m_i: minimum mass required for DNA replication; NLS: nuclear localization signal; P: proline; p9: the smallest subunit forming the cdk trimer in mammalian cells, analogue of $p13^{suc1}$; $p13^{suc1}$: smallest subunit of the mitotic cyclin-dependent kinases in *S. pombe*; p15, p16, p18 and p19: members of the p16 family of cyclin-dependent kinase inhibitors; p21, p27 and p57: members of the p21 family of cyclin-dependent kinase inhibitors; p34 or $p34^{cdc2}$: cyclin-dependent kinase from *S. pombe*; p53: multifunctional protein which functions as a tumor suppressor because is a checkpoint protein and an inducer of apoptosis; p57: member of the p21 family of cyclin-dependent kinase inhibitors; p107 and p130: two checkpoint proteins of the Rb family; PCNA: proliferating cell nuclear antigen = auxiliary factor of the DNA polymerase δ; PI 3 kinase: phosphatidyl inositol lipid kinase. There is a subfamily of them -which includes ATM, DNA-PK and FRAP- which recognize and transduce signals related to the presence of DNA damage; PP2A: phosphatase 2A; pRb: 100 kD negative regulatory protein which is missing or unfunctional in retinoblastoma patients; pre-MPF: pre-mitosis or pre-maturation promoting factor; PSTAIRE: highly conserved motif found in the small lobe of the catalytic subunit of the cdks; R: arginine; Raf: serine-threonine protein kinase which acts on the transduction of mitogenic signals; Ras: GTPase protein which acts on the transduction of mitogenic signals from growth factor receptors; RPA: replication protein A; RF-C: replication factor C; S: serine; Smad: enzyme involved in the transduction of antimitogenic signals leading to induction of the cdk-inhibitor p15; S period: the period of replication of nuclear DNA in the cell cycle; T: threonine; T-14: threonine located in position 14 of the polypeptide chain forming the catalytic subunit of cdk; TFIIH: trancription factor IIH; TGF-ß: transforming growth factor ß (it usually works as antimitogenic); T-loop: structural configuration in the major lobe of the catalytic subunit of the cdks; UV: ultraviolet; Wee1: kinase of *S. pombe* which phosphorylates tyrosine15; XP, xeroderma pigmentosum; XP A, B, C, D, E, G: different DNA helicases encoded by genes which are mutated in xeroderma pigmentosum; Y: tyrosine; Y15: tyrosine in position 15 of the catalytic subunit of cdk.

Acknowledgements. This work has been partially supported by the Dirección General de Investigación Científica y Técnica (Spain) (Projects PB93-0167 and PB94-0167), by the Dirección General de Enseñanza Superior del Ministerio de Educación y Cultura (PB96-0909), by the Fundación Ramón Areces (Spain), by the FONDECYT (Chile) (Project 1930958) and by the European Union (Project BIO4-CT96-0275). We thank Mrs. Beryl Ligus Walker for her revision of the English.

References

Agarwal M.L., Agarwal A., Taylor W.R. and Stark G.R. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc. Natl. Acad. Sci. USA 92, 8493-8497.

Alberts B., Bray D., Lewis J., Raff M., Roberts K. and Watson J.D. (1994). Molecular biology of the cell. Third Edition. Garland Pub. New York, London.

Andreassen P.R. and Margolis R.L. (1992). 2-aminopurine overrides multiple cell cycle checkpoints in BHK cells. Proc. Natl. Acad. Sci. USA 89, 2272-2276.

Bartek J., Bartkova J. and Lukas J. (1996). The retinoblastoma protein pathway and the restriction point. Curr. Op. Cell Biol. 8, 805-814.

Bernhard E.J., Maity A., Muschel R.J. and McKenna W.G. (1995). Effects of ionizing radiation on cell cycle progression. A review. Radiat. Environ. Biophys. 34, 79-83.

Blow J.J. and Laskey R.A. (1988). A role for nuclear envelope in controlling DNA replication within the cell cycle. Nature 332, 546-548.

Brown E.J., Alberts M.W., Shin T.B., Ichikawa K., Keith C.T., Lane W.S. and Schreiber S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 369, 756-758.

Cánovas J.L., Cuadrado A., Escalera M. and Navarrete M.H. (1990). The probability of G1 cells to enter into S increases with their size while S length decreases with cell enlargement in *Allium cepa*. Exp. Cell Res. 191, 163-170.

Cimprich K.A., Shin T.B., Keith C.T. and Schreiber S.L. (1996). cDNA cloning and gene mapping of a candidate human cell cycle checkpoint protein. Proc. Natl. Acad. Sci. USA 93, 2850-2855.

Cremer C., Cremer T. and Simickova M. (1980). Induction of chromosome shattering and micronuclei by ultraviolet light and caffeine. I. Temporal relationship and antagonistic effects of the four deoxiribonucleosides. Environm. Mutagen. 2, 339-351.

Cross S.M., Sánchez C.A., Morgan C.A., Schimke M.K. Ramel S., Idzerda R.L., Raskind W.H. and Reid B.J. (1995). A p53-dependent mouse spindle cell checkpoint. Science 267, 1353-1356.

Cuadrado A., Navarrete M.H. and Cánovas J.L. (1985). The effect of partial protein synthesis inhibition on cell proliferation in higher plants. J. Cell Sci. 76, 97-104.

De la Torre C., González-Fernández A. and Giménez-Martín G. (1989). Stringency at four regions of the plant cell cycle where proteins regulating its progression are synthesized. J. Cell Sci. 94, 259-265.

De Luca A., Esposito V., Baldi A. and Giordano A. (1996). The retinoblastoma gene family and its role in proliferation, differentiation and development. Histol. Histopathol. 11, 1029-1034.

Del Campo A., Giménez-Martín G., López-Sáez J.F., and De la Torre C. (1997). Frailty of two cell cycle checkpoints which prevent entry into mitosis and progression through early mitotic stages in higher plant cells. Eur. J. Cell Biol. 74, 289-293.

Donachie W.D. (1968). Relationship between cell size and the time of initiation of DNA replication. Nature 219, 1077-1079.

Ducommun B., Brambilla P. and Draetta G. (1991). Mutations at sites involved in Suc1 binding inactivate Cdc2. Mol. Cell Biol. 11, 6177-6184.

Edgar B.A., Sprenger F., Duronio R.J., Leopold P. and O'Farrell P.H. (1994). Distinct molecular mechanisms regulate cell cycle timing at succesive stages of *Drosophila* embryogenesis. Genes Dev. 8, 440-452.

El-Deiry W.S., Tokino T., Velculescu V.E., Levy D.B., Parsons R., Trent J.M., Lin D., Mercer E., Kinzler K.W. and Vogelstein B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell 75, 817-825. (WAF1 = p21 cki).

Fan S., Smith M.L., Rivetm II D.J., Duba D., Zhan A., Kohn K.W., Fornace Jr A.J. and O'Connor P.M. (1995). Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. Cancer Res. 55, 1649-1654.

Fisher R.P. (1997) CDKs and cyclins in transition(s). Curr. Op. Genet. Develop. 7, 32-38.

Gallant P. and Nigg E.A. (1992). Cyclin B2 undergoes cell cycle-dependent nuclear translocation and, when expressed as a non-destructible mutant, causes mitotic arrest in HeLa cells. J. Cell Virol. 117, 213-224.

Giménez-Martín. G., Panzera F., Cánovas J.L., De la Torre C. and López-Sáez J.F. (1992). A limited number of chromosomes makes a nucleus competent to respond to inducers of replication and mitosis in a plant. Eur. J. Cell Biol. 58, 163-171.

Gómez-Lechón M.J., Guillén I., Ponsoda X., Fabra R., Trullenque R., Nakamura T. and Castell J.V. (1996). Cell cycle progression proteins (cyclins), oncogene expression, and signal transduction during the proliferative response of human hepatocytes to hepatocyte growth factor. Hepatology. 23, 1012-1019.

González-Fernández A., Hernández P. and López-Sáez J.F. (1985). Effect of caffeine and adenosine on G2 repair: mitotic delay and chromosome damage. Mutation Res. 149, 275-281.

Gorbsky G.J. (1997). Cell cycle checkpoints: arresting progress in mitosis (a review). BioEssays 19, 193-197.

Gottlieb T.M. and Jackson S.P. (1994). Protein kinases and DNA damage. Trends Biol. Sci. 19, 500-503.

Greenwell P.W., Kronmal S.L., Porter S.E., Gassenhuber J., Obermaier B. and Petes T.D. (1995). TEL1, a gene involved in controlling telomere length in *S. cerevisiae*, is homologous to the human ataxia telangiectasia gene. Cell 82, 823-829.

Griffin S. (1996). DNA damage, DNA repair and disease. Curr. Biol. 6, 497-499.

Gu Y., Turck C.W. and Morgan D.O. (1993). Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. Nature 366, 707-710.

Gurley L.R., Walters R.A. and Tobey R.A. (1974). Cell cycle specific changes in histone phosphorylation associated with cell proliferation and chromosome condensation. J. Cell Biol. 60, 356-364.

Hahn S.A., Schutte M., Hoque A.T.M.S., Moskaluk C.A., Da Costa L.T., Rozenblum E., Weinstein C.L., Fischer A., Yeo C.J., Hruban R.H. and Kern S.E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271, 350-353.

Harper J.W., Adami G.R., Wei N., Keyomarsi K. and Elledge S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75, 805-816.

Harper J.W. and Elledge S.J. (1996). Cdk inhibitors in development and cancer. Curr. Op. Genet. Dev. 6, 56-64.

Hartley K.O., Gell D., Smith G.C., Zhang H., Civecha N., Connelly M.A., Admon A., Lees-Miller S.P., Anderson C.W. and Jackson S.P. (1995). DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiestasia gene product. Cell 82, 849-856.

Hartwell L.H. and Kastan M.B. (1994). Cell cycle control and cancer. Science 266, 1821-1828.

Hartwell L.H., Culotti J., Pringle J. and Reid B.J. (1974). Genetic control of the cell division cycle in yeast. Science 183, 46-51.

Herrera R.E., Mäkelä T.P. and Weinberg R.A. (1996). TGFß-induced growth inhibition in primary fibroblasts requires the retinoblastoma protein. Mol. Biol. Cell 7, 1335-1342.

Hervás J.P., López-Sáez J.F. and Giménez-Martín G. (1982). Multinucleate plant cells. II. Requirements for DNA synthesis. Exp. Cell Res. 139, 341-350.

Hinds P.W., Dowdy S.F., Eaton E.N., Arnold A. and Weinberg R.A. (1994). Function of a human cyclin gene as an oncogene. Proc. Natl. Acad. Sci. USA 91, 709-713.

Hittelman W.N. and Rao P.N. (1978). Predicting response of progression of human leukemia by premature chromosome condensation of bone marrow cells. Cancer Res. 38, 416-423.

Hoffman L, Draetta G. and Karsenti E. (1994). Activation of the phosphatase activity of Cdc25A by a cdk2-cyclin E dependent phosphorylation at the G1/S transition. EMBO J. 13, 4302-4310.

Holloway S.L., Glottzer M., King R.W. and Murray A.W. (1993).

Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. Cell 73, 1393-1402.

Howard A. and Pelc S. (1953). Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity 6 (suppl.) 261-273.

Iavarone A. and Massagué, J. (1997). Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-ß in cells lacking the CDK inhibitor p15. Nature 387, 417-426.

Irniger S., Piatti S. and Nasmyth K. (1995). Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. Cell 81, 269-277.

Jackson S.P. (1996). The recognition of DNA damage. Curr. Opin. Genet. Dev. 6, 19-25.

Jayaraman L. and Prives C. (1995). Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. Cell 81, 1021-1029.

Johnston G.C. and Singer R.A. (1985). Novel cell cycle regulation in the yeast *Schizosaccharomyces pombe*. The DNA-division sequence modulates mass accumulation. Exp. Cell Res. 158. 544-553.

Jung J.U., Stager M. and Desrosiers R.C. (1994). Virus-encoded cyclin. Mol. Cell Biol. 14, 7235-7244.

Kharbanda S., Saleem A, Datta R., Yuan Z.M., Weichselbaum R. and Kufe D. (1994). Ionizing radiation induces rapid tyrosine phosphorylation of $p34^{cdc2}$. Cancer Res. 54, 1412-1414.

Killander D. and Zetterberg A. (1965). A quantitative cytochemical investigation of the relationship between cell mass and initiation of DNA synthesis in mouse fibroblasts *in vitro*. Exp. Cell Res. 40, 12-20.

King R.W., Glotzer M. and Kirschner M.W. (1996). Mutagenic analysis of the destruction signal of mitotic cyclins and structural chracterization of ubiquitinated intermediates. Mol. Biol. Cell 7, 1343-1357.

Lagna G., Hata A., Hemmati-Brivanlou A. and Massagué J. (1996). Parthnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. Nature 383, 832-836.

Lee S., Elenbaas B., Levine A. and Griffith J. (1995). p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. Cell 81, 1013-1020.

Li J., Meyer A.N. and Donoghue D.J. (1995). Requirement for phosphorylation of cyclin B1 for *Xenopus* oocyte maturation. Mol. Biol. Cell 6, 1111-1124.

Li R. and Murray A.W. (1991). Feedback control of mitosis in budding yeast. Cell 66, 519-531.

Li X. and Nicklas R.B. (1995). Mitotic forces control a cell-cycle checkpoint. Nature 373, 630-632.

Liu J.J., Chao J.R., Jiang M.C., Ng S.Y., Yen J.J.Y. and Yangyen H.F. (1995). Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. Mol. Cell Biol. 15, 3654-3663.

Lohka M.I., Hayes M.K. and Maller J.L. (1988). Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. Proc. Natl. Acad. Sci. USA 85, 3009-3013.

Lukas J., Bartkova J. and Bartek J. (1996). Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin-D-cyclin-dependent kinase-pRb-controlled G1 checkpoint. Mol. Cell. Biol. 16, 6917-6925.

Makino F. and Okada S. (1975). Effects of ionizing radiation on DNA replication in cultured mammalian cells. Radiat. Res. 62, 37-51.

McClintock B. (1984). The significance of responses of the genome to challenge. In: Les prix Nobel en 1983. The Nobel Foundation. Stockholm. Sweden. pp 174-193.

Meijer L. (1996). Chemical inhibitors of cyclin-dependent kinases. Prog. Cell Cycle Res. 1, 351-363.

Minshull J., Straight A., Rudner A.D., Dernburg A.F., Belmont A. and Murray A.W. (1996). Protein phosphatase 2A regulates MPF activity and sister chromatid cohesion in budding yeast. Curr. Biol. 6, 1609-1620.

Mitchison J.M. (1971). The biology of the cell cycle. Cambridge University Press. London.

Moran E. (1993). Interaction of adenoviral proteins with pRb and p53. FASEB J. 7, 880-885.

Moreno M.L. and De la Torre C. (1985). Increased replication in plant nuclei, whose spread chromatin contains enlarged nucleosomal linkers, is only partially due to a rise in the number of DNA elongating chains. Cell Biol. Int. Rep. 9, 479-489.

Morrow D.M., Tagle D.A., Shiloh Y., Collins F.S. and Hieter P. (1995). TEL1, an *S. cerevisiae* homolog of the human gene mutated in ataxia telangiectasia, is functionally related to the yeast checkpoint gene MEC1. Cell 82, 831-840.

Murray A.W. (1993). Cell cycle. Sunburnt fission yeast. Nature 363, 302.

Murray A.W. (1995). Cyclin-ubiquitination: the destructive end of mitosis. Cell 81, 149-152.

Murray A.W. and Hunt T. (1993). The cell cycle. An introduction. Oxford Univ. Press. New York-Oxford.

Navarrete M.H., Cuadrado A. and Cánovas J.L. (1983). Partial elimination of G_1 and G_2 periods in higher plant cells by increasing the S period. Exp. Cell Res. 148, 273-280.

Nelson W.G. and Kastan M.B. (1994). DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol. Cell. Biol. 14, 1815-1823.

Nigg E.A. (1996). Cyclin-dependent kinase 7: at the cross-roads of transcription, DNA repair and cell cycle control? Curr. Opin. Cell Biol. 8, 312-317.

Nurse P. (1990). Universal control mechanism regulating onset of M-phase. Nature 344, 503- 508.

Painter R.B. and Young B.R. (1980). Radiosensitivity in ataxia-telangiectasia: a new explanation. Proc. Natl. Acad. Sci. USA 77, 7315-7317.

Panzera F., Giménez-Abián M.I., López-Sáez J.F., Giménez-Martín G., Cuadrado A., Cánovas J.L. and De la Torre C. (1997). Competence for nuclear replication and the NOR-chromosomes of *Allium cepa* L. Eur. J. Cell Biol. 72, 9-12.

Parge H.E., Arvai A.S., Murtari D.J., Reed S.I. and Tainer J.A. (1993). Human CksHs2 atomic structure: a role for its hexameric assembly in cell cycle control. Science 262, 387-395.

Patra D. and Dunphy W.G. (1996). Xe-p9, a *Xenopus* Suc1/Cks homolog, has multiple essential roles in cell cycle control. Genes Dev. 10, 1503-1515.

Paulovich A.G. and Hartwell L.H. (1995). A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. Cell 82, 841-847.

Poon R.Y.C., Jiang W., Toyoshima H. and Hunter T. (1996). Cyclin-dependent kinases are inactivated by a combination of p21 and Thr-14/Tyr-15 phosphorylation after UV-induced DNA damage. J. Biol. Chem. 271, 13283-13291.

Porter S.E., Greenwell P.W., Ritchie K.B. and Peter T.D. (1996). The DNA-binding protein Hdf1p (a putative Ku homologue) is required for maintaining normal telomere length in *Saccharomyces cerevisiae*.

Nucleic Acids Res. 24, 582-585.

Porter A.G., Ng P., and Jänicke R. (1997). Death substrates come alive (a review). BioEssays 19, 501-507

Powell S.N., DeFrank J.S., Connell, P., Eogan M., Preffer F., Dombkowski D., Tang W. and Friend S. (1995). Differential sensitivity of $p53^{(-)}$ and $p53^{(+)}$ cells to caffeine-induced radiosensitization and override of G2 delay. Cancer Res. 55, 1643-1648 .

Rao P.N. and Johnson R.T. (1970). Mammalian cell fusion studies on the regulation of DNA synthesis and mitosis. Nature 225, 159-164.

Rao P.N. and Johson R.T. (1971). Mammalian cell fusion. IV. Regulation of chromosome formation from interphase nuclei by various chemical compounds. J. Cell Physiol. 78, 217-223.

Russell P. and Nurse P. (1987). Negative regulation of mitosis by $wee1^{+}$, a gene encoding a protein kinase homolog. Cell 49, 559-567.

Sánchez Y., Wong C., Thoma R.S., Richman R., Wu Z., Piwnica-Worms H. and Elledge S.J. (1997) Conservation of the Chk1 chekcpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science 277, 1497-1501.

Sans J. and De la Torre C. (1979). Proliferative index estimated by chromatin pattern analysis. Cell Biol. Int. Rep. 3, 227-235.

Serrano M., Hannon G.J. and Beach D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 366, 704-707.

Smith M.L., Chen I.T., Zhan Q., Bae I., Chen C.Y., Gilmer T.M., Kastan M.B., O'Connor P.M. and Fornace A.J. (1994). Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 266, 1376-1379.

Taylor S.J. and Shalloway D. (1996a). Src and the control of cell division. BioEssays 18, 9-11.

Taylor S.J. and Shalloway D. (1996b). Cell cycle-dependent activation of Ras. Curr. Biol. 6, 1621-1627.

Van't Hof J. (1976). Replication size and rate of fork movement in early S of higher plant cells (*Pisum sativum*). Exp. Cell Res. 103, 395-403.

Vesely J., Havliçek L., Strnad M., Blow J.J., Donella-Deana A., Pinna L., Letham D.S., Kato J.Y., Detivaud L., Leclerc S. and Meijer L. (1994). Inhibition of cyclin-dependent kinases by purine analogues. Eur. J. Biochem. 224, 771-786.

Waga S., Hannon G.J., Beach D. and Stillman B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369, 574-578.

Walworth N., Davey S. and Beach D. (1993). Fission yeast kinase links the rad checkpoint pathway to cdc2. Nature 363, 368-371.

Wang X.W., Yeh H., Schaeffer L., Roy R., Moncollin V., Egly J.M., Wang Z., Friedberg E.C., Evans M.K., Taffe B.G., Bohr V.A., Weeda G., Hoeijmakers J.H.J., Forrester K. and Harris C.C. (1995). p53 modulation of TFIIH-associated nucleotide excision repair activity. Nature Genet. 10, 188-195.

Wang X.W., Vermeulen W., Coursen J.D., Gibson M., Lupold S.E., Forrester K., Xu G., Elmore L., Yeh, H., Hoeijmakers J.H. and Harris C.C. (1996). The XPB and XPD DNA helicases are components of the p53 mediated apoptosis pathways. Genes Dev. 10, 1219-1232.

Watanabe I. (1974). Radiation effects on DNA chain growth in mammalian cells. Radiat. Res. 58, 541-556.

Weinert T.A., Kiser G.L. and Hartwell L.H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev 8, 652-665.

Weintraub H. (1972). A possible role for histone in the synthesis of DNA. Nature 240, 449-453.

White E. (1996). Life, death and the pursuit of apoptosis. Gen. Dev. 10, 1-15.

Xiao Z.X., Chen J., Levine A.J., Modjtahedl N., Xing J., Sellers W.R. and Livingston D.M. (1995) Interaction between the retinoblastoma protein and the oncoprotein MDM2. Nature 375, 694-697.

Xiong Y., Hannon G.J., Zhang H., Casso D., Kobayashi R. and Beach D. (1993). p21 is a universal inhibitor of cyclin-dependent kinases. Nature 366, 701-704.

Yao S.L., Akhtar A.J., McKenna K.A., Bedi G., Sidransky D., Mabry M., Ravi R., Collector M.I., Jones R.J., Sharkis S.J., Fuchs E.J. and Bedi A. (1996). Selective radiosensitization of p53-deficient cells by caffeine-mediated activation of the $p34^{cdc2}$ kinase. Nature Med. 2, 1140-1143.

Yu H., King R.W., Peters J.M. and Kirschner M.W. (1996). Identification of a novel ubiquitin-conjugating enzyme involved in mitotic cyclin degradation. Curr. Biol. 6, 455-466.

Zörnig M. and Evan G.I. (1996). Cell cycle: on target with myc. Curr. Biol. 6, 1553-1556.