# S-phase patterns of cyclin (PCNA) antigen staining resemble topographical patterns of DNA synthesis

## A role for cyclin in DNA replication?

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The sequence of cyclin (proliferating cell nuclear antigen, PCNA), antigen staining throughout the cell cycle of African green monkey kidney cells (BS-C-1) has been determined by indirect immunofluorescence using PCNA autoantibodies specific for this protein. Patterns of cyclin staining observed between the beginning of S-phase and maximum DNA synthesis are similar to those reported in human AMA cells [(1985) Proc. Natl. Acad. Sci. USA 82, 3262–3266], while those detected thereafter are significantly different; the most striking feature being the continuous staining of the nucleoli up to or very near the $S/G_2$ border of the cell cycle. Using [$^3$H]thymidine autoradiography and indirect immunofluorescence of the same cells we show a remarkable correlation between cyclin antigen distribution and topographical patterns of DNA synthesis. In addition, we present evidence showing that DNase I treatment of Triton-extracted monolayers abolishes cyclin antigen staining but does not result in a substantial release of this protein. Taken together the above observations argue for a role of cyclin in some aspect of DNA replication.

*PCNA antibody* *Cell cycle* *[$^3$H]Thymidine autoradiography* *Two-dimensional gel electrophoresis* *Free cyclin* *Bound cyclin*

## 1. INTRODUCTION

Cyclin ($M_r$ 36000; IEF 49 in the HeLa protein catalogue; [1–3]), also termed PCNA [4,5], is an acidic nuclear non-histone protein of widespread occurrence in vertebrate cells whose rate of synthesis increases specifically during S-phase [6,7], and correlates directly with the proliferative state of normal cultured cells and tissues ([8–11] and references therein). Cyclin is present in very small amounts in normal non-dividing cells (senescent and quiescent cells included) and tissues, but is synthesized by proliferating cells of both normal and transformed origin, including tumors ([8–11] and references therein).

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

Immunofluorescence studies of transformed human amnion cells (AMA) [12,13] and other cells of vertebrate origin [11–14] 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. Recently, Celis and Celis [12,13] have shown that some of the patterns of cyclin antigen staining (nucleolar exclusion, nucleolar labelling) mimic at least in part topographical patterns of DNA synthesis [15,16] implying a role for this protein in some aspect of DNA replication. The possibility however that this correlation may be coincidental could not be excluded.

To address this question we have searched for cell types that may exhibit a sequence of cyclin antigen staining different from that of AMA [12,13] and mouse 3T3 cells [11]. If cyclin antigen staining

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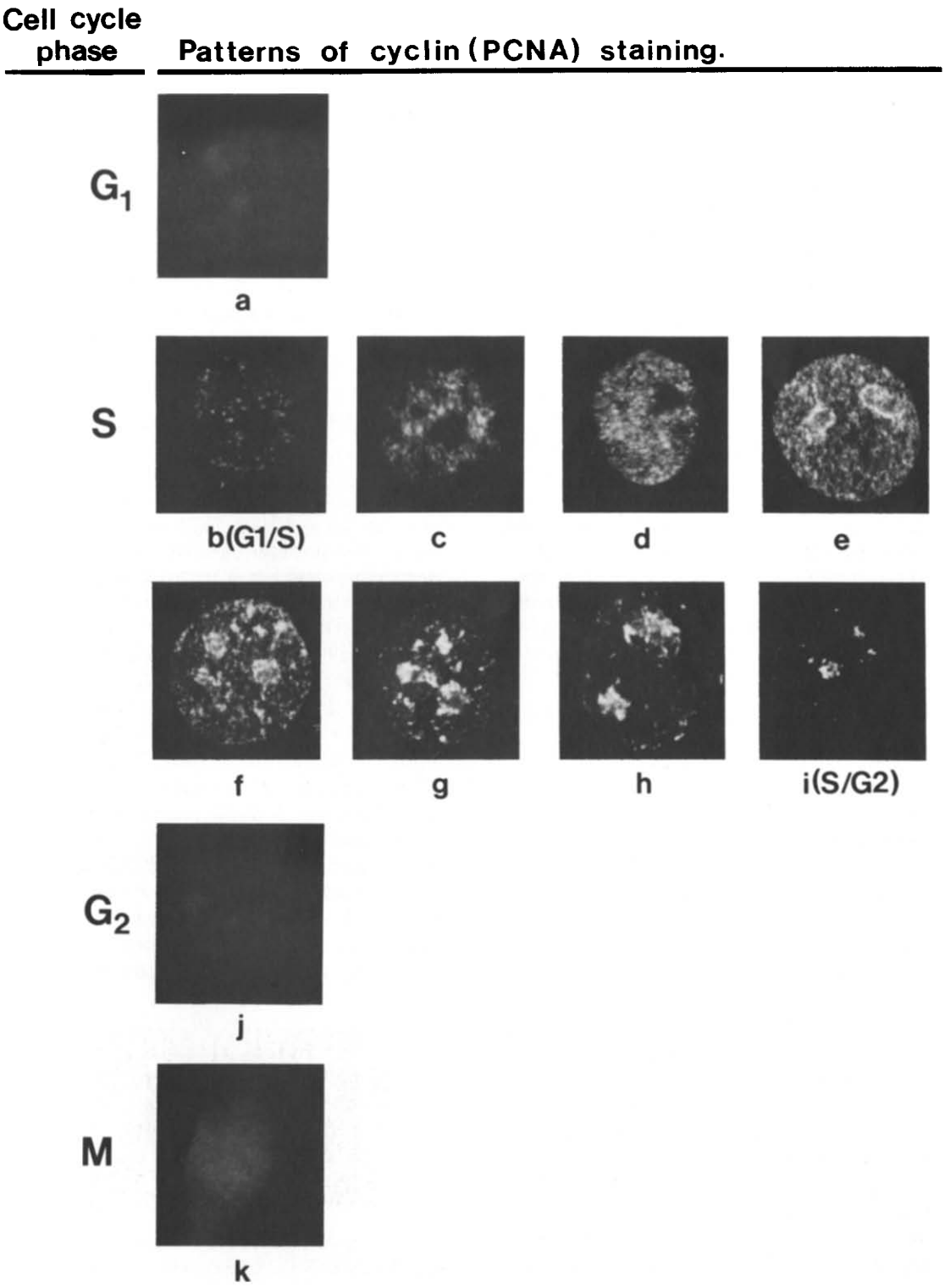


Fig.1. Sequence of cyclin (PCNA) staining patterns during the cell cycle of BS-C-1 cells (× 1150).

in fact follows DNA replication, changes in its distribution should be accompanied by changes in the topographical patterns of DNA synthesis. Here we show that this is indeed the case in African green monkey kidney BS-C-1 cells. In addition, we show that DNase I treatment of Triton-extracted monolayers abolishes cyclin antigen staining but does not result in a substantial release of this protein. Taken together these observations argue for a role of cyclin in some aspect of DNA replication.

## 2. MATERIALS AND METHODS

### 2.1. *Cells*

African green monkey kidney BS-C-1 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).

### 2.2. *Indirect immunofluorescence and autoradiography*

The procedures for indirect immunofluorescence and autoradiography have been detailed in [13]. PCNA antibodies specific for cyclin [5] were kindly provided by M.B. Mathews and R. Bernstein.

### 2.3. *In situ fractionation of monolayers*

Cells (unlabelled or [$^{35}$S]methionine labelled) grown on coverslips were washed twice in Hank's buffered saline and were treated for 90 s with a 0.1% solution of Triton X-100 in Hank's saline. After washing twice with Hank's saline the monolayers were (i) fixed with methanol (−20°C, 5 min), (ii) resuspended in lysis buffer [17] or (iii) extracted at room temperature with either DNase I (10 $\mu$g in 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris-HCl [pH 7.3]) or RNase (10 $\mu$g in 50 mM NaCl, 10 mM Tris-HCl [pH 7.2]). Samples for immunofluorescence were fixed with methanol (5 min at −20°C), while those for two-dimensional gel electrophoresis were resuspended directly in lysis buffer [17].

### 2.4. *Other procedures*

The procedures for labelling somatic cells with [$^{35}$S]methionine (1 mCi/ml) [18] and two-dimensional gel electrophoresis [19] have been described in detail elsewhere.

## 3. RESULTS

### 3.1. *S-phase patterns of cyclic (PCNA) antigen staining resemble topographical patterns of DNA synthesis*

The putative order of the various nuclear staining patterns observed during the cell cycle of BS-C-1 cells was determined by staining synchronized cells obtained at various times after release from a hydroxyurea block (10 mM hydroxyurea for 25 h). $G_1$ (fig.1, pattern a), $G_2$ (fig.1, pattern j) and mitotic cells (fig.1, pattern k) do not react with the antibody while S-phase cells show variable nuclear staining patterns in terms of both the intensity and distribution of the antigen (fig.1, patterns Sb–Si). The sequence of staining patterns from Sb (nucleolar exclusion) to Sf (nucleolar staining) is identical to that described for AMA [12,13] and 3T3 cells [11], while that observed thereafter (patterns Sg–Si) is different: the most striking feature being the continuous staining of the nucleoli up to or very near the S/$G_2$ border (fig.1, see [11–13]). Nucleolar staining was confirmed by double immunofluorescence analysis of cells reacted with PCNA autoantibodies (fig.2A) and a monoclonal antibody that stains nucleoli (fig.2B) [12], and by phase contrast microscopy (not shown). Pattern Si (fig.1) is the last one observed in S-phase and comprises 2% of this phase (see [13]).

The continuous staining of the nucleolus throughout late S-phase prompted us to compare immunofluorescence (PCNA antibodies) and autoradiographic patterns ([$^3$H]thymidine incorporation) of the same cells. As shown in fig.3, there is a remarkable correlation between the granular cyclin nucleolar staining (fig.3A, pattern Sh; fig.3C, pattern Si) and autoradiographic pat-

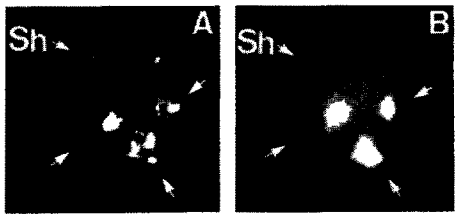


Fig.2. Double immunofluorescence of methanol fixed late S-phase BS-C-1 cells reacted with (A) PCNA antibodies and (B) a mouse monoclonal antibody that stains the nucleoli (× 1050).

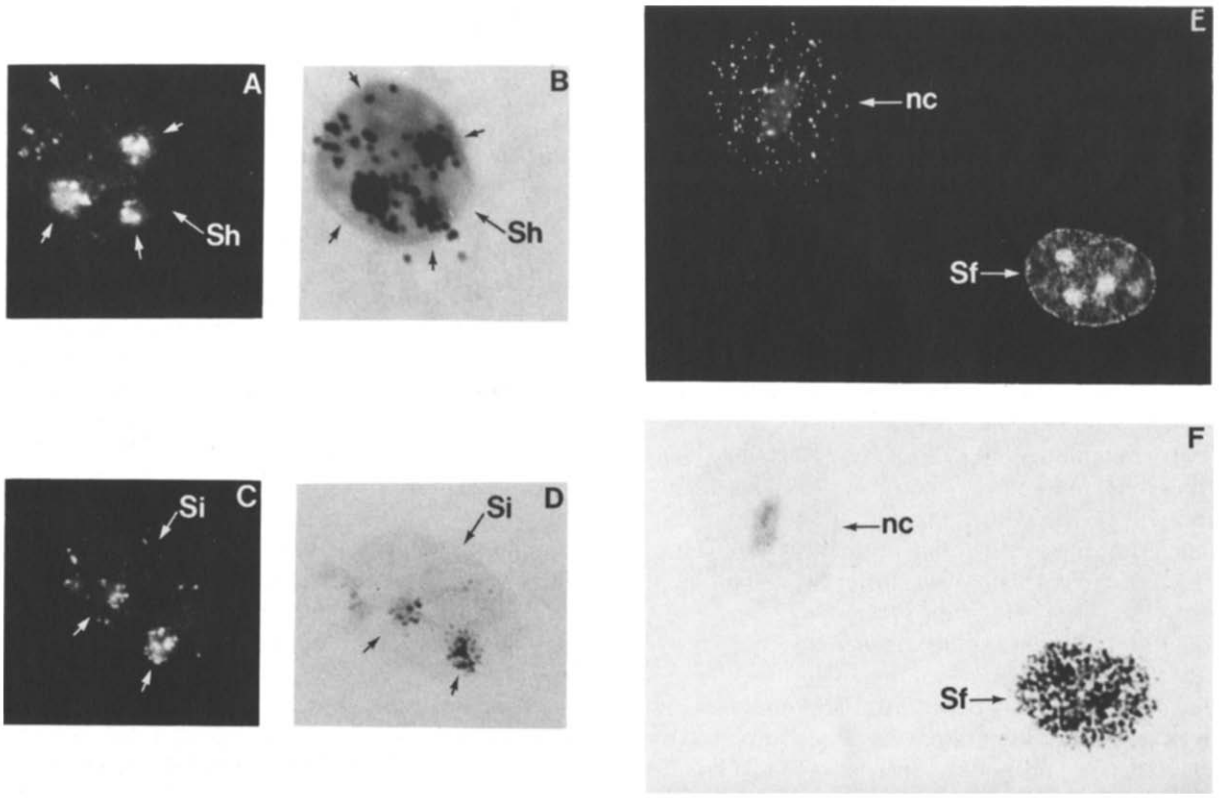


Fig.3. Immunofluorescence (A, C, E; PCNA antibodies) and autoradiography (B, D, F; [$^3$H]thymidine incorporation) of the same field of BS-C-1 cells. Cells were labelled with methyl[$^3$H]thymidine (4 $\mu$Ci/ml, 30 min) and processed for immunofluorescence as described in section 2 ($\times$ 895).

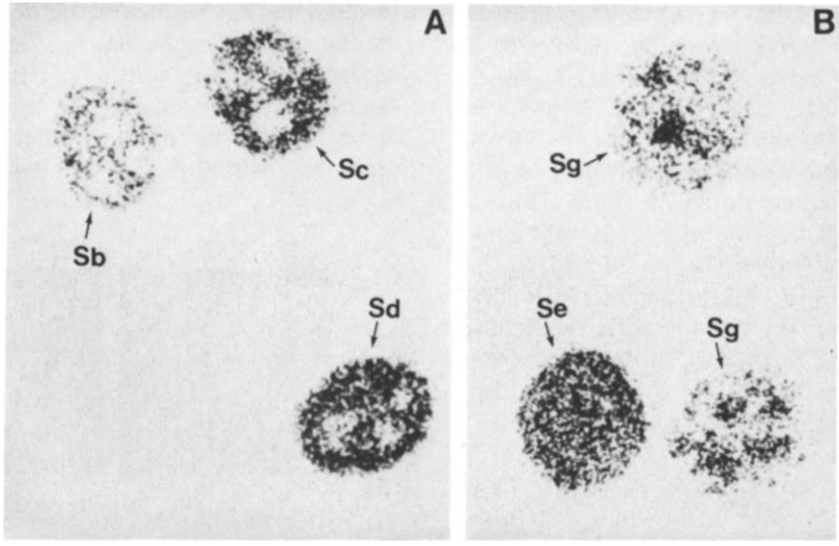


Fig.4. [$^3$H]Thymidine autoradiography of S-phase BS-C-1 cells. Cells were labelled for 3 h with 4 $\mu$Ci/ml of methyl-[$^3$H]thymidine. Autoradiograms were exposed for 24 h ($\times$ 980).

terns of [$^3$H]thymidine incorporation (fig.3B and D).

In fact, under favourable conditions, the flat nature of BS-C-1 cells allows the reading of most cyclin staining patterns directly from [$^3$H]thymidine autoradiograms. Fig.4A and B shows selected autoradiograms (only S-phase cells are shown) of BS-C-1 cells exhibiting most of the cyclin patterns presented in fig.1.

In addition to the patterns discussed above, we observed a bright punctated staining (fig.3E, pattern nc, not cycling) in 5% of the total cell population that may characterize cells withdrawing from the cell cycle. These cells do not incorporate [$^3$H]thymidine as determined by autoradiography (cf. fig.3E and F).

### 3.2. *DNase I treatment abolishes cyclin (PCNA) antigen staining but does not result in a substantial release of this protein*

The remarkable correlation between cyclin antigen staining and topographical patterns of DNA synthesis through S-phase suggested an association (direct or indirect) of this protein with DNA. Accordingly, we extracted BS-C-1 monolayers with 0.1% Triton X-100 followed by 30 min incubation at room temperature with a 10 $\mu$g/ml solution of DNase I in 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.3). Fig.5A and B shows indirect immunofluorescence of methanol-fixed BS-C-1 Triton cytoskeletons (fig.5A) and DNase I-treated BS-C-1 Triton cytoskeletons (fig.5B) reacted with PCNA antibodies. Clearly, treatment with DNase I abolishes all S-phase patterns of cyclin staining (fig.5B), but does not result in a significant release of this protein as judged by two-dimensional gel electrophoresis (IEF, fig.6C; only the appropriate region of the gel is shown; table 1). Triton monolayers, on the other hand (fig.5A), show similar levels of cyclin staining to whole cells (not shown), despite the fact that they contain only 47% (bound cyclin, fig.6B, table 1) of total cyclin (fig.6A, table 1).

Treatment of Triton cytoskeletons with RNase (10 $\mu$g/ml in 50 mM NaCl, 10 mM Tris-HCl [pH 7.2]) for 30 min at room temperature did not result in a significant change in the immunofluorescence patterns (either intensity or quality of the patterns, not shown) or in significant release of cyclin (table 1).

## 4. DISCUSSION

The remarkable correlation between nuclear patterns of cyclin (PCNA) antigen distribution and topographical patterns of DNA synthesis argue for a role for this protein in some aspect of DNA replication [20,21]. Based on $M_r$ estimates cyclin is different from DNA polymerase $\alpha$ [22,23], $\beta$ [24–26], $\gamma$ [27], DNA ligase [28], DNA primase [23,29,30], DNA topoisomerases [31,32] and DNA polymerase $\alpha$ cofactors $C_1C_2$ [33,34]. Single-strand-binding proteins of similar molecular masses have been described [35,36], but their relation to cyclin is unclear. Salt extraction of Triton monolayers ([19], and unpublished) have shown that cyclin is not an integral component of the nuclear matrix.

The DNase I digestion experiments provide indirect evidence for an interaction of cyclin with DNA. Interestingly, loss of cyclin antigen staining following digestion is not accompanied by a

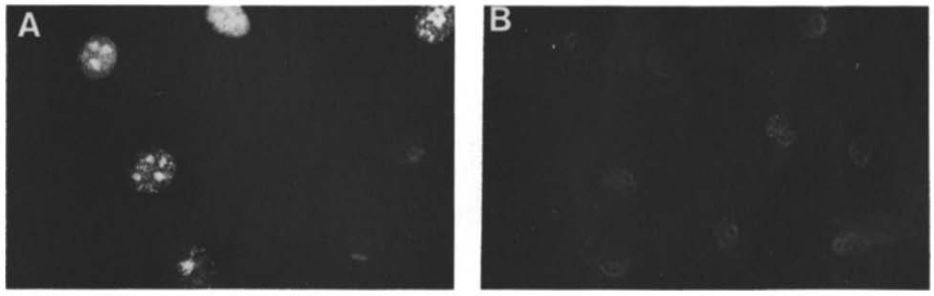


Fig.5. Immunofluorescence staining (PCNA antibodies) of BS-C-1 cytoskeletons. (A) Triton-extracted cells, (B) DNase I-treated Triton cytoskeletons (× 250).

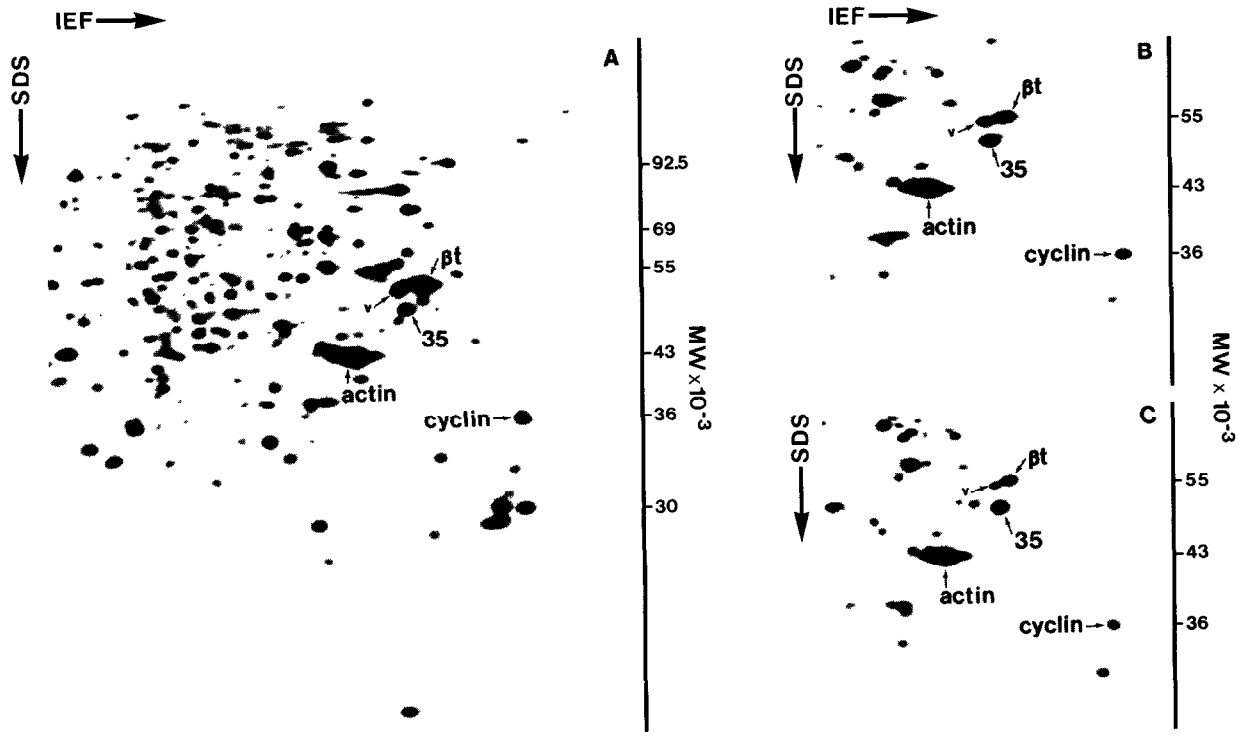


Fig.6. Two-dimensional gel electrophoresis (IEF) of [$^{35}$S]methionine-labelled proteins from BS-C-1 cells and cytoskeletons. (A) Whole cells, (B) Triton cytoskeletons and (C) DNase I-treated Triton cytoskeletons. Only the relevant regions of the gels are shown.

substantial release of this protein, suggesting that masking of antigenic determinants rather than changes in the ratio of free to bound cyclin may determine the levels of immunofluorescence staining with PCNA antibodies. These considerations may also apply to $G_1$,$G_2$ and mitotic cells which contain high amounts of this protein (unpublished; cyclin however is synthesized preferentially during S-phase [6,7]) and yet do not stain with PCNA antibodies.

Table 1

% of cyclin (PCNA) in whole and in situ fractionated BS-C-1 cells

| Cell fraction | % of [$^{35}$S]methionine-labelled cyclin[a] |
|---|---|
| 1. Whole cells | 100 |
| 2. Triton cytoskeletons | 47 |
| 3. DNase I-treated Triton cytoskeletons | 38 |
| 4. RNase-treated Triton cytoskeletons | 40 |

[a] Calculated based on the ratios against IEF 35 (see fig.6), as the % of this protein is not affected by the various treatments used. The identity of cyclin was confirmed by one-dimensional peptide mapping

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