# Changes in the nuclear distribution of cyclin (PCNA) during S-phase are not triggered by post-translational modifications that are expected to moderately affect its charge

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Indirect immunofluorescence studies of HeLa cells using PCNA autoantibodies specific for cyclin have revealed striking changes in the nuclear localization of this protein during S-phase. Two-dimensional gel electrophoretic analysis of the [$^{32}$P]orthophosphate and [$^{35}$S]methionine labelled proteins from synchronized cells showed that phosphorylation, or other post-translational modifications that are expected to moderately affect the charge of cyclin (acetylation, glycosylation, sialylation, etc.) are not likely part of the mechanism(s) triggering the migration of this protein.

*Cell cycle* *Cyclin (PCNA) synthesis* *PCNA antibody* *Immunofluorescence* *[$^{35}$S]Methionine labelling* *[$^{32}$P]Orthophosphate labelling* *Two-dimensional gel electrophoresis*

## 1. INTRODUCTION

Cyclin ($M_r$ = 36000; fig.1) ([1]; review, [2]), also termed PCNA [3–8], is an acidic nuclear nonhistone protein whose rate of synthesis is modulated during the cell cycle (increases in S-phase [9]) and correlates directly with the proliferative state of normal cells ([2,10–13] and references therein). Transformed cells synthesize this protein in elevated, although variable amounts [14–27], and there is evidence indicating that cyclin may be partly phosphorylated [7].

Immunofluorescence studies of transformed human amnion cells (AMA) [28] and of other cells of vertebrate origin [4,28,29] using PCNA autoantibodies specific for cyclin have revealed dramatic changes in the nuclear localization of this protein during the cell cycle, particularly within S-phase. These observations raised important questions concerning the mechanism(s) governing the migration of this protein, and in particular to the nature of the signal that triggers these events during the cell cycle [28,29]. Here, we present evidence showing that phosphorylation, or other post-translational modifications that are expected to moderately affect the charge of cyclin, are not likely part of the mechanism(s) triggering the differential distribution of this protein during S-phase.

*Abbreviations:* IEF, isoelectric focussing; PCNA, proliferating cell nuclear antigen

## 2. MATERIALS AND METHODS

### 2.1. *Cells and cell synchrony*

HeLa cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics (100 units penicillin per ml; 50 $\mu$g streptomycin per ml).

Mitotic cells were prepared by gentle mechanical detachment essentially as previously described [9,30]. One 250 ml flask containing 1–2 $\times$ $10^6$ cells was used. The distribution of phases was determined by phase contrast microscopy [9].

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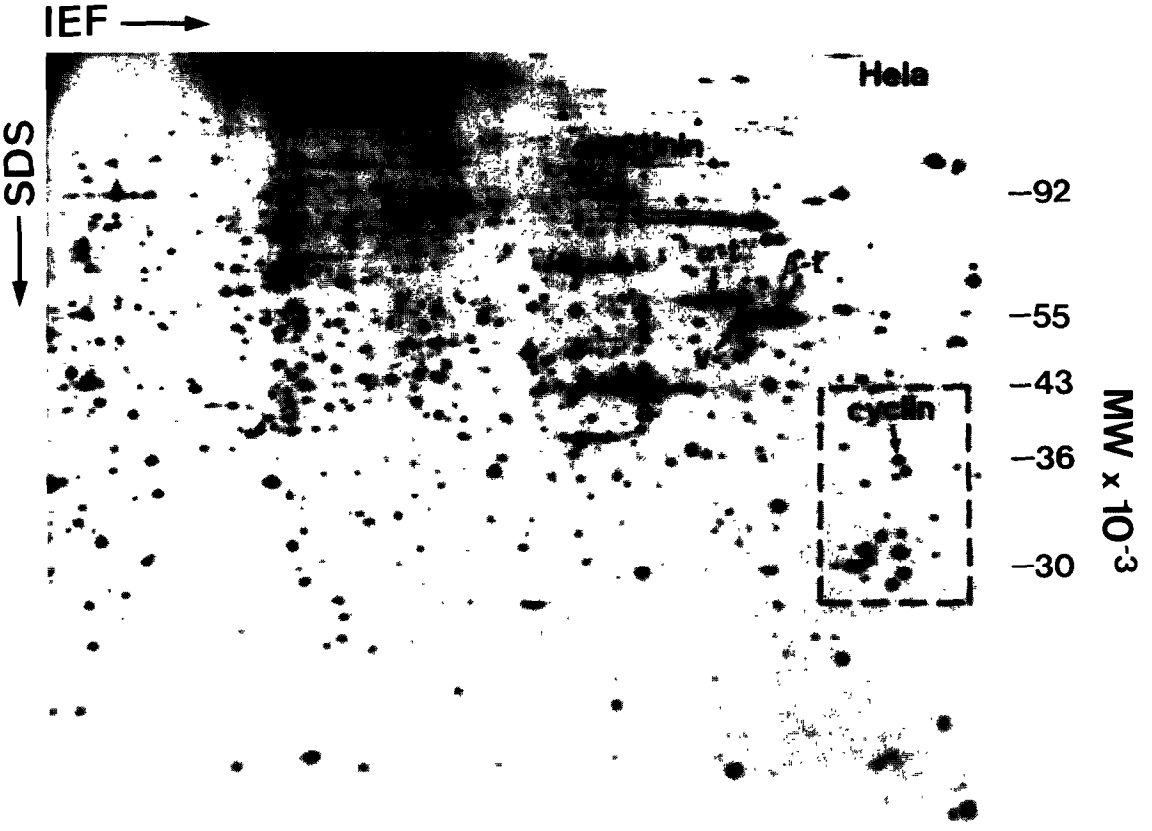


Fig.1. Two-dimensional gel electrophoresis (IEF) of [$^{35}$S]methionine labelled proteins from asynchronous HeLa cells. Cells were labelled for 16 h with [$^{35}$S]methionine (1 mCi/ml). The positions of $\alpha$- and $\beta$-tubulin ($\alpha$-t, $\beta$-t), vimentin (v) and actin (a) are indicated for reference. The area where cyclin migrates has been enclosed in a box. Cyclin corresponds to IEF 49 in the HeLa protein catalogue [36–38].

### 2.2. *Other procedures*

The procedures for labelling cells with [$^{35}$S]methionine, [$^{32}$P]orthophosphate [31], two-dimensional gel electrophoresis [32–34], autoradiography [18], and indirect immunofluorescence [35] have been described in detail elsewhere.

## 3. RESULTS AND DISCUSSION

Fig.2 shows an immunofluorescence micrograph of asynchronously growing HeLa cells treated with methanol and reacted with PCNA antibodies specific for cyclin [7]. About 40% of the total cell population (S-phase cells) stain strongly with the antibody to reveal variable nuclear staining patterns both in terms of the intensity and distribution of the antigen [28,29]. Very little cytoplasmic staining is observed. Various S-phase patterns of cyclin staining are indicated in fig.2 with an S and a letter corresponding to the homologous patterns originally described for AMA cells [28]. Patterns Sb and c (granular staining, nucleolar exclusion) are found at the beginning of S-phase (9–12 h after plating mitotic cells) and their relative order has been determined by the analysis of synchronized cells. The stronger cyclin staining observed in pattern Sc most likely reflects an increased synthesis of cyclin (see fig.3; [28,29]). Patterns Sd (well defined nucleolar exclusion, but less intense than Sc), Se (punctuated pattern with foci of staining throughout the nucleus) and Sf (as Sd but with nucleolar staining) are found later in S-phase (see also [28,29]). There are a few other patterns of cyclin staining (punctuated and of decreasing intensity) which are not present in fig.2 and that are

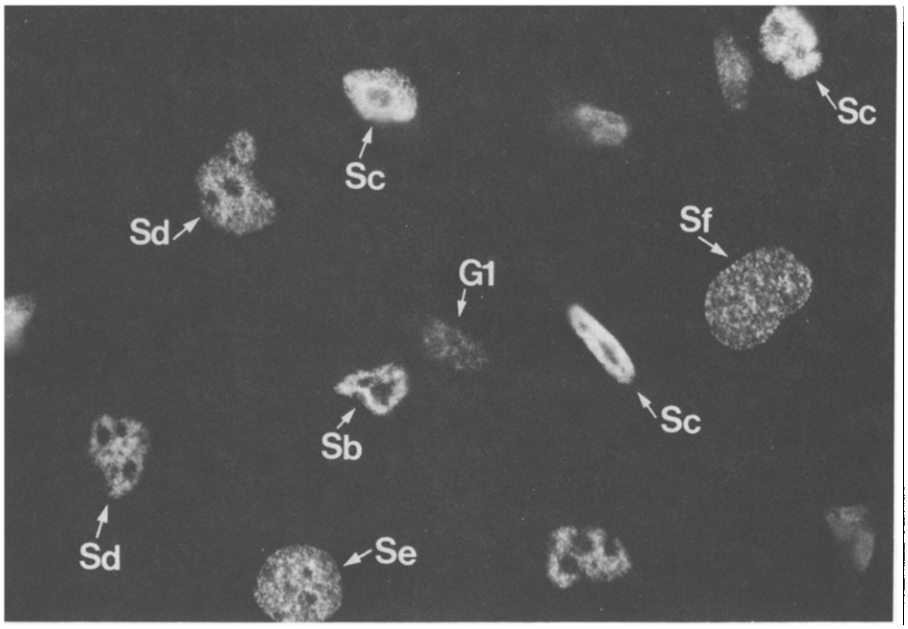


Fig.2. Immunofluorescence patterns of nuclear cyclin staining. Asynchronous HeLa cells grown in coverslips were treated with methanol prior to immunofluorescence using PCNA antibodies specific for cyclin.

observed mainly late in S-phase [28,29]. It should be emphasized that we have not determined the exact order of the late S-phase patterns, although this may be similar to that previously described for AMA [28] and mouse 3T3 cells [29]. $G_1$ (fig.2), $G_2$ (not shown) and mitotic cells (not shown) react only weakly with the antibody, although the $G_1$ pattern is quite characteristic (see also, [28]).

Since there is evidence indicating that cyclin may be partly phosphorylated in HeLa cells [7], it seemed possible that the changes in the nuclear localization of this protein during S-phase may reflect preferential phosphorylation or dephosphorylation of this protein. To explore this possibility we carried out a detailed 2-dimensional gel electrophoresis (IEF) analysis of the [$^{32}$P]orthophosphate labelled proteins produced at various stages of the cell cycle. Fig.3 shows examples (only the appropriate region of the gel is shown) of [$^{32}$P]orthophosphate labelled proteins from synchronized HeLa cells labelled for 2 h beginning at 2 ($G_1$, fig.3a), 10.45 (early S, fig.3b) and 15.30 h (late S, fig.3c) after plating mitotic cells. The position of cyclin (arrowheads; no phosphoprotein is observed at this position) was determined by co-running [$^{35}$S]methionine and [$^{32}$P]orthophosphate labelled samples.

Careful observation of the gels presented in fig.3, as well as of gels exposed for prolonged periods of time, failed to reveal acidic satellite spots that may correspond to phosphorylated variants of cyclin. Similar analysis of [$^{32}$P]orthophosphate labelled proteins from asynchronous (fig.4, overexposed to show details around the position of cyclin) and mitotic cells (not shown) supported the above observations and further suggested that cyclin may not be phosphorylated at any stage of the cell cycle. The reason for the discrepancy between our results and those previously reported [7] are at present unclear. It should be emphasized, however, that we have not detected phosphorylated variants of cyclin in gels of [$^{32}$P]orthophosphate labelled proteins prepared from other cultured cells of human (AMA, Detroit 98, W138, W138-SV40) or mouse origin (3T3, S-180).

Two-dimensional gel electrophoretic analysis of [$^{35}$S]methionine labelled proteins obtained from synchronized HeLa cells [$G_1$ and S (early and late), fig.5] confirmed and extended the above observations as neither acidic nor basic satellite spots could be detected (fig.5). These results imply that post-translational modifications that are expected to moderately affect the charge of cyclin (acetyla-


Fig.3. Two-dimensional gel electrophoresis (IEF) of [$^{32}$P]orthophosphate labelled proteins from synchronized HeLa cells. Mitotic cells obtained by mechanical detachment were plated in Costar plates and labelled for 2 h with [$^{32}$P]orthophosphate starting at (a) 2 ($G_1$); (b) 10.45 (early S) and (c) 15.30 h (late S) after plating. Only the relevant areas of the gels are shown. The arrowheads indicate the position of cyclin as determined by co-running [$^{35}$S]methionine and [$^{32}$P]orthophosphate labelled samples. A few phosphoproteins are indicated for reference [37,38].

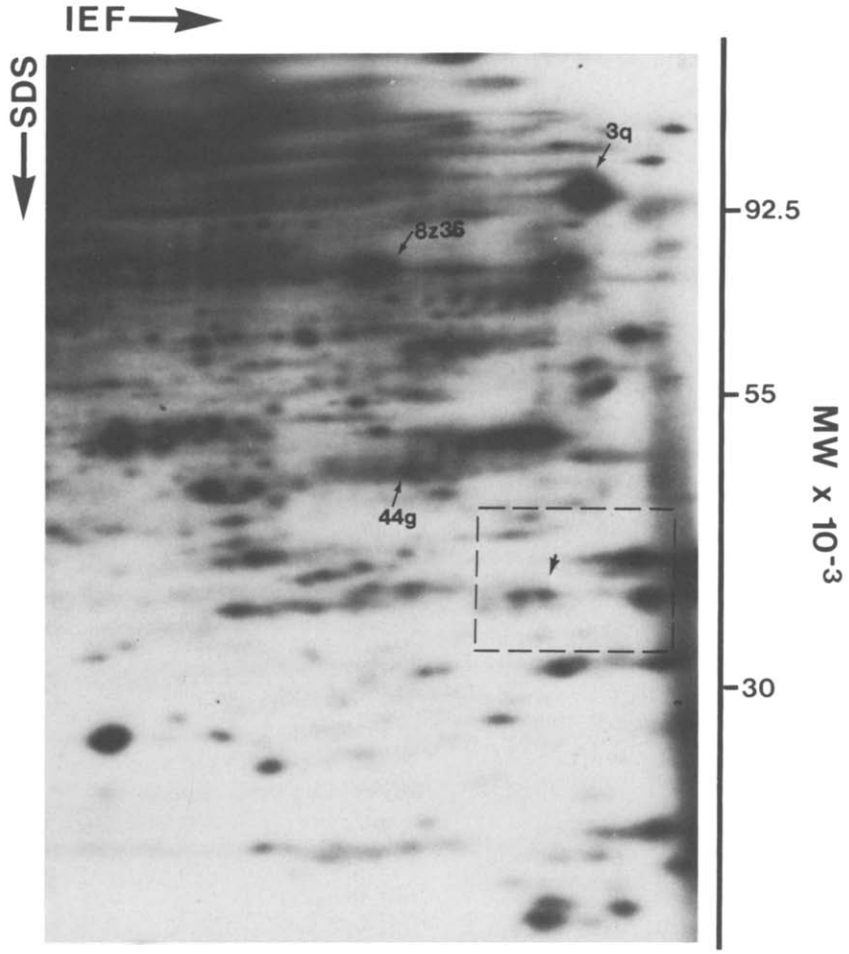


Fig.4. Two-dimensional gel electrophoresis (IEF) of proteins from asynchronous HeLa cells labelled for 2 h with [$^{32}$P]orthophosphate. Only a region of the gel is shown. The arrowhead indicates the position of cyclin. A few phosphoproteins are indicated for reference [37,38].

tion, glycosylation, sialylation, etc.) are not likely part of the mechanism(s) triggering the differential migration of this protein during S-phase. Furthermore, similar gel analysis of asynchronous as well as mitotic HeLa cells (not shown) has suggested that cyclin may not be significantly modified at any particular stage of the cell cycle.

At present, little is known concerning the mechanism(s) that control cyclin migration within defined nuclear compartments during S-phase. Our results, however, raise the possibility that the changes in the nuclear localization of this protein may be driven by association with other macromolecule(s) [28,29].

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## REFERENCES

[1] Bravo, R., Fey, S.J., Bellatin, J., Mose Larsen, P., Arevalo, J. and Celis, J.E. (1981) Exp. Cell Res. 136, 311–319.
[2] Celis, J.E., Bravo, R., Mose Larsen, P. and Fey, S.J. (1984) Leuk. Res. 8, 143–157.
[3] Miyachi, K., Fritzler, M.J. and Tan, E.M. (1978) J. Immunol. 121, 2228–2234.

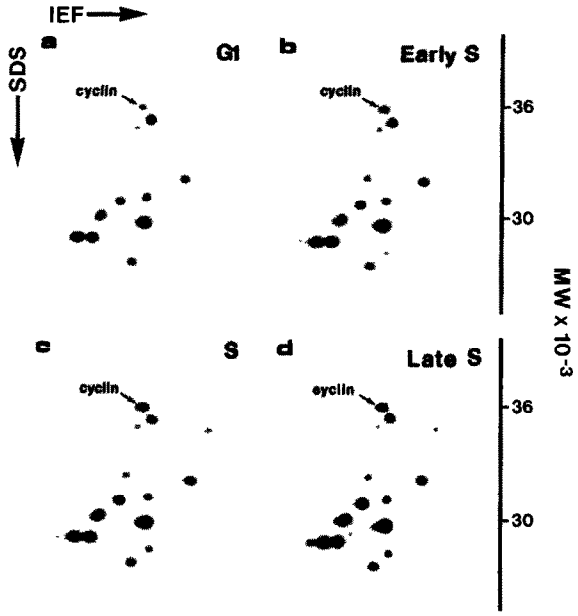


Fig.5. Synthesis of [$^{35}$S]methionine labelled cyclin in synchronized HeLa cells. Mitotic cells obtained by mechanical detachment were plated in microtiter plates and labelled for 2 h with [$^{35}$S]methionine (1 mCi/ml) starting at (a) 3 ($G_1$); (b) 10 (early S); (c) 14 (S) and (d) 18 h (late S-phase) after plating. Only the relevant areas of the gels are shown.

[4] Takasaki, Y., Deng, J.S. and Tan, E.M. (1981) J. Exp. Med. 154, 1899–1909.
[5] Tan, E.M. (1982) Adv. Immunol. 33, 167–240.
[6] Takasaki, Y., Fischwild, D. and Tan, E.M. (1984) J. Exp. Med. 159, 981–982.
[7] Mathews, M.B., Bernstein, R.M., Franza, R. and Garrels, J.I. (1984) Nature 309, 274–276.
[8] Takasaki, Y., Fischwild, D. and Tan, E.M. (1984) J. Exp. Med. 159, 981–985.
[9] Bravo, R. and Celis, J.E. (1980) J. Cell Biol. 48, 795–802.
[10] Celis, J.E., Bravo, R., Mose Larsen, P., Fey, S.J., Bellatin, J. and Celis, A. (1984) in: Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications (Celis, J.E. and Bravo, R. eds) pp.308–362, Academic Press, New York.
[11] Celis, J.E. and Bravo, R. (1983) FEBS Lett. 165, 21–25.
[12] Bravo, R. (1984) FEBS Lett. 169, 185–188.
[13] Bravo, R. and Macdonald-Bravo, H. (1984) EMBO J. 13, 3177–3188
[14] Bravo, R. and Celis, J.E. (1980) Exp. Cell Res. 127, 249–260.
[15] Bravo, R., Fey, S.J. and Celis, J.E. (1981) Carcinogenesis 2, 769–782.
[16] Bravo, R., Fey, S.J., Bellatin, J., Mose Larsen, P. and Celis, J.E. (1982) in: Embryonic Development, Part A (Burger, M. ed.) pp.235–248, Liss, New York.
[17] Bravo, R. and Celis, J.E. (1982) Clin. Chem. (Winston-Salem, NC) 28, 949–954.
[18] Bellatin, J., Bravo, R. and Celis, J.E. (1982) Proc. Natl. Acad. Sci. USA 79, 4367–4370.
[19] Bravo, R., Bellatin, J., Fey, S.J., Mose Larsen, P. and Celis, J.E. (1983) in: Gene Expression in Normal and Transformed Cells (Celis, J.E. and Bravo, R. eds) pp.291–314, Plenum, New York.
[20] Forchhammer, J. and Macdonald-Bravo, H. (1983) in: Gene Expression in Normal and Transformed Cells (Celis, J.E. and Bravo, R. eds) pp.291–314, Plenum, New York.
[21] Celis, J.E., Fey, S.J., Mose Larsen, P. and Celis, A. (1984) Cancer Cells 1, 123–135.
[22] Franza, B.R. and Garrels, J.I. jr (1984) Cancer Cells 1, 137–146.
[23] Bravo, R. (1984) Cancer Cells 1, 147–151.
[24] Bravo, R. (1984) Proc. Natl. Acad. Sci. USA 81, 4848–4850.
[25] Celis, J.E., Fey, S.J., Mose Larsen, P. and Celis, A. (1984) Proc. Natl. Acad. Sci. USA 81, 3128–3132.
[26] Bravo, R. and Macdonald-Bravo, H. (1984) Exp. Cell Res., in press.
[27] Bravo, R. and Graf, T. (1985) Exp. Cell Res., in press.
[28] Celis, J.E. and Celis, A. (1984) Proc. Natl. Acad. Sci. USA, in press.
[29] Bravo, R. and Macdonald-Bravo, H. (1984) EMBO J., in press.
[30] Terasima, T. and Tolmach, L.J. (1963) Exp. Cell. Res. 30, 344–362.
[31] Bravo, R., Fey, S.J., Small, J.V., Mose Larsen, P. and Celis, J.E. (1981) Cell 25, 195–202.
[32] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[33] Bravo, R., Small, J.V., Fey, S.J., Mose Larsen, P. and Celis, J.E. (1982) J. Mol. Biol. 154, 121–143.
[34] Bravo, R. (1984) in: Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications (Celis, J.E. and Bravo, R. eds) pp.3–36, Academic Press, New York.
[35] Mose Larsen, P., Bravo, R., Fey, S.J., Small, J.V. and Celis, J.E. (1982) Cell 31, 681–692.
[36] Bravo, R., Bellatin, J. and Celis, J.E. (1981) Cell Biol. Int. Rep. 5, 93–96.
[37] Bravo, R. and Celis, J.E. (1982) Clin. Chem. (Winston-Salem, NC) 28, 766–781.
[38] Bravo, R. and Celis, J.E. (1984) in: Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications (Celis, J.E. and Bravo, R. eds) pp.445–476, Academic Press, New York.