Existence of Two Populations of Cyclin/Proliferating Cell Nuclear Antigen during the Cell Cycle: Association with DNA Replication Sites

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Abstract. Pulse-chase experiments have revealed that cyclin, the auxiliary protein of DNA polymerase-δ, is stable during the transition from growth to quiescence in 3T3 cells. Immunoblotting together with immunofluorescence analysis has shown that the amount of cyclin after 24 h of quiescence is 30–40% of that of growing cells and that it presents a nucleoplasmic staining. Immunofluorescence studies show the existence of two populations of cyclin during the S phase, one that is nucleoplasmic as in quiescent cells and is easily extracted by detergent, and another that is associated to specific nuclear structures. By using antithymodeoxyuridine immunofluorescence to detect the sites of DNA synthesis, it was shown that the staining patterns of the replicon clusters and their order of appearance throughout the S phase are identical to those observed for cyclin. Two-dimensional gel analysis of Triton-extracted cells show that 20–30% of cyclin remains associated with the replicon clusters. This population of cyclin could not be released from the nucleus using high-salt extractions. This demonstrates that cyclin is tightly associated to the sites of DNA replication and that it must have a fundamental role in DNA synthesis in eukaryotic cells.

1. Abbreviations used in this paper: BrdU, 5-bromodeoxyuridine; PCNA, proliferating cell nuclear antigen.

THE identification of the cellular proteins that are involved in the control of cell proliferation in normal cells is essential for understanding the mechanisms underlying growth regulation and cellular transformation. A nuclear protein, cyclin (mol wt 36,000), whose synthesis correlates with the proliferative state of the cell, may be such a candidate. This protein is present in variable amounts in normal proliferating as well as transformed cells of several species (reviewed in references 3 and 11). The level of cyclin fluctuates during the cell cycle, with a clear increase during the S phase (4). Moreover, a coordinate synthesis of cyclin and DNA has been demonstrated in quiescent cells stimulated with different mitogens (3, 7). The proliferating cell nuclear antigen (PCNA) (18, 27-29) has been shown to be identical to cyclin (17, 20). Immunofluorescence studies of the distribution of cyclin (PCNA) during the cell cycle have revealed dramatic changes in its nuclear localization during the S phase (7, 10, 19). It appears to be located in replicon clusters during DNA synthesis.

Cyclin has been cloned and sequenced and shows homology with DNA binding proteins (1, 18). Recently, it has been proved that cyclin (PCNA) is the auxiliary protein of DNA polymerase-δ (5, 24, 30) and that it is required for SV40 replication in vitro (20).

In this report we present evidence that the cyclin protein is stable during the transition from a growing to a quiescent state of the cell cycle and that a fraction of it is tightly associated with DNA replication sites during the S phase.

Materials and Methods

Cells

Mouse NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DME) supplemented with 5% fetal calf serum (FCS) and antibiotics (100 U/ml of penicillin, 50 μg/ml of streptomycin).

Labeling of Cells with [35S]Methionine

Cells were grown in 24-well plates with 2 ml of medium supplemented with 5% FCS. The medium was changed to DME containing 0.5% FCS and cultures were used 3 d later. >98% of the cells presented no DNA synthesis after this period. To obtain a synchronous population of cells reaching DNA synthesis, quiescent cultures were stimulated with 20% FCS after 20 h of starvation and 90% of the cells were engaged in DNA replication. Labeling of cells was carried out for the indicated time in 250 μl of medium lacking methionine and in the presence of 250 μCi of [35S]methionine (SJ 204, Amersham Ltd., Amersham, United Kingdom).

Indirect Immunofluorescence

Cells were grown on glass coverslips (9 x 9 mm) and washed twice with Hank’s buffered salt solution (HBSS) before fixation. Methanol fixation was carried out at −20°C for 5 min. Formaldehyde treatment was done at 4°C for 30 min using 3.5% formaldehyde in HBSS. Then cells were permeated with 0.1% Triton X-100 for 2 min at room temperature. After washing extensively with HBSS, the coverslips were covered with 20 μl of human anti-PCNA antibody (1:100 in HBBS) and incubated for 1 h at 37°C in a humid environment. Coverslips were then washed in HBSS and covered with 20 μl of rhodamine-conjugated rabbit anti-human immunoglobulin (1:100 in HBBS, DAKO). After 1 h of incubation at 37°C, the coverslips were washed thoroughly with HBSS and mounted on Moviol 4-88 (Hoechst AG, Frankfurt, Federal Republic of Germany).

For detection of DNA replication, cells were incubated with 20 μM 5-bromodeoxyuridine (BrdU) in the presence of 2 μM fluorodeoxyuridine to inhibit thymidylate synthetase (34) for 15 min before fixation. Cells were washed twice in HBSS and fixed with 3.5% formaldehyde as described...
Triton X-100 and Salt Extraction

The [35S]methionine-labeled cells were rinsed in HBSS and 0.2 ml of 0.1% Triton X-100 in Pipes cytoskeleton buffer (Ca2+-free HBSS solution containing 2 mM MgCl2, 2 mM EGTA, 5 mM Pipes, pH 6.1) was added (8). Treatment with Triton X-100 was carried out for 60-90 s at room temperature. Then the solution was carefully removed and the extracted cells (Triton cytoskeletons) were resuspended in 100 μl of two-dimensional gel lysis buffer (22).

For salt extractions the Triton cytoskeletons were treated for 2 min at room temperature in phosphate buffer containing different concentrations of NaCl (10, 30, 50, 120, 240, 500 mM). The salt-extracted cytoskeletons were resuspended in 100 μl of lysis buffer.

Two-dimensional Gel Electrophoresis

The procedures used have been previously described (2, 22). Briefly, the first-dimension separations (IEF) were performed in 230 x 1.2 mm 4% (wt/vol) polyacrylamide gels containing 2% ampholytes (1.6% pH 5-7; 0.4% pH 3.5-9.5) at 1,200 V for 20 h. The second dimension was run in 15% polyacrylamide gels (25 x 25 cm) for 16 h at 13 mA.

The gels were processed for fluorography as described (16). Approximately 106 trichloroacetic acid-precipitable counts per minute were applied to each gel.

Immunoblotting

Proteins resolved by two-dimensional gels were transferred at 130 mA for 1 h with [35S]methionine as they reached maximum DNA synthesis (Fig. 1 A) as determined by [3H]thymidine incorporation. As previously demonstrated cyclin and DNA synthesis increase coordinately (6). The label was chased by transferring the cells to a serum-free medium with 10 times the normal amount of methionine. Insulin was also added to the medium to allow completion of the cell cycle without a new round of division (see Fig. 1 A). Every 2 h for a period of 24 h, duplicate cultures were resuspended in lysis buffer and their proteins were analyzed in two-dimensional gels to determine the amount of radioactive cyclin. Parallel cultures were labeled every 2 h for 1 h with [3H]thymidine to determine DNA synthesis after transfer to the serum-free medium during a 24-h period. Cells were also counted to verify that most of them had divided. The observation that cyclin is a stable protein indicates that it should be detected in quiescent cells at least for a period of 24-48 h after cells have stopped dividing. This contrasts with earlier studies showing the cyclin was undetectable in quiescent 3T3 cells by immunofluorescence (6). Therefore, this problem was reexamined determining the presence of cyclin in growth-arrested cells by immunoblotting analysis together with immunofluorescence using different fixation procedures. Cells were also labeled with [35S]methionine to prove that cyclin synthesis was reduced. Fig. 2 A shows that quiescent cells synthesize cyclin at approximately one-tenth the rate of growing cells (Fig. 2 B). In contrast, the immunoblotting analyses demonstrated that the amount of cyclin

Results

Stability of Cyclin

Immunofluorescence analyses have demonstrated that cyclin (7) presents a strong nuclear staining in growing 3T3 cells but is undetectable in quiescent cells, suggesting that it is rapidly degraded upon growth arrest. It is therefore important to determine the stability of cyclin during the transition from a growing to a quiescent state of the cell cycle. We used synchronized cultures of mouse 3T3 cells and labeled them for 1 h with [35S]methionine as they reached maximum DNA synthesis (Fig. 1 A) as determined by [3H]thymidine incorporation. As previously demonstrated cyclin and DNA synthesis increase coordinately (6). The label was chased by transferring the cells to a serum-free medium with 10 times the normal amount of methionine. Insulin was also added to the medium to allow completion of the cell cycle without a new round of division (see Fig. 1 A). Every 2 h for a period of 24 h, duplicate cultures were resuspended in lysis buffer and their proteins were analyzed in two-dimensional gels to determine the amount of radioactive cyclin. Parallel cultures were labeled every 2 h for 1 h with [3H]thymidine to determine DNA synthesis after transfer to the serum-free medium during a 24-h period. Cells were also counted to verify that most of them had divided. The observation that cyclin is a stable protein indicates that it should be detected in quiescent cells at least for a period of 24-48 h after cells have stopped dividing. This contrasts with earlier studies showing the cyclin was undetectable in quiescent 3T3 cells by immunofluorescence (6). Therefore, this problem was reexamined determining the presence of cyclin in growth-arrested cells by immunoblotting analysis together with immunofluorescence using different fixation procedures. Cells were also labeled with [35S]methionine to prove that cyclin synthesis was reduced. Fig. 2 A shows that quiescent cells synthesize cyclin at approximately one-tenth the rate of growing cells (Fig. 2 B). In contrast, the immunoblotting analyses demonstrated that the amount of cyclin
24–48 h after growth arrest is 30–40% of that present in growing cells (Fig. 2, C and D). The amount of cyclin in long-term quiescent cells such as those in organs is probably negligible because it is undetectable in nonproliferative tissues by immunoblotting analyses (unpublished observations).

The immunofluorescence studies show that cyclin is undetectable in methanol-fixed quiescent cells (Fig. 3 B, 0 h) in agreement with our previous findings, however, cells fixed with formaldehyde and permeabilized with Triton X-100 present a homogenous nuclear staining with the exception of the nucleoli (Fig. 3 C, 0 h), thus supporting the evidence that cyclin is present in quiescent cells and confined to the nucleus.

Two Distinct Populations of Cyclin Are Revealed by Immunofluorescence

Immunofluorescence analysis of methanol-fixed cells at various times after induction of growth of quiescent cells by serum stimulation (Fig. 3, A and B) show that cyclin (PCNA) can be detected in the nuclei after 10 h, reaching a maximum at 16–18 h (corresponding to 90–95% of the cells). A clear decrease in the percentage of immunofluorescent nuclei is detected after 24 h. This correlates closely with the percentage of cells in the S phase as determined by autoradiography (Fig. 3 A). The immunofluorescent staining of cyclin varies during the period of DNA replication presenting several patterns in a sequential order. Some of them are shown in Fig. 4. Similar immunolocalization of cyclin has been observed using rabbit anti-cyclin antibody.

When an identical study was performed in formaldehyde-fixed cells cyclin was observed in nonstimulated quiescent cells as a nucleoplasmic staining which remained unaltered through G1 (Fig. 3 C). During DNA replication the nucleoplasmic background remained and certain more intensely stained nuclear areas were observed. A detailed analysis of these strong fluorescent nuclear areas show that they are identical to those present in methanol-treated cells and that they also appear sequentially. We have found by two-dimensional gel analysis that the amount of cyclin present in the cells does not decrease after methanol treatment (not shown). This finding suggests that after methanol fixation the antibody recognizes only a population of cyclin that interacts with specific nuclear structures and that formaldehyde fixation allows the recognition of at least two populations. Interestingly, when cells were extracted with Triton X-100 before formaldehyde fixation only the cyclin present in the defined nuclear structures remained (Fig. 3 D), suggesting that the two populations of cyclin may correspond to distinct associations with nuclear components.

Cyclin Is Tightly Associated with DNA Replication Sites

Previous observations have shown that the nuclear immunofluorescent patterns of cyclin roughly correspond to the sites...
Figure 4. Immunofluorescence staining patterns of BrdU and cyclin (PCNA). Cells were fixed with methanol and treated as described in Materials and Methods.

Figure 5. Immunofluorescent staining patterns of cyclin (PCNA) in S-phase cells untreated (control) or treated with detergent (Triton X-100) before methanol fixation. Cells were processed for immunofluorescence as described in Materials and Methods.
shortly before DNA replication (3), it would suggest that during S phase the cellular content of cyclin duplicates in order to maintain a constant amount of this protein in the nucleus after cell division.

Immunofluorescence analysis in formaldehyde-fixed quiescent cells showed that the localization of cyclin is nucleoplasmic, indicating that the protein does not shuttle between cytoplasm and nucleus during the transition from G0 to S phase. The immunoblotting analyses of several nonproliferating tissues have revealed that the amount of cyclin is negligible, suggesting that after a long period of quiescence this protein disappears in the cells.

Our immunofluorescence studies revealed two populations of cyclin, one probably associated with the sites of DNA replication and another that is homogeneously spread in the nucleoplasm. After formaldehyde fixation of both quiescent and G1 cells, the nucleoplasmic distribution of cyclin can be clearly detected, however, it is undetectable after methanol treatment. Extraction of quiescent cells with Triton X-100 before formaldehyde fixation eliminates completely the nucleoplasmic staining, suggesting that this population of cyclin is loosely bound to nuclear structures or free in the nucleoplasm.

During the S phase formaldehyde-fixed cells show both types of cyclin populations, whereas after methanol treatment cells present only the one bound to the DNA replication sites. Triton eliminates the nucleoplasmic staining without affecting the one associated to the sites of DNA replication. These results may suggest, first, that not all cyclin is recruited at once for DNA synthesis, and second, that cyclin forms a stable complex with the DNA replication sites. The latter agrees with the previous observation that the changes in the nuclear distribution of cyclin during S phase depend on DNA synthesis (7).

The conclusions drawn from our immunofluorescence studies are further supported by the analysis of the Triton-insoluble material from [35S]methionine labeled cells by two-dimensional gels. The results demonstrate that 70–80% of cyclin is extracted by Triton X-100 treatment of S-phase cells as determined by autoradiography and immunoblotting. This suggests that the previously existing cyclin and the newly synthesized one follow the same pattern of distribution.

The extraction of Triton cytoskeletons with different salt concentrations showed that the population of cyclin (20–30% of the total) that is associated with the DNA replication sites forms a stable complex in that concentrations as high as 0.5 M NaCl did not release significant amounts of the protein.

Many of the properties previously described for cyclin and the ones presented here have been reported for DNA polymerase-α (13, 15, 31). These observations, together with the recent evidence that cyclin is the auxiliary protein of DNA polymerase-δ (5, 24) and that it is required for SV40 DNA replication in vitro (23), lead us to believe that cyclin belongs to a replicative complex (replisase 21, 25, 26) that possibly assembles during G1/S, permitting DNA synthesis and disperses at the end of the S-phase.

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References

1. Almendral, J. M., D. Huebsh, P. A. Blundell, H. Macdonald-Bravo, and R. Bravo. 1987. Cloning and sequence of the human nuclear protein cyclin: homology with DNA binding proteins. Proc. Natl. Acad. Sci. USA. 84:1575–1579.

2. Bravo, R. 1984. Epidermal growth factor inhibits the synthesis of the nuclear protein cyclin in A431 human carcinoma cells. Proc. Natl. Acad. Sci. USA. 81:4848–4851.
3. Bravo, R. 1986. Synthesis of the nuclear protein cyclin (PCNA) and its relationship with DNA replication. Proc. Cell Res. 163:287–293.

4. Bravo, R., and J. E. Celis. 1980. A search for differential polypeptide synthesis throughout the cell cycle of Hela cells. J. Cell Biol. 84:795–802.

5. Bravo, R., P. A. Blundell, and H. Macdonald-Bravo. 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. Nature (Lond.). 326:515–517.

6. Bravo, R., and H. Macdonald-Bravo. 1984. Induction of the nuclear protein cyclin in quiescent mouse 3T3 cells stimulated by serum and growth factors: correlation with DNA synthesis. EMBO (Eur. Mol. Biol. Organ.) J. 3:3177–3181.

7. Bravo, R., and H. Macdonald-Bravo. 1985. Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. EMBO (Eur. Mol. Biol. Organ.) J. 4:655–661.

8. Bravo, R., J. V. Small, S. J. Fey, P. Mose Larsen, and J. E. Celis. 1982. Architecture and polypeptide composition of Hela cytoskeletons. J. Mol. Biol. 154:121–143.

9. Burnette, W. N. 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein. Anal. Biochem. 112:195–203.

10. Celis, J. E., and A. Celis. 1985. Cell cycle-dependent variations in the distribution of the nuclear protein cyclin in cultured cells. Proc. Natl. Acad. Sci. USA 82:3262–3266.

11. Celis, J. E., P. Madsen, S. Nielsen, and A. Celis. 1986. Nuclear patterns of cyclin (PCNA) antigen distribution subdivide S-phase in cultured cells. Leuk. Res. 10:237–249.

12. Dolbeare, F., H. Gratzner, M. G. Pallavicini, and J. W. Gray. 1983. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc. Natl. Acad. Sci. USA 80:5573–5577.

13. Fry, M., and L. A. Loeb. 1986. Animal cell DNA polymerases. CRC Press, Inc., Boca Raton, FL.

14. Gratzner, H. G. 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science (Wash. DC). 218:474–475.

15. Koizumi, T., T. Seno, and T. Yagure. 1986. Activity levels of mouse DNA polymerase α-primase complex (DNA replicase) and DNA polymerase-δ, free from primase activity in synchronized cells, and a comparison of their catalytic properties. Eur. J. Biochem. 157:251–259.

16. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of 3H and 14C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335–341.

17. Mathews, M. B., R. M. Bernstein, B. R. Franza, and J. I. Garrels. 1984. The identity of the proliferating cell nuclear antigen and cyclin. Nature (Lond.). 309:374–376.

18. Matsumoto, K., T. Moritoh, T. Koj, and P. Nakane. 1987. Molecular cloning of cDNA coding for rat proliferating nuclear antigen (PCNA)/cyclin. EMBO (Eur. Mol. Biol. Organ.) J. 6:637–642.

19. Miyashita, K., M. J. Fritzer, and E. M. Tan. 1978. An autoantibody to a nuclear antigen in proliferating cells. J. Immunol. 121:2228–2234.

20. Nakamura, H., T. Morita, and C. Sato. 1986. Structural organizations of replicon domains during DNA synthetic phase in the mammalian nucleus. Exp. Cell Res. 165:291–297.

21. Noguchi, H., G. P. Reedy, and A. B. Pardee. 1983. Rapid incorporation of label from ribonucleoside disphosphates into DNA by a cell-free high molecular weight fraction from animal cell nuclei. Cell. 32:443–451.

22. O’Farrell, P. H. 1975. High-resolution two dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007–4021.

23. Prelich, G., M. Kostura, D. R. Marshall, M. B. Mathews, and B. Stillman. 1987. The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication in vitro. Nature (Lond.). 326:471–475.

24. Prelich, G., C. K. Tan, M. Kostura, M. B. Mathews, A. G. So, K. M. Downey, and B. Stillman. 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. Nature (Lond.). 326:517–520.

25. Reedy, G. P., and A. B. Pardee. 1980. Multienzyme complex for metabolic channeling in mammalian DNA replication. Proc. Natl. Acad. Sci. USA 77:3312–3316.

26. Reedy, G. P., and A. B. Pardee. 1983. Inhibitor evidence for allosteric interaction in the replicase multienzyme complex. Nature (Lond.). 304:86–88.

27. Takasaki, J., J. S. Deu, and E. M. Tan. 1981. A nuclear antigen associated with cell proliferation and blast transformation. Its distribution in synchronized cells. J. Exp. Med. 156:1899–1909.

28. Takasaki, J., D. Fischwild, and E. M. Tan. 1984. Characterization of proliferating cell nuclear antigen recognized by autoantibodies in lupus sera. J. Exp. Med. 159:981–992.

29. Tan, E. M. 1982. Autoantibodies to nuclear antigens (ANA): their immunobiology and medicine. Adv. Immunol. 33:167–240.

30. Tan, C. K., C. Castillo, A. G. So, and K. M. Downey. 1986. An auxiliary protein for DNA polymerase-δ from <i>foetal calf</i> thymus. J. Biol. Chem. 261:12310–12316.

31. Thommesen, R., T. Reiter, and R. Knippers. 1986. Synthesis of DNA polymerase-α analyzed by immunoprecipitation from synchronously proliferating cells. Biochemistry. 25:1308–1314.