# Estimation of PCNA mRNA Stability in Cell Cycle by a Serum-Deprivation Method

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**Abstract**    A simple scheme was developed to study the mRNA stability of the proliferating cell nuclear antigen (PCNA) gene during cellular transition from the $G_1/S$ boundary to a quiescent state. By this scheme, CHO.K1 cells were grown to about 80% confluence and then serum-starved for 40 h for synchronization in a quiescent state. The quiescent cells were serum-stimulated for a period of time (between 8 h and 12 h) and then grown in serum-free medium until being harvested for further analyses. The cellular PCNA mRNA level was analyzed by Northern blotting. As compared with that in cells which were continuously incubated in serum-containing medium, the decline of the mRNA level, after reaching the peak, in these serum-deprived cells was virtually devoid of mRNA synthesis. Thus, this mRNA decay was taken for the measurement of mRNA stability. The advantage of the scheme is that, unlike the treatment of transcription inhibitors, it does not prevent the cells from completing the rest of the cell cycle before returning to the resting state, and so the mRNA stability observed is cell cycle dependent. In contrast with the previous report that the stability of PCNA mRNA in quiescent cells is less by severalfold than that in S phase cells, our study shows that the mRNA stability of PCNA remained constant during the cellular transition from $G_1/S$ boundary to quiescent state.    © 1995 Wiley-Liss, Inc.

**Key words:** PCNA, mRNA stability, cell cycle, serum-deprivation

Proliferating cell nuclear antigen (PCNA), also known as an auxiliary factor of DNA polymerase δ, is required for quiescent cells to reenter the cell cycle and cellular DNA replication [Celis et al., 1987; Tan, 1989; So and Downey, 1992; Jaskulski et al., 1988; Liu et al., 1989]. Although in actively growing cells or tissues the PCNA gene is constitutively expressed, in quiescent cells or tissues the expression of PCNA gene is low and can be induced many times after mitogen stimulation or other treatments (such as partial hepatectomy) [Morris and Mathews, 1989; Almendral et al., 1987; Matsumoto et al., 1987; Liu et al., 1993]. Previous studies on the regulation of PCNA gene expression following serum stimulation of quiescent cells have shown that posttranscriptional but not transcriptional level control plays an important role [Chang et al., 1990; Baserga, 1991]. PCNA mRNA level is

low in quiescent cells and is inadequate for estimating the mRNA stability by using the transcription inhibitors, such as actinomycin D. However, when human PCNA gene does not contain intron 4, its mRNA level in the quiescent cells becomes high and suitable for stability analysis [Chang et al., 1990]. It has been found previously [Chang et al., 1990] that in Balb/c-3T3 cells transfected with human PCNA gene without intron 4, the human PCNA mRNA has a shorter half-life in Go stage (2 h) than in S phase (8 h). However, also by the use of actinomycin D to study the mRNA stability, PCNA mRNA in human diploid fibroblasts was found to have a half-life of 12 h when cells were blocked at the $G_1/S$ interphase [Stewart and Dell,orco 1992]. Actinomycin D is known to perturb the cell cycle [Baserga, 1985]. In this report, we estimated the PCNA mRNA stability during cell phase progression by a simple protocol avoiding actinomycin D, and we found that the PCNA mRNA stability is very constant during the cell phase transition.

## MATERIALS AND METHODS
### Cell Culture

The Chinese hamster ovary cell line (CHO-K1) was obtained from the American Type Cul-

ture Collection (Rockville, MD). Cells were maintained as monolayer cultures at 37°C in a humidified atmosphere of 5% $CO_2$ in air and were grown in McCoy's 5A medium supplemented with 2.2% sodium bicarbonate (pH 7.5), 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 10% FCS. For exponential cultures, cells were removed from the flasks for transfer by treatment with 0.25% (wt/vol) trypsin in phosphate buffered saline containing 0.25 mM EDTA. The cells were plated at a density of $10^5$ cells per 75 cm² tissue culture flask and were harvested for analysis at the third day after plating. To obtain synchronous populations of cells, CHO-K1 cells were plated at $5 \times 10^5$ cells per 100 mm (diameter) tissue culture dish and were grown in complete medium to reach 80% confluence. Then the medium was removed, and the cells were washed with 1× PBS and incubated in serum-free medium for 40 h. After 40 h of serum starvation, cells were considered to be arrested at $G_0$ phase. For serum stimulation, the medium of quiescent cells was changed at time 0 to fresh McCoy's 5A medium containing 10% FCS. When cells were treated with actinomycin D to block transcription, 5 μg/ml actinomycin D (Sigma, St. Louis, MO) was used.

## Thymidine Incorporation

Thymidine incorporation was performed virtually as described in Freshney [1983]. Briefly, cells were grown in 12-well culture dishes. For each well, there were about $2 \times 10^5$ cells. When cells reached 80% confluence, cells were cultured in serum-free medium for 40 h for synchronization. Cells were stimulated by 10% FCS. Labeling of the cells was performed by incubating cells at different periods after stimulation for 1 h with 1 μCi/ml [Met-$^3$H]thymidine (50 Ci/mmole). Cells were extracted with 200 μl of 0.1 N NaOH followed by TCA precipitation in the cold. The precipitate was filtered through Whatman GF/A filters, which were washed with 70% ethanol, and 10% TCA. Radioactivity on the filter was measured using a liquid scintillation counter.

## RNA Extractions and Northern Blotting

Total RNA was prepared from cultured cells by the method described in Chomczynski and Sacchi [1987]. The concentration of RNA was determined by absorbance at 260 nm by spectrophotometer. Equal amounts of RNA (usually 10–20 μg) were used for each gel electrophoresis

sample. RNA samples were separated in 1% agarose gels. The gels contained 18% formaldehyde, 10× MOPS. Details of hybridization and washing steps in Northern blotting analysis were conducted as described in Liu and Bambara [1989]. Radioactive probes were prepared by the random priming method as described in Feinberg and Volgelstein [1984]. The rat PCNA cDNA probe was obtained as described in Liu et al. [1989]; the histone H3.2 cDNA probe was provided by Dr. W. Marzluff (Chapel Hill Laboratory, Chapel Hill, NC). For autoradiography, the filter was air-dried and exposed to X-ray film at −70°C or phosphorscreens. Quantitation of the autoradiography signals was done by a computing densitometer or by a phosphorimager (Molecular Dynamics Co., Sunnyvale, CA).

## RESULTS

### Growth Regulation of PCNA Gene Expression

Expression of the PCNA gene in Chinese hamster ovary cells CHO.K1 is cell cycle dependent, as shown in Figure 1. Cells in late log phase were serum-starved for 40 h to become quiescent and then serum-stimulated again to enter the cell cycle. Expression of PCNA mRNA reached the maximum at the $G_1/S$ transition, as indicated by the timing of maximal expression of histone 3 gene and $^3$H-thymidine incorporation.

### Apparent Half-Life of PCNA mRNA

CHO.K1 cells in late log phase (nearly 80% confluence) were deprived of serum. While the cells were entering quiescence, the level of PCNA mRNA decayed, as shown in Figure 2. The apparent half-life of PCNA mRNA was about 8 h. Since this experiment started with a nonsynchronous population, the decay of PCNA mRNA level seen in Figure 2 merely reflected that the total synthesis rate is less than the degradation rate (i.e., it was not a pure measurement of mRNA stability). To obtain a mRNA decay curve without the involvement of RNA synthesis, the usual method is to block the transcription by inhibitors such as actinomycin D. The change of PCNA mRNA level in the exponential cells after serum deprivation and treatment with actinomycin D is shown in Figure 3, giving an average half-life of less than 4 h for PCNA mRNA in cells of various phases. Similar results were seen in a duplicated analysis.
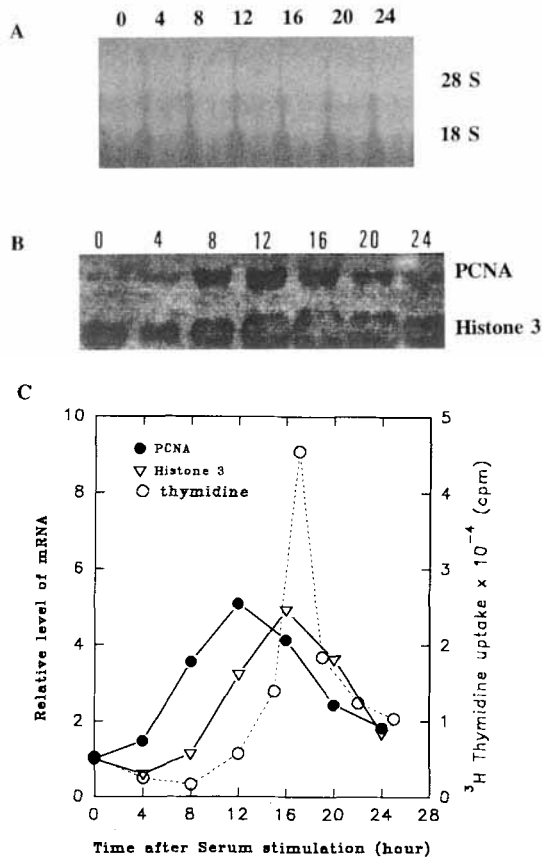
Fig. 1. Expression of PCNA mRNA in quiescent CHO.K1 cells after serum stimulation. Cells were plated in 100 mm culture dishes at 5 × 10⁵ cells per dish in medium with 10% FCS. When the cells reached 80% confluence, they were washed once with 1 × PBS and then grown in 10 ml of medium deprived of serum. After serum starvation of 40 h, the cells became arrested and were considered to be a quiescent culture. The quiescent cells were restimulated with 10% FCS at time zero. A: The ethidium bromide staining pattern of RNA samples after electrophoresis to indicate the same amount of RNA loaded into each lane. The number on top of each lane is the time after serum stimulation (in hours). B: Images of Northern blot by exposure to phosphorscreens. The number on top of each lane is the time after serum stimulation (in hours). C: Relative levels of mRNA were determined by phosphorimager scanning and plotted against the duration of the cell culture. The histone mRNA expression curve and ³H-thymidine incorporation curve were used to mark the timing of S phase.

Fig. 2. Decay of PCNA mRNA in late log cells after serum starvation. Cells were plated in 100 mm culture dishes at a density of 5 × 10⁵ cells per dish in medium with 10% FCS. When the cells reached 80% confluence, they were grown in medium without serum. At the times indicated, cells were harvested and the amount of mRNA determined by Northern blotting. Typical results of the repeated experiments are shown. A: The ethidium bromide staining pattern of RNA samples after electrophoresis to indicate the same amount of RNA loaded into each lane. The number on top of each lane is the time after serum starvation (in hours). B: Images of Northern blot by exposure to phosphorscreens. The number on top of each lane is the time after serum starvation (in hours). C: Relative levels of mRNA were determined by phosphorimager scanning and plotted against the duration of the cell culture.

## Estimation of PCNA mRNA Half-Life in the Cell Cycle by a Serum-Deprivation Scheme

Cells were synchronized to quiescent state and serum-stimulated to reenter the cell cycle. At a certain period after stimulation, the cells were serum-deprived again and were continuously incubated in the serum-free medium for the rest of the time until harvest for analysis. The cells, although in serum-deprived medium,
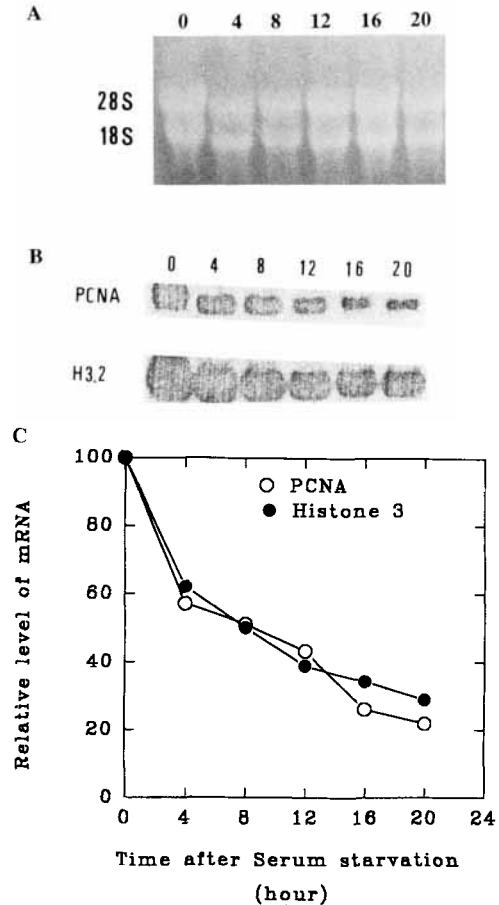
were able to progress through the S phase, as indicated by the comparable ³H-thymidine uptake seen in the control cells (see Fig. 1 for the control cells and Fig. 4 for the serum-deprived cells). The timing of the last serum deprivation appeared to be essential, because if the serum deprivation was performed at 4 h or earlier after serum stimulation, the major thymidine incorporation was not seen. This finding seemed to be consistent with the model of restriction (R) point [Pardee, 1974]. The expression of the PCNA
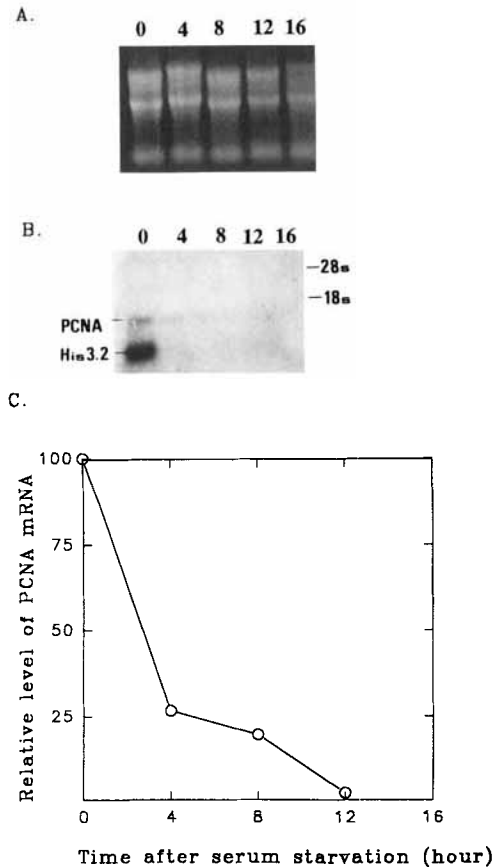
A.

0  4  8  12 16

B.

0  4  8  12 16

—28₅
—18₅

PCNA

Hₐ3.2

C.

Relative level of PCNA mRNA

Time after serum starvation (hour)

**Fig. 3.** Effect of actinomycin D on the decay of PCNA mRNA in late log cells after serum starvation. The cells were grown and processed as described in Figure 2, except that during serum starvation the cells were also treated with 5 μg/ml actinomycin D (Sigma). Typical results of the repeated experiments are shown. A–C: Similar to their counterparts in Figure 2. The number on top of each lane in A and B is the time after serum starvation (in hours).

$^3$H–thymidine incorporation (cpm)

Time after serum stimulation (hour)

**Fig. 4.** $^3$H-thymidine incorporation in quiescent cells after serum stimulation for a short period of time. Cells were grown in culture dishes and made quiescent as described in Figure 1. These quiescent cells were then restimulated with serum for the indicated interval. Cells were labeled with [Met-$^3$H]thymidine, and the amount of $^3$H-thymidine incorporation was measured as described in Materials and Methods. Open circles, serum stimulation for 4 h; filled circles, serum stimulation for 8 h; open triangles, serum stimulation for 12 h. Quiescent cells after 8–12 h (but not 4 h) serum stimulation were able to progress through S phase.

gene, as might be expected from the previous studies, was induced in the serum-deprived cells which were able to enter the S phase (Fig. 5A). However, the decay of the PCNA mRNA in these cells (see Fig. 5B, curves with triangles) was much faster than in the control cells which were grown in serum containing medium (Fig. 5B, curve with open circles), suggesting that the decay of PCNA mRNA in these serum-deprived cells contains little or no mRNA synthesis. This suspicion was supported by seeing comparable decay rates in both serum-deprived cells and cells treated with actinomycin D (see Fig. 5B, curve with closed circles). Hence, the mRNA decay curve seen by the serum-deprivation scheme allows the estimation of PCNA mRNA stability. More importantly, because the scheme does not prevent cells from completing a cell
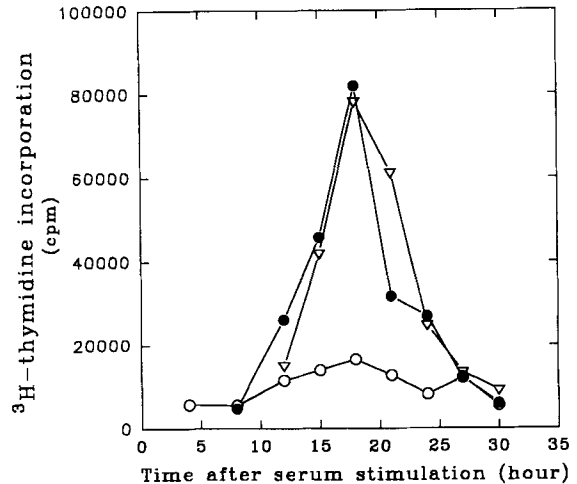
cycle before entering another resting stage, the mRNA stability observed thereby is cell cycle related. For a first order irreversible reaction A → B, the decay rate of substance A is $-d(A)/dt = k$ (A), or, in the integrated form, $\log A/A_0 = -kt/2.303$, where $A_0$ is the initial concentration and A the concentration after time t. Hence, the plot of log A vs. t is a linear curve. The PCNA mRNA decay was a first order, irreversible reaction, as suggested by the data fitted to the best lines of least squares shown in Figure 6. In other words, PCNA mRNA stability was constant during the cell cycle (precisely, from the $G_1/S$, S, $G_2/M$ until quiescency). The half-life of PCNA mRNA was calculated to be 3.45 h (by use of the slope of the best line for estimating constant $k$, and the half-life is $0.693/k$). Similar results were seen in a repeat experiment and also if serum was withdrawn at 10 h after serum stimulation (data not shown). This observation is different from the previous finding that stability of PCNA mRNA was four times lower in $G_0$ cells (half-life of 2 h) than in serum-stimulated cells (half-life of 8 h) [Chang et al., 1990].

## DISCUSSION

Gene expression of PCNA in quiescent cells is induced after serum stimulation. PCNA mRNA

A

8 12 16 20 24 28 32
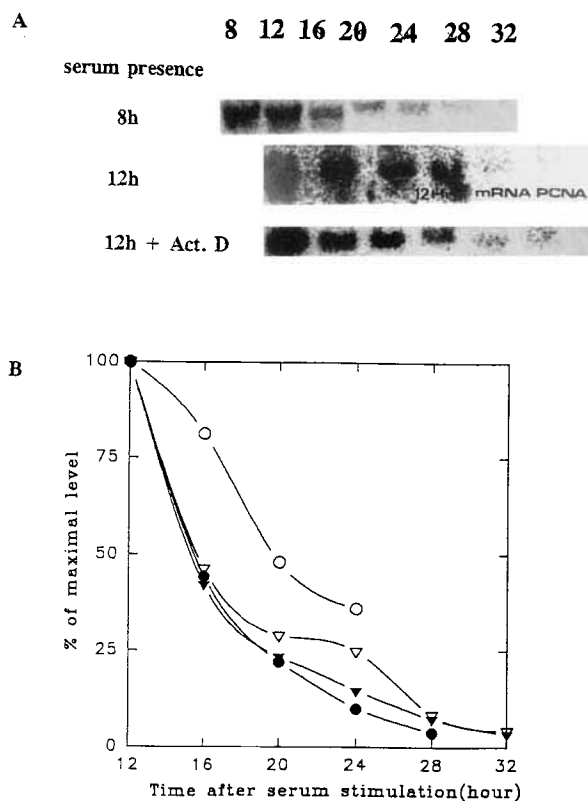
serum presence

8h

12h

12h + Act. D

B



Fig. 5. Expression of PCNA mRNA in quiescent CHO.K1 cells after serum stimulation for a short period of time. Cells were grown in culture dishes and made quiescent as described in Figure 1. These quiescent cells were then restimulated with serum for 8 h or 12 h. After 8 or 12 h serum stimulation, cells were incubated in serum-free medium until harvested for analysis. Typical results of the repeated experiments are shown. A: Northern analysis of PCNA mRNA in the cells under the indicated situations: serum presence in culture medium for 8 h and 12 h with or without actinomycin D. The number on top of each lane is the time after serum stimulation (in hours). B: Comparison of PCNA mRNA decay in the cells under the indicated situations. The mRNA level was represented as the relative level to that in 12 h cells after serum stimulation. Open circles, control cells which were continuously grown in serum-containing medium; filled circles, same as the control cells except with actinomycin D treatment at 12 h after serum stimulation; open triangles, cells with serum stimulation for only 8 h; filled triangles, cells with serum stimulation for only 12 h.



Fig. 6. Semilogarithmic plot of PCNA mRNA decay. The data shown in Figure 5B was used in this plot. The log $A/A_0$ stands for the logarithmic value of relative mRNA level in cells at time (t) after serum stimulation. Filled circles, cells with actinomycin D treatment; open triangles, cells with serum stimulation for 8 h; filled triangles, cells with serum stimulation for 12 h. The curves were the best lines fitted by the method of least squares.

stability is a potential target in regulation of the gene expression. The common protocol to estimate mRNA half-life involves the use of transcription inhibitors such as actinomycin D, which perturbs the cell cycle, giving an estimation which may not represent the stability in reality. Besides the protocol using actinomycin D, another method [Schibler et al., 1978], using pulse labeling RNA followed by hybrid selecting the specific mRNA, is also available, and the method is suitable for examining mRNA stability in the
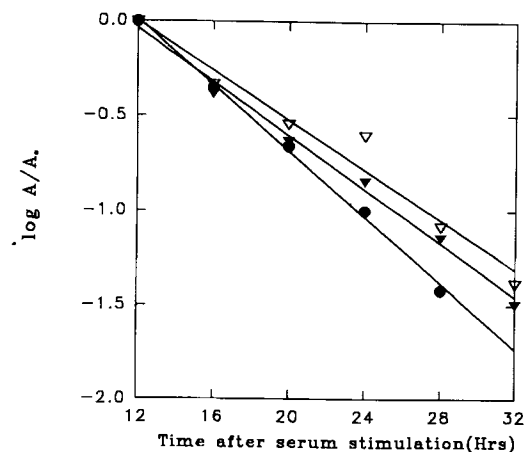
cell cycle. However, the technical difficulties and unreliability of the technique prevent it from becoming a popular procedure. Here, we report a simple scheme which allowed the estimation of mRNA stability during the cell progression after serum deprivation. The key points in the scheme are (1) that the decay of mRNA under the serum-free condition did not contain mRNA synthesis (an effect that can be produced by the transcription inhibitors) and (2) that the cellular progression continued before entering the resting state (an effect that cannot be produced using the transcription inhibitors). By this scheme, we found that the stability of PCNA mRNA was constant during the transition of cell phase from the $G_1/S$ boundary to the quiescent state. This finding seems to be different from the previous observation that stability of PCNA mRNA was low in $G_0$ cells and high in serum-stimulated cells [Chang et al., 1990]. It may be concerned with whether the cells, after 24 h serum deprivation in our experimental scheme, really reached quiescence and/or whether the cells progressed from S to $G_2/M$. As the absence of PCNA expression is widely accepted as a marker for the cells in the resting state, and since it was shown (Fig. 2) that the cells after serum deprivation could enter S phase, it is unlikely that the cells would not progress through $G_2/M$ before reaching quiescence. As mentioned in the introductory paragraphs of this article, the previous assessment of human PCNA mRNA stability in $G_0$ was

indirectly made by studying the mRNA decay of human PCNA gene without introns 4 and 5 stably transfected into mouse cells in the presence of actinomycin D. It is not unlikely that the discrepancy might result from different experimental procedures and environments. On the other hand, our estimation for the half-life of PCNA mRNA in $G_1/S$, S, and $G_2/M$ cells was about 4 h, and yet the average half-life of PCNA in an asynchronous, exponential cell population was less than 4 h (about 3 h) (see Fig. 3). Hence, the stability in early or mid-$G_1$ might be slightly lower than in S and $G_2/M$. In summary, our data did not support the previous observation that PCNA mRNA stability in quiescent cells is severalfold less than that in dividing cells, nor did the data rule out the possibility that PCNA mRNA may undergo a mild degree of stability change during early stage after serum stimulation. In contrast with the finding of the relatively constant mRNA stability during the cell phase transition, our recent study indicated that the rat PCNA gene promoter activity was significantly increased during the course of serum stimulation of quiescent cells [unpublished results], suggesting that transcriptional control is essential in growth regulation of PCNA gene expression.

Although our main interest was on PCNA mRNA stability, we have also attempted to use the scheme developed here to monitor histone 3 mRNA stability in the cell cycle. Previous study has indicated that histone mRNA stability is severalfold lower in $G_2$ than in $G_1$ and S cells [Harris et al., 1991]. We found that the semilogarithmic plot of histone mRNA decay was apparently not fitted to the best lines of least squares (data not shown), suggesting that the histone mRNA decay is not a simple first order irreversible reaction, namely, the histone mRNA stability is not constant during the transition from S to quiescence.

In conclusion, by using a simple protocol without blocking the cell progression, we estimated PCNA mRNA stability in the cell cycle and found that mRNA stability was constant during the transition of $G_1/S$ to quiescent state. The protocol seems to be potentially adaptable to study the mRNA stability of other growth-regulated genes.

## ACKNOWLEDGMENTS

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