

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 anti-

bromodeoxyuridine 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.

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)¹ (18, 27–29) has been shown to be identical to cyclin (17, 28). 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* (23).

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.

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

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 (10 U/ml of penicillin, 50 μ g/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 ~90% of the cells were engaged in DNA replication. Labeling of cells was carried out for the indicated time in 250 μ l of medium lacking methionine and in the presence of 250 μ Ci of [³⁵S]methionine (SJ 204, Amersham Ltd., Amersham, United Kingdom).

Indirect Immunofluorescence

Cells were grown on glass coverslips (9 \times 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 μ l of human anti-PCNA antibody (1:100 in HBSS) and incubated for 1 h at 37°C in a humid environment. Coverslips were then washed in HBSS and covered with 20 μ l of rhodamine-conjugated rabbit anti-human immunoglobulin (1:100 in HBSS, 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 μ M 5-bromodeoxyuridine (BrdU) in the presence of 2 μ M fluorodeoxyuridine to inhibit thymidylate synthetase (14) for 15 min before fixation. Cells were washed twice in HBSS and fixed with 3.5% formaldehyde as described

above. Then cells were treated with 1.5 N HCl for 30 min at room temperature to denature the DNA and washed several times in HBSS. Cells were covered with 20 μ l of a mouse monoclonal antibody anti-BrdU (1:100 in HBSS, Partec) and incubated for 1 h at 37°C in a humidified atmosphere. After washing a rhodamine-conjugated rabbit anti-mouse antibody (1:50, DAKO) was added and cells were incubated for another hour at 37°C. Then coverslips were mounted as described above.

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 μ l 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 μ l 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 \times 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 \times 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 8 h onto nitrocellulose filters as described (9). The filters were incubated in PBS containing 10% bovine serum and rabbit anti-cyclin antibody diluted 1:500 for 1 h at room temperature. The filters were then incubated with peroxidase-conjugated anti-rabbit IgG for another hour in the same buffer. The immunocomplexes were visualized using diaminobenzidine as a substrate.

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. Fig. 1 A shows that DNA synthesis was undetectable 20 h after serum deprivation and >90% of

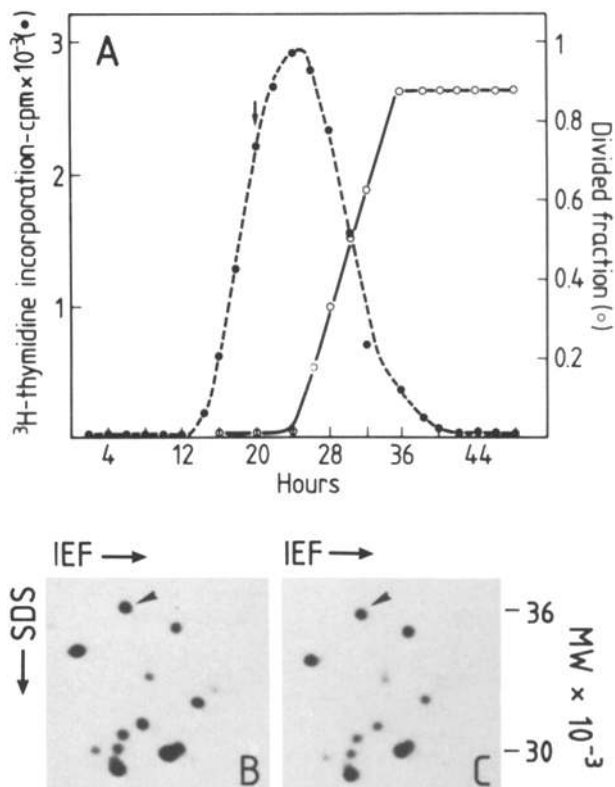


Figure 1. (A) Quiescent cells after 18 h of stimulation with 20% FCS (indicated by arrow) were transferred to serum-free medium. Cells were labeled with 1 μ Ci/ml of [³H]thymidine for 1 h every 2 h for 48 h. Divided fraction represents $n/n_0 - 1$ so that a true doubling of the cell population would appear as an increase from 0 to 1.0 on the ordinate axis. n , number of cells at any given time in the experiment; n_0 , initial cell number. (B) Quiescent cells pulse-labeled for 1 h with [³⁵S]methionine after 18 h stimulation. (C) Labeled cells as in B left for 24 h in serum-free medium. The position of cyclin is indicated by arrowhead.

the cells had divided. Analysis of the cells after the pulse reveal that the amount of cyclin does not decrease more rapidly than other cellular proteins after a 24 h chase (Fig. 1, B and C). These were further proved by direct counting of the spots (not shown). These results suggest that cyclin is stable. The transition from the growing to the quiescent state of the cell cycle does obviously not result in a rapid degradation of the protein. The half-life of cyclin is \sim 20 h.

Presence of Cyclin in Quiescent Cells

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

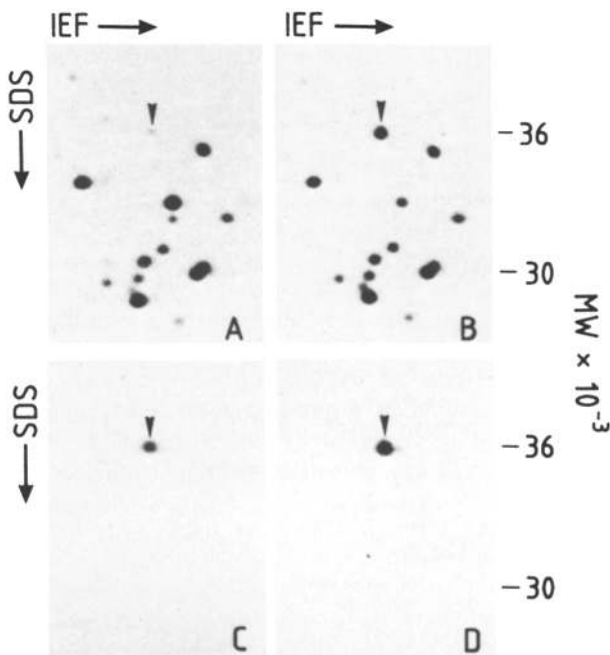


Figure 2. Two-dimensional gel electrophoresis (IEF) of [³⁵S]methionine-labeled (A) quiescent and (B) growing cells. Immunoblot of (C) quiescent and (D) growing cells. Cyclin was detected using specific rabbit anti-cyclin antibodies. The same amount of radioactivity or protein was applied on the gels. The arrowhead indicates the position of cyclin.

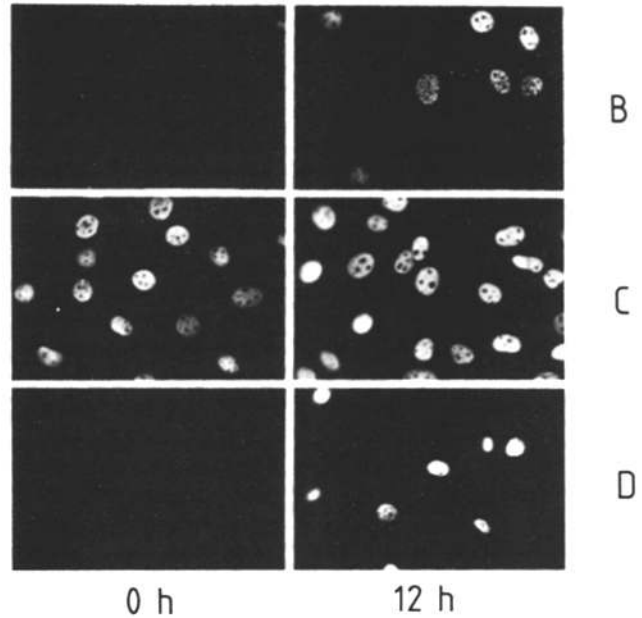
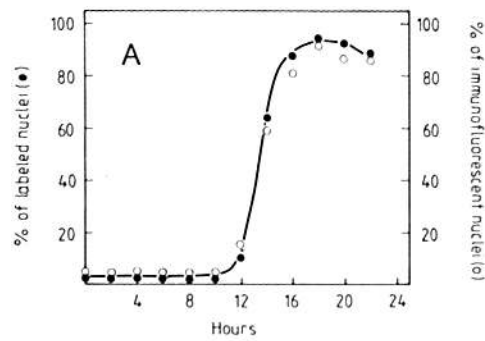


Figure 3. (A) Kinetics of appearance of positive nuclei for cyclin (PCNA) staining and [³H]thymidine incorporation in serum-stimulated quiescent cells. Immunofluorescent staining of cyclin after (B) methanol, (C) formaldehyde, and (D) Triton-formaldehyde fixation of quiescent cells (0 h) and after 12 h serum stimulation.

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

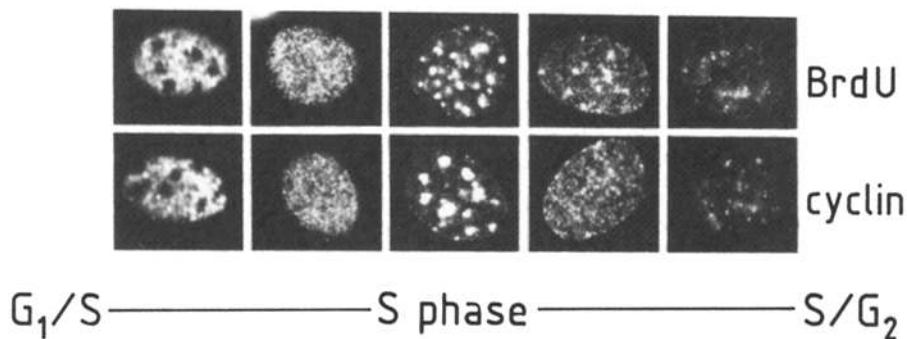


Figure 4. Immunofluorescence staining patterns of BrdU and cyclin (PCNA). Cells were fixed with methanol and treated as described in Materials and Methods.

of DNA replication as determined by *in situ* autoradiography of [³H]thymidine-labeled cells (7, 10, 11). We have extended these studies by using BrdU an artificial analogue of thymidine (12, 14) as a precursor for DNA synthesis and detecting the sites of incorporation by immunofluorescence using a monoclonal antibody to BrdU (20). This procedure gives a better resolution than autoradiography. Synchronized 3T3 cells were incubated for 15 min with BrdU and then processed for immunofluorescence. Fig. 4 shows the immunofluorescence staining of BrdU (sites of DNA replication) and those of cyclin throughout the S phase in methanol-fixed cells. Cells were fixed in methanol so as to detect only the population of cyclin that interacts with specific nuclear structures (see Fig. 3). The patterns observed are similar if not identical, indicating that during S phase, one population of cyclin is present in the sites of DNA replication.

Studies with DNA polymerase- α have demonstrated that a low percentage of the total cellular activity is present in the sites of DNA replication and that it is tightly associated as determined by detergent and salt extractions (reviewed in reference 13). Therefore, if cyclin is a component of the DNA replication system (5, 23, 24), it would be interesting to establish the strength of its association with the replicons. For this, S-phase cells were initially extracted with 0.1% Triton X-100 in low-salt buffer and methanol-fixed before immunofluorescence staining. As shown in Fig. 5, the staining patterns observed are identical in Triton-extracted and control cells throughout the S phase, suggesting that the cyclin population detected after methanol fixation is associated with the DNA replication sites. To establish that cyclin was not released by Triton treatment, S-phase cells were labeled for 4 h with [³⁵S]methionine and extracted with Triton X-100 in low-salt buffer followed by two-dimensional gel analysis. The presence of cyclin in Triton-extracted cells (Triton cytoskeletons) was determined by autoradiography and im-

munoblotting. Around 20–30% of cyclin remained in Triton cytoskeletons (Fig. 6, B and D) compared with nontreated cells (Fig. 6, A and C). This finding together with the immunofluorescence analyses indicates that 70–80% of cyclin is nucleoplasmic extractable with Triton during S phase but the form interacting with DNA replication sites remains in the Triton cytoskeletons.

Similar experiments done with quiescent cells demonstrated that all cyclin is extracted by Triton inasmuch as it was undetectable in immunoblots of Triton cytoskeletons (not shown), thus confirming the immunofluorescence studies illustrated in Fig. 3.

To study the strength of the association of cyclin with the DNA replicons, S-phase cells labeled for 4 h with [³⁵S]methionine were extracted with Triton X-100 followed by increasing salt extractions, from 30 to 500 mM NaCl. The results illustrated in Fig. 7 show that the cyclin remaining in the Triton cytoskeletons is tightly associated in that high concentrations of NaCl do not release significant amounts of the protein (Fig. 7, B and C). In all cases, the intermediate filament protein vimentin was used as an internal control (not shown) because it is known to be very insoluble (8) (the ratio of vimentin to cyclin does not change significantly during the salt extractions).

Discussion

We have demonstrated by pulse-chase experiments and immunoblotting analysis that cyclin is stable and is not degraded during the transition of the cell from a growing to a quiescent state.

Synthesis of cyclin is barely detectable in quiescent cells, but its amount is 40% of that present in growing cells 24–48 h after cells have entered G₀. In that synthesis of cyclin increases severalfold in serum-stimulated quiescent cells

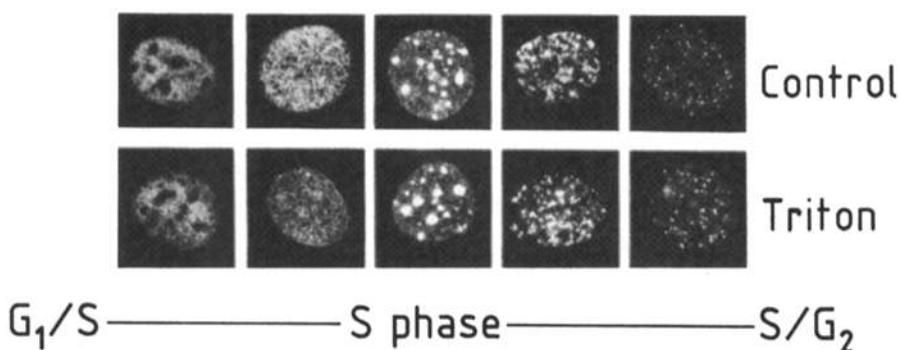


Figure 5. Immunofluorescent staining patterns of cyclin (PCNA) in S-phase cells untreated (control) or treated with detergent (Triton X-100) before methanol fixation. Cells were processed for immunofluorescence as described in Materials and Methods.

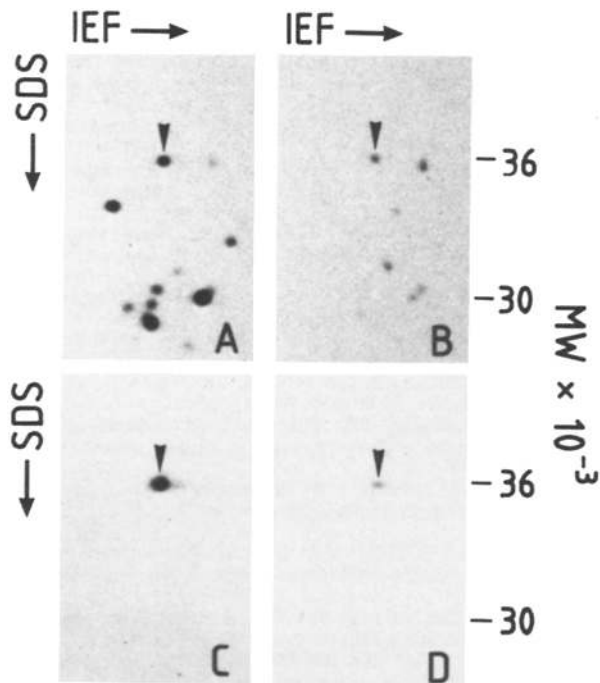


Figure 6. Two-dimensional gel electrophoresis (IEF) of [³⁵S]methionine polypeptides from (A) total and (B) Triton cytoskeletons from S-phase cells. (C and D) Respective immunoblots. Arrowhead indicates the position of cyclin.

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 nu-

cleoplasmic 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 (replisome 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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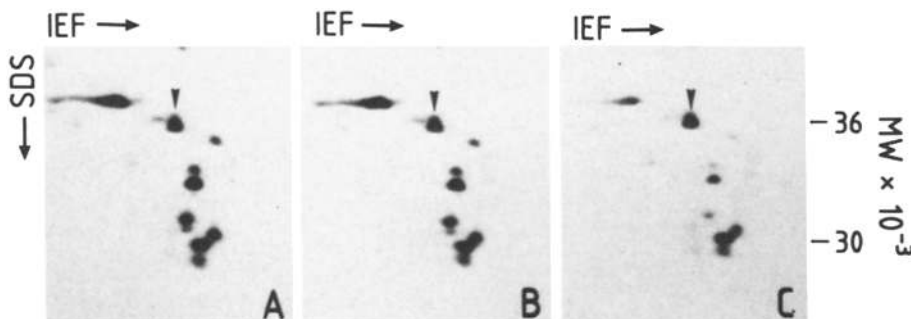


Figure 7. Two-dimensional gel electrophoresis (IEF) of [³⁵S]methionine-labeled polypeptides from S-phase cells (A) after Triton treatment followed by salt extraction with (B) 240 mM, and (C) 500 mM NaCl. Arrowhead indicates position of cyclin.

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