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Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication

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Synthesis of cyclin in serum-stimulated quiescent 3T3 cells increases shortly before DNA synthesis after 10 h of stimulation, reaching a maximum after 16 h. Inhibition of DNA synthesis by hydroxyurea does not affect the increase of cyclin following stimulation, as determined by quantitative two-dimensional gel electrophoresis. The levels of cyclin decrease dramatically at the end of the S-phase. Cells kept in the presence of hydroxyurea (G_1/S boundary) do not show this decrease in cyclin, indicating that its amounts are regulated by events occurring during the S-phase. Immunofluorescence studies of serum-stimulated quiescent cells in the presence of hydroxyurea, using proliferating cell nuclear antigen (PCNA) autoantibodies, confirm the results obtained by protein analysis. They also reveal that there are dramatic changes in the nuclear distribution of cyclin and that these depend on DNA synthesis or events occurring during the S-phase. Cyclin (PCNA) is no longer detectable at the end of the S-phase. However, pulse-chase experiments indicate that this protein is very stable, suggesting that it possibly interacts with other macromolecules rendering it inaccessible to the antibody. These results strengthen the notion that cyclin is an important component of the events leading to DNA replication and cell division.

Key words: acidic nuclear protein/immunofluorescence/S phase/PCNA antibodies/two-dimensional electrophoresis

Introduction

The identification of proteins that are preferentially synthesized in proliferating cells is essential for understanding the mechanisms underlying growth regulation and cellular transformation. The nuclear protein cyclin (mol. wt. 36 kd) is a potential candidate, since its synthesis correlates directly with the proliferative state of the cell and it has been identified in several cell types of human, mouse, hamster and avian origin (Bravo *et al.*, 1981a, 1981b, 1981c, 1982a; Bravo, 1984d; Bravo and Graf, 1985; Celis *et al.*, 1984a). This protein is synthesized by normally proliferating as well as transformed cells and tumours but it is present in very small amounts in non-dividing cells and tissues. The levels of cyclin fluctuate during the cell cycle with a specific increase in the S-phase (Bravo and Celis, 1980a). Furthermore, there is a close correlation between the levels of cyclin and DNA synthesis induced by serum and purified growth factors in quiescent mouse 3T3 cells (Bravo, 1984b; Bravo and Macdonald-Bravo, 1984). So far all the properties of cyclin are shared by the proliferating cell nuclear antigen (PCNA, Miyashi *et al.*, 1978; Takasaki *et al.*, 1981, 1984; Tan, 1982) a human protein that has recently been shown to be identical to cyclin (Mathews *et al.*, 1984; Takasaki *et al.*, 1984). Immunofluorescence studies of the distribution of cyclin (PCNA) during the cell cycle have revealed dramatic changes in its localization at S-phase (Miyashi *et al.*, 1978; Celis and Celis, 1985).

As previous studies have suggested that cyclin synthesis and DNA replication are closely related (Bravo and Celis, 1980a; Bravo, 1984b; Bravo and Macdonald-Bravo, 1984; Macdonald-Bravo and Bravo, 1985), we thought it important to investigate their possible relationship in more detail. For this, we have studied the effect of hydroxyurea on cyclin synthesis and its nuclear distribution after serum-stimulation of quiescent 3T3 cells. The results show that the synthesis of cyclin is not triggered by DNA replication, but that its changes in nuclear localization depend on events occurring during the S-phase.

Results

Effect of hydroxyurea on cyclin synthesis

A two-dimensional gel analysis of the acidic (IEF, Bravo *et al.*, 1982b) [^{35}S]methionine-labelled polypeptides of asynchronous 3T3 cells is shown in Figure 1. The position of the nuclear protein cyclin (Bravo *et al.*, 1981a, 1982a), vimentin, total actin and α- and β-tubulins are indicated. Cyclin corresponds to 0.1% of the total ^{35}S-labelled protein in proliferating 3T3 cells (Bravo and Celis, 1980b). The identity of cyclin in these cells has been previously confirmed by one-dimensional peptide mapping (Bravo, 1984b).

The induction of cyclin and DNA synthesis are closely related in both serum- or growth factor-stimulated quiescent 3T3 cells (Bravo, 1984b; Bravo and Macdonald-Bravo, 1984). As hydroxyurea blocks DNA synthesis by inhibiting ribonucleotide reductase and stops the progression from G_1 to S (Adams and Lindsay, 1976) we thought it important to establish whether this agent could have an effect on cyclin induction. For this, quiescent 3T3 cells were stimulated with 20% foetal calf serum (FCS) and labelled with [^{35}S]methionine for 2 h every 2 h for a period of 24 h. Hydroxyurea (1 mM) was added 8 h after stimulation to minimize the period of treatment. Parallel cultures were labelled with [^{3}H]-thymidine to follow DNA synthesis in the presence or absence of hydroxyurea. Cyclin synthesis was determined by quantitative two-dimensional gel electrophoresis analysis of the [^{35}S]-methionine-labelled polypeptides. Some of these results are shown in Figure 2 and the quantitative data are presented in Figure 3. As shown in Figure 2 hydroxyurea has no clear effect on the induction of cyclin. The quantitative analysis (Figure 3) demonstrates that the kinetics and intensity of the induction of cyclin synthesis is identical in both cases. An increase in cyclin synthesis can be observed 10–12 h after serum stimulation, reaching a maximum at 16–18 h (corresponding to a 7-fold increase over the level found in quiescent cells). DNA synthesis (as determined by [^{3}H]thymidine incorporation) increases after 12 h of serum-stimulation in control cells (Figure 3A), while hydroxyurea-treated cells show an insignificant incorporation of [^{3}H]thymidine (Figure 3B).

Cyclin synthesis during the S-phase

As hydroxyurea has no effect on the induction of cyclin, we have

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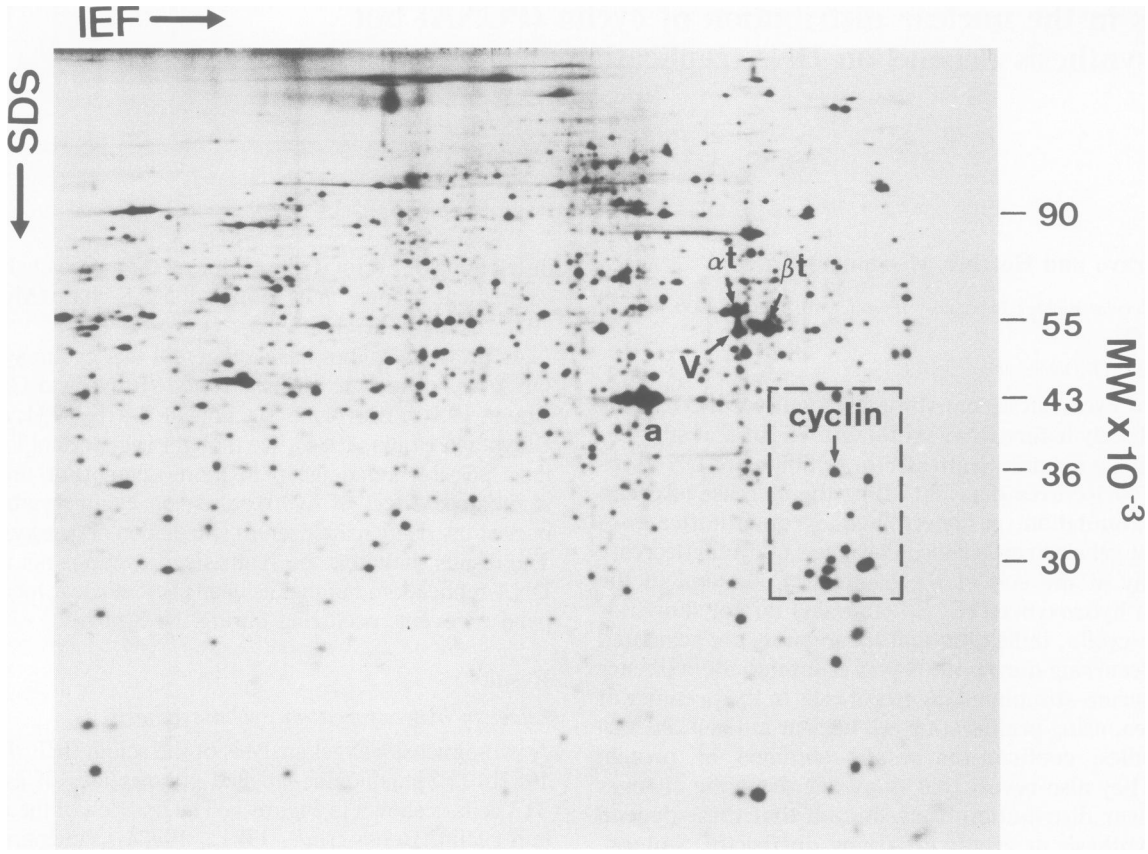

Fig. 1. Two-dimensional gel electrophoresis (IEF) of [^{35}S]methionine-labelled polypeptides from asynchronous mouse 3T3 cells. Cells were labelled for 16 h with [^{35}S]methionine (1 mCi/ml) as described (Bravo *et al.*, 1982b). a = actin; αt = α-tubulin; βt = β-tubulin; v = vimentin. The area of interest has been enclosed in a box.

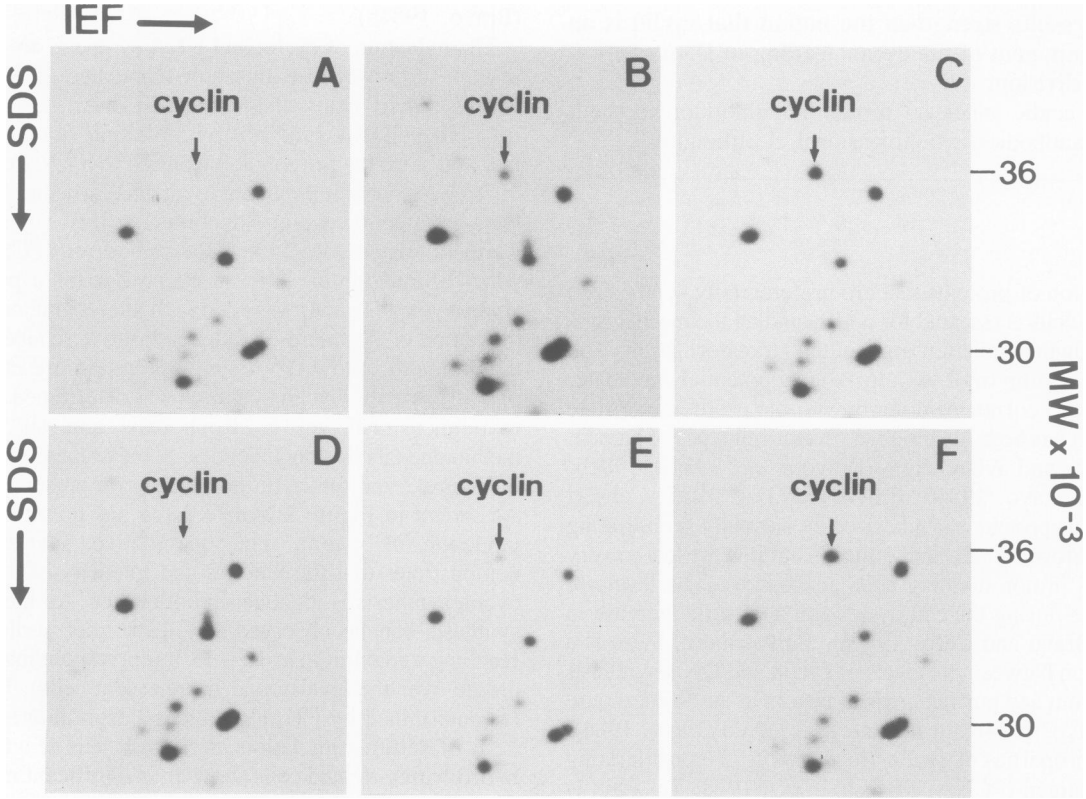

Fig. 2. Effect of hydroxyurea on cyclin induction in serum-stimulated quiescent 3T3 cells. Control cells after: (**A**) 8 h stimulation; (**B**) 12 h stimulation; (**C**) 16 h stimulation. Cells treated with 1 mM hydroxyurea after: (**D**) 8 h stimulation; (**E**) 12 h stimulation; (**F**) 16 h stimulation. In all cases cells were labelled for 2 h. Only the area of interest is shown (see Figure 1).

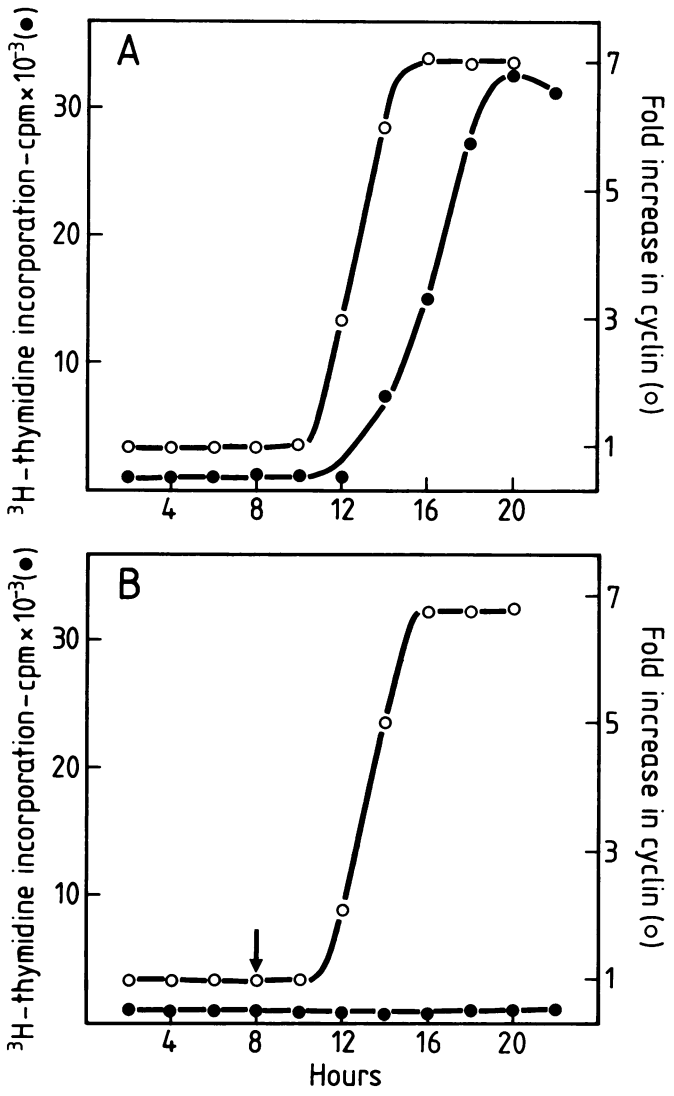

Fig. 3. Synthesis of cyclin and DNA after serum-stimulation of quiescent 3T3 cells. Cells were labelled for 2 h with [^{35}S]methionine or [^{3}H]thymidine at each indicated time following serum stimulation. Quantitation of radioactive cyclin was performed as described (Bravo *et al.*, 1982b). The data are given as the fold increase of cyclin compared with non-stimulated quiescent 3T3 cells. Equal amounts of incorporated radioactivity were applied to the gels. [^{3}H]Thymidine incorporation was determined as described (Macdonald-Bravo and Bravo, 1984). (**A**) Serum-stimulated quiescent cells in the absence of hydroxyurea. (**B**) Serum-stimulated quiescent cells in the presence of 1 mM hydroxyurea. The arrow indicates the time at which hydroxyurea was added.

used this drug to synchronize cells at the G_1/S boundary. Quiescent cells were stimulated with 20% FCS and after 8 h of stimulation 1 mM hydroxyurea was added. To ensure that cells have indeed traversed G_1 in the presence of hydroxyurea, they were washed with DMEM after 12 h of treatment and left in complete medium. At 2 h intervals thereafter cells were counted or labelled with [^{3}H]thymidine to follow cell division and DNA synthesis. The results in Figure 4 indicate that, although DNA synthesis is essentially zero at the time of removal of hydroxyurea, the cells synchronously move into the S-phase immediately after elimination of the drug from the medium. The growth data indicate that cells start to divide 10 h after the removal of hydroxyurea and that nearly all cells have accomplished division after 14 h (not shown). Cyclin synthesis was investigated by labelling parallel cultures with [^{35}S]methionine for

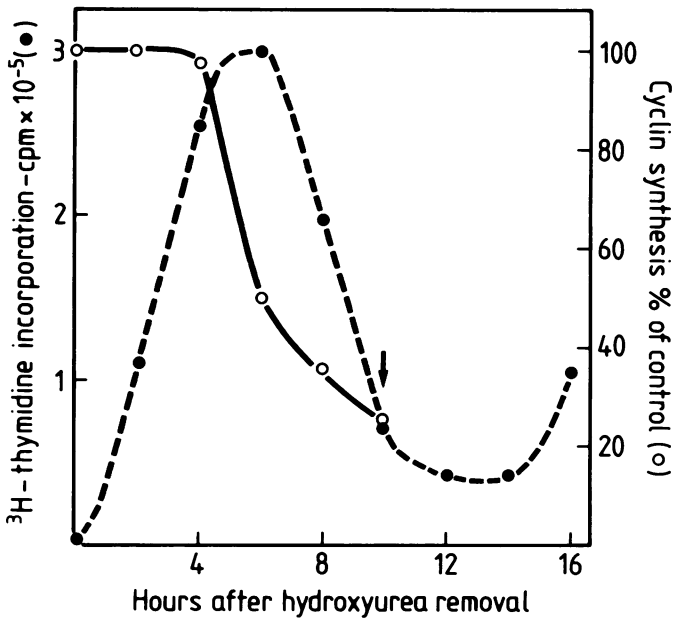

Fig. 4. Cyclin and DNA synthesis after hyroxyurea release. Quiescent cells were stimulated with 20% FCS in the presence of 1 mM hydroxyurea for 20 h. Then cells were washed and incubated at 37°C in fresh medium without hydroxyurea. At times thereafter cells were labelled with [^{35}S]-methionine or [^{3}H]thymidine. [^{3}H]Thymidine incorporation was determined as described (Madconald-Bravo and Bravo, 1984). Quantitation of radioactive cyclin was performed as previously published (Bravo *et al.*, 1982b). The arrow indicates the time at which first cell divisions are detected. At 14 h all cells have duplicated.

2 h every 2 h after hydroxyurea removal for a period of 10 h. Levels of cyclin were determined by two-dimensional gel electrophoresis as described (Bravo, 1984c). The quantitative data are illustrated in Figure 4. As shown in the figure cyclin synthesis starts decreasing afer 4 h of hydroxyurea removal (DNA synthesis peak) and reaches a basal level after 10 – 12 h (end of S-phase). No changes are detected in cyclin synthesis if the cells are kept in the presence of hydroxyurea (G_1/S boundary) for a similar period (not shown).

Cyclin (PCNA) changes in nuclear localization depend on DNA synthesis

Cyclin (PCNA) migrates within defined nuclear compartments during the cell cycle, specifically during the S-phase (Miyashi *et al.*, 1978; Celis and Celis, 1985). The changes in distribution of this protein follow a specific pattern in spontaneously transformed human amnion cells (AMA) as well as in several cell types of vertebrate origin (Celis and Celis, 1985). However, no studies have yet been done to demonstrate a correlation between these changes and DNA synthesis. To approach this problem, we have analyzed the effect of hydroxyurea on the nuclear distribution of cyclin (PCNA). Quiescent 3T3 cells were stimulated with 20% FCS and hydroxyurea was added 8 h after stimulation. Cells were further incubated for 10 – 12 h before being processed for immunofluorescence. In Figure 5A and B are shown some of the typical nuclear distributions of cyclin (PCNA) in an asynchronous culture of 3T3 cells (see also Figure 9). A selected field has been chosen in order to have several nuclear patterns together. In general, in an asynchronous culture 40 – 45% of the cells present nuclear staining. As illustrated in Figure 5 (letters assigned according to sequence presented in Figure 9; see also Celis and Celis, 1985), cyclin (PCNA) can

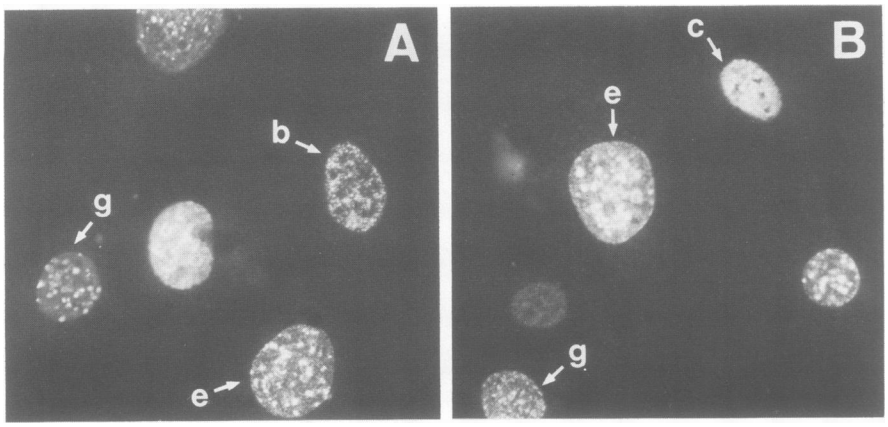

Fig. 5. Nuclear localizations of cyclin (PCNA) in asynchronous mouse 3T3 cells (**A**) and (**B**). Cells were prepared for immunofluorescence as described in Materials and methods. Selected areas are shown. Only 40–45% of the cells in an asynchronous culture present immunofluorescent staining.

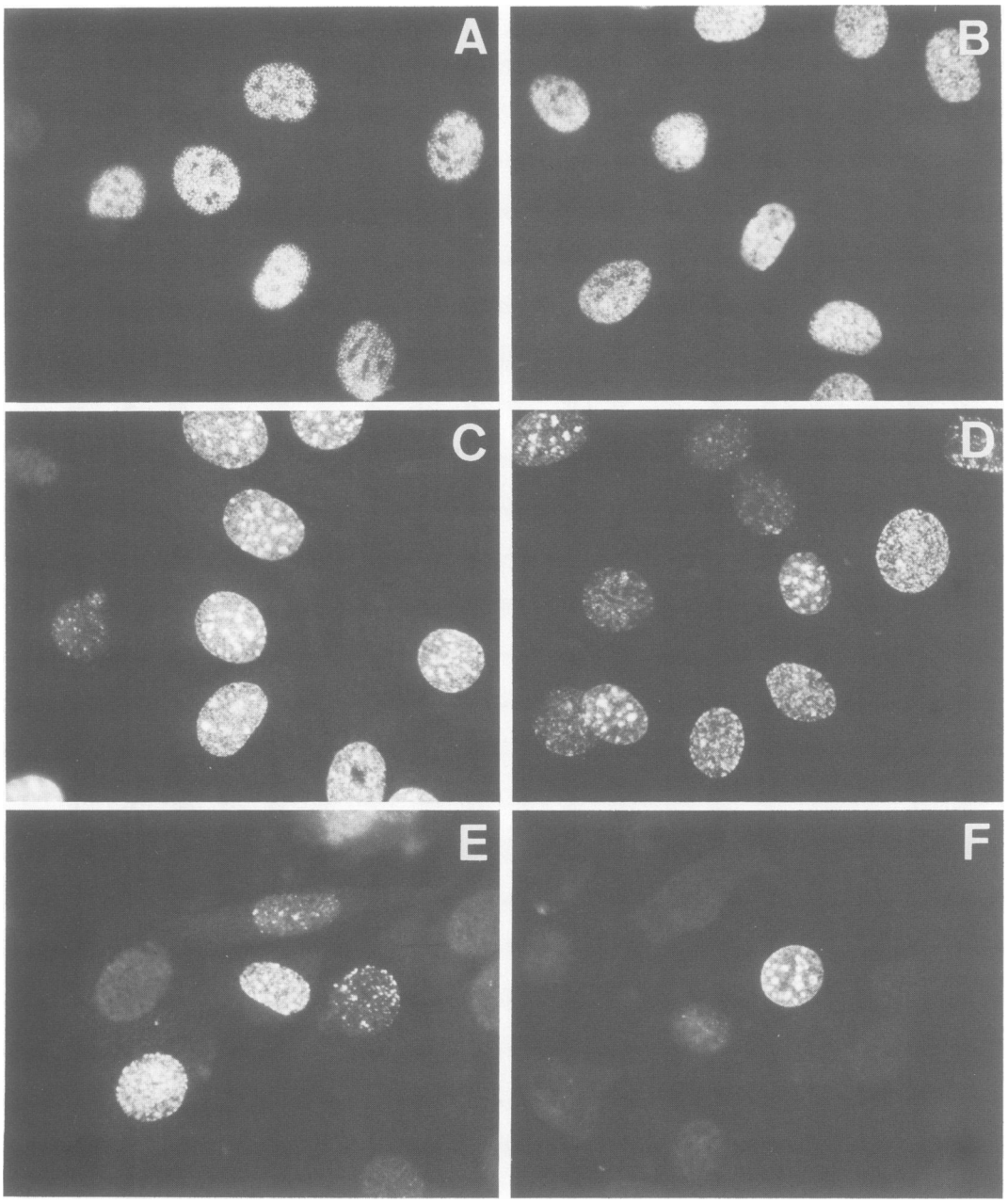

Fig. 6. Nuclear distribution of cyclin (PCNA) in 3T3 cells after removal of hydroxyurea. Indirect immunofluorescence of cyclin (PCNA antibodies) (**A**) before hydroxyurea removal; (**B**) 2 h after; (**C**) 4 h after; (**D**) 6 h after; (**E**) 8 h after; (**F**) 10 h after.

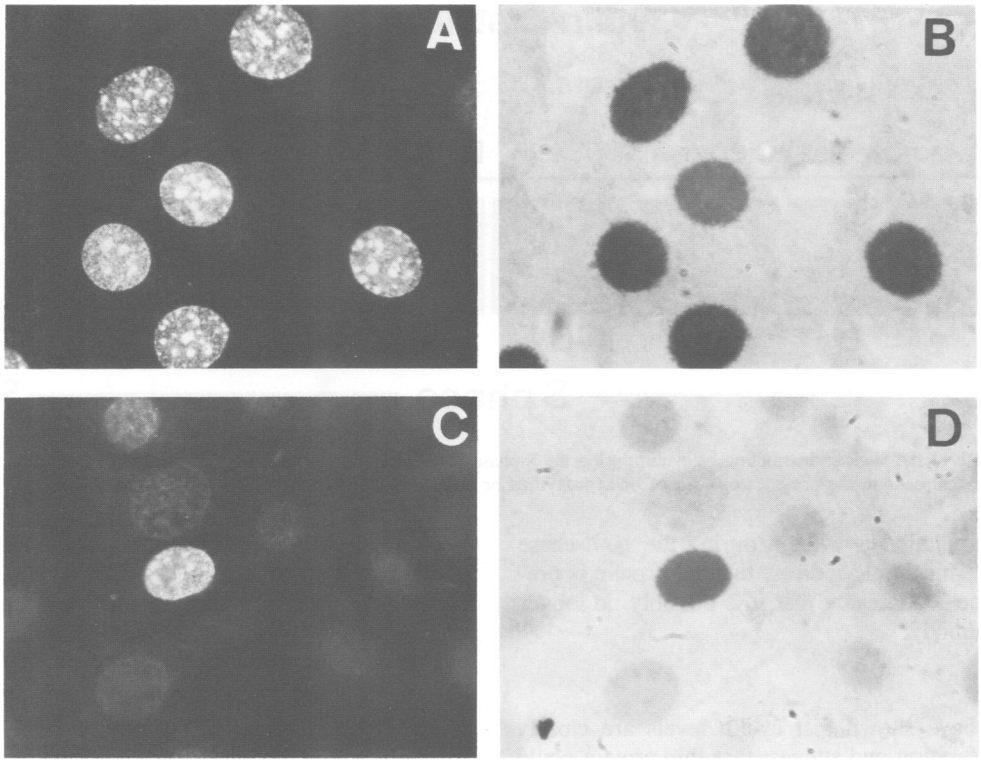

Fig. 7. Immunofluorescence of cyclin (PCNA) 4 h (**A**) and 10 h (**C**) after hydroxyurea removal. The corresponding autoradiographies are shown in (**B**) and (**D**), respectively. Immunofluorescence pictures were taken before being processed for autoradiography.

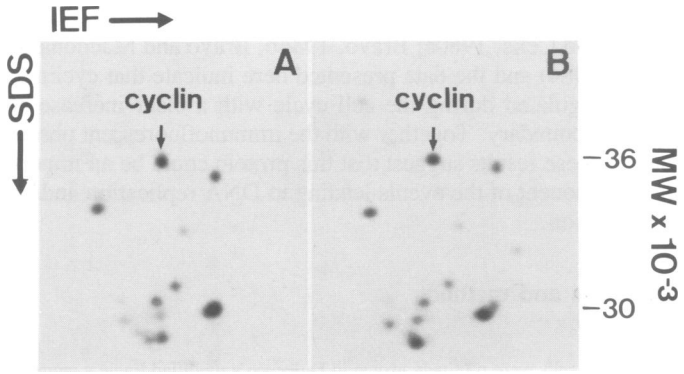

Fig. 8. Pulse-chase analysis of cyclin following hydroxyurea release. Cells were labelled for 30 min with [^{35}S]methionine before removal of the agent. (**A**) Cells before removal of hydroxyurea; (**B**) Cells after 10 h chase.

present a granular pattern confined to only certain nuclear structures (b); a granular pattern throughout the nucleus with the exception of the nucleoi (c); a granular pattern with nucleolar staining (e) and a punctuated pattern of different intensities (g). If quiescent cells are stimulated with 20% FCS in the presence of hydroxyurea (therefore remaining in the G_1/S boundary) they only present the granular pattern through the nuclei with the exception of the nucleoli (Figure 6A). This distribution does not change so long as hydroxyurea is present in the medium. Very similar results have been obtained with aphidicolin, a drug known to inhibit specifically DNA synthesis by interacting with DNA polymerase α (Ikemani *et al.*, 1978; Ohashi *et al.*, 1978; Wist and Prydz, 1979) with no effect on protein or RNA synthesis.

As the results indicated that possibly DNA synthesis itself or other events triggered by DNA replication are needed for the changes in nuclear localization of cyclin (PCNA) we followed these after hydroxyurea release. After removal of the agent cells were labelled every 2 h with [^{3}H]thymidine and processed for immunofluorescence followed by autoradiography. Figure 6 illustrates the changes in nuclear distribution of cyclin (PCNA) after removal of hydroxyurea. At 2 h clear changes in nuclear distribution were detected. Cyclin (PCNA) redistributes revealing a punctuated pattern of staining throughout the nucleus (Figure 6B). A strong staining in globular structures, possibly the nucleoli, can be seen 4 h after hydroxyurea relase (Figure 6C). Thereafter the nuclear staining of cyclin (PCNA) becomes punctuated and the intensity decreases rapidly after 6 h, always showing a clear punctuated pattern (Figure 6C, D). At the end of the S-phase only very few cells show a positive nuclear staining (Figure 6E). A good correlation has been found between the percentage of cells showing immunofluorescence staining and those presenting labelled nuclei as determined by autoradiography. Figure 7 shows the immunofluorescence of cells at the middle (Figure 7A) and at the end of the S-phase (Figure 7C) with their corresponding autoradiographies. It is clear from these studies that cells presenting no cyclin staining are no longer engaged in DNA synthesis. Similar results have also been reported by Celis and Celis (1985).

It was considered possible that cyclin (PCNA) was no longer detected by immunofluorescent staining at the end of the S-phase due to either very rapid degradation during this period, or interaction with other macromolecules that make the protein inaccessible to the antibody. To test this, cells were pulse-labelled with [^{35}S]methionine for 30 min after 12 h of hydroxyurea treatment and chased in the absence of hydroxyurea in complete medium containing 10 times the normal concentration of methionine. Samples were prepared every 2 h up to 10 h and analysed by two-dimensional gel electrophoresis. The results obtained show that cyclin is a very stable protein at least during

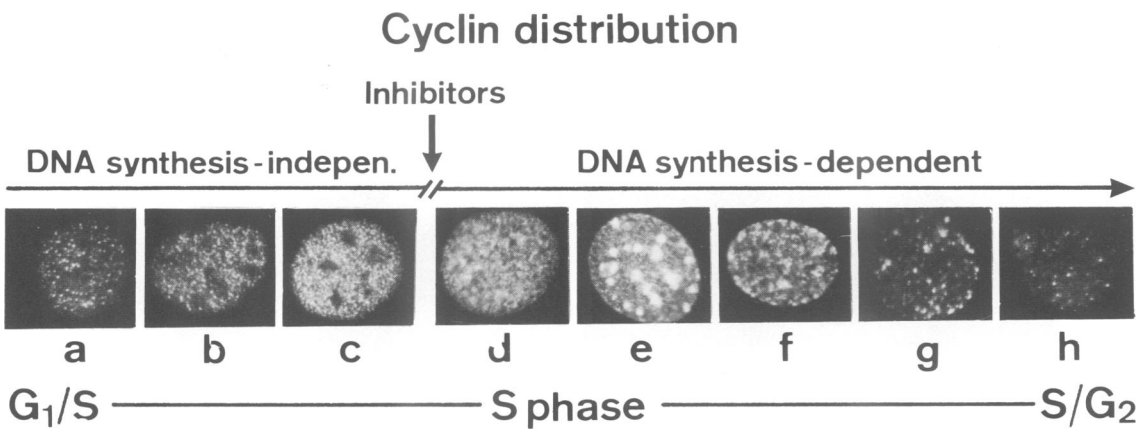

Fig. 9. Proposed sequence of the nuclear distribution of cyclin during the S-phase of 3T3 cells. The sequence was deduced from immunofluorescence studies of serum-stimulated quiescent cells in the presence or absence of hydroxyurea or aphidicolin, and of cells after hydroxyurea release.

the S-phase, no degradation is evident during the 10 h chase period (Figure 8B). This would indicate that the protein is present at the end of the S-phase but that it is possibly no longer accessible to the antibody.

Discussion

Previously studies have shown that cyclin levels are closely related to cell proliferation and suggest that this protein could be an important component in the events leading to DNA replication and cell division (Bravo, 1984b, 1984d; Bravo and Graf, 1985; Celis *et al.*, 1984b). Furthermore there is a close correlation between the levels of cyclin and DNA synthesis induced by serum and purified factors in quiescent 3T3 cells (Bravo and Macdonald-Bravo, 1984). We have shown here that cyclin synthesis can be induced in serum-stimulated 3T3 cells in the presence of hydroxyurea, indicating that the synthesis of this protein is not triggered by DNA replication. Its synthesis is possibly induced very late in G_1 or in the G_1/S boundary. These results are in line with our previous observations that show that aphidicolin does not inhibit induction of cyclin in serum-stimulated quiescent 3T3 cells (Macdonald-Bravo and Bravo, 1985).

Studies on the synthesis of cyclin following hydroxyurea release have shown that its synthesis decreased several fold at the end of S-phase. This corroborates and extends previous observations that revealed a coordinate synthesis of cyclin and DNA after serum-stimulation of quiescent 3T3 cells (Bravo, 1984b; Bravo and Macdonald-Bravo, 1984). The fact that the synthesis of this protein remains high in the presence of hydroxyurea or aphidicolin suggests that the events occurring during the S-phase are relevant for the regulation of cyclin expression and that its synthesis may not be self-regulated.

The immunofluorescence studies confirm that cyclin (PCNA) remains induced in the presence of hydroxyurea and also show that some of its nuclear distribution depends on DNA synthesis itself, or events triggered by DNA replication. Figure 9 shows the tentative sequences of the changes in nuclear localization of cyclin during the S-phase (see also Celis and Celis, 1985). Its early distribution is independent of DNA synthesis, as shown by the hydroxyurea and aphidicolin (not shown) studies. Shortly after DNA synthesis inhibitors are removed, cyclin changes its nuclear localization. At present it is not known if cyclin follows the sites at which DNA replication occurs, as has been recently described for a viral DNA-binding protein (Quinlan *et al.*, 1984). No changes in nuclear localization were found in the presence of hydroxyurea several hours after cyclin induction. Similar results were obtained with aphidicolin suggesting that nuclear migration of cyclin is not due solely to an increase in the amount of protein present in the nucleus. The fact that cyclin (PCNA) is no longer detected by immunofluroescence at the end of the S-phase and that the protein is stable during this period, suggest that cyclin is interacting with other macromolecules that render it inaccessible to the antibody. Possibly, the changes in nuclear localization are driven by the association with specific nuclear proteins and these could be related to the activity of cyclin.

At present there is no clear evidence of the possible role of cyclin in DNA replication or cell proliferation. Previous studies (Bravo and Celis, 1980a; Bravo, 1984b; Bravo and Macdonald-Bravo, 1984) and the data presented here indicate that cyclin is tightly regulated during the cell cycle with a clear increase at the G_1/S boundary. Together with the immunofluorescent observations these results suggest that this protein could be an important component of the events leading to DNA replication and/or cell division.

Materials and methods

Cells

Mouse 3T3 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics (penicillin, 100 units/ml, streptomycin 50 μg/ml).

Labelling of cells with [^{35}S]methionine

Cells were grown in 0.3 cm^2 microtiter plates with 0.2 ml of medium supplemented with 10% FCS. The medium was changed to DMEM containing 0.5% FCS and the cells were used 3 days later. Cells were stimulated by adding DMEM with 20% FCS. Labelling of the cells was carried out for 2 h at the indicated time after stimulation, in 50 μl of medium lacking methionine in the presence of 100 μCi [^{35}S]methionine (Amersham SJ204, UK).

Indirect immunofluorescence

Cells grown on glass coverslips (12 x 12 mm) were washed twice with Hank's and treated for 5 min at −20°C with absolute methanol. After washing extensively in Hank's, the coverslips were covered with 20 μl of anti-PCNA antibody (1:80 dilution in Hank's) and incubated for 1 h at 37°C in a humid environment. The coverslips were washed several times in Hank's and covered with 20 μl of rhodamine-conjugated rabbit anti-human immunoglobulins (DAKO, 1:100 in Hank's). After 1 h incubation at 37°C in a humid environment, the coverslips were washed thoroughly with Hank's and mounted in Mowiol 4-88 (Hoechst).

Two-dimensional gel electrophoresis (Bravo, 1984a, 1984c; Bravo *et al.*, 1982b; O'Farrell, 1975), quantitation of radioactive spots from gels (Bravo *et al.*, 1982b) and DNA synthesis assays (Macdonald-Bravo and Bravo, 1985) have all been described elsewhere.

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References

Adams,R.L.P. and Lindsay,J.G. (1967) *J. Biol. Chem.*, **242**, 1314-1317.
Bravo,R. (1984a) in Celis,J.E. and Bravo,R. (eds.), *Two-dimensional Gel Electrophoresis: Methods and Applications*, Academic Press, NY, pp. 3-36.
Bravo,R. (1984b) *FEBS Lett.*, **169**, 185-188.
Bravo,R. (1984c) in Levine,A., Topp,W., van de Woude,G. and Watson,J.D. (eds.), *The Cancer Cell*, Vol. **1**, Cold Spring Harbor Laboratory Press, NY, pp. 147-151.
Bravo,R. (1984d) *Proc. Natl. Acad. Sci. USA*, **81**, 4848-4850.
Bravo,R. and Graf,T. (1985) *Exp. Cell Res.*, **156**, 450-454.
Bravo,R. and Macdonald-Bravo,H. (1984) *EMBO J.*, **3**, 3177-3181.
Bravo,R. and Celis,J.E. (1980a) *J. Cell Biol.*, **84**, 795-802.
Bravo,R. and Celis,J.E. (1980b) *Cell Res.*, **127**, 249-260.
Bravo,R., Celis,A., Mosses,D. and Celis,J.E. (1981a) *Cell Biol. Int. Rep.*, **5**, 479-489.
Bravo,R., Fey,S.J., Bellatin,J., Mose Larsen,P., Arevalo,J. and Celis,J.E. (1981b) *Exp. Cell Res.*, **136**, 311-319.
Bravo,R., Fey,S.J. and Celis,J.E. (1981c) *Carcinogenesis*, **2**, 769-782.
Bravo,R., Fey,S.J., Bellatin,J., Mose Larsen,P. and Celis,J.E. (1982a) in Burger,M. (ed.), *Progress in Clinical and Biological Research*, Vol. **854**, Allan R.Liss, pp. 235-248.
Bravo,R., Small,J.V., Fey,S.J., Mose Larsen,P. and Celis,J.E. (1982b) *J. Mol. Biol.*, **154**, 121-143.
Celis,J.E. and Celis,A. (1985) *Proc. Natl. Acad. Sci. USA*, in press.
Celis,J.E., Bravo,R., Mose Larsen,P., Fey,S.J., Bellatin,J. and Celis,A. (1984a) in Celis,J.E. and Bravo,R. (eds.), *Two-dimensional Gel Electrophoresis: Methods and Applications*, Academic Press, NY, pp. 307-362.
Celis,J.E., Bravo,R., Mose Larsen,P. and Fey,S.J. (1984b) *Leukaemia Res.*, **8**, 143-157.
Ikegami,S., Taguchi,T., Ohashi,M., Oguro,M., Nagano,H. and Mano,J. (1978) *Nature*, **275**, 458-460.
Macdonald-Bravo,H. and Bravo,R. (1985) *Exp. Cell Res.*, **156**, 455-461.
Mathews,M.B., Bernstein,R.M., Franza,B.R. and Garrels,J.I. (1984) *Nature*, **309**, 374-376.
Miyashi,K., Fritzler,M.J. and Tan,E.M. (1978) *J. Immunol.*, **121**, 2228-2234.
O'Farrell,P.H. (1975) *J. Biol. Chem.*, **250**, 4007-4021.
Ohashi,M., Taguchi,T. and Ikegami,S. (1978) *Biochem. Biophys. Res. Commun.*, **82**, 1084-1090.
Quinlan,M.P., Chen,L.B. and Knipe,D.M. (1984) *Cell*, **36**, 857-868.
Takasaki,Y., Deng,J.-S. and Tan,E.M. (1981) *J. Exp. Med.*, **154**, 1899-1909.
Takasaki,Y., Fischwild,D. and Tan,E.M. (1984) *J. Exp. Med.*, **159**, 981-992.
Tan,E.M. (1982) *Adv. Immunol.*, **33**, 167-240.
Wist,E. and Prydz,H. (1979) *Nucleic Acids Res.*, **6**, 1583-1590.

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