

## Overexpression of Proliferating Cell Nuclear Antigen in Mammalian Cells Negates Growth Arrest by Serum Starvation and Cell Contact

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**Proliferating cell nuclear antigen (PCNA) functions as a processivity factor for DNA polymerase  $\delta$ , and is expressed at high levels in growing normal and tumor cells. To clarify the relationship between cell proliferation and PCNA expression, we generated NIH-3T3 cells that overexpress PCNA and analyzed the phenotype of these cells. The resulting 3T3-PCNA cells, which overexpressed PCNA, were found to proliferate beyond the saturation density of the parental NIH-3T3 cells. Although NIH-3T3 cell proliferation is arrested under serum starvation conditions, 3T3-PCNA cell proliferation is not arrested by serum starvation. The expression levels of *cdk2*, *cdk4* and *cdk6* were the same in 3T3-PCNA and NIH-3T3 cells. The activity of *cdk4* was identical for both cell types. However, the activity of *cdk2* was higher in serum-starved 3T3-PCNA cells than in NIH-3T3 cells, although the expression of cyclin E decreased in both types of cells, suggesting that increases in *cdk2* activity are related to negation of growth arrest in 3T3-PCNA cells. These results indicate that increases in PCNA expression lead to the disruption of growth control and may lead to malignant transformation.**

Key words: PCNA — Growth arrest — Contact inhibition

Proliferating cell nuclear antigen (PCNA) was identified by Miyachi and his collaborators as an autoantigen in patients with systemic lupus erythematosus,<sup>1</sup> and Bravo and Celis identified the same protein as one that is expressed during the S phase of the cell cycle.<sup>2</sup> The expression of PCNA is low in the G1 and G2 phases, but at S phase its expression is elevated and localized in the nuclei.<sup>3</sup> PCNA is expressed highly in many tumor cells and tissues, where it is also localized in the nuclei.<sup>4</sup>

PCNA, a 35-kDa protein, is essential for DNA replication, acting as a processivity factor for DNA polymerase  $\delta$ . It forms a toroidal homotrimer that is capable of interacting nonspecifically with double-stranded DNA by encircling it, thus forming a sliding clamp that tethers the polymerase to the DNA strand.<sup>5,6</sup> PCNA is highly conserved in many species and has been identified as an essential gene required for DNA replication in yeast.<sup>7,8</sup> In addition to its role in replication, PCNA is also required for nucleotide excision repair in cell-free systems<sup>9,10</sup> and plays a role in one pathway of base excision repair.<sup>11</sup>

The cell cycle is regulated by many factors, including cyclin-dependent kinase (*cdk*), and *cyclin* family genes have recently received much attention. The *cdk* forms a complex with a specific cyclin, which functions as a protein kinase for a specific protein and promotes the cell

cycle.<sup>12</sup> It has been reported that retinoblastoma gene product (RB) is one of the substrates phosphorylated by *cdk/cyclin* complexes.<sup>12</sup> The phosphorylation of RB is seen as one of the key factors in the transition of the G1(G0)/S boundary.<sup>12,13</sup> Therefore, the specific *cdk/cyclin* complexes are considered to be key regulators in the G1(G0)/S transition.

The activity of *cdk* is regulated by its binding to cyclin, but recently it has been shown that this activity is also regulated by *cdk* inhibitors, encoded by *p21* and *p16/INK4* family genes.<sup>14</sup> The *p21<sup>cip1/waf1</sup>* gene product inhibits the activity of most *cdk/cyclin* complexes by binding to them and is thought to function as a *cdk* inhibitor in the growth arrest induced by *p53*.<sup>15,16</sup> The *p16<sup>INK4A/MTS1</sup>* gene product, which was identified in cells lacking functional RB, represses the activity of *cdk4* and *cdk6* by binding to them.<sup>17,18</sup> The *p15<sup>INK4B</sup>* gene product also represses the activity of *cdk4* and *cdk6* by binding to them in the same manner as *p16*, and is thought to function in the growth arrest induced by TGF- $\beta$ .<sup>19</sup>

Increases in PCNA expression in tumor cells are regarded as a consequence of rapid cell proliferation, because PCNA is one of the components in DNA synthesis, and its expression is at a peak in the S phase of the cell cycle. However, to characterize further the function of PCNA, we generated NIH-3T3 cells that overexpress exogenous PCNA and analyzed these cells. We discovered that exogenous overexpression of PCNA transforms NIH-

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3T3 cells by causing a change in cell morphology and a negation of growth arrest by serum starvation and contact inhibition. Here, we discuss this new finding and the relationship between PCNA expression and malignant transformation.

## MATERIALS AND METHODS

**Cell culture** NIH-3T3 cells, including the transformant cells, were maintained by general methods. Growth-arrested (serum-starved) NIH-3T3 and transformant cells (G0) were prepared by treatment in a medium containing low serum (0.5% newborn cow serum [NCS; GIBCO, Rockville, MD]), and growth-stimulated cells (24 h) were prepared by treating young G0 cells in a medium containing 5% NCS for 24 h.

**Generation of 3T3-PCNA cells** cDNA encoding human PCNA was subcloned into pH $\beta$ APr-1neo<sup>20</sup> (containing  $\beta$ -actin promoter). 3T3-PCNA cells were generated by transfection of the PCNA expression vector (pH $\beta$ APr-1-PCNA) into NIH-3T3 cells by electroporation.<sup>20</sup> After transfection the cells were plated in Dulbecco's modified Eagle's medium (DMEM) containing 5% NCS and incubated at 37°C for the first 48 h. They were then selected for 2 weeks in medium with 300  $\mu$ g/ml of G418 (GIBCO). Some of the G418-resistant colonies that subsequently appeared were picked up, and their expression of PCNA was confirmed.

**Analysis of DNA synthesis** The cells, plated on 18-mm round coverslips in 12-well multiplates, were labeled with bromodeoxyuridine (BrdU) for 24 h. The cells incorporating BrdU were detected with a Cell Proliferation Kit (Amersham, Buckinghamshire, England). BrdU-positive and -negative cells were counted by microscopy.

**Analysis of cell growth** Culture dishes (60 mm) were seeded with  $5 \times 10^4$  cells, and the growth medium was changed every 2 days. Total cell numbers were determined periodically by the trypan blue method. For each time point, three dishes of cells were counted, and each dish was counted only once.

**Soft-agar colony assay** Three thousand cells were seeded onto each 60-mm culture dish in DMEM containing 5% NCS and 0.33% noble agarose (DIFCO, Detroit, MI) over a base of 0.5% agarose. Two weeks after seeding, colonies were stained with 0.05% *p*-iodonitrotetrazolium violet (Sigma, St. Louis, MO) before they were counted.

**Western blot analysis** Western blot analysis was done as previously reported.<sup>20</sup> Standardization of each sample was performed by normalizing the amount of electrophoresed protein. Anti-cdk4, anti-p21 and anti-p15 antibodies were supplied by Santa Cruz Inc., Santa Cruz, CA; anti-cyclin E and anti-cdk2 antibodies were supplied by UBI Co., Lake Placid, NY; anti-cyclin D1, anti-p27 and anti-PCNA antibodies were supplied by MBL, Nagoya.

**Northern blot analysis** Total RNA for northern blot analysis was isolated from NIH-3T3 and 3T3-PCNA cells by the use of Trizol (GIBCO), and northern blot analysis of cellular RNA was done as previously reported.<sup>20</sup> Standardization of each sample was performed by normalizing the amount of electrophoresed total RNA. Plasmids used were as follows: pT7 PCNA carrying human PCNA cDNA sequence was a gift from Dr. T. Tsurimoto, pcdk2-H carrying human cdk2 cDNA sequence was a gift from Dr. K. Oda, pCB204.1 carrying mouse cyclin D1 cDNA sequence was a gift from Dr. C. J. Sherr, pFO422 carrying human histone H1 cDNA sequence was a gift from Dr. R. Baserga, pTK11 carrying human thymidine kinase (TK) cDNA sequence was a gift from Dr. T. Ide and pHcyclinA carrying human cyclin A cDNA sequence was a gift from Dr. H. Yasuda.

**Protein kinase assay of immunoprecipitant** Antigen-antibody complexes on Protein A Sepharose beads (Phar-

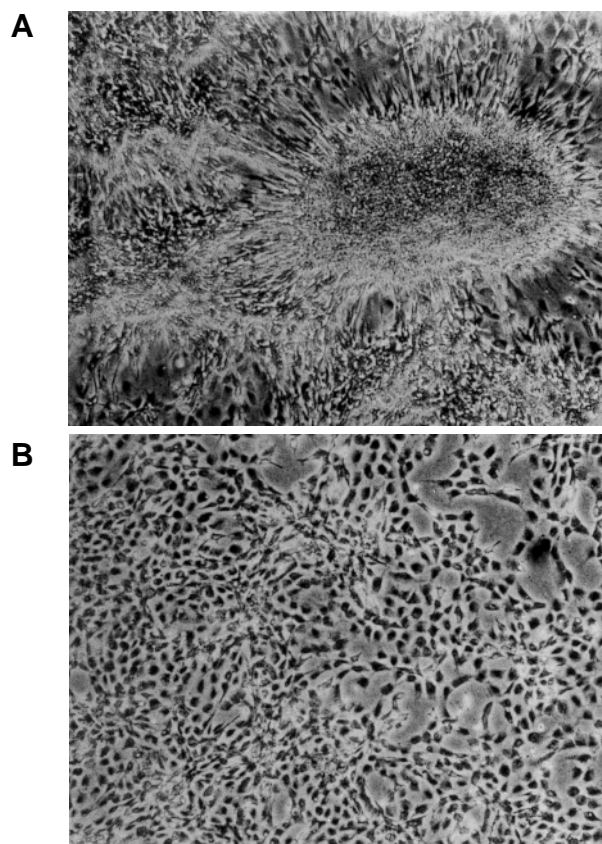


Fig. 1. The morphology of colonies formed by transfection with the PCNA expression vector. NIH-3T3 cells were transfected by the PCNA expression vector (A) or mock vector (B). Colonies observed with a phase contrast microscope after selection with G418 for 2 weeks.

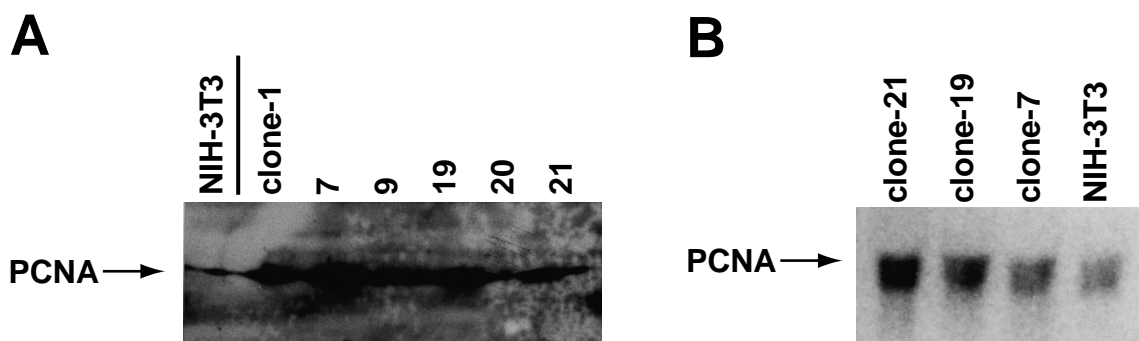


Fig. 2. Expression of PCNA in the NIH-3T3 cells transfected with the PCNA expression vector. (A) Western blot, (B) northern blot.

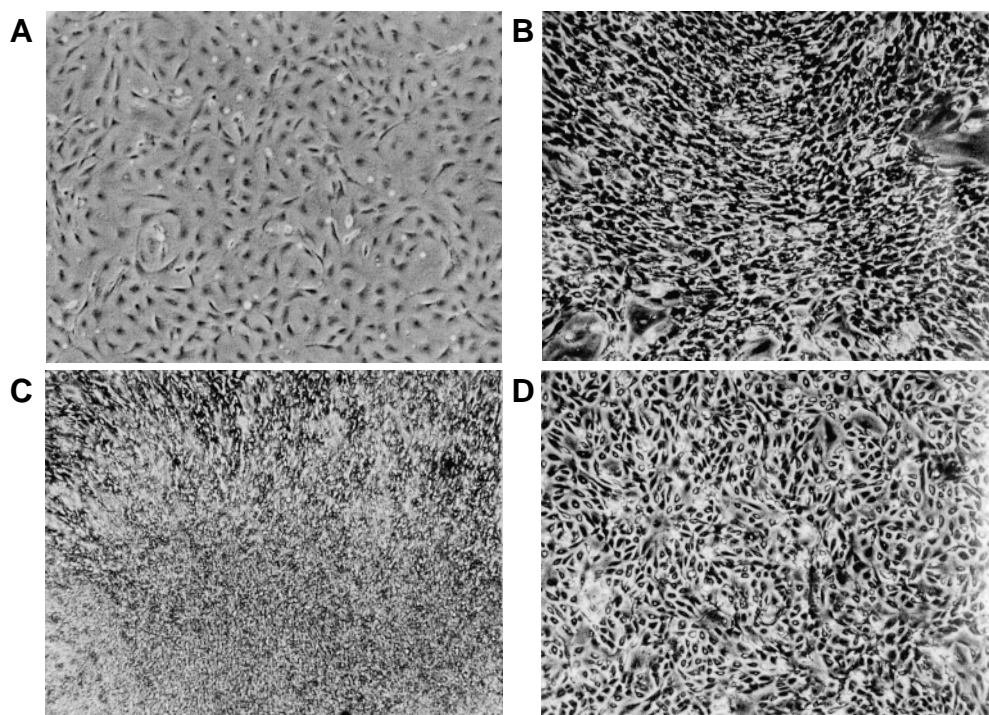


Fig. 3. Morphology of 3T3-PCNA cells. Confluent 3T3-PCNA and NIH-3T3 cells observed with a phase contrast microscope. (A) NIH-3T3, (B) 3T3-PCNA7, (C) 3T3-PCNA19, and (D) 3T3-PCNA21.

macia Biotech, Uppsala, Sweden) were prepared from the cell lysate of NIH-3T3 and 3T3-PCNA cells with anti-cyclin D1 and anti-cdk2 antibodies (Santa Cruz Inc.), as previously reported.<sup>20</sup> The complexes were suspended in 40  $\mu$ l of kinase buffer (20 mM Tris-HCl; pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM ethyleneglycol bis(2-aminoethylether)tetraacetic acid, 4.5 mM 2-mercaptoethanol), 20  $\mu$ M ATP, 0.2  $\mu$ g/ml RB (substrate; Santa Cruz Inc.) and 185 kBq of [ $\gamma$ -<sup>32</sup>P]ATP (220 TBq/nmol; Amersham). After incubation for 20 min at 30°C, the samples were added to 12.5  $\mu$ l

Laemmli's buffer, boiled at 100°C for 5 min and electrophoresed on sodium dodecyl sulfate-acrylamide gel. Phosphorylated RB was analyzed with a BAS2000 (Fuji Film Co., Tokyo).

## RESULTS

**Generation of 3T3-PCNA cells** To characterize the function of PCNA, we generated NIH-3T3 cells that over-express exogenous PCNA by transfection with a PCNA

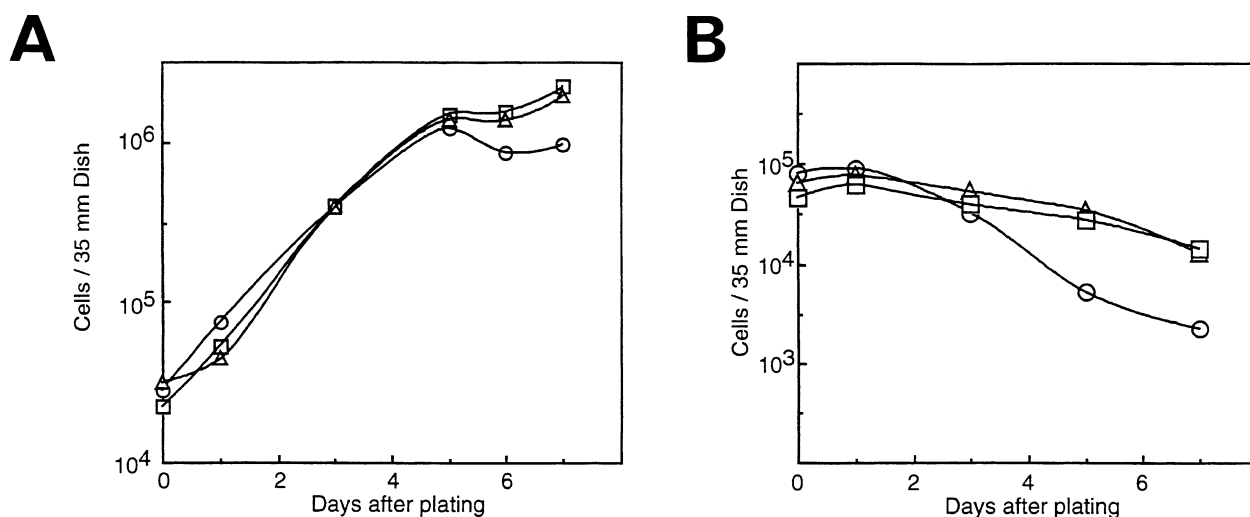


Fig. 4. Growth curves for 3T3-PCNA and NIH-3T3 cells. Cells in medium containing 5% NCS (A) or 0.5% NCS (B) were counted as described in "Materials and Methods." Open circles, NIH-3T3 cells; open triangles, PCNA-7; open squares, PCNA-19.

expression vector. Marked foci were formed by the PCNA-transfected cells, but not by the cells transfected with the mock vector (Fig. 1). After G418 selection, we isolated several colonies, and PCNA expression was confirmed by western and northern blot analyses (Fig. 2). We named the cells in which the overexpression of PCNA was confirmed 3T3-PCNA cells (clones 7, 19, and 21) and used these cells to determine the role of PCNA.

**Overexpression of PCNA transforms NIH-3T3 cells** Ordinarily, NIH-3T3 cells are deneoplastic cells, and they proliferate in a monolayer. However, the transfected NIH-3T3 cells formed foci (Fig. 1), which suggests that the overexpression of PCNA transformed these cells.

The morphology of 3T3-PCNA cells was distorted compared with that of the parental cells, and they proliferated by piling up, whereas NIH-3T3 cells proliferated in a monolayer (Fig. 3). 3T3-PCNA cells also proliferated beyond the saturated density of NIH-3T3 cells in medium containing 5% NCS (Fig. 4A). The number of 3T3-PCNA cells was maintained in medium containing 0.5% NCS (serum-starved condition), whereas the number of NIH-3T3 cells decreased 3 days after plating under these conditions (Fig. 4B). Marked detachment of 3T3-PCNA cells from the culture dish was also observed in the serum-starved environment. Despite detachment of 3T3-PCNA cells, the cell number was maintained in the serum-starved situation, which suggests that 3T3-PCNA cells are able to proliferate under this condition. NIH-3T3 cells are known to be growth-arrested (G0) by culture in medium containing 0.5% NCS for 2 days (serum starvation procedure).<sup>21</sup> Hence, their labeling index was repressed to

Table I. 3T3-PCNA Cells Overcome Growth Arrest Induced by Serum Starvation

Cells	% of labeled nuclei	
	G0	Stimulation
NIH-3T3	3.08	59.7
3T3-PCNA7	16.4	71.4
3T3-PCNA19	38.3	70.2
3T3-PCNA21	24.0	88.8

These cells were serum-starved (G0) as described in "Materials and Methods." G0: G0-arrested cells were cultured in medium containing 0.5% NCS and BrdU for 24 h. Stimulation: G0-arrested cells were cultured in medium containing 5% NCS and BrdU. Following this, cells incorporating BrdU were detected by using a Cell Proliferation Kit (Amersham).

below 5% in serum starvation (Table I). However, the labeling index of 3T3-PCNA cells was repressed less than that of NIH-3T3 cells. These results suggest that it is difficult to induce growth arrest in 3T3-PCNA cells by serum starvation. Thus, a transformed phenotype, namely a change in cell growth, was observed in 3T3-PCNA cells, even though 3T3-PCNA cells do not proliferate as well as NIH-3T3 cells in soft agar (data not shown). In 3T3-PCNA cells a remarkable detachment was observed with over-confluent proliferation in medium containing 5% NCS, as well as in 0.5% NCS. We investigated whether the viability of these detached cells by the trypan

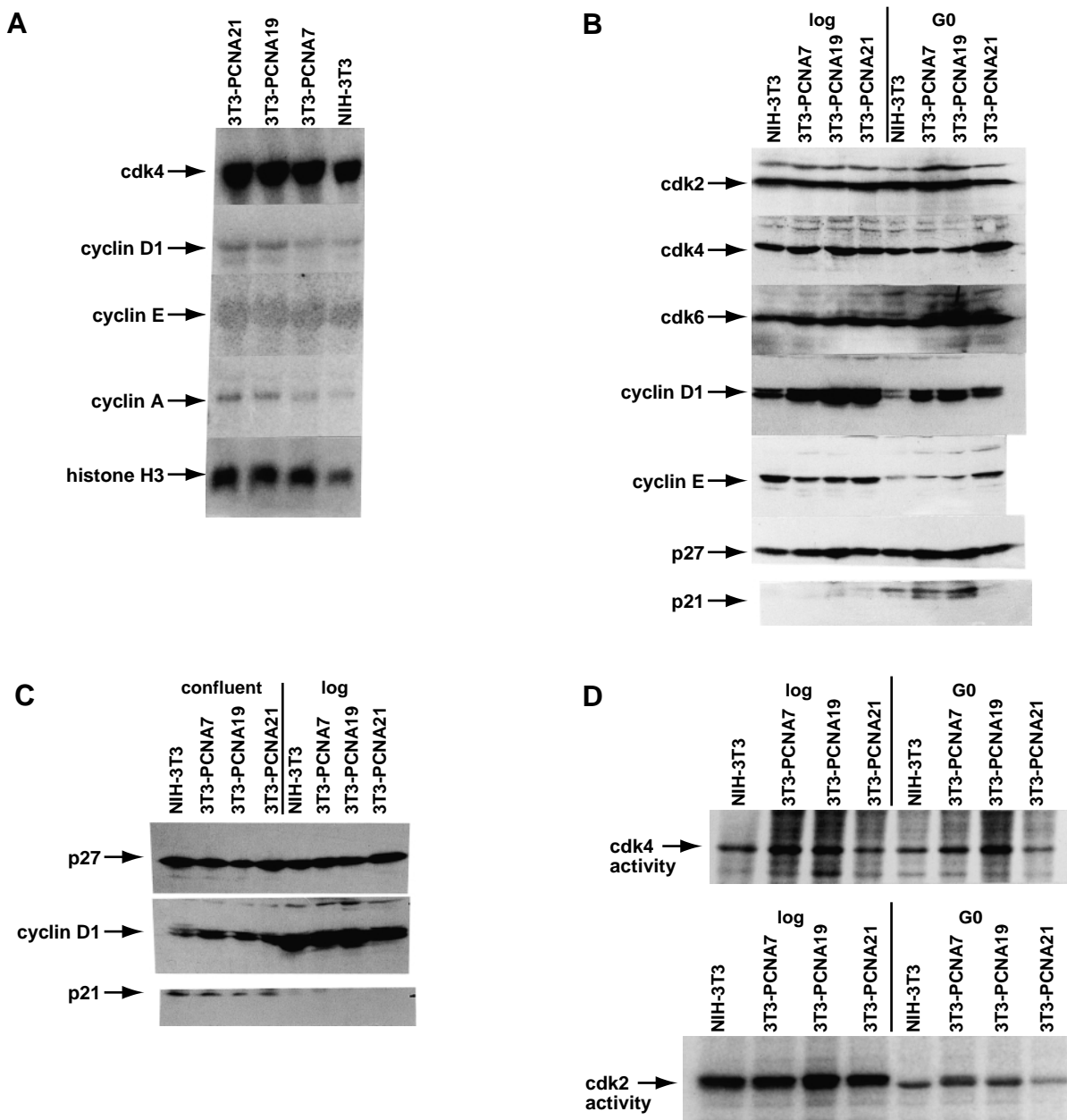


Fig. 5. Expression of cell cycle-related genes in 3T3-PCNA cells. (A) Expression of these genes at the growth phase demonstrated by northern blotting as described in "Materials and Methods." (B) Detection of the gene products in the growth (log) phase and growth-arrest (G0) phase induced by serum starvation demonstrated by western blotting as described in "Materials and Methods." (C) Expression of p27 and cyclin D1 in confluent 3T3-PCNA and NIH-3T3 cells demonstrated by western blotting as described in "Materials and Methods." (D) Activity of cdk2 and cdk4 in 3T3-PCNA cells in the growth phase (log) and growth-arrest phase (G0) investigated as described in "Materials and Methods."

blue method and by DNA ladder analysis. In the serum-starved and over-confluent situations, the ratio of viable cells was the same for attached and detached 3T3-PCNA cells, and no DNA ladder was detected in any of the cells (data

not shown). These results suggested that 3T3-PCNA cells are alive and able to proliferate in these environments.

**Activity of cdk2 in 3T3-PCNA cells in growth-arrested conditions** Our results suggest that 3T3-PCNA cells are

able to suppress growth arrest during serum starvation and contact inhibition. To clarify the mechanism of this phenomenon, we investigated the expression of cell cycle-related genes.

In 3T3-PCNA cells the expression of cyclin A and histone H3 mRNAs, which are induced at the S phase, was greater than in the parental NIH-3T3 cells (Fig. 5A). However, cyclin D1, cyclin E and cdk4 mRNAs, which are expressed in the late G1 phase, appeared in 3T3-PCNA cells at the same levels as in NIH-3T3 cells (Fig. 5A). Moreover, the expressions of cdk2, cdk4 and cdk6 at the protein level were the same in the growth phase (log) for both types of cells and were also similar in the serum-starved situation (G0) (Fig. 5B). The expressions of cyclin E and cyclin D1 were the same in 3T3-PCNA and NIH-3T3 cells in the log phase, but their expression in the G0 phase was lower than that in the log phase in both cell types (Fig. 5B). The expression of p21, one of the cdk inhibitors, increased in the G0 phase in both cell types (Fig. 5B), which suggests that p21 is responsible for the growth arrest by serum starvation in NIH-3T3 cells. However, the expression of p27, another cdk inhibitor, was the same for 3T3-PCNA and NIH-3T3 cells and its expression levels remained similar in the log and G0 phases (Fig. 5B). In addition, p15 was expressed at low levels in both cell types (data not shown). The expression of p16/INK4 was undetectable in these cells (data not shown). The expression of p27 was the same in confluent 3T3-PCNA and NIH-3T3 cells (Fig. 5C), although it has been reported that p27 regulates growth arrest by contact inhibition.<sup>22</sup> However, the expression of p21 increased in confluent NIH-3T3 and 3T3-PCNA cells (Fig. 5C). The expression of cyclin D1 decreased in confluent cells (Fig. 5C). These results suggest that the growth arrest by cell contact in NIH-3T3 cells is dependent on p21 rather than p27, and the negation of contact inhibition in 3T3-PCNA cells is not due to changes in p27 expression.

The activities of cdk4, cdk6 and cdk2 are regarded as important at the G1 (G0)/S transition.<sup>12, 13</sup> Therefore, we investigated the activities of these kinases in 3T3-PCNA and NIH-3T3 cells. Cdk4 activity was equivalent in 3T3-PCNA and NIH-3T3 cells at the log phase. In the G0 phase, this activity was also the same for both cell types (Fig. 5D), although the expression of cyclin D1 decreased in the G0 phase as compared with that in the log phase (Fig. 5B). Cdk2 activity was detected at the same levels in all cells in the log phase, but its activity in 3T3-PCNA cells, particularly clone-7 and -19, did not decrease in the G0 phase to the same level as that in NIH-3T3 cells (Fig. 5D). Besides, it seemed that cdk2 activity decreased in the G0 phase in 3T3-PCNA21 as well as NIH-3T3 cells (Fig. 5D). However, a decline in the mobility of RB as a substrate was seen in 3T3-PCNA21 and not in NIH-3T3 cells, which suggested that cdk2, which was extracted

from serum-starved 3T3-PCNA21 cells, phosphorylated RB, and 3T3-PCNA21 cells possess cdk2 activity in the serum-starved situation. Fig. 5B shows that the expression of cyclin E, a regulating factor of cdk2 activity, decreased at the G0 phase in both 3T3-PCNA cells and NIH-3T3 cells. This finding suggests that the level of cdk2 activity is maintained by means other than the regulation of cdk2 and cyclin E expression. These observations imply that the maintenance of cdk2 activity level in a serum-starved situation is important for the negation of growth arrest in 3T3-PCNA cells.

## DISCUSSION

Earlier reports have suggested that the increased expression of PCNA in many tumor cells is a result of rapid cell proliferation.<sup>2-4</sup> However, we found that the overexpression of PCNA suppresses growth arrest in conditions of contact inhibition and serum starvation, suggesting that increases in PCNA expression in tumor cells not only reflect rapid cell growth, but actually contribute to the disruption of cell proliferation.

In 3T3-PCNA cells, increased levels of cyclin A and histone H3, which are expressed in the S phase, were observed in the growth phase as compared with NIH-3T3 cells (Fig. 5A), but the expression of other genes was identical for 3T3-PCNA and NIH-3T3 cells in the growth phase (log phase) or in the growth arrest phase (G0) induced by serum starvation (Fig. 5, A and B). Cdk4 activity was the same for 3T3-PCNA and NIH-3T3 cells in the log and G0 phases (Fig. 5D). Cdk2 activity was identical for these cells in the log phase, but decreased more at G0 in NIH-3T3 cells than in 3T3-PCNA cells (Fig. 5D). These results suggest that the regulation of cdk2 activity is important for the introduction to the G0 phase in NIH-3T3 cells.

PCNA directly binds not only DNA replication factors, but also cyclin D1 and p21.<sup>23, 24</sup> The overexpression of p21 induces G1 arrest and inhibits DNA synthesis in both normal and tumor cells by binding to cdk2 and inhibiting their activity.<sup>25, 26</sup> These findings suggest that p21 controls the G1/S transition and the induction of growth arrest. In other words, the induction of growth arrest is regulated by the expression of p21. In NIH-3T3 cells the expression of p21 increased in serum starved and over-confluent situations (Fig. 5, B and D), which suggests that p21 regulates growth arrest induced by serum starvation and cell contact in NIH-3T3 cells.

Replication factor C (RF-C), one of the DNA replication accessory proteins, is necessary for the loading of PCNA onto DNA and for the subsequent synthesis of the leading DNA strand by DNA polymerase  $\delta$ .<sup>27</sup> Overexpression of the p140 subunit of RF-C accelerates cellular proliferation in NIH-3T3 cells,<sup>28</sup> suggesting the possibil-

ity that this subunit regulates cell proliferation directly. That finding also suggests that PCNA regulates cell proliferation by essentially the same mechanisms as the p140 subunit of RF-C, because PCNA, like RF-C, is a DNA replication accessory protein. Thus, our results, along with earlier findings, suggest that increases in PCNA expression in tumor cells not only reflect rapid cell growth, but also are related to the disruption of cell proliferation. In other words, the regulation of PCNA expression is important for normal cell proliferation and the overexpression of PCNA leads to malignant transformation. Therefore, it is necessary to reevaluate the significance of PCNA expression in tumor cells.

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