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# Individual nuclei in polykaryons can control cyclin distribution and DNA synthesis

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**Nuclear patterns of cyclin (PCNA) distribution that subdivide S-phase (determined using PCNA autoantibodies specific for this protein) as well as [³H]thymidine incorporation followed by autoradiography have been used to determine the S-phase synchrony of homophasic polykaryons produced by polyethylene glycol (PEG)-induced fusion of populations of mitotic transformed human amnion cells (AMA) exhibiting the following average distribution of phases: prophase, 9%, metaphase, 60% (including early and late prometaphase), anaphase, 3.8%, telophase, 26.2% and interphase, 1%. Both synchronous and asynchronous polykaryons were generated from these fusions; the latter being frequently observed only amongst populations of multinucleated cells having three or more nuclei. These results are taken to imply that individual nuclei in these polykaryons can control cyclin distribution and DNA synthesis in spite of the fact that they share a common cytoplasm.**

*Key words:* mitotic cells/cell fusion/PCNA autoantibodies/subdivision of S-phase/immunofluorescence/[³H]thymidine incorporation/autoradiography

## Introduction

Immunofluorescence studies using proliferating cell nuclear antigen (PCNA) autoantibodies (Miyachi *et al.*, 1978; Mathews *et al.*, 1984) specific for the growth rate-sensitive protein 'cyclin' (Bravo *et al.*, 1981c; Bravo and Celis, 1982b; Celis *et al.*, 1984a, 1984b; IEF 49 in the HeLa protein catalogue; Bravo *et al.*, 1981a; Bravo and Celis, 1982a, 1984) have revealed dramatic changes in the nuclear distribution of this protein during the S-phase of the cell cycle (Takasaki *et al.*, 1981; Celis *et al.*, 1984c; Celis and Bravo, 1984b; Celis and Celis, 1985; Bravo and Macdonald-Bravo, 1985; see also Bravo and Celis, 1980, 1985). Several patterns of cyclin staining that subdivide S-phase have been observed; some of which (nucleolar exclusion; nucleolar labelling) are strikingly similar to those detected in autoradiograms of cells labelled with [³H]thymidine (Celis and Celis, 1985; Madsen and Celis, in preparation; this article). These observations as well as recent evidence showing that the nuclear localization of this protein is determined at least in part by the status of DNA replication (Bravo and Macdonald-Bravo, 1985; this article) have lent support to the notion that cyclin activity may be related to a specific aspect of DNA replication (Celis and Bravo, 1984a; Bravo, 1984; Celis and Celis, 1985; Bravo and Macdonald-Bravo, 1984, 1985), and have emphasized its usefulness as a marker to assess S-phase synchrony.

In an effort to gain a better understanding of the mechanisms controlling the initiation of DNA replication during the cell cycle, we present here experiments in which we have used PCNA antibodies as well as [³H]thymidine autoradiography to determine the S-phase synchrony of homophasic polykaryons produced by polyethylene gycol (PEG)-induced fusion of populations of mitotic transformed human amnion cells (AMA) that are very close in their cell cycle stage. The results show that individual nuclei in these polykaryons can control cyclin distribution and DNA synthesis in spite of the fact that they share a common cytoplasm.

## Results

### *Patterns of cyclin staining during the cell cycle of AMA cells: subdivision of S-phase*

For reference purposes and to help the identification of the various cyclin staining patterns observed in the polykaryons described below, Figure 1 (Celis and Celis, 1985) shows the sequence of cyclin immunofluorescence staining patterns observed throughout the cell cycle of AMA cells which were treated with methanol prior to immunofluorescence with PCNA autoantibodies specific for this protein (Mathews *et al.*, 1984). $G_1$ (Figure 1a), $G_2$ (Figure 1j) and mitotic cells (Figure 1k; chromosomes are not stained)

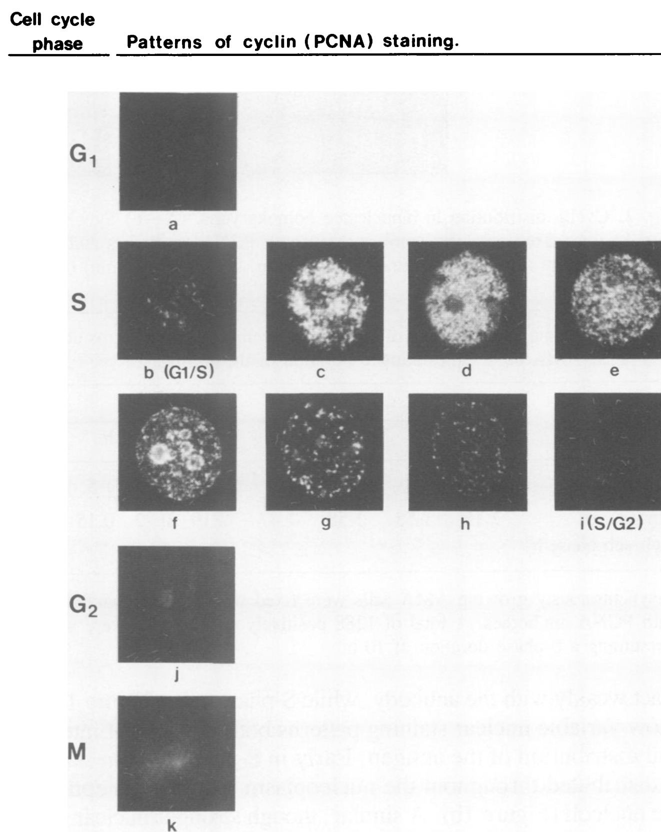


**Fig. 1.** Sequence of cyclin (PCNA) staining patterns during the cell cycle of AMA cells. The transition between the different staining patterns does not take place simultaneously in all cells (Celis and Celis, 1985). x 484.

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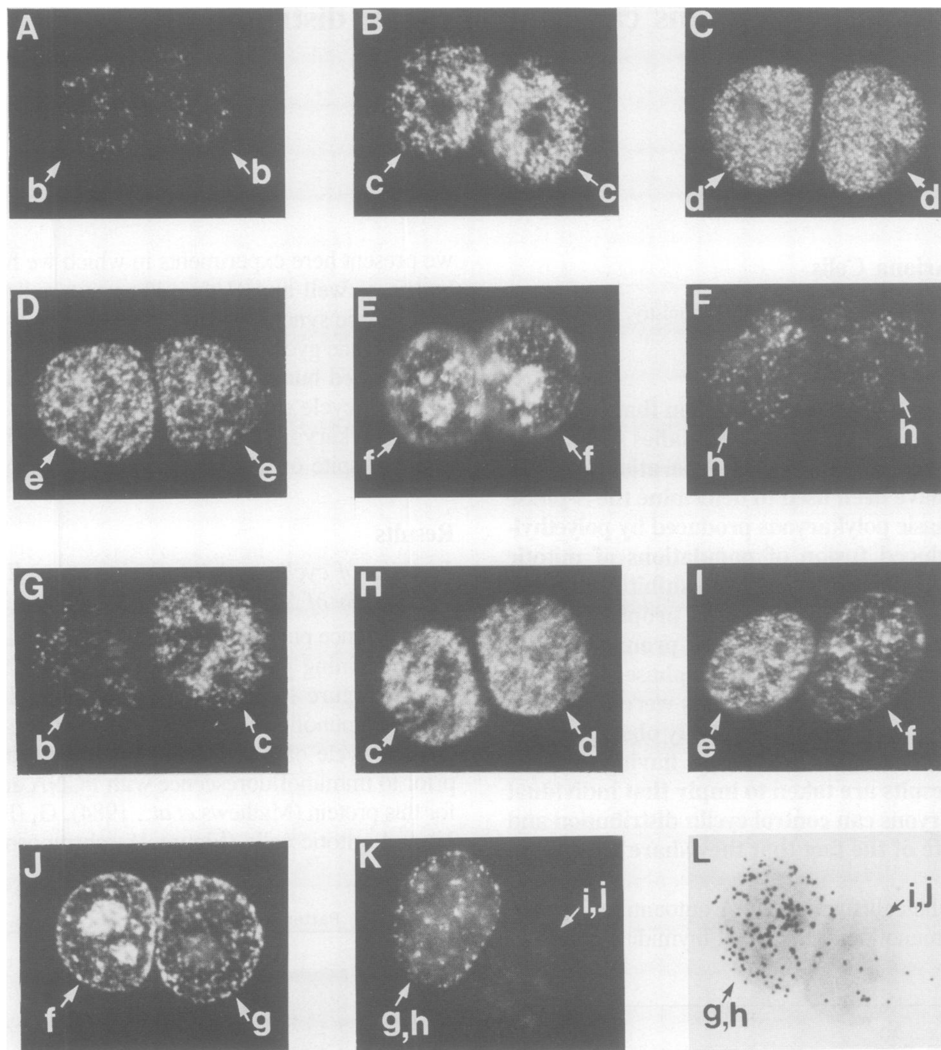


**Fig. 2.** Cyclin distribution in binucleated homokaryons. **(A–F)** Synchronous (early, **A–C**; and late, **D–F**, during S-phase) and **(G–K)** asynchronous homokaryons fixed with methanol, reacted with PCNA antibodies and analyzed by indirect immunofluorescence. **(K,L)** Immunofluorescence **(K)**, and autoradiography (**L**, [$^3$H]thymidine incorporation; 2 $\mu$Ci/ml, 30 min) of the same asynchronous homokaryon analyzed late during S-phase. x 980.

**Table I.** Percentage distribution of the various cyclin staining patterns observed in S-phase AMA cells. Approximate duration of the sub-phases

| | S-phase pattern | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | b | c | d | e | f | g | h | i |
| % of total[a] | 21.5 | 15.8 | 15.8 | 14.7 | 21.9 | 7.2 | 1.5 | 1.6 |
| Duration of each sub-phase/h[b] | 2.15 | 1.58 | 1.58 | 1.47 | 2.19 | 0.72 | 0.15 | 0.16 |

[a]Asynchronously growing AMA cells were fixed with methanol and stained with PCNA antibodies. A total of 1288 positively stained cells were scored.
[b]Assuming a S-phase duration of 10 h.

**Table II.** S-phase synchrony of AMA homophasic polykaryons as judged by the pattern of cyclin staining

| Number of nuclei per polykaryon | S-phase synchrony – %[a] | |
|---|---|---|
| | Synchronous | Asynchronous |
| 2[b] | 94 | 6 |
| 3[c] | 64 | 36 |
| 4[a] | 50 | 50 |
| 5 or more | nd | nd |

[a]These values were calculated from cell populations containing at least one nucleus in S-phase. Most cells, however, had 50% or more of their nuclei in S-phase. No signs of deterioration were observed in these polykaryons. At present, we cannot exclude the possibility that asynchrony in some polykaryons may be caused by PEG-induced damage at the time of fusion.
[b]Based on the analysis of 300 binucleated cells.
[c]Based on the analysis of 157 trinucleated cells.
[d]Based on the analysis of 70 tetranucleated cells.

react weakly with the antibody, while S-phase cells (Figure 1b–i) show variable nuclear staining patterns both in terms of intensity and distribution of the antigen. Early in S-phase, cyclin staining is distributed throughout the nucleoplasm with the exception of the nucleoli (Figure 1b). A similar, though stronger nuclear staining pattern (most likely reflecting an increased synthesis of cyclin; Bravo and Celis, 1980, 1985; Bravo and Macdonald-Bravo, 1985) is observed as the cells progress through S-phase (Figure 1c and d). Late in S-phase, before maximum DNA synthesis, cyclin staining redistributes to reveal a punctuated pattern with foci of staining throughout the nucleus (Figure 1e). In many cases, it is also possible to see a light rim of cyclin staining around the nucleolus (Figure 1e). This pattern precedes a major change in the distribution of this protein, which is then detected in the nucleolus, the nucleoplasm and in distinct foci located close to

the nuclear membrane (Figure 1f). At this stage, DNA synthesis is at or near a maximum. Thereafter, there are further changes in the staining distribution of this protein, with the pattern becoming punctuated and of decreasing intensity (Figure 1g – i). Table I gives the percentage distribution of the various S-phase patterns described above (see also Figure 1). Furthermore, an approximate duration for each subphase (as defined by the various patterns described above) has been calculated assuming an S-phase duration of 10 h (Table I).

Of the various immunofluorescence patterns of cyclin staining (Figure 1b – i), the ones showing nucleolar exclusion (Figures 1b and c) and nucleolar labelling (Figure 1f) are remarkably similar to those observed in autoradiograms of [$^{3}$H]thymidine-labelled cells (Celis and Celis, 1985; Madsen and Celis, in preparation; see also Figure 3A – D), suggesting that the distribution of this protein mimics at least in part some of the topographical patterns of DNA replication.

### *S-phase synchrony of homophasic polykaryons produced by PEG-induced fusion of mitotic cells*

Typical mitotic preparations used for PEG-induced fusions exhibited the following average distribution of phases: prophase, 9%, metaphase, 60% (including early and late prometaphase), anaphase, 3.8%, telophase, 26.2% and interphase, 1%. Complete synchrony of mitotic phases cannot be achieved at present. Following cell fusion, the cell population was plated onto coverslips and samples were withdrawn at various times during the cell cycle (mainly early and late during S-phase) for immunofluorescence (PCNA autoantibodies) and autoradiographic analysis ([$^{3}$H]thymidine incorporation). In a typical fusion, ~21% of the total population of cells that attached to the coverslips corresponded to polykaryons (two or more nuclei) as judged by phase contrast microscopy, and of these 66.5% corresponded to binucleated cells, 19.5% to trinucleated, 7.6% to tetranucleated and 6.4% to cells having five or more nuclei.

As expected from fusions of cells at the same stage of the cell cycle, most binucleated homokaryons (94%; Table II) analyzed early (see for example Figure 2A – C) or late (see for example Figure 2D – F) during S-phase exhibited a synchronous pattern of cyclin staining. To aid the identification of the various patterns, each nucleus in a given homokaryon (Figure 2) is indicated with a letter corresponding to the homologous pattern shown in Figure 1. Synchrony of DNA synthesis has in many cases been confirmed by [$^{3}$H]thymidine autoradiography (not shown but see Figure 3). Interestingly, ~6% of the binucleated cells (Table II) exhibited an asynchronous pattern of cyclin staining (see for example Figure 2G – K), a fact that was confirmed by autoradiographic analysis ([$^{3}$H]thymidine incorporation) of the same cells (see for example Figure 2K and L).

A higher percentage of asynchronous polykaryons was, however, observed amongst cells that contained three or more nuclei, and a summary of these results is given in Table II (average of six fusions). The data were calculated based on the analysis of polykaryons having at least one nucleus in S-phase, although most polykaryons had >50% of their nuclei in this phase. Examples of both synchronous (Figure 3) and asynchronous (Figure 4) polykaryons are presented in Figures 3 and 4, respectively. Corresponding patterns of cyclin distribution and [$^{3}$H]thymidine incorporation are shown in Figure 3A and B (nucleolar exclusion), C and D (nucleolar labelling) and Figure 4C and D (nucleolar labelling).

## Discussion

Nuclear patterns of cyclin distribution that subdivide S-phase and that, in some instances, distinguish nuclei that may be 10 – 20 min apart in their cell cycle stage (late S-phase; see for example Figure 4H and Table I) have been used to determine the synchrony of homophasic polykaryons obtained by fusing populations of mitotic cells that are very close in their cell cycle stage. The results showed that both synchronous and asynchronous homokaryons are generated from these fusions; the latter being frequently observed only amongst populations of multinucleated cells having three or more nuclei.

To discuss the nature of the asynchrony, we would like to take as an example the tri-nucleated polykaryon shown in Figure 4A (immunofluorescence) and B (autoradiography). Clearly, one nucleus in this polykaryon (indicated with a b; see also Figure 1) is at a very early stage during S-phase while the other two are more advanced in this phase (indicated with a c; see also Figure 1). There are at least two possibilities that may account for the differential cyclin staining: (i) RNA synthesized by all three nuclei (albeit at different rates between nuclei b and c) direct the synthesis of cyclin which enters all nuclei. The intensity and distribution of this protein is then determined intranuclearly and (ii), as (i) but the amount of cyclin entering nuclei b and c is different. Two lines of evidence support the first possibility.

First, entry of cyclin into the nucleus takes place even in the absence of DNA synthesis (see also Bravo and Macdonald-Bravo, 1984, 1985). This has been shown by determining the distribution of this protein in synchronized AMA cells treated with inhibitors such as thymidine and hydroxyurea which block cells at the $G_1$/S border of the cell cycle. Figure 5B (thymidine-treated cells, 2 mM) and C (hydroxyurea-treated cells, 10 mM) show immunofluorescence micrographs of representative sister AMA cells treated with PCNA antibodies 18 h after plating mitotic AMA cells in the presence of the inhibitors. In both cases, the nuclei reacted positively with PCNA antibodies to reveal bright foci of staining that may correspond to prereplicative sites of cyclin localization (Quinlan *et al.*, 1984), and that are similar to those observed at the very beginning of S-phase (see Figures 1b and 4E; Celis and Celis, 1985; Bravo and Macdonald-Bravo, 1985). Control, untreated sister AMA cells, on the other hand, showed mainly nucleolar staining (Figure 5A).

Secondly, enucleation of [$^{35}$S]methionine-labelled asynchronous cells with cytochalasin B have revealed very low levels of cyclin in cytoplasts (Bravo *et al.*, 1981b), implying that once synthesized this protein rapidly migrates into the nucleus.

Clearly, the above considerations would also explain the differential cyclin staining observed in the other AMA polykaryons presented in Figure 4. Asynchronous cyclin staining has also been observed in a significant proportion of bi- (17%) and trinucleated cells (65%) present in asynchronously growing cultures of AMA cells (not shown), suggesting that asynchrony is not generated artificially as a result of PEG-induced fusion. Since asynchrony of cyclin distribution has so far always been observed to be accompanied by asynchronous DNA synthesis it seems reasonable to conclude that individual nuclei in these multinucleated cells can control cyclin distribution and DNA synthesis. Even though these nuclei may share common cytoplasmic factors, some intranuclear events seem to be needed for both replication and cyclin distribution. These results are consistent with the notions that intranuclear events determine the initiation and temporal order of DNA replication (Graves, 1972; Yanishevsky and Prescott,

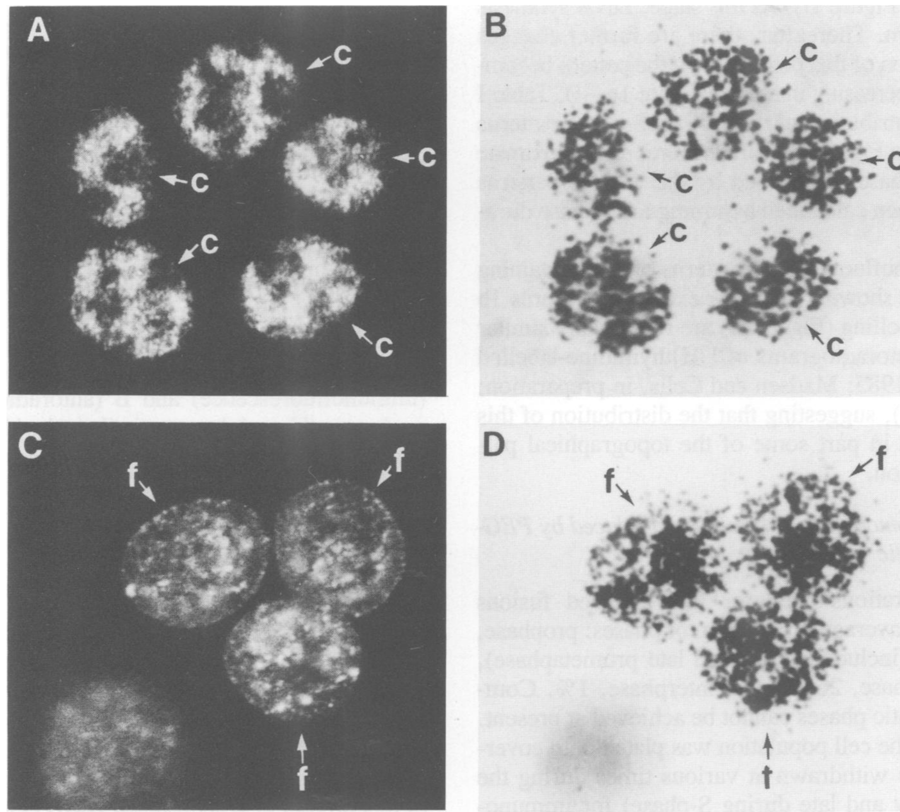


**Fig. 3.** Immunofluorescence (**A,C**; PCNA antibodies) and autoradiography (**B,D**; [$^3$H]thymidine incorporation) of the same field of synchronous AMA homophasic polykaryons. Cells were labelled with [$^3$H]thymidine (2 $\mu$Ci/ml; 30 min) and processed for immunofluorescence early (**A,B**) and late (**C,D**) during S-phase. x 1050.

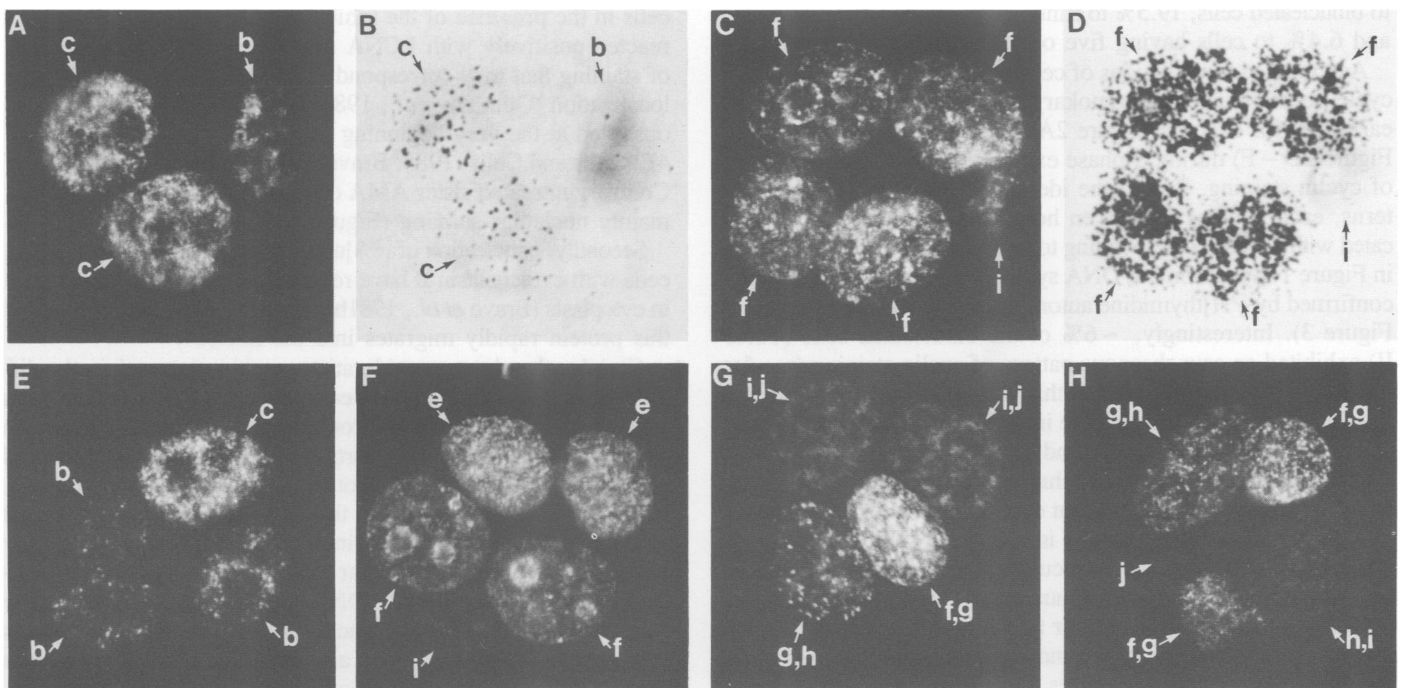


**Fig. 4.** Cyclin distribution and [$^3$H]thymidine incorporation in asynchronous AMA polykaryons. (**A,C**) Immunofluorescence (PCNA antibodies) and (**B,D**) autoradiography ([$^3$H]thymidine incorporation; 2 $\mu$Ci/ml; 30 min) of the same field of asynchronous polykaryons analyzed early (**A,B**) and late (**B,D**) during S-phase. (**E – H**) Immunofluoresence (PCNA antibodies) of polykaryons analyzed early (**E**) and late (**B – H**) during S-phase. Each nuclei in a given polykaryon is indicated with a letter corresponding to the homologous pattern shown in Figure 1. Nuclei indicated with two letters exhibit intermediate patterns of cyclin distribution. x 1050.

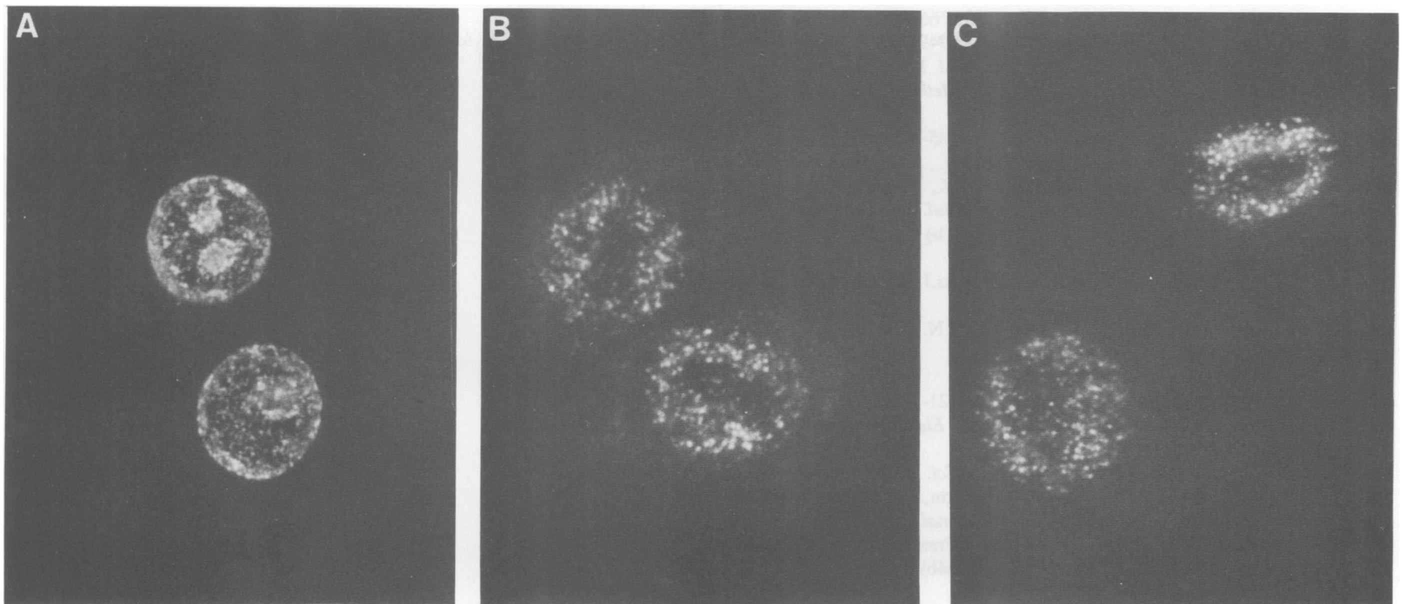


**Fig. 5.** Cyclin distribution in synchronous AMA cells treated with thymidine and hyroxyurea. **(A)** Synchronous sister cells fixed with methanol and reacted with PCNA antibodies 16–18 h after plating mitotic cells. **(B)** as **(A)** but with thymidine (2 mM) added 1 h after plating. **(C)** as **(A)** but with hydroxyurea (10 mM) added 1 h after plating. x 980.

1978 and references therein) and that cyclin distribution is determined at least in part by the status of DNA replication (Bravo and Macdonald-Bravo, 1985; this article). At present, it is not clear why nuclei respond differently to cytoplasmic signals, although it is possible that this may reflect differences in their state (composition, programming, etc.) at the time of fusion.

Examples of asynchronous DNA synthesis have been reported by several laboratories, namely by Gallardo *et al.* (1970) in giant cell osteoclastomas, by Sandberg *et al.* (1966) in binucleated cells from patients with acute myeloid leukemia, by Burns (1971) in multinucleated Ehrlich ascites tumour cells, by Sheehy *et al.* (1974) in multiovarian cancer cells and by Johnson and Harris (1969c) in HeLa-Ehrlich ascites heterokaryons. Moreover, asynchrony of DNA synthesis has been observed in chicken erythrocyte-HeLa heterokaryons (Bolund *et al.*, 1969; Johnson and Harris, 1969b; Johnson and Mullinger, 1975) as well as in senescent human diploid cells fused with replicative transformed cells of different types (see Stein *et al.*, 1982 and references therein).

Synchrony of DNA synthesis has been reported both in hetero- and homokaryons (Yamanaka and Okada, 1966; Johnson and Harris, 1969a, 1969b; Westerveld and Freeke, 1971; Graves, 1972; see also Ringertz and Savage, 1976 and references therein), and Rao and Johnson (1970) first provided evidence for the existence of cytoplasmic transmissible factors (putative initiators of DNA synthesis present in early S-phase cells) that induce $G_1$ nuclei to enter prematurely into S-phase (see also Graves, 1972; Johnson and Mullinger, 1975; Yanishevsky and Prescott, 1978; Brown *et al.*, 1985). Even though these factors may be present in AMA polykaryons, their function is not dominant as one would have expected perfect synchrony of DNA synthesis and cyclin distribution in polykaryons made by fusing cells that are so close in their cell cycle stage. Whether asynchrony of DNA initiation and replication in polykaryons represent peculiarities of the cell types studied or in fact reflect an alternative mechanism by which cells control the initiation of DNA synthesis during the cell cycle is at present unknown.

## Materials and methods

### *Cells*

AMA cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics (100 units of penicillin/ml; 50 μg of streptomycin/ml).

### *Mitotic cells*

Mitotic cells were obtained by gentle mechanical detachment essentially as described by Bravo and Celis (1980). One 250 ml flask containing $1-2 \times 10^6$ cells was used. The distribution of phases in these preparations was determined by phase-contrast microscopy.

### *Cell fusion*

Mitotic cells obtained from a 250 ml flask were washed twice in Hank's-buffered saline and treated for 2.5 min with 0.3 ml of 50% PEG 6000. After fusion, 5 ml of DMEM containing 20% fetal calf serum was added and the cells were recovered by centrifugation. These were then washed once with DMEM containing 20% sera and once with DMEM containing 10% sera. Finally, they were resuspended in DMEM (10% serum) and plated in 5 cm Petri dishes containing coverslips.

### *Indirect immunofluorescence and autoradiography*

The procedures for indirect immunofluorescence and autoradiography have been described in detail elsewhere (Bellatin *et al.*, 1982; Mose Larsen *et al.*, 1982). For preparations to be analyzed by immunofluorescence and autoradiography the following protocol was used: cells grown in marked coverslips were labelled with [methyl-$^3$H]thymidine (30 min; 2 μCi/ml), fixed with methanol (−20°C) and reacted with PCNA antibodies specific for cyclin (Mathews *et al.*, 1984, kindly provided by M.B.Mathews and R.Bernstein). Immunofluorescence pictures were taken prior to autoradiography. Autoradiographies were exposed for 5 days.

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