Journal of Cellular Biochemistry 52:362–372 (1993)

Cell Cycle Dependent Distribution of Proliferating Cell Nuclear Antigen/Cyclin and cdc2-Kinase in Mouse T-Lymphoma Cells

David A. Brott, James D. Alvey, Michael R. Bleavins, Felix A. de la Iglesia, and Narendra D. Lalwani

Department of Pathology and Experimental Toxicology, Parke-Davis Pharmaceutical Research, Warner-Lambert Company, Ann Arbor, Michigan 48106

Abstract The aim of the present study was to investigate bromodeoxyuridine (BrdU) uptake and coordinated distribution of proliferating cell nuclear antigen (PCNA) and p34-cdc2-kinase, two important proteins involved in cell cycle regulation and progression. Flow cytometric analysis of marker proteins in freshly plated mouse T-lymphoma cells (Yac-1 cells), using fluorescein isothiocyanate (FITC)-labeled specific antibodies, showed PCNA distributed throughout the cell cycle with increased intensity in S-phase. PCNA is essential for cells to cycle through S-phase and its synthesis is initiated during late G1-phase before incorporation of BrdU and remains high during active DNA replication. The intensity of PCNA fluorescence increases with the duration of incubation after plating. The cdc2-kinase was detectable in all phases of the cell cycle and the G2-M-phase appears to have the maximum concentrations. The cell cycle analysis of high dose colcemid (2 μg/ml) treated Yac-1 cells showed an aneuploid or hypodiploid population. Although the G2-M-phase seems to be the dominating population in aneuploid cells, the concentrations of cdc2-kinase were variable in this phase of cell cycle. The colcemid treatment at 25 ng/ml arrested 96% of cells in S-phase and G2-M-phase, but PCNA expression was evident in a portion of the cell population in G2-M-phase. Although cells blocked in M-phase seem to have high levels of cdc2-kinase, colcemid renders them inactive. From these data, it appears that the down regulation and/or inactivation of cdc2-kinase could be responsible for the colcemid arrest of cells in M-phase.

Key words: flow cytometry, BrdU incorporation, S-phase, DNA synthesis, p34-cdc2, colcemid, mitotic inhibitors, aneuploidy

Numerous proteins have been identified in the nucleus and cytoplasm of eukaryotic cells which are synthesized or activated in specific phases of the cell cycle. The proteins such as proliferating cell nuclear antigen (PCNA/cyclin) [Bravo and Macdonald-Bravo, 1985, 1987; Bravo et al., 1987; Celis et al., 1987], p105 nuclear associated antigen [Bauer et al., 1986; Turner et al., 1989; Swanson and Brooks, 1990], Ki-67 antigen [Swanson and Brooks, 1990; van Dierendonck et al., 1991], p110 and p85 [Black et al., 1987], and p120 and p145 [Bolton et al., 1992] are known to be differentially distributed throughout the cell cycle at various levels and are used as markers for localizing cells in specific phase. The identification of these proteins involved in the control of cell proliferation has provided new insights into the understanding of mechanisms underlying control of cell growth and transformation.

An acidic nuclear polypeptide of the molecular weight 36,000 daltons identified as PCNA/cyclin is an auxiliary protein for processivity of DNA polymerase-delta [Tan et al., 1986; Bravo et al., 1987]. Immunofluorescence analysis of synchronously growing cell populations using antibodies against PCNA/cyclin has revealed dramatic changes in the nuclear distribution of this protein during the S-phase of the cell cycle [Celis and Celis, 1985; Morris and Mathews, 1989; Bolton et al., 1992]. Cells in Go-G1, G2, and mitosis exhibit weak antibody affinity, while S-phase cells show variable patterns of staining in terms of both intensity and distribution of the antigen [Bravo and Macdonald-Bravo, 1985; Celis and Celis, 1985; Miyashi et al., 1978]. PCNA/cyclin is present in very small amounts in senes-

Received November 30, 1992; accepted February 17, 1993.

Address reprint requests to Dr. Narendra D. Lalwani, Parke-Davis Pharmaceutical Research, Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48106.

cent and quiescent cells and tissues, but it is synthesized actively in proliferating cells, particularly in S-phase, of both normal and transformed phenotypes including tumors [Celis and Celis, 1985; Robbins et al., 1987; Garcia et al., 1989; Morris and Mathews, 1989; Kawakita et al., 1992].

Changes in PCNA/cyclin levels also point to coordinated regulation in other phases of the cell cycle. For example, the onset of M-phase in eukaryotic cells is determined by cyclin-associated activation of protein kinase activity of cdc2, a cell division control-2 gene encoding a 34 kD protein kinase (cdc2-kinase) [Nurse, 1990]. Three mitotic cyclins, A, B1, and B2, form complex with cdc2-kinase during the cell cycle. B-type cyclins form a complex with cdc2-kinase that is most active as an H1-kinase at metaphase during the human somatic cell cycle [Pines and Hunter, 1989]. cdc2-Kinase along with B-type cyclin have been shown to be the main components of *Xenopus* maturation promoting factor and M-phase specific histone H1-kinase [Arion et al., 1988; Gautier et al., 1988; Dunphy et al., 1988]. The kinase activity of cdc2-cyclin complex is also regulated by a phosphorylation site located in the ATP-binding site of cdc2 at tyrosine 15 [Draetta and Beach, 1988; Solomon et al., 1990; Nurse, 1990; Pines and Hunter, 1991]. The dephosphorylation of this site activates the cdc2-kinase, which then causes cells to enter mitosis [Fang and Newport, 1991]. Microinjection of cdc2-kinase promotes the transition of cells from G2 into the early mitosis [Lamb et al., 1990]. These findings suggest that the cdc2-kinase is a key enzyme acting when cells enter and complete mitosis or meiosis. Phosphorylation of some of the key proteins leads to the major M-phase events, including chromosome condensation, cytoskeletal reorganization, envelope breakdown, and cell shape changes [Nurse, 1990; Maller, 1990; Lewin, 1990].

In this paper we present evidence that PCNA/cyclin protein is stable during the cell cycle and is expressed at its peak in G1-S-phase populations as determined by fluorescence-labeled anti-PCNA antibodies in exponentially growing Yac-1 cells. The experiments using anti-cdc2-kinase antibodies demonstrate that cdc2-kinase is highly expressed in mitosis or at the interphase of G2- and M-phase and could be involved in degradation of PCNA during the transition from a growing to a quiescent cell. The accumulation of PCNA/cyclin and cdc2-kinase may play a role in the arrest of cells in M-phase upon colcemid treatment.

MATERIALS AND METHODS

Cell Line

Yac-1 murine T-lymphoma cells were obtained from American Tissue Type Culture Collection (Rockville, MD). The cells were grown and maintained in RPMI-1640 (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (Sigma Chemical Company, St. Louis, MO), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The apparent doubling time of Yac-1 cells, at a density of 0.33×10^6 cells/ml, was approximately 10–12 h. The viability of cells was approximately 90–95% as assessed by trypan blue exclusion. All experiments were performed on cells during the exponential phase of their growth.

S-Phase Labeling With Bromodeoxyuridine (BrdU)

Cell progression through the cell cycle was measured by labeling with 10 μM BrdU in medium for 2 h. The progression of BrdU-labeled populations was followed for 48 h in BrdU-free medium. In parallel experiments cells were continuously labeled with BrdU up to 24 h. The BrdU incorporation in cells was detected using fluorescein isothiocyanate (FITC)-labeled anti-BrdU monoclonal antibodies (Boehringer Mannheim, Indianapolis, IN) according to the procedure outlined by Schutte et al. [1987]. The proportions of cells in different phases of the cell cycle were then detected by staining with propidium iodide (PI) (50 μg/ml) in phosphate buffered saline (PBS) for 30 min and analyzed by flow cytometry. The rate of progression was estimated based on decrease in number of cells in G1-phase and increase in S- and G2-M-phases [Darzynkiewicz et al., 1987].

Detection of PCNA and cdc2-Kinase

Fresh cultures of Yac-1 cells were incubated in the presence of 25 ng/ml or 2 μg/ml colcemid (Sigma). Control incubations for the same periods were devoid of colcemid. Cells subjected to flow cytometric analysis were fixed in a suspension of 70% ethanol for 30 min. Cellular DNA and specific proteins were measured by rehydrating cells in PBS followed by staining with antibodies or PI. The cell cycle distribution of PCNA

was measured by localization of anti-PCNA monoclonal antibodies (clone #19F4, Coulter Cytometry, Hialeah, FL), detected by FITC-labeled anti-mouse IgM raised in goat. The cdc2-kinase was measured by localization of polyclonal anti-mouse cdc2-kinase against 264–288 residues at the C-terminus [Samiei et al., 1991], raised in rabbit (Upstate Biotechnology, Inc., Lake Placid, NY). Antibodies were detected by FITC-labeled secondary antibodies against rabbit IgG raised in donkey (Chemicon, Temecula, CA).

Flow Cytometry

Fluorescence of individual cells was measured with an Epics® Elite Flow Cytometer (Coulter Electronics, Inc., Hialeah, FL). The single cell population detected by forward and side scattering was selected for collecting data on red and green fluorescence emitted by PI and FITC. The fluorescence dyes were excited at 488 nm by a 15 mW argon ion laser. The FITC fluorescence was collected by means of a 525 nm band pass filter and PI fluorescence by means of a 575 nm long pass filter. The data collected in list mode were further analyzed using Multiplus® software (Phoenix Flow System, San Diego, CA), which incorporates Multicycle analysis and Multi2D software for further analysis of PCNA, cdc2-kinase, and BrdU-labeled cell populations.

RESULTS

BrdU Incorporation in Exponentially Growing Cells

The progression of cell populations through various phases of the cell cycle was first monitored by labeling with BrdU after plating fresh cultures. Cells were either labeled for 2 h and followed for 24 h in label-free medium, or were continuously labeled with BrdU for 24 h in BrdU-containing medium. As shown in Figure 1A, cells entering in S-phase are first labeled with BrdU as detected by FITC-labeled anti-BrdU monoclonal antibodies. The BrdU-positive cells after removal of BrdU from medium slowly progress through G2-M and enter in Go-G1 (Fig. 1B,C). The cells labeled during the initial labeling period of 2 h are located in S-phase only (Fig. 1D) and at the 8 h time interval after BrdU labeling 95% of cells were progressing through G2-M-phase (Fig. 1E). BrdU-positive cells were present throughout the cell cycle 18 h after labeling (Fig. 1F). The distribution of cells cycling through each phase remained constant throughout the 24 h BrdU chase period. The percent distribution of cells in various phases of the cell cycle varied between different experiments within acceptable limits. However, BrdU-positive cells progressed through each phase in the first 6–8 h after labeling and the cells entered into resting phase (Go-G1).

The cells continuously grown in BrdU-containing medium, all of the cells in S- and G2-M-phase, were labeled in the first 4–6 h (Fig. 2A,B), and then the cells cycled through the resting phase (Go) between 6 and 24 h. At the 14 h interval, as shown in Figure 2C and Figure 2F, all of the cells irrespective of their position in the cell cycle were labeled with BrdU. The cells in these conditions were found to be exponentially growing for at least 12–18 h and were in plateau between 24 and 48 h. A large percentage was found in the Go-G1 peak at 48 h after plating in fresh medium.

Distribution of PCNA During the Cell Cycle

Samples taken at similar time points in exponential phase of growth as in BrdU labeling experiments described in Figures 1 and 2 were analyzed for quantitation of PCNA/cyclin in each phase of the cell cycle. The bivariate histogram for PCNA/cyclin labeling and single parameter DNA histograms are shown in Figure 3. After 4 h of incubation following fresh plating, about 70% of the cells were detected in S-phase and G2-M-phase and showed high content of PCNA/cyclin. Approximately 20% of PCNA-positive cells were in Go-G1-phase and showed low levels of PCNA/cyclin (Fig. 3A,B). The population of PCNA-positive cells after subtracting fluorescence of isotype control (IgM) was specifically located in S-phase only (Fig. 3C). The analysis of single parameter DNA histograms indicated that 55% of total cell population was in S-phase and bivariate analysis showed 87% of PCNA-positive cells in S-phase. There was a significant increase in PCNA/cyclin concentrations from G1- to early S-phase and these levels were sustained or increased further in late S- and early G2-M-phase (Fig. 3A). The PCNA/cyclin concentrations increased continuously during exponential growth (Fig. 4), which could represent a characteristic associated with transformation phenotype of rapidly dividing Yac-1 lymphoma cells in culture.

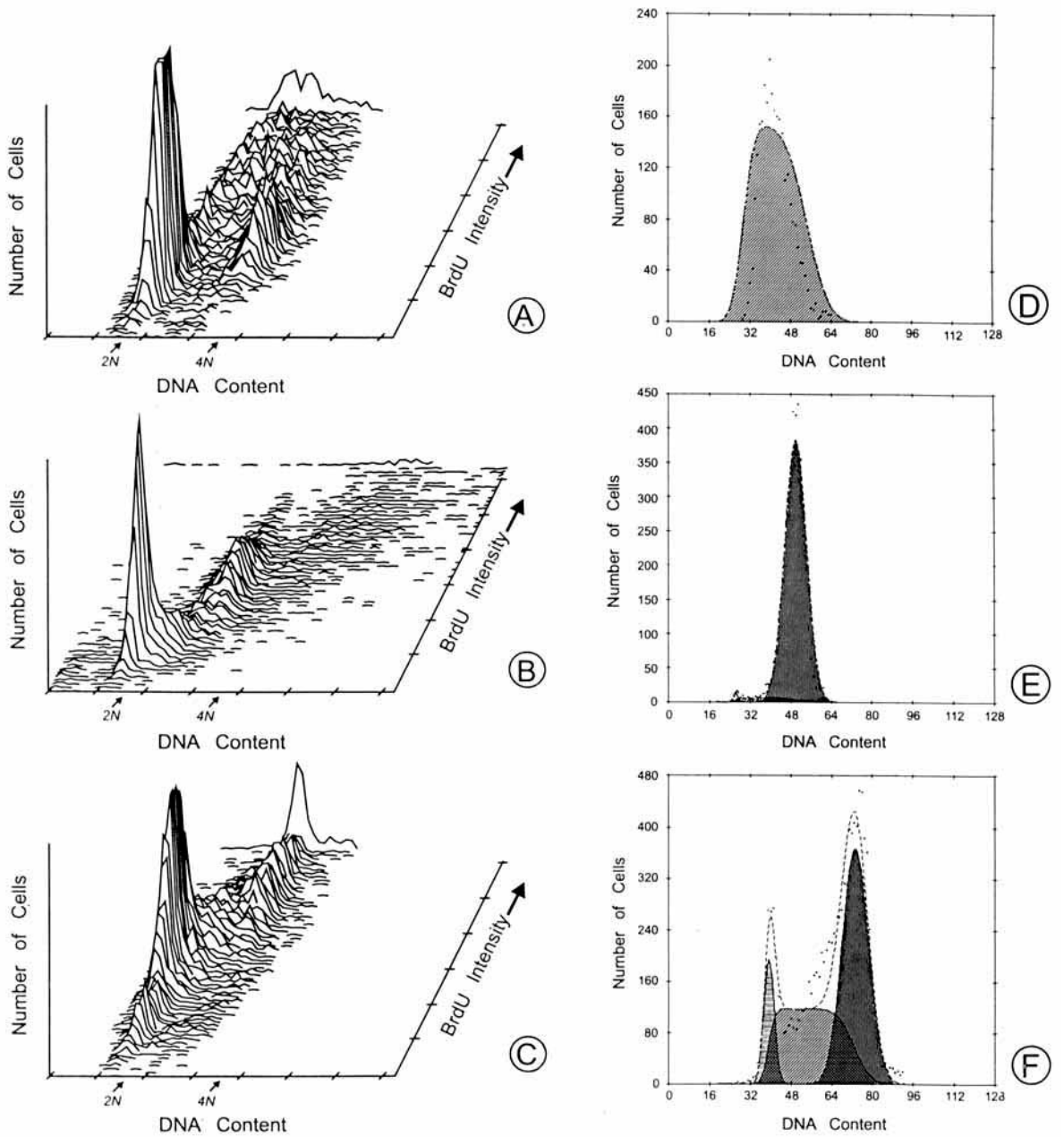

Fig. 1. Bivariate and cell cycle analysis of Yac-1 cells labeled for 2 h pulse in the presence of BrdU after plating in fresh medium. After incubating for 2 h in the presence of 10 μM BrdU, cells were kept in fresh medium without BrdU and harvested at 2 h (**A**), 8 h (**B**), and 18 h (**C**) after labeling and stained with PI for detection of DNA and FITC-labeled anti-BrdU. **D–F** show the distribution of BrdU-positive cells in each phase of cell cycle: S = 100% at 2 h (D), S = 5%, G2-M = 95% at 8 h (E), and Go-G1 = 12%, S = 39%, and G2-M = 49% at 18 h (F) after pulse-labeling.

Localization of cdc2-Kinase in M-Phase

The dual parameter analysis of exponentially growing cells in medium after 6 h showed that the cdc2-kinase content, as determined by anti-cdc2-kinase antibodies, in Go-G1-phase and in S-phase of cell cycle was lower than in G2-M-phase (Fig. 5). As shown in Table I, only 12.8% of the cell population was positive for cdc2-kinase content and 50.8% of these positive cells were in G2-M-phase. This finding was confirmed when the cells were incubated with low colcemid concentration (25 ng/ml) in cultures for 2–6 h to arrest the cells in M-phase. After 6 h of incubation in the presence of colcemid (25 ng/ml), 91.5% of cells were in G2-M-phase and the cdc2-kinase content was approximately 10–

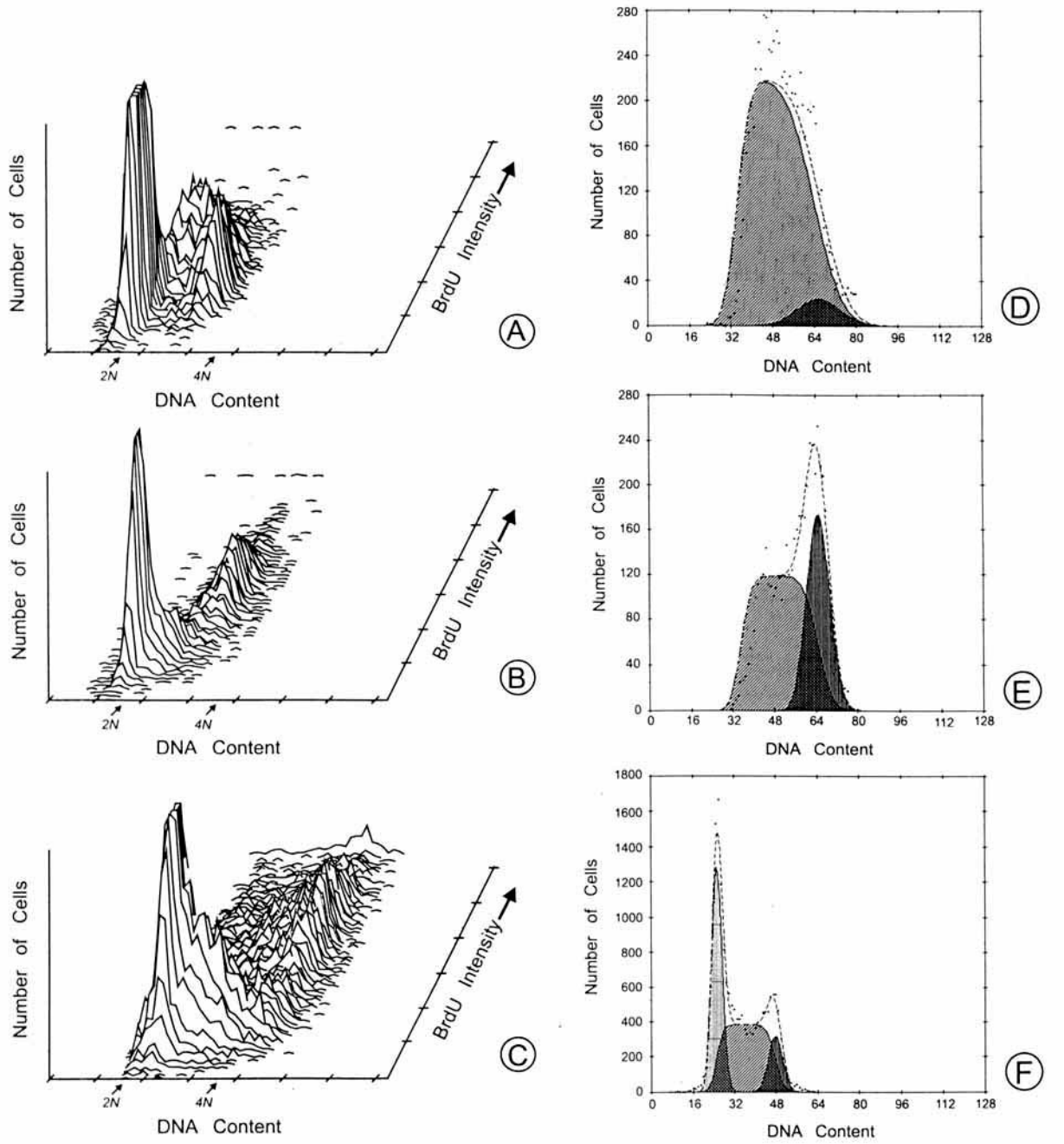

Fig. 2. Bivariate and cell cycle analysis of Yac-1 cells continuously labeled in the presence of 10 μM BrdU for 2 h **(A)**, 6 h **(B)**, and 14 h **(C)**. The corresponding distribution of BrdU-positive cells in each phase of cell cycle is S = 90% and G2-M = 10% at 2 h **(D)**, S = 70% and G2-M = 30% at 6 h **(E)**, and Go-G1 = 32%, S = 52%, and G2-M = 16% at 14 h **(F)**.

100 times higher in terms of fluorescence intensity (Fig. 5C,D). Although 77.5% of the total population of colcemid-treated cells were positive for cdc2-kinase, only 71.7% of cdc2-kinase-positive cells were in G2-M-phase. From the above data, the cdc2-kinase-positive cells detected in G2-M-phase were most likely in M-phase.

PCNA/Cyclin and cdc2-Kinase Content in Colcemid-Treated Cells

Cells incubated in the presence of low colcemid concentration (25 ng/ml) expressed higher fluorescence intensity of cdc2-kinase than cells incubated in control medium. Upon colcemid treatment, 69.2% of cells were arrested in G2-M-

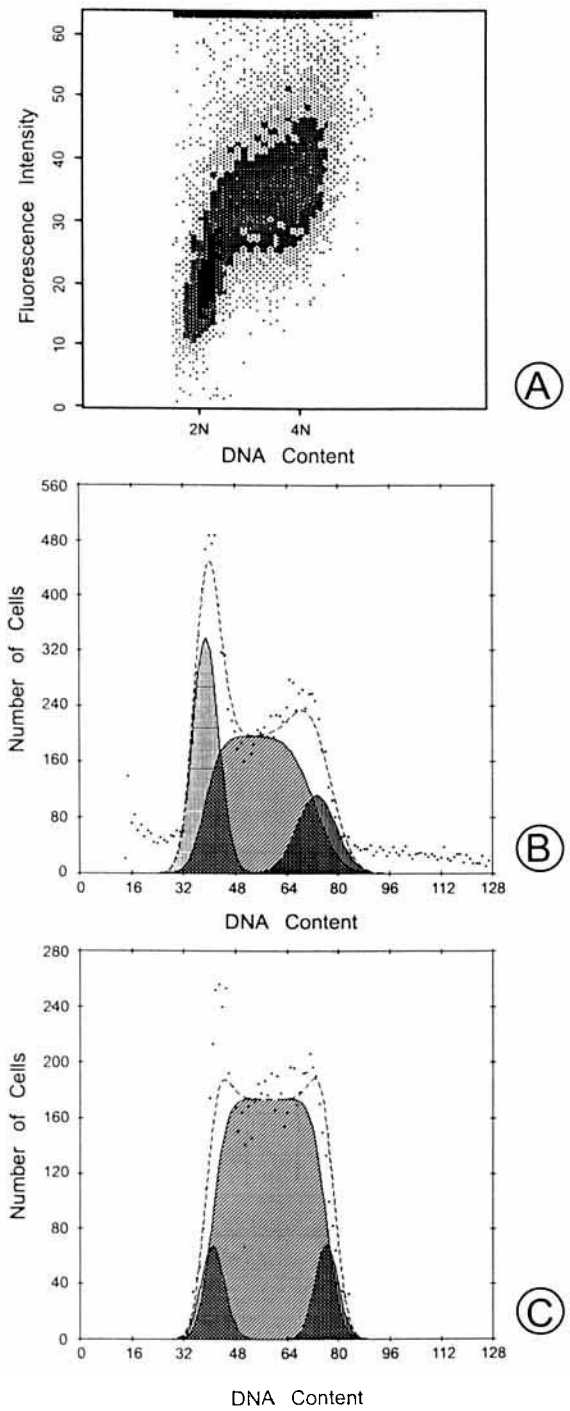

Fig. 3. Distribution of PCNA in cell populations distributed in various phases of cell cycle. Yac-1 cells were incubated in fresh medium for 4 h after plating. **A:** Bivariate distribution of PCNA detected by localization of anti-PCNA against DNA content. **B:** DNA distribution of entire population analyzed by multicycle parameters. The distribution of cell population in this analysis is Go-G1 = 28%, S = 55%, and G2-M = 17%. **C:** Distribution of PCNA-positive cells after subtraction of background fluorescence detected by control IgM. About 87% of PCNA-positive cells are present in S-phase and 13% are distributed in other phases of the cell cycle.

phase, the number of PCNA-positive cells decreased, and the number of S-phase cells also declined (Table I). Whereas PCNA-positive cells were almost equally distributed in S- and G2-M-phases, PCNA-positive cells were found only in S-phase in control incubations. Expression of PCNA/cyclin in cells arrested in G2-M-phase was detected in 16.9% of the total cell population and also showed high content of cdc2-kinase protein (Fig. 5C,D).

Treatment of Yac-1 cells up to 6 h with colcemid at 2 μg/ml revealed a population of fast growing cells having escaped arrest by colcemid and entered in Go-G1- and S-phases. The viability of cells in the presence of colcemid up to 6 h was >90% as determined by trypan blue exclusion. This cell population had lower (80%) DNA content than diploid cells, a characteristic of hypodiploid cells (Fig. 6). Approximately 67–70% of the cell population was hypodiploid after colcemid treatment at 2 μg/ml. Multicycle DNA analysis on cdc2-kinase-positive cells allowed the separation of these cells into compartments of *low, medium,* and *high* fluorescence intensity. The results revealed that 60% of the diploid cells showed *high* cdc2-kinase content and were in G2-M-phase (Fig. 6A). Although cdc2-kinase was detected throughout the cell cycle, all cells with *low* cdc2-kinase content were in Go-G1-phase. DNA analysis of the hypodiploid population showed that 95% of cells were in S-phase or G2-M-phase. These hypodiploid cells arrested in G2-M-phase formed a distinctive compartment with varying levels of cdc2-kinase content (Fig. 6B). These results show that aneuploid cells arrested in M-phase due to colcemid treatment had lower DNA content than diploid cells and also lower levels of cdc2-kinase protein.

DISCUSSION

The results of this study demonstrate a quantitative correlation in the distribution of PCNA/cyclin and cdc2-kinase and BrdU uptake kinetics during S-phase as indicators of various phases of cell cycle in mouse T-lymphoma cells exponentially growing in cultures. Anti-PCNA antibodies recognize a nuclear protein, cyclin (mol wt 36,000), whose synthesis correlates with the proliferative state of the cell. Bravo et al. [1987] demonstrated that PCNA and cyclin have many common properties and it has been shown that these two are identical auxiliary proteins for DNA polymerase-delta.

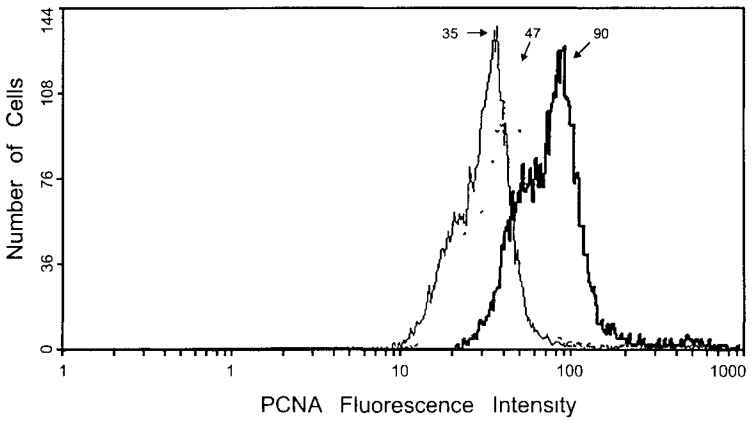

Fig. 4. Single parameter analysis of PCNA-positive Yac-1 cells after plating in fresh medium The intensity of PCNA increased with incubation time after plating Cells harvested at 6 h (solid line), 10 h (dotted line), and 26 h (boldface line) demonstrated mean fluorescence intensities of 30 8, 38 5, and 75 0, respectively Peak fluorescence intensities are indicated by the arrows

BrdU uptake and PCNA/cyclin are the markers used extensively in the detection of cell proliferation of various cell types [Coltrera and Gown, 1991; Bolton et al., 1992; Fogt et al., 1991; Weghorst et al., 1991]. BrdU, a thymidine analog, is incorporated in newly synthesized DNA during S-phase of the cell cycle and can be detected by using anti-BrdU monoclonal antibodies. From the data presented in Figures 1 and 2, BrdU uptake, either by pulse-labeling or by continuous labeling, can be used to study cells moving through the different phases of the cell cycle. The pulse-labeling technique could be used to measure kinetic permutations after treatment with various test chemicals, and Fogt et al. [1991] proposed specific calculations based on growth kinetics of Walker-256 carcinosarcomas in rats.

PCNA is a well-characterized marker differentially distributed in various phases of cell cycle with the highest level of expression in the G1-S interface. PCNA is elevated in a variety of solid tumors [Robbins et al., 1987] and also in chronic myeloid leukemia [Takasaki et al., 1984]. Detection of PCNA using specific monoclonal antibodies as a measure of S-phase activity has been demonstrated in tumors as well as in normal cells by light microscopy [Robbins et al., 1987; Coltrera and Gown, 1991; van Dierendonck et al., 1991; Kawakita et al., 1992]. Flow cytometry provides an extremely sensitive tool to detect logarithmic concentration differences and distribution in various phases of cell cycle with the possibility of detecting more than one parameter simultaneously. Our data indicate that PCNA is present in very high concentrations in S-phase (Fig. 3), but its synthesis might have started earlier, either at the interphase of G1-S-phase or immediately before cells entering S-phase. The data suggest that PCNA/cyclin synthesis precedes BrdU incorporation which takes place only during DNA synthesis. However, translocation of PCNA/cyclin to the nucleus could be the crucial requirement for cells entering into S-phase. The fluorescence intensity expressing PCNA/cyclin synthesis in exponentially growing cells after fresh plating is a characteristic associated with abnormal growth rates of transformed phenotypes or reduced degradation of PCNA/cyclin protein. In this study a large number of PCNA-positive cells were present in S-phase (Table I). However, PCNA-positive cells in Go-G1-phase are probably cells in late G1-phase entering active DNA synthesis and those PCNA-positive cells in G2-M-phase are in transition between S- and G2-phase (Fig. 3C). Although cells in Go-G1- and G2-M-phase were PCNA-positive, most of the PCNA/cyclin protein is normally regulated by degradation before entering M-phase and the concentrations are reduced to a minimum in cells before entering Go-phase [Celis et al., 1987]. The sustained increases in PCNA in rapidly dividing Yac-1 cells in cultures could be due to reduced PCNA/cyclin degradation during G2-M-phase. It could also be interpreted that cells at the G1-S-phase transition were actively synthesizing PCNA/cyclin (Fig. 4).

Studies of cdc2-kinase localization in the cell have also been relevant to understanding its role in M-phase onset. An immunofluorescence and cell fractionation study of mammalian cells lo-

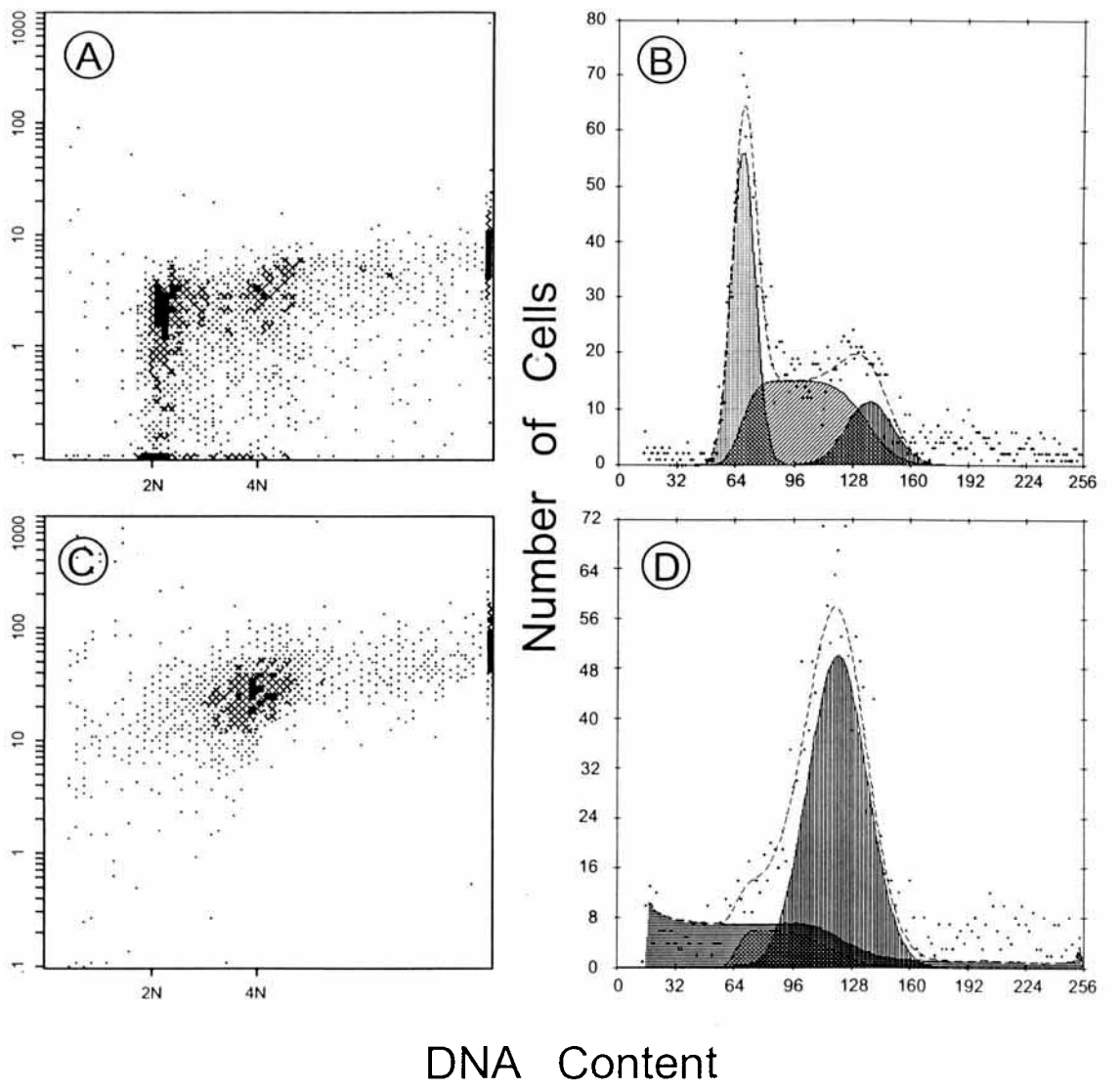

Fig 5 Comparison of cdc2 kinase distribution and cell cycle kinetics in control and colcemid arrested Yac 1 cells Cells were incubated for 6 h in the control medium (**A,B**) and colcemid (25 ng/ml) containing medium (**C,D**) The number of cells distributed in different phases of the cell cycle for control cells (B) are Go G1 = 39 5% S = 44 7% and G2 M = 15 7% and for colcemid treated cells (D) are Go G1 = 0 5% S = 8% and G2 M = 91 5%

cated the cdc2-kinase protein in both the nuclear and cytoplasmic fractions [Bailly et al , 1989] As reported earlier by several investigators [Lewin, 1990, Nurse, 1990, Roy et al , 1991, Solomon et al , 1990], cdc2-kinase is known to form a complex with mitotic cyclin (A, B1, and B2) which results in the degradation of B-type cyclins during the transition stage from G2- and M-phase The cyclin A-associated kinases are active during S- and G2-phases and earlier in the division cycle, and apparently cdc2-kinase activity is not required during the G1- and S-phases of the cell cycle [Roy et al , 1991, Hamaguchi et al , 1992], but plays a crucial role during the M-phase activity of the cell cycle It is possible that the cells are kept in M-phase by means of high cdc2-kinase activity [Yasuda et al , 1990], and the data presented here agree with the assumption that upregulation or activation of cdc2-kinase is essential for M-phase activity Cells in G2-M-phase have maximum levels of cdc2-kinase protein compared to cells in Go-G1- or S-phase (Fig 6A), and cdc2-kinase protein increased when cells were synchronized in G2-M-phase upon colcemid treatment (25 ng/ml) (Fig 5C) Colcemid might have arrested cells in M-

TABLE I. Distribution of PCNA and cdc2-Kinase in Phases of Cell Cycle With and Without Colcemid Treatment*

	Distribution in cell cycle phases (%)[a]							
	Control				Colcemid-treated[b]			
Parameter	Positives[c]	Go-G1	S	G2-M	Positives	Go-G1	S	G2-M
DNA	100	28.0	55.1	16.8	100	4.3	26.6	69.2
PCNA	53.1	7.9	86.6	5.5	32.8	0.0	48.5	51.5
cdc2-Kinase	12.8	6.8	42.3	50.8	77.5	5.5	22.8	71.7

*Yac-1 cells were cultured in RPMI containing 10% fetal calf serum, with and without 25 ng/ml colcemid, for 6 h. The aliquots of cells were stained with anti-PCNA/DNA or anti-cdc2-kinase/DNA and analyzed by flow cytometry.
[a]Distribution of cells in various phases of cell cycle was determined using Multiplus®. The background fluorescence was subtracted using control antibodies.
[b]Viability of cells was > 90% upon colcemid treatment.
[c]Percentage of cells stained with marker dye or antibodies in the total cell population analyzed.

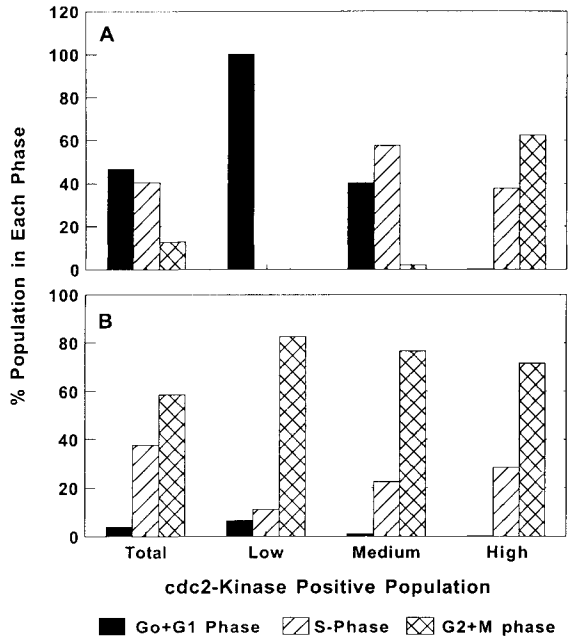

Fig. 6. Histograms representing cell cycle distribution of diploid and aneuploid cell populations after 6 h incubation with colcemid (2 μg/ml) in medium. Diploid cells **(A)** and aneuploid (hypodiploid) cells **(B)** are subdivided into Go-G1-phase (solid bars), S-phase (hatched bars), and G2-M-phase (cross-hatched bars). The cell populations are divided into *low, medium,* and *high* cdc2-kinase content based on percent fluorescence intensity on the linear scale.

phase by inhibiting the protein kinase activity of cdc2-kinase. The loss of kinase activity would have resulted in the dislocation of other events associated with cdc2-kinase, such as degradation of mitotic cyclins in G2-M-phase. In fact, the data show accumulation of both PCNA and cdc2-kinase in colcemid-treated cells, which could be virtually in inactive state (Table I).

Although the DNA content of normal cell populations and colcemid-arrested cell populations appears to be in the same range, it is evident that due to colcemid at 2 μg/ml, the daughter cells entering in Go-G1-phase after mitotic division have lower DNA content. Bhuyan et al. [1987] observed a similar phenomenon with B16 melanoma cells, where newly divided cells had about 80% of original DNA content after mitotic division, a characteristic of aneuploidy (hypoploidy). In the experiments reported here colcemid also induces pseudoaneuploidy in cultures, evident in cells exposed to higher concentration of colcemid (2 μg/ml). Most of the aneuploid cells are arrested in G2-M-phase with wide variation of cdc2-kinase content (Fig. 6B). However, in normal diploid cells, only cells in M-phase have high cdc2-kinase activity (Fig. 6A). The large percentage of cells having *low* or *medium* cdc2-kinase levels points to the fact that cdc2-kinase synthesis is inhibited or down regulated in the presence of colcemid. Although from the above results it can be deducted that colcemid arrests cells in mitosis or premitotic phase by blocking synthesis of cdc2-kinase, there are no data to support this hypothesis. Yasuda et al. [1990] showed that cdc2-kinase activity increases severalfold in the nuclear fraction compared to the cytoplasmic fraction in mouse mammary carcinoma cells. Protein phosphorylation is generally increased during M-phase, as a direct consequence of the cdc2-kinase or as an indirect consequence of cdc2-kinase activating other protein kinases. Fang and Newport [1991] proposed that different forms of cdc2 proteins in higher eukaryotes are responsible for G1-S (DNA replication) and G2-M (mitosis) transitions.

In summary, these findings support that PCNA is upregulated or present in high concen-

trations during S-phase and cdc2-kinase in M-phase. PCNA is synthesized during G1-S transition permitting DNA synthesis and degrades at the end of the S-phase or probably in late G2-phase due to, albeit unproven, complexing with cdc2-kinase. Further investigations should address the differential regulation of isoforms of cdc2-kinase and PCNA and other cyclins [Roy et al., 1991] at transcriptional and translational levels in order to delineate if these proteins are down regulated at mRNA level or inactivated by proteolysis.

REFERENCES

Arion D, Meijer L, Brizuela L, Beach D (1988) cdc2 is a component of the M phase-specific histone H1 kinase Evidence for identity with MPF Cell 55 371–378

Bailly E, Doree M, Nurse P, Bornens M (1989) $p34^{cdc2}$ is located in both nucleus and cytoplasmic parts centrosomally associated at G_2/M and enters vesicles at anaphase EMBO J 8 3985–3995

Bauer KD, Clevenger CV, Endow RK, Murad T, Epstein AL, Scarpelli DG (1986) Simultaneous nuclear antigen and DNA content using paraffin-embedded colonic tissue and multiparametric flow cytometry Cancer Res 46 2428–2434

Bhuyan BK, Adams EG, Badiner GJ, Trzos RJ (1987) Colcemid progression and aberrant mitotic division J Cell Physiol 132 237–245

Black A, Freeman JW, Zhou G, Busch H (1987) Novel cell cycle-related nuclear proteins found in rat and human cells with monoclonal antibodies Cancer Res 47 3266–3272

Bolton WE, Mikulka WR, Healy CG, Schmittling RJ, Kenyon NS (1992) Expression of proliferation associated antigens in the cell cycle of synchronized mammalian cells Cytometry 13 117–126

Bravo R, Macdonald-Bravo H (1985) Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication EMBO J 4 655–661

Bravo R, Macdonald-Bravo H (1987) Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle Association with DNA replication sites J Cell Biol 105 1549–1554

Bravo R, Frank R, Blundell PA, Macdonald-Bravo H (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta Nature 326 515–517

Celis JE, Celis A (1985) Cell cycle dependent variations in the distribution of nuclear protein cyclin proliferating cell nuclear antigen in cultured cells Subdivision of S phase Proc Natl Acad Sci USA 82 3262–3266

Celis JE, Madsen P, Celis A, Nielsen HV, Geaser B (1987) Cyclin (PCNA, auxiliary protein of DNA polymerase delta) is a central component of the pathway(s) leading to DNA replication and cell division FEBS Lett 220 1–7

Coltrera MD, Gown AM (1991) PCNA/cyclin expression and BrdU uptake define different subpopulations in different cell lines J Histochem Cytochem 39 23–40

Darzynkiewicz Z, Traganos F, Kimmel M (1987) Assay of cell cycle kinetics by multivariate flow cytometry using the principle of stathomokinesis In Gray JW, Darzynkiewicz Z (eds) "Techniques in Cell Cycle Analysis" Clifton, NJ Humana Press, pp 291–336

Draetta G, Beach D (1988) Activation of cdc2 protein kinase during mitosis in human cells Cell cycle-dependent phosphorylation and subunit rearrangement Cell 54 17–26

Dunphy WG, Brizuela L, Beach D, Newport J (1988) The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis Cell 54 423–431

Fang F, Newport JW (1991) Evidence that the G1-S and G2-M transition are controlled by different cdc2 proteins in higher eukaryotes Cell 66 731–742

Fogt F, Wan J, O'Hara C, Bistrian BR, Blackburn GL, Istfan NW (1991) Flow cytometric measurement of cell cycle kinetics in rat Walker-256 carcinoma following in vivo and in vitro pulse labelling with bromodeoxyuridine Cytometry 12 33–41

Garcia RL, Coltrera MD, Gown AM (1989) Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues Comparison with flow cytometric analysis Am J Pathol 134 188–194

Gautier J, Nobury C, Lohka M, Nurse P, Maller J (1988) Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene $cdc2^+$ Cell 54 433–439

Hamaguchi JR, Tobey RA, Pines J, Crissman HA, Hunter T, Vradbury EM (1992) Requirement of $p34^{cdc2}$ kinase is restricted to mitosis in the mammalian cdc2 mutant FT210 J Cell Biol 117 1041–1053

Kawakita N, Seki S, Sakaguchi H, Yanai A, Kuroki T, Mizoguch Y, Kobayashi K, Monna T (1992) Analysis of proliferating hepatocytes using a monoclonal antibody against proliferating cell nuclear antigen/cyclin in embedded tissues from various liver diseases fixed in formaldehyde Am J Pathol 140 513–520

Lamb NJC, Fernandez A, Watrin A, Labbe J-C, Cavadore J-C (1990) Microinjection of $p34^{cdc2}$ kinase induces marked changes in cell shape, cytoskeletal organization, and chromatin structure in mammalian fibroblasts Cell 60 151–165

Lewin B (1990) Driving the cell cycle M phase kinase, its partners, and substrates Cell 61 743–752

Maller JL (1990) *Xenopus* oocytes and the biochemistry of cell division Biochemistry 29 3157–3166

Miyashi KM, Fritzer J, Tan EM (1978) An autoantibody to a nuclear antigen in proliferating cells J Immunol 121 2228–2234

Morris GF, Mathews MB (1989) Regulation of proliferating cell nuclear antigen during the cell cycle J Biol Chem 264 13856–13864

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

Pines J, Hunter T (1989) Isolation of a human cyclin cDNA Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$ Cell 58 833–846

Pines J, Hunter T (1991) Cyclin-dependent kinases A new cell cycle motif? Trends Cell Biol 1 117–121

Robbins BA, de la Vega D, Ogakata K, Tan EM, Nakamura RM (1987) Immunohistological detection of proliferating cell nuclear antigen in solid human malignancies Arch Pathol Lab Med 111 841–845

Roy LM, Swenson KI, Walker DH, Gabrielli BG, Li RS, Ouwbuca-Worms H, Maller JL (1991) Activation of $p34^{cdc2}$ kinase by cyclin A J Cell Biol 113 507–514

Samiei M, Daya-Makin M, Clark-Lewis I, Pelech SL (1991) Platelet-activating factor- and thrombin-induced stimulation of $p34^{cdc2}$-cyclin histone H1 kinase activity in platelets J Biol Chem 266 14889–14892

Schutte B, Reynders MMJ, van Assche CLMVJ, Hupperets PSGJ, Bosman FT, Blijham GH (1987) An improved method for the immunocytochemical detection of bromodeoxyuridine labeled nuclei using flow cytometry Cytometry 8 372–376

Solomon MJ, Glotzer M, Lee T, Phillipe M, Kirschner MW (1990) Cyclin activation of $p34^{cdc2}$ Cell 63 1013–1024

Swanson SA, Brooks JJ (1990) Proliferation markers Ki-67 and p105 in soft tissue lesions Correlation with DNA flow cytometric characteristics Am J Pathol 137 1491–1500

Takasaki Y, Robinson WA, Tan EM (1984) Proliferating cell nuclear antigen in blast crisis cells of patients with chronic myeloid leukemia J Natl Cancer Inst 73 655–659

Tan CK, Castillo C, So AG, Downey KM (1986) An auxiliary protein for DNA polymerase-delta from fetal calf thymus J Biol Chem 261 12310–12316

Turner RR, Layfield LJ, Bishop PC, Epstein AL, Parker JW (1989) Flow cytometric measurements of proliferation-associated nuclear antigen p105 and DNA content in non-Hodgkin's lymphoma Arch Pathol Lab Med 113 907–911

van Dierendonck JH, Wijsman JH, Keijzer R, van de Velde CJH, Cornelisse CJ (1991) Cell-cycle-related staining patterns of anti-proliferating cell nuclear antigen monoclonal antibodies Comparison with BrdUrd labelling and Ki-67 staining Am J Pathol 138 1165–1172

Weghorst CM, Henneman JR, Ward JM (1991) Dose response of hepatic and renal DNA synthetic rates to continuous exposure of bromodeoxyuridine (BrdU) via slow-release pellets or osmotic minipumps in male B6C3F1 mice J Histochem Cytochem 39 177–184

Yasuda H, Kamijo M, Honda R, Nagahara M, Ohba Y (1990) The difference in murine cdc2 kinase activity between cytoplasmic and nuclear fractions during the cell cycle Biochem Biophys Res Commun 172 371–376