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Induction of the nuclear protein 'cyclin' in quiescent mouse 3T3 cells stimulated by serum and growth factors. Correlation with DNA synthesis

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The effect of serum and growth factors [platelet-derived growth factor (PDGF), fibroblast growth factor (FGF)] on the synthesis of the nuclear protein cyclin and its correlation with DNA synthesis has been studied in quiescent mouse 3T3 cells by means of quantitative two-dimensional gel electrophoresis. Serum must be present in the medium for at least 8–12 h to induce maximal synthesis of cyclin (6- to 7-fold increase compared with quiescent cells). The stimulation of cyclin synthesis is dose-dependent and correlates directly with DNA synthesis. In addition, partially purified PDGF and FGF also induce cyclin and DNA synthesis in a coordinate way. Both growth factors, like serum, exhibit a similar lag phase to induce maximal cyclin (6- to 7-fold) and DNA synthesis (90% of the cells). Pure PDGF at a concentration as low as 10 ng/ml has the same effect as 10% serum. The coordinate induction of cyclin and DNA synthesis can only be observed with growth factors that induce DNA synthesis. These results strengthen the notion that cyclin is an essential component of the events leading to DNA replication.

Key words: polypeptide synthesis/platelet-derived growth factor/[^{35}S]methionine labelling/two-dimensional gel 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 acidic 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 human, mouse, hamster and avian cell types (Bravo *et al.*, 1981a, 1981b, 1981c, 1982a; Bravo and Graf, 1984; Celis *et al.*, 1984a). This protein is synthesized by normal proliferating cells as well as transformed cells and tumours but it is present in very small amounts in non-dividing cells and tissues (see Celis *et al.*, 1984b). The levels of cyclin fluctuate during the cell cycle with a specific increase in the S phase (Bravo and Celis, 1980a). Furthermore, the synthesis of cyclin and DNA has bee shown to be closely related in serum-stimulated cells (Bravo, 1984b). Many of the properties of cyclin were known to be shared by PCNA, the human proliferating cell nuclear antigen (Miyashi *et al.*, 1978; Takasaki *et al.*, 1981, 1984; Tan, 1982); PCNA has recently been shown to be cyclin (Mathews *et al.*, 1984).

As suggested previously (Bravo and Celis, 1980a; Bravo, 1984b; Macdonald-Bravo and Bravo, 1984), cyclin seemed to be an important component of the events leading to DNA synthesis. Therefore we have analysed in detail the relationship between the induction of cyclin synthesis and DNA replication in quiescent mouse 3T3 cells stimulated by serum or by purified growth factors.

Results

Induction of cyclin parallels induction of DNA synthesis in serum-stimulated quiescent cells

The two-dimensional gel analysis of the acidic [isoelectric focussing (IEF), Bravo *et al.*, 1984c] [^{35}S]methionine-labelled polypeptides of asynchronous mouse 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. The amount of labelled cyclin in proliferating 3T3 cells corresponds to ~0.1% of the total radioactive proteins (Bravo and Celis, 1980b). The identity of cyclin in these cells has been confirmed by one-dimensional peptide mapping (Bravo, 1984b).

Autoradiography shows that the induction of quiescent cells to synthesize DNA is dependent on serum concentration (see Figure 3). Since cyclin and DNA synthesis are closely related (Bravo and Celis, 1980a; Bravo, 1984b) we studied whether the induction of cyclin followed a dose response similar to DNA synthesis. For this, quiescent cells were stimulated for 22 h with different concentrations of serum, labelled for 2 h with [^{35}S]methionine and analysed by quantitative two-dimensional gel electrophoresis (Bravo, 1984a, 1984c; Bravo *et al.*, 1982b; O'Farrell, 1975). These results are shown in Figure 2 and their quantitative analysis is presented in Figure 3. Cyclin synthesis was slightly induced by 0.5% serum and reached a maximum with 5% serum. This represents a 6- to 7-fold increase when compared with quiescent cells. These studies demonstrate that the induction of cyclin and DNA synthesis in serum-stimulated quiescent 3T3 cells shows a similar dose response.

To establish that the induction of DNA synthesis in serum-stimulated quiescent cells was directly related to the capacity of serum to induce cyclin synthesis, we did similar experiments with platelet-poor plasma, (PPP, Pledger *et al.*, 1977) since its capacity to induce DNA synthesis in quiescent cells is markedly decreased (Pledger *et al.*, 1977). These studies show that 5% PPP induces a very small increase in cyclin synthesis compared with 5% serum. Simiarly, platelet-poor plasma is a weak inducer of DNA synthesis when compared with serum (Figure 3). The results indicate that a component(s) present in serum but not in platelet-poor plasma, [possibly platelet-derived growth factor (PDGF)], is needed for both the induction of cyclin and DNA synthesis.

With most cell types studied, there is a prerequisite to expose the cell to the growth factors for at least 8–12 h to commit the majority of cells to DNA synthesis (Aharonov *et al.*, 1978; Goldberger and Yamamoto, 1982; Guroff, 1983; Haigler and Carpenter, 1980). The notion that cylcin could be a late component of the events leading to DNA replication (Bravo, 1984b; Macdonald-Bravo and Bravo, 1984) made it interesting to establish whether cyclin induction also required

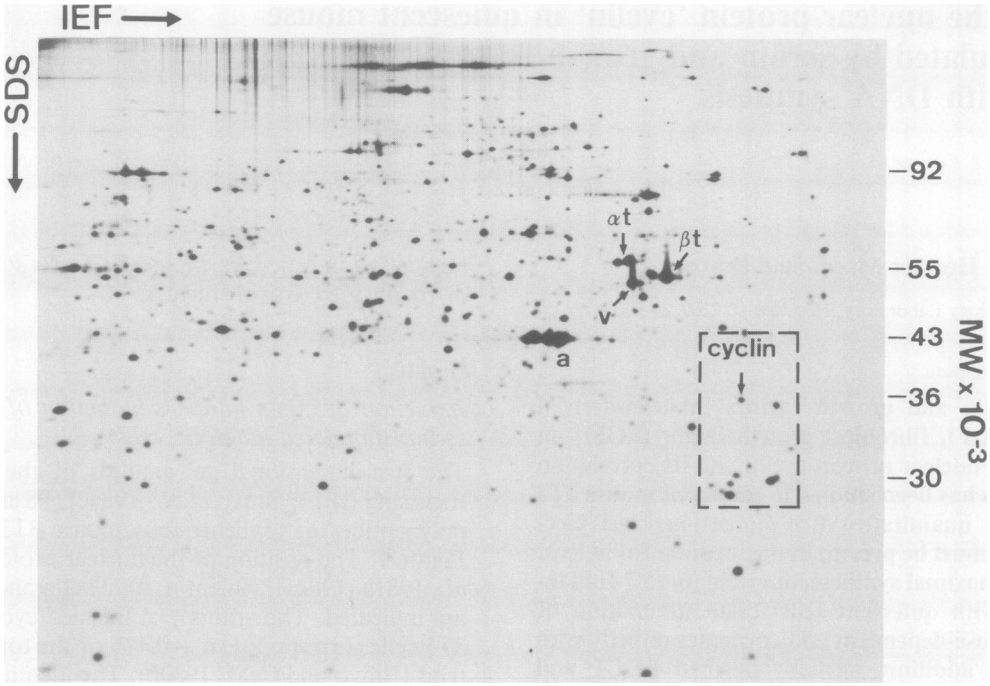

Fig. 1. Two-dimensional gel electrophoresis (IEF) of [^{35}S]methionine-labelled polypeptides from asynchronous mouse 3T3 cells. Cells were labelled for 16 h with [^{35}S]methionine (1 mCi/ml) as described (Bravo *et al.*, 1982b). a, actin; αt, α-tubulin; βt, β-tubulin; v, vimentin. The area of interest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

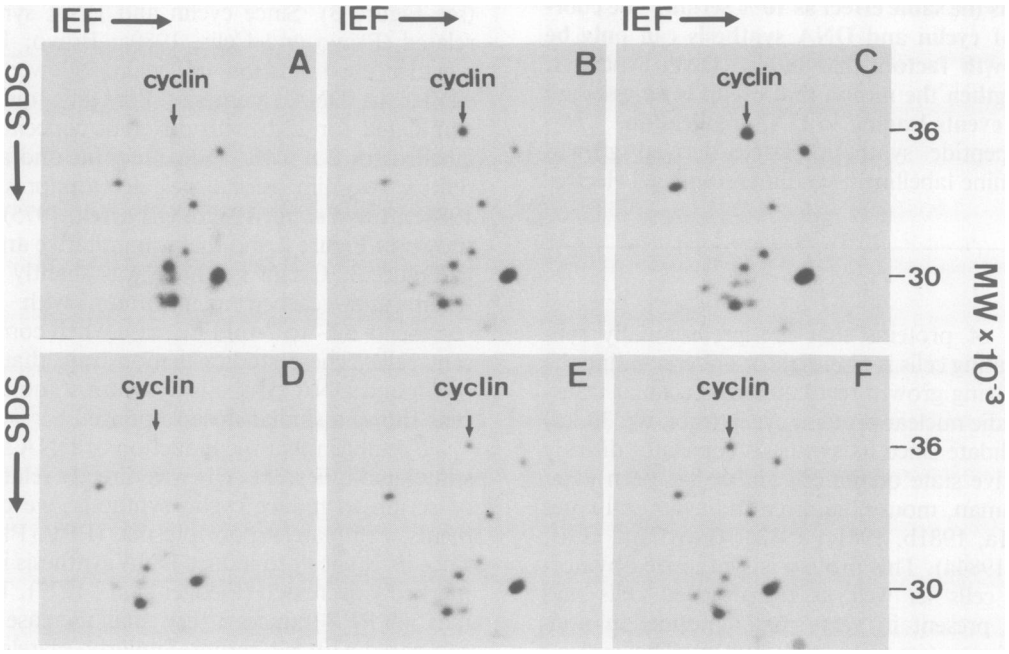

Fig 2. Effect of FCS or PPP on cyclin synthesis in quiescent mouse 3T3 cells. **(A)** Quiescent non-stimulated cells. Quiescent cells stimulated with **(B)** 1% FCS, **(C)** 5% FCS, **(D)** 1% PPP, **(E)** 5% PPP, **(F)** 10% PPP. Cells were labelled for 2 h after 22 h stimulation. Only the area of interest of the gel is shown.

the presence of serum for several hours to reach a maximal response. For this, quiescent cells were stimulated with serum for different times, then cultured in serum-free Dulbecco's modified Eagle's medium (DMEM) and labelled for 2 h with [^{35}S]methionine always 22 h after the onset of stimulation. The labelled proteins were analysed for induction of cyclin synthesis as described before. No induction of cyclin synthesis was detected after 24 h when quiescent cells were exposed to serum for <4 h and a maximal stimulation of cyclin was obtained only if the quiescent cells were exposed to serum for 12 h or more (not shown). These results are similar to those obtained for the induction of DNA synthesis (not shown), suggesting that induction of cyclin synthesis and DNA replication are closely related.

Growth factors induce cyclin synthesis in quiescent cells

From the observations described above and as some growth factors stimulate the initiation of DNA synthesis in cultures of quiescent cells in a concentration-dependent manner, we decided to investigate if these factors also induce cyclin syn-

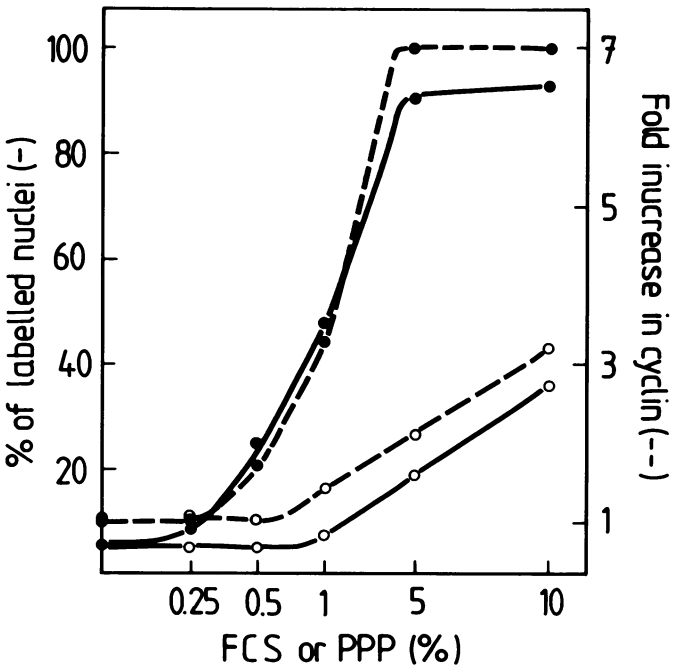

Fig. 3. Induction of cyclin and DNA synthesis in quiescent mouse 3T3 cells stimulated with different concentrations of FCS (●) or PPP (○). Cells were labelled for 24 h with 1 μCi/ml of [^{3}H]thymidine and processed for autoradiography according to standard procedures. About 500 cells were counted in each case in duplicate. 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 cells. Equal amounts of incorporated radioactivity were applied to the gels.

thesis. Quiescent mouse 3T3 cells were treated with different concentrations of growth factor for 22 h before being labelled for another 2 h with [^{35}S]methionine. Figure 4 shows the results obtained with semi-purified PDGF and fibroblast growth factor (FGF). Induction of cyclin is maximal (6- to 7-fold the synthesis found in quiescent cells) with 100 ng/ml PDGF or FGF. A similar increase is obtained with optimal serum concentration (see Figure 2). Determination by autoradiography shows that this concentration of growth factor induces the majority of the cells (85–90%) to DNA synthesis (not shown). Other growth factors like insulin, hydrocortisone and $PGF_{2\alpha}$ which do not induce DNA synthesis in the cells did not stimulate cyclin synthesis (not shown).

To eliminate the possibility that contaminants of the growth factors are responsible for stimulating cyclin synthesis we have done similar studies with pure PDGF. Some of these results are presented in Figure 5 and their quantitative analysis is shown in Figure 6. In this case a maximal induction of cyclin and DNA synthesis is obtained with 5 ng/ml of growth factor. Again, a good correlation between cyclin induction and DNA synthesis was observed at the different doses used.

An increase in cyclin synthesis occurs 8–10 h after serum stimulation, reaching a maximum at 16–18 h (Bravo, 1984b; Macdonald-Bravo and Bravo, 1984). To see if PDGF induces cyclin in a similar manner, we stimulated quiescent cells with pure PDGF (10 ng/ml) and labelled them for 2 h every 2 h. The levels of cyclin were determined by quantitative two-dimensional gel electrophoresis analysis of the [^{35}S]-methionine-labelled polypeptides at various times after PDGF stimulation. Some of these results are illustrated in

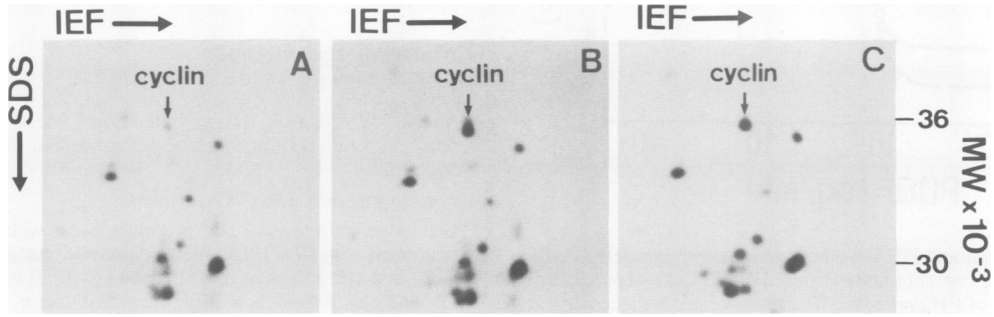

Fig. 4. Effect of growth factors on cyclin synthesis in quiescent mouse 3T3 cells. **(A)** Quiescent non-stimulated cells. Quiescent cells stimulated with **(B)** 100 ng/ml FGF, **(C)** 100 ng/ml PDGF. Cells were labelled for 2 h after 22 h stimulation.

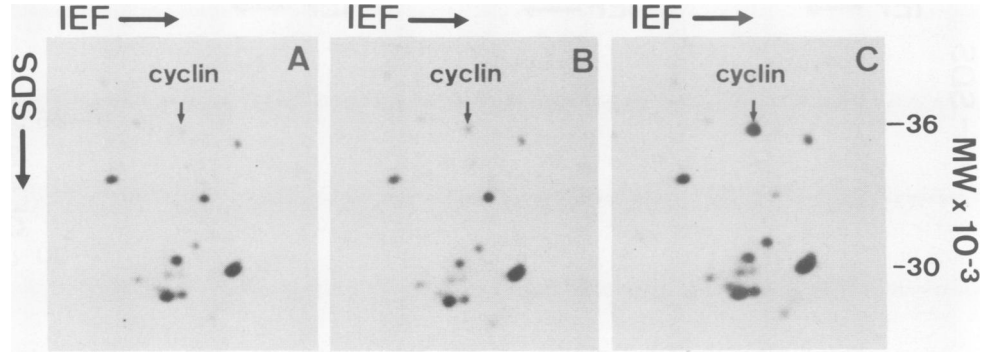

Fig. 5. Effect of pure PDGF on cyclin synthesis in quiescent mouse 3T3 cells. **(A)** Quiescent non-stimulated cells. Quiescent cells stimulated with pure PDGF **(B)** 1 ng/ml, **(C)** 5 ng/ml. Cells were labelled with [^{35}S]methionine for 2 h after 22 h stimulation.

Figure 7 and the quantitative data is presented in Figure 8. The results show that induction of cyclin after PDGF stimulation of quiescent cells follows similar kinetics to those previously described for serum (Macdonald-Bravo and Bravo, 1984). The same kinetics of induction are obtained when FGF is used (not shown). As for serum, the culture must be exposed to PDGF or FGF for at least 8–12 h to induce the majority of the cells to cyclin and DNA synthesis (not shown). Quiescent cells treated with epidermal growth factor (EGF) only show a weak response compared with serum as determined by DNA and cyclin synthesis. At concentrations as high as 100 ng/ml only 40–50% of the cells are stimulated (not shown).

Discussion

Cyclin levels are closely related to cell proliferation and this suggests that cyclin could be an important component of the events leading to DNA replication and cell division (Bravo, 1984b, 1984d; Bravo and Graf, 1984; Celis *et al.*, 1984b). The present results show that an exposure time of at least 8–12 h to serum or purified growth factors is required to induce maximal cyclin synthesis and this correlates closely with the time needed to commit the majority of the cells to DNA synthesis. Cyclin induction shortly precedes DNA synthesis in stimulated quiescent 3T3 cells (Macdonald-Bravo and Bravo, 1984). Furthermore, induction of cyclin does not require DNA synthesis (Macdonald-Bravo and Bravo, 1984). Studies using hydroxyurea (Tobey, 1973) indicate that cyclin induction is triggered in late G_1 or at the G_1/S boundary (R.Bravo and H.Macdonald-Bravo, unpublished observation). Together these findings suggest that the induction of cyclin is possibly one of the latest events ultimately leading to DNA replication.

Our present data show a close correlation between the levels of cyclin and DNA synthesis in 3T3 cells induced by serum and purified growth factors. A431 cells inhibited to grow by EGF show a decrease in cyclin and DNA synthesis (Bravo, 1984d). On the other hand, A431 cells that are resistant to the growth inhibitory effect of EGF do not exhibit changes in the level of this protein after EGF treatment (Bravo, 1984d). Taken together, these results demonstrate that induction or inhibition of cyclin and DNA synthesis closely parallel each other in cultured cells.

Evidence indicating that cyclin induction could be an obligatory step for DNA synthesis has come from the observations that microinjection of DNA from adenoviruses and SV40 in quiescent mouse 3T3 cells induces cyclin together with DNA synthesis (S.Stabel and R.Bravo, unpublished observations). Work is now in progress to elucidate the possible mechanisms that control the levels of cyclin during the cell cycle.

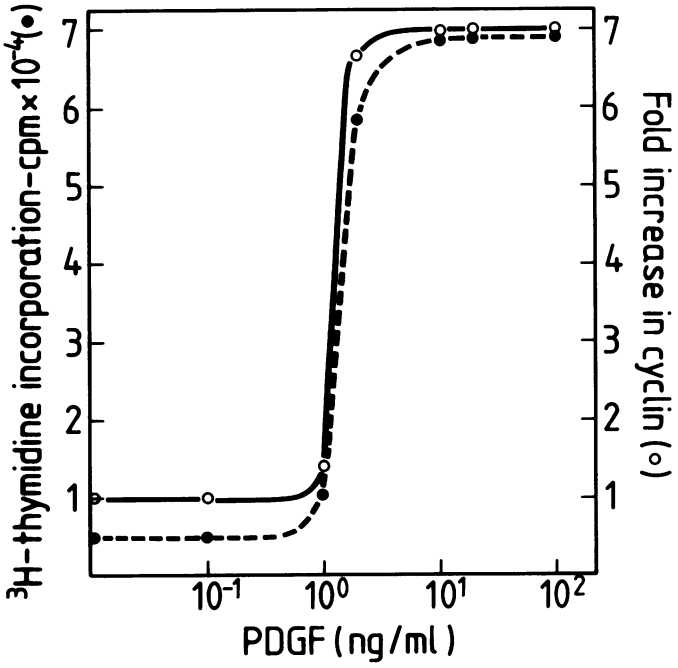

Fig. 6. Induction of cyclin and DNA synthesis in quiescent mouse 3T3 cells stimulated with different concentrations of pure PDGF. Cells were labelled for 24 h with 1 μCi/ml of [^{3}H]thymidine. The incorporated radioactivity was determined as described (Macdonald-Bravo and Bravo, 1984). Labelling with [^{35}S]methionine after 22 h stimulation was as described in Materials and methods.

Materials and methods

Cells

Mouse 3T3 cells were routinely grown in DMEM supplemented with 10% fetal calf serum (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% and the cells were used 3 days later. Cells were stimulated by adding DMEM with 20% FCS or a specific growth factor. 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).

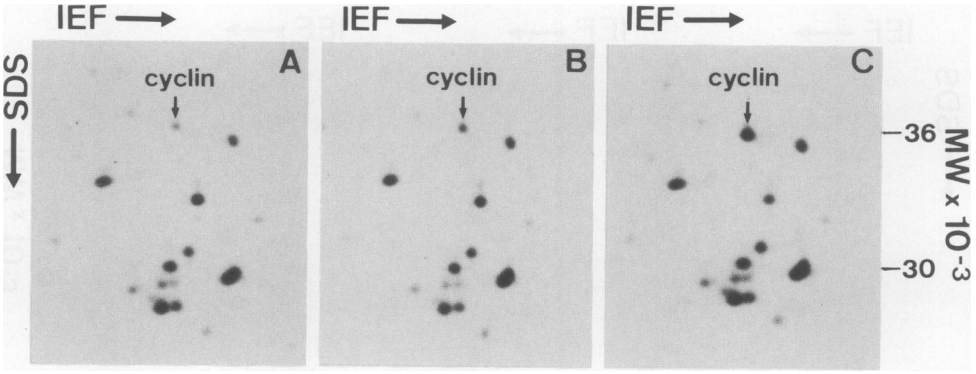

Fig. 7. Cyclin synthesis in PDGF-stimulated quiescent 3T3 cells for different periods. Cells after **(A)** 6 h stimulation, **(B)** 10 h stimulation, **(C)** 16 h stimulation. The cells were always labelled for 2 h.

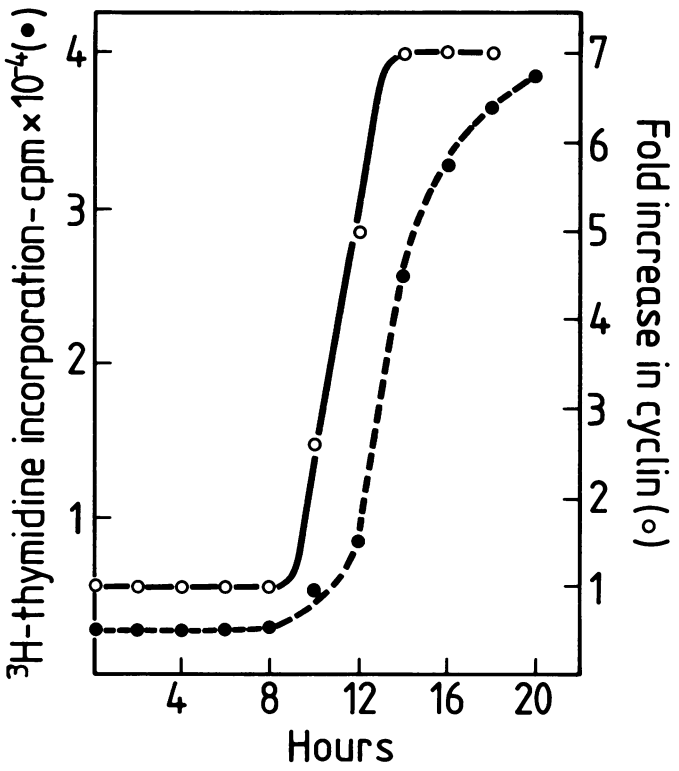

Fig. 8. Synthesis of cyclin and DNA after PDGF stimulation of quiescent 3T3 cells. Cells were labelled for 2 h with [^{35}S]methionine and [^{3}H]thymidine at each indicated time following PDGF stimulation. Cyclin synthesis was quantitated as described (Bravo *et al.*, 1982b). The data are given as the fold increase of cyclin compared with non-stimulated quiescent cells. [^{3}H]thymidine incorporation was determined as described (Macdonald-Bravo and Bravo, 1984).

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, 1984) have all been described elsewhere.

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