Requirement for Proliferating Cell Nuclear Antigen Expression during Stages of the Chinese Hamster Ovary Cell Cycle†

Yin-Chang Liu,‡ Robert L. Marraccino,§ Peter C. Keng, Robert A. Bambara, Edith M. Lord, Wen-Gang Chou, and Sayeeda B. Zain*

Departments of Biochemistry, Microbiology, and Immunology and Cancer Center, University of Rochester Medical Center, Rochester, New York 14642

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ABSTRACT: Proliferating cell nuclear antigen (PCNA/cyclin) is a nuclear protein that can stimulate purified DNA polymerase δ in vitro, and its synthesis correlates with the proliferation rate of cells. We have attempted to determine whether synthesis of PCNA/cyclin in Chinese hamster ovary cells is necessary to regulate entry into S phase. We have measured cellular PCNA/cyclin concentration of the mRNA or protein throughout the cell cycle. Cells were separated by centrifugal elutriation into populations enriched for G-1, S, and G-2/M phases. Quantitative Northern hybridization analysis was performed on RNA isolated from each cell population by using a cDNA clone of PCNA/cyclin as a probe. Results demonstrated that although intact PCNA/cyclin mRNA is present during all phases of the cell cycle, an induction of about 3-fold occurs during S phase. Two-parameter staining for PCNA/cyclin and DNA, and analysis by flow cytometry, confirmed that the quantity of PCNA/cyclin protein in the cells increases severalfold in G-1 or early S phase but generally is invariant in S and G-2/M phases. This cell cycle dependence of PCNA/cyclin expression suggests that the observed synthesis is a prerequisite for initiation of DNA replication. Introduction of an antisense oligonucleotide complementary to the PCNA/cyclin mRNA to inhibit PCNA/cyclin synthesis effectively prevented entry of G-1 phase cells into S phase. A complementary sense oligonucleotide used as a control did not have an inhibitory effect. This result suggests that a threshold concentration of PCNA/cyclin is necessary for entry into S phase.

Replicative DNA synthesis in the S phase of the cell cycle is a discrete event in eukaryotic cells. The transition to the S phase may involve the synthesis or activation of regulatory proteins that trigger the progression from G-1 to S phase and of proteins involved in the actual metabolic process of DNA synthesis. An approach to determine the mechanism of regulation and to identify these proteins is the quantitation of synthesis or activation of suspected regulatory proteins during the cell cycle, with the expectation that a maximum would be reached at the G-1/S transition.

By such a criterion PCNA,[1] also known as cyclin (Mathews et al., 1984), may be an important cellular protein involved in this S-phase transition. The rate of synthesis of PCNA/cyclin positively correlates with the proliferation rate of normal and transformed cells from a variety of vertebrate species (Celis et al., 1984a). As assessed by quantitative two-dimensional gel electrophoresis, the quantity of PCNA–cyclin protein increases coordinately with the percentage of normal or transformed cells in the S phase (Bravo & Celis, 1980; Bravo et al., 1981a,b, 1982; Bravo, 1984a,b; Celis et al., 1984a,b; Celis & Bravo, 1984). In a synchronized HeLa cell culture, PCNA/cyclin apparently is preferentially synthesized in S phase, but this protein is also present in the G-1 and G-2 phases (Bravo & Celis, 1980) and is stable throughout the cell cycle (Bravo & Macdonald-Bravo, 1985, 1987). The prevention of DNA synthesis by specific inhibitors that block the G-1/S transition has no affect upon the induction of PCNA/cyclin synthesis (Bravo & Macdonald-Bravo, 1985; Macdonald-Bravo & Bravo, 1985; Bravo, 1986; Kurki et al., 1987). These results indicate that PCNA/cyclin regulation is not dependent on DNA replication, but is controlled by events prior to the initiation of S phase.

Indirect immunofluorescence with monoclonal antibodies or human antisera directed specifically against PCNA/cyclin indicates that the expression of the protein is observable in the nucleus and that it precedes DNA synthesis, reaching a maximum during S phase after mitogenic stimulation (Miyachi et al., 1978; Takasaki et al., 1981; Kurki et al., 1986, 1987, 1988). Large changes are observed in the nuclear distribution of PCNA/cyclin during late G-1 phase and throughout S phase (Takasaki et al., 1981; Chan et al., 1983; Celis & Celis, 1985a,b; Sadaie & Mathews, 1986). These changes are dependent upon DNA replication (Bravo & Macdonald-Bravo, 1985, 1987). Furthermore, PCNA/cyclin clusters around active centers of replication in the nucleus (Madsen & Celis, 1985; Bravo & Macdonald-Bravo, 1987). This topographical association of PCNA/cyclin and replicating nuclear DNA suggests that PCNA/cyclin may be an important component in DNA replication.

An auxiliary subunit of the eukaryotic DNA polymerase δ has been identified as PCNA/cyclin (Bravo et al., 1987; Prelich et al., 1987a; Tan et al., 1987). This subunit increases the binding and processivity of DNA polymerase δ on specific

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* Address correspondence to this author at the Cancer Center and Department of Biochemistry, Box 704, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642.

‡ Present address: Institute of Life Science, National Tsing Hua University, Hsinchu, Taiwan 30043, R.O.C.

§ Present address: Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104.

[1] Abbreviations: PCNA, proliferating cell nuclear antigen; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; FBS, fetal bovine serum; FITC, fluoroscein isothiocyanate; PI, propidium iodide.

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DNA templates. Recently, monospecific antiserum to PCNA/cyclin has been demonstrated to inhibit both DNA polymerase δ activity on specific templates and nuclear DNA synthesis in cell-free systems (Wong et al., 1987; Tan et al., 1987). PCNA/cyclin is also required for efficient leading-strand DNA synthesis of Simian virus 40 in vitro (Prelich et al., 1987b; Prelich & Stillman, 1988). These results suggest that both PCNA/cyclin and DNA polymerase δ may function during leading-strand synthesis at eukaryotic cell DNA replication forks (Downey et al., 1988). Although the relationship between PCNA/cyclin and DNA replication is circumstantial, it is multifaceted and always consistent with the possibility that its synthesis triggers initiation of replication and is required throughout the S phase of the cell cycle.

The regulation of PCNA/cyclin is controlled, at least in part, at the transcriptional level (Almendral et al., 1987; Celis et al., 1987; Matsumoto et al., 1987). In these studies, the regulation of PCNA/cyclin was determined in cell populations undergoing G_0- to S-phase transitions. In order to examine the role of PCNA/cyclin in the initiation and propagation of S-phase DNA synthesis, however, it is important to examine its regulation in an exponential cell population. In this way regulatory changes accompanying the onset of S phase can be distinguished from those involving the shift from quiescence to proliferation. Consequently, we have measured the regulation of PCNA/cyclin transcript in enriched populations of G-1, S, and G-2/M cells, separated from an exponential CHO culture by centrifugal elutriation (Keng et al., 1981). In addition, PCNA/cyclin protein content was determined in an exponential CHO population by quantitative indirect immunofluorescence with a flow cytometer.

In a second approach, we have used an antisense oligonucleotide to PCNA/cyclin mRNA to inhibit expression of PCNA/cyclin protein. This experiment was designed to determine whether variation in PCNA/cyclin expression could affect progression of early G-1-phase cells into S phase. Inhibition of entry into S phase would imply a necessary role of PCNA/cyclin for the phase transition.

Materials and Methods

Chemicals and Reagents. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. All radioactive nucleotides and antibodies and Hybond-N transfer membrane were supplied by Amersham (Arlington Heights, IL). Analytical-grade, mixed-bed resin (AG 501-X8D) was obtained from Bio-Rad Laboratories (Richmond, CA). Formaldehyde, guanidinium thiocyanate, and formamide were obtained from Fluka (Rononkong, NY). Yeast RNA, agarose (NA), and Sepharose 4B-CL were from Pharmacia (Piscataway, NJ). The nick-translation reagents, Sanger dideoxynucleotide sequencing reagents, cesium chloride, RNA standard size markers, restriction enzymes, and other enzymes that were used in the preparation of the cDNA library were supplied by Bethesda Research Laboratory (Gaithersburg, MD). Polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). The X-ray film (XAR-5) was from Kodak (Rochester, NY). The 7-deaza-GTP was from Boehringer Mannheim Biochemicals (Indianapolis, IN). The Cronex intensifying screen was obtained from Du Pont (Wilmington, DE).

Monoclonal antibody against PCNA/cyclin, 19F4 (Ogata et al., 1987), was supplied by American Biotech (Plantation, FL). Goat antimouse antiserum, conjugated to fluorescein isothiocyanate (20 mg mL^{-1}), was supplied by Cappel (West Chester, PA). The actin-specific cDNA was prepared by the method of Fryberg et al. (1980). Dr. Fu-Zon Chung of the National Institutes of Health (Bethesda, MD) kindly provided the oligonucleotide used for cloning cDNA for PCNA/cyclin and inhibiting its expression. The complementary sense oligonucleotide was obtained from Genetic Designs (Houston, TX).

Cells and Culture Conditions. CHO cells were cultured as monolayers in 150-cm^2 plastic flasks from Costar (Cambridge, MA) in F-10 medium (30 mL) supplemented with sodium bicarbonate (0.15%, pH 7.5), L-glutamine (2 mM), penicillin (100 units mL^{-1}), streptomycin sulfate (100 μg mL^{-1}), and 10% fetal bovine serum from J. R. Scientific (lot no. 9550, Woodland, CA). Media and supplements were obtained from Gibco (Grand Island, NY) unless otherwise noted. Cell cultures were routinely demonstrated to be *Mycoplasma* free by the Mycotrim assay from Hana Biologics (Alemeda, CA). For exponential cultures, cells (1×10^6/flask) were inoculated and grown as monolayers for 48 h prior to an experiment. For plateau cell cultures, cells (2.5×10^5) were cultured in flasks (75 cm^2) for 7 days, and the media and supplements were replaced daily in culture flasks after 3 days. The cell cultures were maintained at 37 °C in a humidified incubator with an atmosphere of 95% air and 5% carbon dioxide. CHO cells were harvested with 0.25% trypsin in Hank's balanced salt solution buffer, pH 7.5. After trypsinization, cells were suspended in complete F-10 medium with supplements.

Synchronization of Cells by Centrifugal Elutriation. After harvesting, exponential cultures of CHO cells (1×10^8) were elutriated in DMEM medium with 1% neonatal bovine serum from Biocell (lot no. 2112003, Carson, CA), as described by Keng et al. (1981) using a Beckman JE-6 elutriation rotor driven by a Beckman 21-C centrifuge. The cells were fractionated to subpopulations, primarily by size. As cells progress through the cell cycle, the volume of the cells increases proportionally with the different stages of the cell cycle (Keng et al., 1981). Subpopulations that have approximate cellular volume ratios of 1.0:1.5:2.0 represent G-1, S, and G-2/M stages of the cell cycle, respectively. The plateau cells were also elutriated, and the cells in the G-1 phase of the cell cycle were obtained from this population. The G-1 subpopulation from plateau cultures had a cell volume of 0.85 relative to the G-1 cells isolated from exponential cultures and is described herein as the early G-1 cell (G_0/G-1) population. The cell viability was similar for the G-1 cells isolated from either exponential or plateau populations. The number of cells and their size distribution was determined by a Coulter Counter and Channelyzer system from Coulter Electronics (Haileah, FL).

Treatment of Early G-1 (G_0/G-1) CHO Cells with an Antisense Oligodeoxynucleotide to PCNA/Cyclin mRNA. Early G-1 (G_0/G-1) CHO cells isolated by centrifugal elutriation were inoculated into 60 × 15 mm Petri dishes from Costar (Cambridge, MA). These cells were cultured as described for exponential CHO cells in the absence or presence of an antisense or sense oligodeoxynucleotide corresponding to a segment of PCNA/cyclin mRNA (30 μM). The antisense oligodeoxynucleotide was complementary to the sequence that encodes the third through twelfth codons of the rat PCNA/cyclin mRNA (Matsumoto et al., 1987). The correct sense oligonucleotide, exactly complementary to the antisense oligonucleotide, was also used in a control experiment. The sense oligonucleotide had an identical length and similar nucleotide composition to that of the antisense oligonucleotide. Portions of this cell population were harvested at 0 and 12 h after inoculation and analyzed for PCNA/cyclin and DNA content.

Flow Cytometry. Flow cytometry was used to verify the homogeneity of cell populations separated by centrifugal elutriation, and for indirect immunofluorescence measurement. From each elutriated cell population, cells (1.0×10^6) were fixed in 70% methanol. Cells were washed and stained with chromomycin A3 (100 μg mL^{-1}), in 12.5 mM $MgCl_2$ and potassium phosphate buffer (pH 7.2). After the cells were stained for at least 2 h, the DNA content was quantitated for each cell with an EPICS V flow cytometer (Coulter Electronics). A 5-W argon ion laser operated at 457 nm and 150 mW of power was used for excitation. The percentages of G-1, S, and G-2/M phase cells for each population were analyzed by the cell cycle program CCYCLE in a Terak 8600 microcomputer (Luk et al., 1985).

The PCNA/cyclin content was quantitated by flow cytometry in CHO populations by indirect immunofluorescence, and simultaneously the DNA content of these cells was determined. The CHO cell populations were fixed in 70% methanol for at least 12 h at 25 °C. These cells were collected by centrifugation (1000*g*; 10 min) and resuspended in 1% paraformaldehyde solution followed by a 10-min treatment with a detergent, Nonidet P-40, at 4 °C. The cells were prepared for staining by the addition of mouse monoclonal antibody to PCNA/cyclin, 19F4 (Ogata et al., 1987) for 30 min at 25 °C. The cells were washed in phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS). Then goat antimouse antiserum, conjugated to fluorescein isothiocyanate (FITC), was diluted in PBS (1:100) and added to the cells for 30 min. The cells were washed twice in PBS with 2% FBS, and the cell pellet then resuspended in PBS with RNase (1 mg mL^{-1}) for 10 min. The cells were washed in PBS and resuspended in propidium iodide solution (10 μg mL^{-1}).

After this procedure, these cells were then analyzed on the EPICS-PROFILE flow cytometer with an argon ion laser operated at 488 nm. After passing through a 550-nm dichroic filter, the green fluorescence from FITC was collected through a 530-nm short-wavelength-pass filter and recorded as a measure of PCNA protein content in each cell. Red fluorescence from PI was passed through a 610-nm long-wavelength-pass filter and was recorded as a measure of DNA content in each cell. The data were collected by using a linear amplifier for PI fluorescence and both a linear and logarithmic amplifier for FITC fluorescence. The dual-parameter histograms, combining the fluorescence of FITC and PI, were analyzed on an IBM XT microcomputer with the CYTOLOGO program (Coulter Electronics, Haileah, FL).

The titers of antibodies used in this procedure were quantitated by the highest serial dilution that produced specific immunofluorescence. The nonspecific binding of the goat antiserum was detected by repeating the procedure, except the PCNA/cyclin antibody was removed.

Preparation of the cDNA Library and Screening for PCNA/Cyclin-Specific DNA. The cDNA library was constructed in the λ-gt-11 vector from a rat thyroid radiation-induced anaplastic adenocarcinoma, designated IR6. (Details will be published elsewhere.) The procedure for construction was essentially the same as we have described previously (Zain et al., 1988). Screening was performed according to Benton and Davis (1977). The filters were hybridized for 16 h at 42 °C with an oligodeoxynucleotide complementary to the sequence that encodes the third through twelfth codons of the rat PCNA/cyclin mRNA (Matsumoto et al., 1987). Positive clones having a portion of the PCNA/cyclin DNA sequence were identified and isolated at a frequency of approximately 1 out of 25 000.

Isolation and Analysis of the PCNA/Cyclin cDNA Insert. A restriction digestion analysis of the PCNA/cyclin cDNA insert was performed, and the products were analyzed by agarose gel electrophoresis (Maniatis et al., 1982). The products resulting from *Pvu*II digestion have mobilities corresponding to 770 and 560 nucleotides. The *Pst*I products are approximately 910 and 450 nucleotides long, and *Sau* 96I products have mobilities corresponding to 740, 430, and 200 nucleotides in length. The undigested, cloned PCNA/cyclin insert is approximately 1300 nucleotides in length. The sizes of all of the DNA segments are consistent with the sequence of rat PCNA/cyclin reported by Matsumoto et al. (1987). Overall, this analysis demonstrates that this cDNA clone is approximately 100 nucleotides longer at the 5′-side of the sense strand than the cDNA clone reported by Matsumoto et al. (1987).

Sequence analysis (Sanger et al., 1977) was performed after ligation of the insert into M13mp18 (Norrander et al., 1983), for further verification that the cloned DNA represents the PCNA/cyclin mRNA. The sequenced region was found to be complementary to positions 987–1093 of the sequence of rat PCNA/cyclin mRNA in the 3′-untranslated region (Matsumoto et al., 1987). Since we used a 5′-end probe for cloning (nucleotide positions: 7–40), the determination of this 3′-end sequence and the total length of the clone suggest that we have isolated a full-length coding sequence and 3′-untranslated sequence region. This conclusion is fully consistent with the restriction endonuclease digestion patterns.

Northern Hybridization Analysis. Total cytoplasmic RNA was isolated from CHO cells in each phase of the cell cycle by the method of Chirgwin et al. (1979). The concentration of total RNA was based on absorbance at 260 nm. Equal amounts of RNA were used for each gel electrophoresis sample. RNA samples were separated in 1.3% agarose gels (24 × 21.5 cm). The gels contained 18% formaldehyde and 20 mM sodium phosphate (pH 7.0). The electrophoresis buffer was 20 mM sodium phosphate (pH 7.0) and was circulated continuously for 18 h at 40 V. The fractionated RNA was transferred to a nitrocellulose membrane (0.45 μm) according to Maniatis et al. (1982). The membrane was dried and stored.

In preparation for Northern hybridization analysis with the cDNA of PCNA/cyclin, the filter was immersed in a solution containing 6 × SSC, 50% formamide, 50 μg mL^{-1} yeast tRNA, 50 mM sodium phosphate (pH 6.5), 0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% poly(vinyl pyrrolidine) for 10 h at 42 °C. PCNA/cyclin cDNA was labeled with ^{32}P by nick translation to 2.0×10^6 cpm mL^{-1} and then incubated with the filter for 20 h at 42 °C. The filter was washed once in 2 × SSC and 0.1% sodium lauryl sulfate at 22 °C, and then thrice in 1 × SCC and 0.1% sodium lauryl sulfate at 42 °C. The filter was wrapped in plastic without drying and exposed to preflashed Kodak XAR-5 film according to Laskey and Mills (1977). The film was preflashed to 0.1–0.15 absorbance at 540 nm above the background of unexposed, developed film. The linear range of the film was determined by a sensitometer (Model 331) from Xrite (Grandville, MI). Absorbances were quantitated from the autoradiograph by a laser densitometer (Ultrascan XL) supplied by LKB (Paramus, NJ) and were within the linear range of the film.

In order to correct the measurement of PCNA/cyclin mRNA for variations attributed only to an increase in cell volume during exponential growth, the quantitation of the mRNA for a structural protein that is not cell cycle regulated was also required. Actin was chosen as this internal control because the mRNA (Thompson et al., 1985) and protein

Table I: Evaluation of Synchronization of Elutriated Cell Subpopulations

cell subpopulations[a]	% of the subpopulation in each phase		
	G-1	S	G-2/M
(I) asynchronous, exponential	43 ± 4	37 ± 5	20 ± 4
(II) G-1	94 ± 4	2 ± 2	4 ± 2
(III) S	11 ± 3	73 ± 3	16 ± 7
(IV) G-2/M	8 ± 0.5	39 ± 2	53 ± 2

[a] Exponential CHO cells (I) were elutriated into subpopulations (II–IV) as described by Keng et al. (1981). A sample of each subpopulation was stained with chromomycin A3 for the quantitation of each DNA content by flow cytometry. The percentages of each subpopulation that contained G-1, S, and G-2/M DNA content were analyzed by the CCYCLE program in a Terak 8600 microcomputer. The results given are the percentages of subpopulations from three elutriations. The standard deviations are given.

(Riddle & Pardee, 1980) are present throughout the cell cycle and change essentially in proportion to the cell volume. When samples in the Northern hybridization analysis are equalized with respect to total RNA, which also increases in proportion to cell volume, then the actin mRNA content should be constant in the G-1, S, and G-2/M populations. The measurement of actin mRNA was also a control for any unequal amounts of RNA that were transferred to the nitrocellulose membrane for Northern hybridization analysis. The procedure for Northern analysis was repeated, except that cDNA for actin (Fryberg et al., 1980) was added instead of the cDNA for PCNA/cyclin.

Results

Northern Hybridization Analysis. To determine whether PCNA/cyclin mRNA is regulated during the cell cycle, a Northern hybridization analysis was performed comparing the PCNA/cyclin mRNA content from an exponentially growing CHO cell population versus cells in different phases of the cell cycle isolated from the same exponential population.

Initially, a single population of cells undergoing asynchronous, exponential growth was harvested. A portion of this population (1.0×10^7 cells) was retained as the control sample, and the remaining portion (1.0×10^8 cells) was fractioned by centrifugal elutriation (Keng et al., 1981). The G-1, S, and G-2/M subpopulations were separated from an exponential population, so that mRNA levels could be measured at all cell cycle phases. The total RNA was isolated from each of these subpopulations. Samples of total RNA from three separate centrifugal elutriations were combined in order to have sufficient quantities of RNA from the S and G-2/M populations for Northern hybridization analysis. After a single elutriation we obtained approximately 1×10^7 G-1 cells, 6×10^6 S cells, and 3×10^6 G-2/M cells. The degree of synchrony of these subpopulations combined from three separate elutriations is shown in Table I.

For Northern analysis the concentration of total RNA was estimated by 260-nm absorbance, and approximately equal amounts were separated by formaldehyde–agarose gel electrophoresis. The ribosomal RNAs were visible in the formaldehyde–agarose gels after staining with acridine orange (Figure 1, panel A). The ribosomal RNAs appear intact after separation in each of the samples used for analysis. The total RNA was transferred to a nitrocellulose membrane from these gels. The nitrocellulose membrane was then probed sequentially with cDNA representing PCNA/cyclin (Figure 1, panel B) and actin (Figure 1, panel C).

A single species of PCNA/cyclin mRNA, approximately 1600 nucleotides in length, was found in the RNA samples

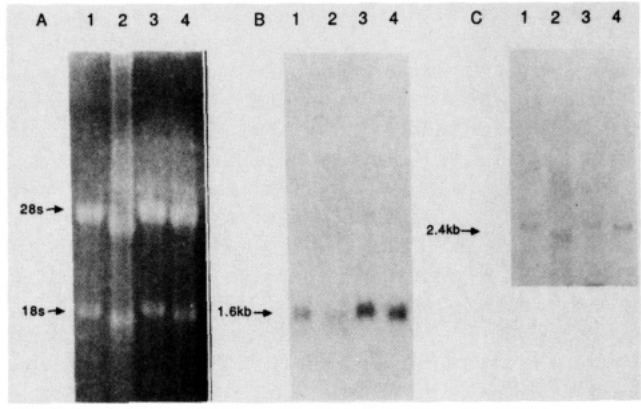

FIGURE 1: PCNA/cyclin and actin mRNA content throughout the cell cycle of cells separated by centrifugal elutriation. Total RNAs (approximately 20 μg) from an asynchronous population (lane 1) and from subpopulations representing G-1 phase (lane 2), S phase (lane 3), and G-2/M phase (lane 4) of cell cycle were separated and blotted onto nitrocellulose. Panel A shows a 1.3% agarose–formaldehyde gel that was stained with acridine orange, such that the ribosomal RNAs are visible. The autoradiograms of the Northern blots that were probed sequentially with PCNA/cyclin cDNA (panel B) and actin cDNA (panel C) are depicted.

from both the exponential population and subpopulations isolated by centrifugal elutriation (Figure 1, panel B). A single species of RNA for actin, approximately 2400 nucleotides in length, was also observed in each sample (Figure 1, panel C). This figure also shows a direct comparison of the levels of PCNA/cyclin and actin mRNA throughout the cell cycle. The PCNA/cyclin mRNA from G-1, S, and G-2/M cells was quantitated by densitometry from the autoradiograph of the Northern hybridization analysis (Figure 1, panel B) as the ratio of each signal relative to the G-1 signal. The ratio of signals from S- versus G-1-phase cell mRNA is 1.88, G-2/M- versus G-1-phase mRNA is 1.55, and asynchronous versus G-1-phase mRNA is 1.32. The ratio of signals from an autoradiograph of mRNA of actin mRNA was determined in a similar way (Figure 1, panel C); the specific signal from the actin mRNA in S- versus G-1-phase cell mRNA is 0.75, G-2/M versus G-1 phase is 0.76, and asynchronous versus G-1phase is 0.83. The variations in steady-state level of mRNA of PCNA/cyclin can, in part, be attributed to an unequal loading of the RNA in the gel, and these variations can be corrected by standardization with the actin results.

A further correction has been made to compensate algebraically for the known percentages of cross contamination of the purified cell populations with cells in other phases of the cell cycle, using the contamination values given in Table I. The final ratio of PCNA/cyclin mRNA in S-phase mRNA versus G-1-phase mRNA is 3.1 and in G-2/M-phase mRNA versus G-1-phase mRNA is 1.6. Therefore, the levels of PCNA/cyclin are substantially higher in S phase than in other cell cycle phases and appear to decline through G-2/M phase into G-1 phase.

Quantitation of PCNA/Cyclin by Flow Cytometry. The PCNA/cyclin protein and DNA content of nonsynchronized, exponential CHO cells is given in Figure 2, panel A. The distribution of staining in this CHO cell population is represented on a three-dimensional plot of flow cytometry data. The fluorescein isothiocyanate (FITC) fluorescence results from the specific reaction between the goat antiserum, conjugated to FITC, and the murine monoclonal antibodies to PCNA/cyclin, 19F4 (Ogata et al., 1987). The fluorescence intensity from PI is indicative of the DNA content of each cell. In Figure 2, panel B, the nonspecific binding of the goat an-

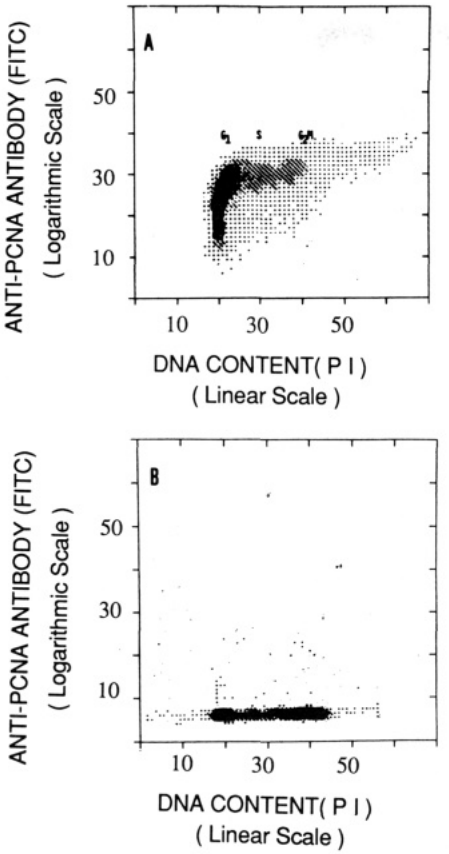

FIGURE 2: A representative three-dimensional scatter dot plot obtained from exponentially growing CHO cells stained with anti-PCNA/cyclin antibody and propidium iodide. CHO cells were incubated with monoclonal antibody to PCNA/cyclin 19F4 (Ogata et al., 1987), followed by second-step staining with a goat anti-mouse antibody conjugated to FITC. The cells were then counterstained with propidium iodide (PI) for DNA content. The fluorescence from the FITC and PI was quantified by flow cytometry (panel A). The procedure was repeated except that the anti-PCNA antibody was removed to determine the nonspecific binding of the goat anti-mouse serum, conjugated to FITC (panel B). The x and y axes represent channel numbers on the flow cytometer. The y axis values are graphed on a logarithmic scale but can be converted to linear values by the formula: (linear y value) = $10^{0.0469y}$, where y is the value shown on the graph.

tiserum, conjugated to FITC, was determined by the removal of only the antibody to PCNA/cyclin, 19F4, from the procedure.

The data represented in Figure 2 were analyzed by the CYTOLOGO program (Coulter Electronics, Haileah, FL) with an IBM XT microcomputer. Analysis of the PI staining was used to determine the position on the plot of cells in the G-1, S, and G-2/M phases of the cell cycle (as indicated). The fluorescence intensity representing the cellular PCNA/cyclin protein content was calculated as the difference between the specific PCNA/cyclin staining of cells in each phase of the cell cycle (Figure 2, panel A) and the nonspecific binding of goat antiserum in the control samples for each phase of the cell cycle (Figure 2, panel B). A summary of the FITC fluorescence intensities representing the average PCNA/cyclin protein content in the G-1, S, and G-2/M cell subpopulations is given in Table II.

In general, the data represented in Figure 2 show that PCNA/cyclin protein levels vary in cells in G-1 and early S phase but then are raised to maximum and stable steady-state concentration throughout middle to late S phase and G-2/M phase. If the total PCNA/cyclin protein as determined by indirect immunofluorescence is integrated on a linear scale, then the average PCNA/cyclin level is approximately 2.5-fold higher in S phase compared to G-1 phase as summarized in Table II.

Table II: Quantitation of the PCNA/Cyclin Content in Asynchronous Exponential CHO Cell Subpopulations during the Cell Cycle[a]

cell subpopulations[b]	net fluorescence intensity of PCNA/cyclin staining[c]
G-1	11[d]
S	27
G-2/M	26

[a] All of the data reported in this table were analyzed by the CYTOLOGO program from Coulter Electronics (Haileach, FL) with an IBM XT microcomputer. [b] The cell subpopulations representing G-1, S, and G-2/M phases of the cell cycle were defined from the propidium iodide staining of the cell populations depicted in Figure 2. [c] The net fluorescence intensity representing the PCNA/cyclin content is obtained by subtracting the immunofluorescence intensity of each cell subpopulation (Figure 2, panel A) from the control samples (Figure 2, panel B). [d] The average intensity (mean channel number) of the net PCNA/cyclin immunofluorescence intensity for each subpopulation is given.

A logical assumption is that, in individual cells, the PCNA/cyclin protein level rises with time during G-1 phase and then reaches a stable maximum for the later phases. If this were the case, the total change in PCNA/cyclin protein occurring throughout G-1 phase would be greater than 2.5-fold.

Inhibition of Entry into S Phase by an Antisense Oligodeoxynucleotide to PCNA/Cyclin. Early G-1 (G_0/G-1) CHO cells were separated from plateau cell culture by centrifugal elutriation and cultured in the presence of 30 μM antisense oligodeoxynucleotide to PCNA/cyclin mRNA. At 0 and 12 h after oligomer treatment, a portion of the cell population was harvested. At these times, the PCNA/cyclin protein and DNA content were simultaneously analyzed by flow cytometry (Figure 3). Panels A and B of Figure 3 represent the analysis of the normal progression of early G-1 cells through the cell cycle with respect to PCNA/cyclin and DNA content at the indicated times after inoculation. Both PCNA/cyclin and DNA content increased as the cells progressed through the cell cycle. Panels C and D of Figure 3 show a sample of the same early G-1 cell subpopulation incubated with 30 μM antisense oligomer to PCNA/cyclin mRNA. In the cell subpopulation incubated with the antisense oligomer, neither PCNA/cyclin nor DNA content significantly increased with time. Panels E and F of Figure 3 show the samples of the same subpopulation incubated with 30 μM sense oligomer complementary to the antisense oligomer and having a similar nucleotide composition. Progression through the cell cycle essentially identical with that depicted in panels A and B was observed, demonstrating no detectable effect of the sense oligomer.

The data in Figure 3 indicate that treatment with the antisense oligomer impedes the progression of early G-1 cells to S phase in the cell cycle and blocks the synthesis of PCNA/cyclin protein as determined by indirect immunofluorescence. This result implies that PCNA/cyclin is a necessary component in the pathway leading to replicative DNA synthesis.

DISCUSSION

PCNA/cyclin is a protein regulated during the cell cycle that may control DNA replication [reviewed by Celis et al. (1987)]. Our study is an attempt to quantitate its temporal expression during exponential cell growth. Centrifugal elutriation was used to separate CHO cells into subpopulations,

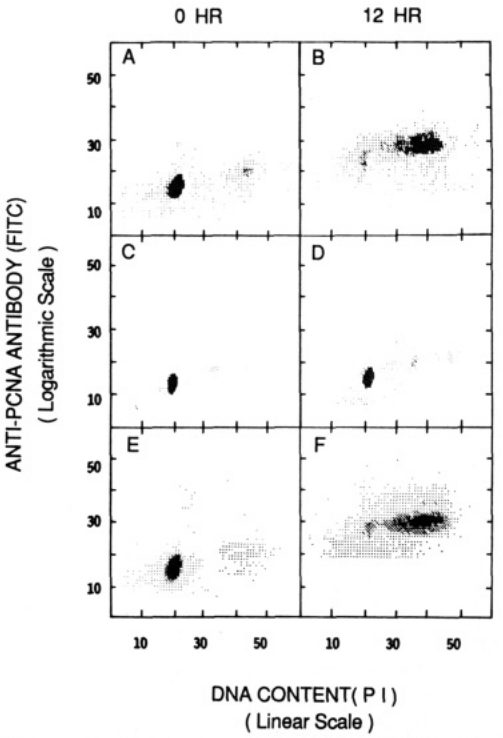

FIGURE 3: An antisense oligodeoxynucleotide to PCNA/cyclin mRNA inhibits early G-1 CHO cells (G_0/G-1) from entering S phase of the cell cycle. Early G-1 cells were isolated from plateau cultures by centrifugal elutriation and allowed to progress through the cell cycle. Flow cytometric analysis was used to quantitate simultaneously the PCNA/cyclin and DNA content in the CHO cell populations after the initiation of the cell cycle progression. Panels A and B represent the flow cytometric analysis of cells without exposure to the antisense oligodeoxynucleotide to PCNA/cyclin mRNA, panels C and D represent cells incubated with 30 μM antisense oligodeoxynucleotide, and panels E and F represent cells incubated with 30 μM complementary sense oligonucleotide. The cell population samples were analyzed at 0 and 12 h, shown in panels A and B, C and D, and E and F, respectively. The x and y axes represent channel numbers. The logarithmic y values can be converted to linear values as described in the legend to Figure 2.

representing G-1, S, and G-2/M phases of the cell cycle. The regulation of PCNA/cyclin mRNA and protein expression with respect to the initiation of DNA synthesis in the S phase was determined. In addition, early G-1 (G_0/G-1) CHO cells were treated with an antisense oligomer to block PCNA/cyclin protein synthesis, and the effects of this treatment were analyzed.

To study the expression of PCNA/cyclin, a cDNA-containing PCNA/cyclin-specific mRNA sequence was isolated. The analysis of the restriction endonuclease digestion products of the PCNA/cyclin cDNA indicates that we have isolated a nearly full-length cDNA clone, 1300 nucleotides in length. It is similar to the clone (1195 base pairs long) isolated by Matsumoto et al. (1987), except that ours is approximately 100 nucleotides longer at the 5′-end. Partial sequence analysis confirmed the identity of this clone. Isolation of this rat tumor cDNA clone of PCNA/cyclin has allowed us to quantitate the steady-state mRNA expression throughout the cell cycle.

In Northern analysis, the cDNA of PCNA/cyclin hybridized to a single species of mRNA from CHO cells, approximately 1600 nucleotides in length (Figure 1). The sizes of human and mouse PCNA/cyclin mRNAs have been reported to be approximately 1300 nucleotides in length (Almendral et al., 1987). Matsumoto et al. (1987) had identified two mRNA species for PCNA/cyclin of approximate length of 1100 and 980 nucleotides in human peripheral lymphocytes. The variations in the estimated lengths of mRNA may result from species differences or from the variations in electrophoresis conditions during the separation of total RNA.

The Northern hybridization analysis was also used to measure the levels of PCNA/cyclin mRNA throughout the cell cycle (Figure 1). The G-1, S, and G-2/M subpopulations were separated from an asynchronous, exponential CHO cell population by centrifugal elutriation (Keng et al., 1981), and the degree of homogeneity of each subpopulation was determined by flow cytometry (Table I). RNA was isolated from these separated subpopulations, and the level of PCNA/cyclin mRNA relative to the level of actin mRNA was quantitated. Since the actin mRNA level is nearly constant throughout the cell cycle (Thompson et al., 1985), any relative change of the PCNA/cyclin mRNA would indicate a cell cycle specific regulation of PCNA/cyclin. Results demonstrate that PCNA/cyclin mRNA levels are about 3-fold higher in S phase than in G-1 phase and about 1.6-fold higher in G-2/M phase than in G-1 phase. They suggest that the majority of synthesis of PCNA/cyclin mRNA occurs between the beginning of G-1 phase and the end of S phase. They are consistent with a preferential regulation of PCNA/cyclin transcription so that the translated protein may be used in S-phase DNA replication.

Previous studies demonstrated that the expression of PCNA/cyclin mRNA increases as quiescence cells are stimulated by serum (Almendral et al., 1987) or by mitogens (Matsumoto et al., 1987). These results have been interpreted as evidence of a coordinate regulation of PCNA/cyclin and DNA synthesis. Our measurements of cell cycle regulation of PCNA/cyclin transcription even more strongly support this interpretation.

The level of PCNA/cyclin protein in asynchronous, exponential cells was assessed by indirect immunofluorescence and quantitated by flow cytometry. Results showed that the PCNA/cyclin protein content of G-1- and early S-phase cells varies 3–4-fold from cell to cell, but essentially all cells that have clearly entered S phase have a PCNA/cyclin content similar to that in the G-1 cells having the maximum levels of PCNA/cyclin. This high level is unchanged in middle to late S-phase cells and G-2/M-phase cells. The observation is consistent with the assumption that PCNA/cyclin is steadily synthesized during G-1 phase, reaches a critical level necessary for a role in S phase, and then is held at that level into G-2/M phase.

In contrast to these immunofluorescence studies, Kurki et al. (1988) reported that PCNA/cyclin is expressed primarily in the S phase relative to both G-1 and G-2/M phases when monoclonal antibodies to PCNA/cyclin (Ogata et al., 1987) were used for quantitation. Although the monoclonal antibody 19F4 (Ogata et al., 1987) was used both by Kurki et al. (1988) and for our work, differences in the methods for fixation of the cells, and cell line characteristics, could account for the difference in results.

An additional consideration is that quantitation by indirect immunofluorescence could depend on modification of the PCNA/cyclin protein. Since no cell cycle dependent modifications of PCNA/cyclin have been detected (Bravo & Celis, 1985; Sadaie & Mathews, 1986), the most likely possibility is modified immunogenicity resulting from association with other cellular components. The possibility of such interactions is of interest because of a potential regulatory role.

To determine whether PCNA/cyclin synthesis is required for the progression into S phase, early G-1 cells (G_0/G-1) were treated with an antisense oligodeoxynucleotide to PCNA/

cyclin mRNA. The PCNA/cyclin and DNA content were simultaneously quantitated in each cell. The antisense oligomer inhibited both PCNA/cyclin protein synthesis, as determined by indirect immunofluorescence, and DNA synthesis (Figure 3, panels C and D). The flow cytometric analysis revealed essentially no progression into S phase. Neither partial entry nor entry of a significant subpopulation of cells was observed, even though a portion of the cells still displayed a significant PCNA/cyclin content. This suggests that the cells will not even attempt entry into S phase unless PCNA/cyclin is at a critical level. Normal PCNA/cyclin and DNA synthesis occurred in control cell populations, either treated with a complementary sense oligomer or not exposed to an oligonucleotide. These results suggest that PCNA/cyclin expression at a critical level is a necessary prerequisite for DNA replication.

Recently, exponentially growing Balb/c 3T3 cells were exposed to either sense or antisense strand oligodeoxynucleotides to PCNA/cyclin mRNA. The antisense oligomer suppressed labeled thymidine incorporation into DNA, whereas corresponding sense oligomer had no inhibitory effects (Jaskulski et al., 1988). The amount of PCNA/cyclin was also quantitated in the cells exposed to sense or antisense oligomers. From indirect immunofluorescence studies, there was a significant decrease of PCNA/cyclin in the nuclei of cells treated with antisense oligomers. These results also suggest that the initiation of replicative DNA synthesis requires a critical concentration of PCNA/cyclin.

The study of the regulation of proteins putatively involved in DNA replication is important because fusion experiments between mammalian cells in different phases of the cell cycle have shown that inducers of DNA synthesis are concentrated during the S phase (Brown et al., 1985). This evidence implies that proteins that control the progression into S phase of the cell cycle are expressed in a temporal manner preceding DNA synthesis. This regulation may be controlled at the level of transcription. As suggested by Thompson et al. (1985), there may be two broad classes of mRNA expression related to the control of the cell cycle progression. One class is mRNA for proteins that are expressed irrespective of the cell cycle but required for proliferation competency. The second class is expressed at a specific phase in the cell cycle, and this expression has regulatory significance. Some oncogenes may function as regulatory proteins required for the competency of a cellular population to proliferate (Bishop, 1983). The mRNAs of nuclear protooncogenes c-*myc* (Thompson et al., 1985), c-*fos* (Muller et al., 1985), and c-*myb* (Thompson et al., 1986) increase transiently as cell populations are shifted from quiescent to exponential growth. Unlike c-*myb*, neither c-*myc* nor c-*fos* is regulated at the G-1/S transition during exponential growth, making them examples of the first class of mRNA expression related to cellular proliferation. The mRNA of histone 2b and thymidine kinase increases specifically during the S phase of the cell cycle in exponential growth (Groudine et al., 1984; Thompson et al., 1985). Histone 2b and thymidine kinase are examples of the second class of proteins, which are regulated at the transcriptional level during a specific phase of the cell cycle and are involved in DNA synthesis associated metabolism. Yet, the biochemical functions of these proteins suggest that their regulation is a consequence of S phase and that they are unlikely to function in an initiation capacity. The presence of some PCNA/cyclin transcript and protein in all cell cycle phases suggests a requirement for proliferation competency. However, the apparent 2–3-fold change of both mRNA and protein within each cell cycle is consistent with an additional role in regulation of the period of chromosomal DNA synthesis.

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Domain- and Sequence-Specific Phosphorylation of Vimentin Induces Disassembly of the Filament Structure†

Shoji Ando,‡ Kazushi Tanabe,‡ Yasunori Gonda,§ Chikako Sato,§ and Masaki Inagaki*,§

Biophysics Unit and Laboratory of Experimental Radiology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan

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ABSTRACT: We reported that stoichiometric phosphorylation by either cAMP-dependent protein kinase or protein kinase C induces disassembly of vimentin filaments [Inagaki, M., Nishi, Y., Nishizawa, K., Matsuyama, M., & Sato, C. (1987) *Nature 328*, 649-652; Inagaki, M., Gonda, Y., Matsuyama, M., Nishizawa, K., Nishi, Y., & Sato, C. (1988) *J. Biol. Chem. 263*, 5970-5978]. In the present work, we attempted to identify the sites of vimentin phosphorylated by each protein kinase. Sequential analysis of the purified phosphopeptides, together with the known primary sequence, revealed that Ser-8, Ser-9, Ser-20, Ser-25, Ser-33, and Ser-41 were specifically phosphorylated by protein kinase C, whereas Ser-46 was phosphorylated preferentially by cAMP-dependent protein kinase. Both kinases reacted with Ser-6, Ser-24, Ser-38, Ser-50, and Ser-65. Specific phosphorylation sites for protein kinase C are mostly located close to the amino-terminal side of arginine while those for cAMP-dependent protein kinase are located close to the carboxyl-terminal side of arginine. The phosphorylation sites exclusively occur in the amino-terminal non-α-helical head domain, particularly at the β-turn region. These results provide clues to the molecular mechanisms of phosphorylation-dependent disassembly of vimentin filaments.

The cytoskeletons of most animal cells are composed of three major fiber systems that can be distinguished by electron microscopy: microfilaments (6 nm in diameter), microtubules (24 nm in diameter), and intermediate filaments (10 nm in diameter). Although intermediate filaments appear to play a significant role in maintenance of the organization of cytoplasmic space (Ishikawa et al., 1968; Lazarides, 1980), the cytoplasmic regulatory mechanisms that govern the assembly–disassembly of these structures are not well characterized. Until recently, most information on the regulatory mechanisms of filament assembly–disassembly has come from the other two classes of cytoskeletal filaments, microfilaments and microtubules, which together with batteries of associated proteins have been extensively characterized (Mohri, 1976;

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* To whom correspondence should be addressed.
‡ Biophysics Unit.
§ Laboratory of Experimental Radiology.

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