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Intranuclear co-location of newly replicated DNA and PCNA by simultaneous immunofluorescent labelling and confocal microscopy in MCF-7 cells

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Summary

The intranuclear distribution of newly replicated DNA and of the proliferating cell nuclear antigen (PCNA) was mapped by confocal laser scanning microscopy after simultaneous immunofluorescent labelling of incorporated bromodeoxyuridine (BrdUrd) and PCNA. A mild hydrolysis with HCl followed by an enzymic digestion of DNA was used to produce single-stranded DNA required for BrdUrd immunorevelation, since this procedure preserves PCNA antigenicity. Optical sections obtained with a laser scanning microscope clearly showed a similar distribution of PCNA and BrdUrd within the nuclei, thus confirming previous observations on parallel labelled synchronized cultures. The intranuclear distribution of PCNA and BrdUrd varies concomitantly during the S phase of MCF-7 cells.

Key words: bromodeoxyuridine, PCNA, immunofluorescence, MCF-7 cells, CLSM.

Introduction

The proliferating cell nuclear antigen (PCNA), also known as cyclin, is an acidic nuclear protein with an apparent molecular mass of 35 kDa (Mathews et al., 1984). Extensive studies have demonstrated the importance of PCNA in the pathway of DNA replication. Indeed, PCNA is required for SV40 DNA synthesis in vitro (Prelich et al., 1987), and seems to be a universal protein necessary for eukaryotic DNA replication (see Celis et al., 1987, for review). The PCNA gene was also found in higher plants (Susuka et al., 1989), showing a strong sequence similarity between mammals and plants (Susuka et al., 1991). Finally, PCNA has been identified as an auxiliary protein of DNA polymerase δ (Bravo et al., 1987).

Immunofluorescence studies of the distribution of PCNA during the cell cycle have revealed dramatic changes in its intranuclear localization during S phase (Bravo and Macdonald-Bravo, 1985; Celis and Celis, 1985; see Celis et al., 1986, for review). Furthermore, association between PCNA and replication sites was suggested on the basis of comparison between the PCNA and bromodeoxyuridine (BrdUrd) immunofluorescent labelling patterns from parallel synchronous cell cultures (Bravo and Macdonald-Bravo, 1987; van Dierendonck et al., 1991). Although informative, these studies, as well as those using a combination of autoradiography and immunocytochemistry for simultaneous detection of newly replicated DNA and PCNA (Celis and Celis, 1985), did not provide direct evidence for co-location of active replicon clusters and this antigen.

A concomitant location of replicated DNA and PCNA was shown at the electron microscope level by Raska et al. (1991), after simultaneous BrdUrd and PCNA immunolabelling. This type of revelation provides a better spatial resolution than autoradiography. However, electron microscopy, although providing very high-resolution images, is not well suited for large-scale quantitative analyses. It is also difficult to correlate BrdUrd images obtained by electron microscopy with the known BrdUrd incorporation patterns that have been accurately characterized throughout S phase by optical microscopy (van Dierendonck et al., 1989; Fox et al., 1991; Usson and Humbert, 1992; Humbert and Usson, 1992). Furthermore, BrdUrd immunofluorescence revelation can also be associated with DNA quantification by image cytometry (Humbert et al., 1990; Fox et al., 1991).

BrdUrd immunorevelation requires a DNA denaturation step in order to make the incorporated BrdUrd accessible to the antibodies. Raska et al. (1991) have shown that simultaneous BrdUrd and PCNA immunolabelling is possible after acidic DNA denaturation. Galand and Degraef (1989) also proposed an HCl (2 M) pretreatment before PCNA revelation in order to improve the immunostaining and to combine it with

BrdUrd procedures. We could not find any PCNA staining on monolayer MCF-7 cell cultures using this procedure. Kill et al. (1991), postulating that simultaneous revelation of incorporated BrdUrd and PCNA was precluded because of the HCl hydrolysis, found an alternative solution: microinjection of biotin-11-dUTP. We show here that a simultaneous revelation of BrdUrd and PCNA by immunofluorescence is possible, using a method derived from the enzymic procedure of Dolbeare and Gray (1988) to produce single-stranded DNA, since this procedure preserves PCNA antigenicity. In this way it was possible to follow the concomitant intranuclear distribution of BrdUrd and PCNA during S phase. The temporal progression during S phase was assessed according to the known ordering of various BrdUrd incorporation patterns (van Dierendonck et al., 1989; Fox et al., 1991; Usson and Humbert, 1992; Humbert and Usson, 1992), and to observations made on parallel synchronized cultures stained for BrdUrd or PCNA. BrdUrd vs. PCNA intranuclear co-location was estimated from nuclear optical sections obtained with a confocal laser scanning microscope.

Materials and methods

Cell culture

MCF-7 human breast cancer cells, kindly provided by Dr. Mercier Baudart (INSERM U33, Hôpital Bicêtre, Paris), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine (4 mM), insulin (0.6 mg/l), penicillin/streptomycin (24 μl/ml), amphotericin (1.5 μg/ml), and non-essential amino acids (1%, v/v). Cells were grown as monolayers cultures in 25 cm^2 Corning flasks in a humidified 5% CO_2/air incubator at 37°C. Cells were passaged weekly using 0.25% trypsin, 1 mM EDTA in Ca^{2+},Mg^{2+}-free phosphate buffered saline. After trypsinization, the cell suspension was distributed on tissue culture slides, at a density of 2500 cells in 750 μl of fresh growth medium per chamber.

BrdUrd labelling and fixation

The medium was removed from exponentially growing cells 76 hours after plating out on microscope slides, and replaced with fresh growth medium containing 20 μM BrdUrd for 1 hour. The cells were then washed 3 times in phosphate buffered saline (PBS), fixed for 1 hour in methanol at −20°C and processed for double staining.

Double-staining procedures

In order to make the BrdUrd accessible to the antibody and to preserve PCNA antigenicity, cells were subjected to DNA digestion according to modified procedures of the endonuclease/exonuclease method described by Dolbeare and Gray (1988). Among the procedures we tested, two were used: one including a mild hydrolysis with HCl before enzymic digestion, called here the $HCl^{(+)}$ procedure; and one excluding this step, called the $HCl^{(-)}$ procedure.

$HCl^{(+)}$ procedure

Methanol-fixed cells were washed several times with PBS at room temperature (RT). Cells were then incubated for 8 minutes in 0.05 M HCl at 4°C, washed 3 times with Tris-HCl buffer at RT, and incubated in detergent solutions (5 minutes in PBS+0.1% Tween 20, 5 minutes in PBS+0.1% NP40) at RT. After 3 washes with Tris-HCl buffer at RT, cells were treated for 30 minutes with 90 units *Eco*RI in 300 μl buffer per slide at 37°C and washed twice with PBS at RT. They were then treated for 30 minutes at 37°C with 600 units Exo III, and quickly washed in 10-fold diluted PBS and twice with PBS containing 0.1% Tween 20. Following this step, cells were labelled with antibodies: 30 minutes with 100 μl drops per chamber of anti-BrdUrd mouse IgG antibody (ascites fluid of clone 76-7, kindly provided by Dr. T. Ternynck, Institut Pasteur, Paris) diluted to 1/250 in PBS+0.1% Tween 20, washed 3 times in PBS+0.1% Tween 20; incubated for 30 minutes with Texas red (TR)-conjugated goat anti-mouse IgG-specific antibodies diluted to 1/80, washed; incubated for 1 hour with anti-PCNA mouse IgM antibody (clone 19A2) diluted to 1/50, washed; incubated for 30 minutes with FITC-conjugated goat anti-mouse IgM-specific antibodies diluted to 1/50. Finally, cells were washed, mounted in glycerol, and stored in the dark at 4°C.

$HCl^{(-)}$ procedure

In this case, the hydrolysis with 0.05 M HCl was omitted and PCNA indirect immunofluorescence revelation steps preceded the enzymic digestion (Tris-HCl, *Eco*RI, Exo III).

Controls

In order to verify the temporal sequence of PCNA and BrdUrd incorporation patterns during S phase, two series of synchronized cell populations were singly labelled for either PCNA or BrdUrd. Cells were synchronized by means of mitotic selection. The flasks were shaken gently to detach mitotic cells from the culture surface. The supernatant was removed and spun down at 500 revs/min (90 *g*, Jouan E96) for 4 minutes. The cell pellets were pooled in warm medium. Cells were counted and viability was tested by the trypan blue exclusion test. Cells were then seeded into plastic tissue culture slides at a concentration of 5000 cells per chamber. The use of plastic slides was mandatory, since detached mitotic cells did not re-adhere to glass slides. Four hours after this step, slides were fixed at 90 minute intervals. For one series of slides, cells were incubated for 30 minutes with 20 μM BrdUrd in fresh medium prior to fixation (1 hour in ethanol 70% at room temperature), and then processed for BrdUrd immunolabelling. For another series of slides, cells were fixed for 2 minutes in 1% paraformaldehyde at room temperature followed by 10 minutes in methanol at −20°C before PCNA immunolabelling (without any denaturation step).

Microscopy and image processing

For high-resolution and simultaneous acquisition of images at two fluorescence emission wavelengths, digital images of central optical sections through nuclei labelled for BrdUrd and PCNA were collected with a Zeiss confocal laser scanning microscope (model LSM10) fitted with a double photodetector (courtesy of Zeiss France), using a ×63, NA 1.4, Plan-apochromat oil immersion objective. Fluorescein (FITC, PCNA labelling) and Texas red (TR, BrdUrd labelling) were simultaneously excited at 488 nm with an internal air-cooled argon ion laser. Emission filter for FITC was 515-545 nm bandpass; for TR a dichroic filter of 580 nm was used in combination with a 590 nm longpass. The theoretical optical section thickness was 0.5 μm and 0.8 μm for the FITC and TR images, respectively. The double photodetector made it possible to collect the two fluorescence emissions from the

same optical section simultaneously. This is a necessary condition for a precise comparison of the location of two dyes. However, since 488 nm is far from the optimum excitation wavelength for TR, we checked on test samples that the use of a 488 nm excitation wavelength, instead of 514 nm, also provides all the expected information on the TR images. These test samples were processed for BrdUrd indirect immunofluorescence staining using a mixture of FITC- and TR-conjugated goat anti-mouse Igs as secondary antibodies, thus ensuring that both fluorescent dyes were co-localized within the cell nucleus. The two fluorescence emissions (BrdUrd, FITC-labelled) and (BrdUrd, TR-labelled) were then observed using the optical procedure described above. The FITC images, optimally excited, were used as a reference and compared with TR images. We verified that the TR fluorescence emission images did not significantly differ from the FITC fluorescence emission images, and therefore that the 488 nm excitation provided a satisfactory TR emission.

Image processing and display were carried out on a Compaq 386/20 personal computer fitted with a Matrox MVP/AT frame storage board (SAMBA™ 2005, Alcatel-TITN Co., France). Specialized routines for noise filtering (median filter), image enhancement (level normalization) and pseudo three-dimensional representation mode of fluorescence intensities were written in C.

Reagents and materials

DMEM, FCS, L-glutamine, amphotericin, *Eco*RI, Exo III, EDTA (Boehringer, France); insulin, BrdUrd, NP40 (SIGMA, France); penicillin/streptomycin, non-essential amino acids (Gibco, France); trypsin (BioMérieux, France); Tween 20 (Merck-Clévenot, France); anti-BrdUrd mouse IgG antibody (ascites fluid of clone 76-7 kindly provided by Dr. T. Ternynck, Institut Pasteur, Paris); anti-PCNA mouse IgM antibody (clone 19A2, Coulter, Florida); FITC-conjugated goat anti-mouse IgM-specific antibodies and TR-conjugated goat anti-mouse IgG-specific antibodies (Immunotech, France); 25 cm^2 Corning flasks (Falcon, France); tissue culture chamber-slides (Miles, France).

Results

BrdUrd versus PCNA location during S phase

Following the $HCl^{(+)}$ procedure, we observed PCNA staining only in those cells that had a positive reaction for BrdUrd (Fig. 1). We scanned more than a thousand nuclei, repeated the experiments several times, and never saw cells labelled for either PCNA alone or BrdUrd alone. In order to compare BrdUrd and PCNA distributions through S phase, the temporal progression was assessed according to the known ordering of various BrdUrd incorporation patterns (van Dierendonck et al., 1989; Fox et al., 1991; Usson and Humbert, 1992; Humbert and Usson, 1992). The percentages of the different patterns were: 13.7% for the very early S phase nuclei (few foci of replication); 55.5% for the subsequent homogeneous distribution of replication; 23% for the later perinuclear and perinucleolar distribution; and 7.8% for the last patterns (few large foci). We also verified that the sequence of patterns was consistent with observations made on parallel synchronized cultures stained for BrdUrd or PCNA. The BrdUrd vs. PCNA intranuclear co-location was estimated from central optical sections obtained with a confocal laser scanning microscope. Fig. 2 shows the various typical PCNA and BrdUrd patterns of doubly stained nuclei observed through S phase (typical patterns, from top to bottom). The left column of Fig. 2 shows confocal images of the PCNA labelling, the right column the corresponding images of the BrdUrd labelling within the same nuclei. In order to give a better estimation of the intranuclear co-location of the two labellings, Fig. 3 is a pseudo three-dimensional representation of the fluorescence relative intensities in the nuclei represented in Fig. 2. For both labellings (PCNA vs. BrdUrd), the peaks showing the location and the relative intensity of fluorescence are similarly

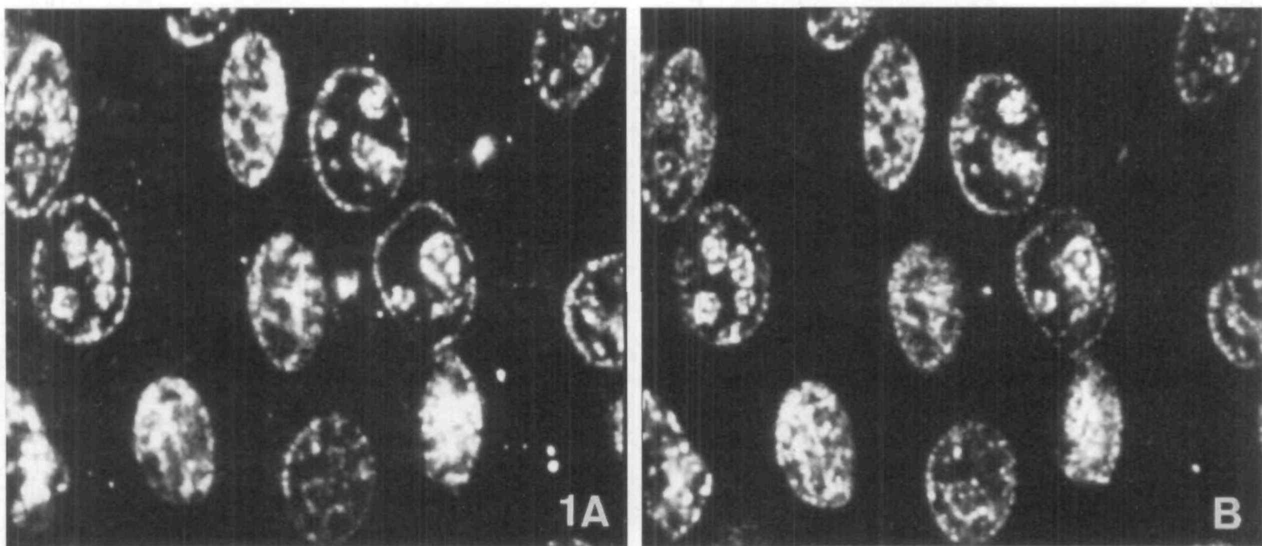


Fig. 1. PCNA (A) versus BrdUrd (B) immunolabelling of MCF-7 cells. The $HCl^{(+)}$ procedure was used prior to simultaneous immunofluorescent labelling. Images were acquired using a confocal laser scanning microscope (model Zeiss LSM10). FITC-PCNA: excitation wavelength, 488 nm, emission filter, 515-565 bandpass; TR-BrdUrd: excitation wavelength, 514 nm, emission filter, 590 longpass. Final magnification, ×755.

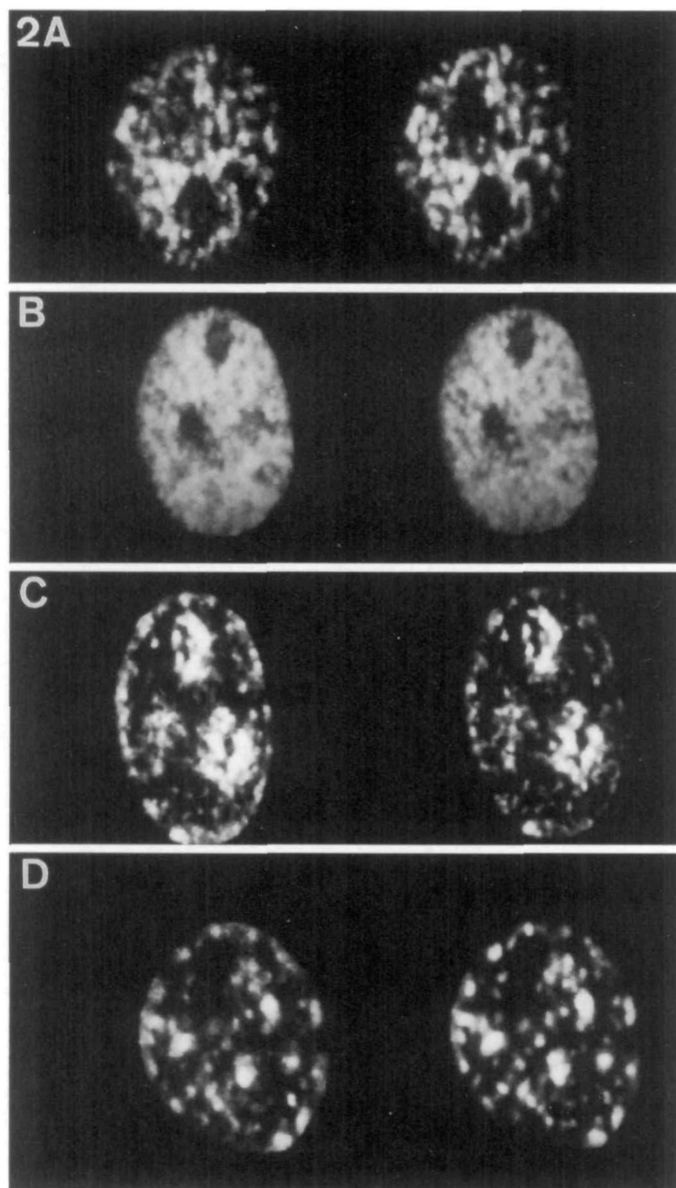


Fig. 2. Typical PCNA (left side) and BrdUrd (right side) patterns of doubly-stained nuclei, through the S phase (from A to D). The $HCl^{(+)}$ procedure was used prior to simultaneous immunofluorescent labelling. Central optical sections acquired with a confocal laser scanning microscope fitted with a double photodetector (grey level images). Excitation wavelength, 488 nm. Emission filters, 515-545 nm bandpass for FITC; dichroic filter of 580 nm with a 590 nm longpass for TR. Final magnification, ×1320.

distributed, and their distributions vary concomitantly during S phase. At the beginning of S phase (Fig. 3A), DNA replication is restricted to a few sites, mainly located in the interior of the nucleus except for the regions occupied by the nucleoli, and are seldom located close to the nuclear periphery. Thereafter (Fig. 3B), DNA replication occurs all over the nucleus, except in the nucleoli (the optical section passed through 3 nucleoli). Later in S phase (Fig. 3C), DNA replication sites are essentially located close to the boundary of the nucleus and nucleoli (the optical section passed through the middle of 2 nucleoli, a third nucleolus outcropped). At the end of S phase (Fig. 3D), DNA replication is restricted to a few well-segregated sites, located close to the nuclear boundary but also in the nuclear interior. The stained sites seem larger than the sites observed in previous nuclei (see also Fig. 2). A thorough examination of these images shows that there are differences in the relative amplitudes of some peaks between the two images of a pair.

When the $HCl^{(+)}$ procedure was used we did not observe nuclei stained for PCNA alone in exponentially growing cell populations. However, in single staining experiments (parallel synchronous cultures) in which the HCl hydrolysis was omitted from the PCNA labelling procedure, we found that PCNA staining could be observed before the appearance of any BrdUrd staining, therefore before S phase. In those cells, PCNA staining distribution was rather homogeneous and covered the whole nuclear area (except the nucleoli). This observation has already been reported (Celis and Celis, 1985; Hutchison and Kill, 1989; Nakane et al., 1989; van Dierendonck et al., 1991). Since in these experiments no acidic treatment was used before PCNA staining, we assume that the absence of cells labelled only for PCNA in our doubly-stained preparations was due to this hydrolysis. We therefore tested another procedure ($HCl^{(-)}$) on exponentially growing cell populations. The same PCNA and BrdUrd patterns as observed in experiments using the $HCl^{(+)}$ procedure were observed in the doubly-stained cells. Figure 4 shows an example of a late S phase nucleus (compare with Fig. 3D). In addition, we also observed that about 39% of the PCNA-stained nuclei were negative for BrdUrd labelling (Fig. 5). Among these nuclei two types of PCNA patterns were observed: (i) in most of the nuclei (91.5%), PCNA distribution was rather homogeneous (Fig. 5) and very similar to that of the nuclei that were PCNA labelled before S phase in the synchronized cell cultures; (ii) in a few nuclei (8.5%), PCNA distribution was very similar to that of early S phase intranuclear distribution (Fig. 6). Fig. 6A shows PCNA distribution within two neighbouring nuclei: one of the nuclei (arrow) is negative for BrdUrd labelling (Fig. 6B), the other nucleus (arrowhead) is at the beginning of S phase (BrdUrd-labelled, Fig. 6B). In this figure, one can observe that the PCNA distribution of the BrdUrd negative nucleus is similar to that of the nucleus at the beginning of S phase. Fig. 6 (arrow) also demonstrates that the cross talk between FITC and TR is negligible.

Discussion

The proliferating cell nuclear antigen, PCNA, co-localizes with newly replicated DNA within the cell nucleus during S phase. This was monitored by simultaneous BrdUrd and PCNA immunofluorescent labelling. The immunocytochemical method that we used ($HCl^{(+)}$ procedure) is derived from the enzymic procedure used by Dolbeare and Gray (1988) to produce single-stranded DNA for detection of incor-

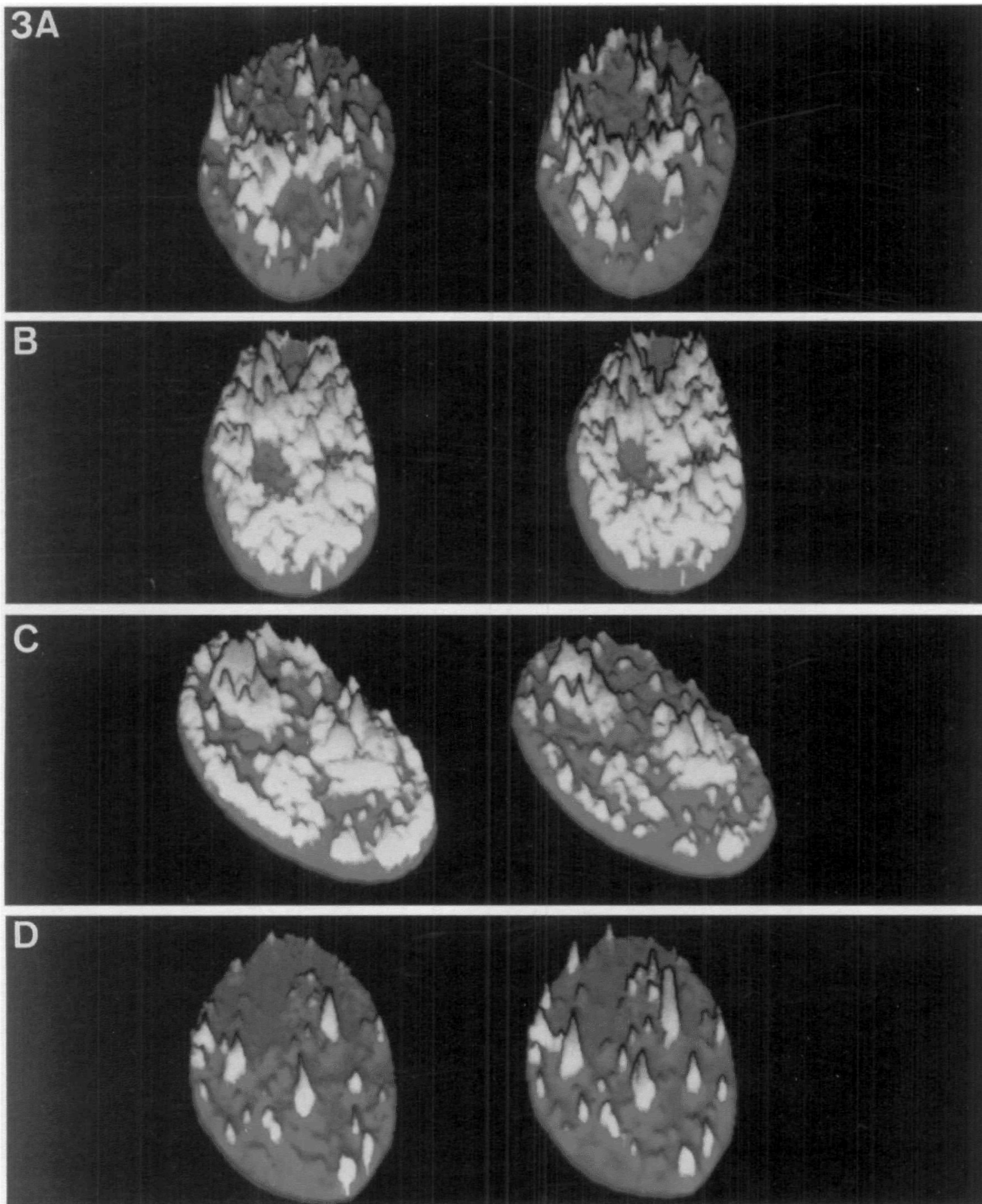
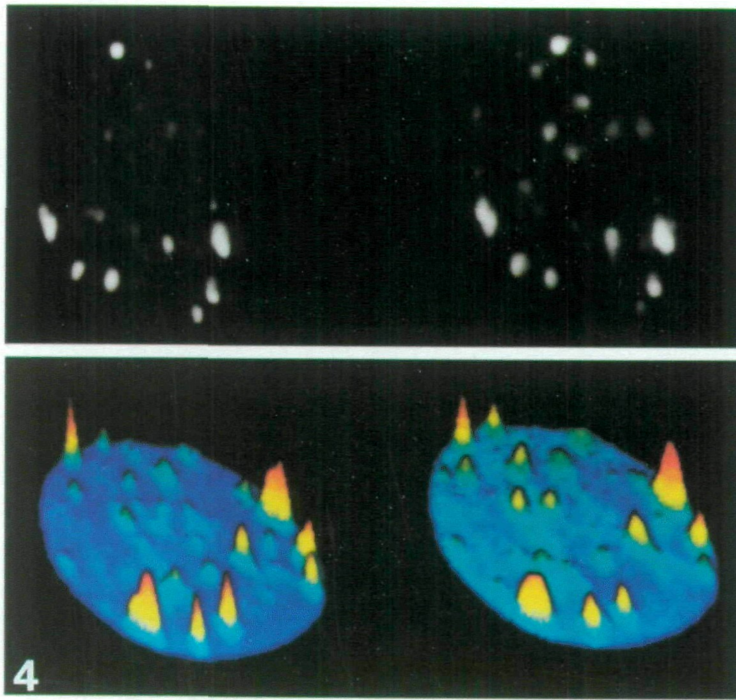


Fig. 3. Three-dimensional representation of the relative fluorescence intensities of the nuclei presented in Fig. 2. The height of the peaks and colours are representative of the relative fluorescence intensities (blue, low fluorescence; yellow, medium fluorescence; red, high fluorescence). Left side, PCNA distribution; right side, BrdUrd distribution; from A to D, progression of S phase. Final magnification, ×1685.



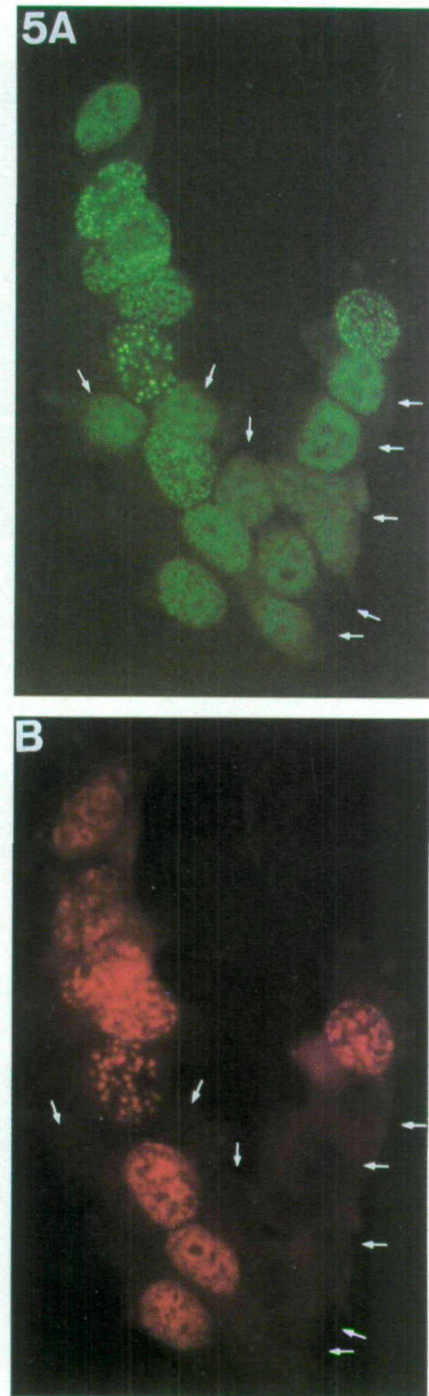


Fig. 5. PCNA (A, green) versus BrdUrd (B, red) immunolabelling of MCF-7 cells. The $HCl^{(-)}$ procedure was used prior to simultaneous immunofluorescent labelling. Some PCNA positive cells are BrdUrd negative (arrows). Conventional fluorescence microscopy, Zeiss Axiophot. Final magnification, ×520.

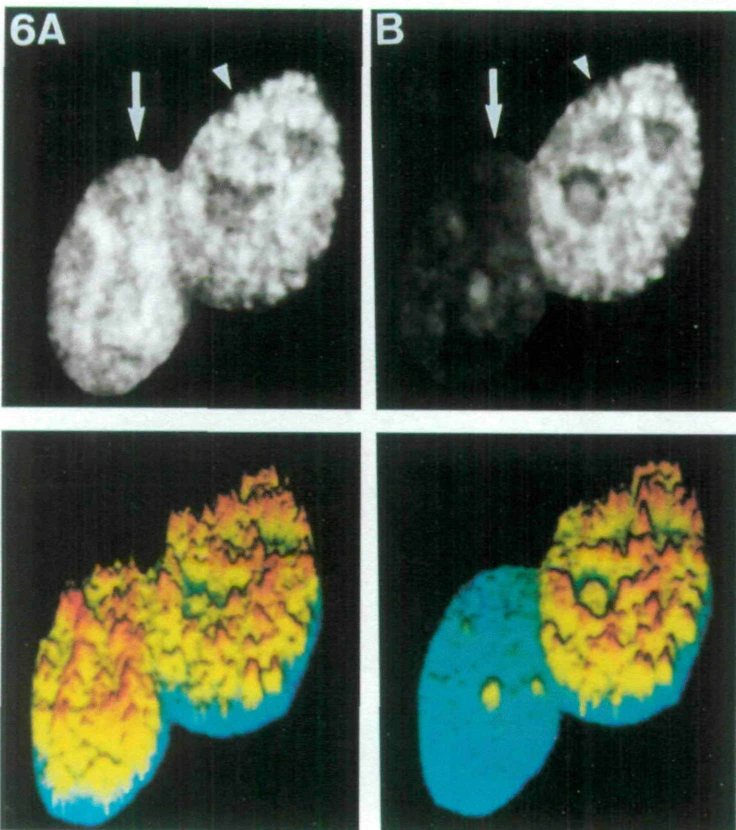


Fig. 4. Late S phase nucleus. Top: central optical sections acquired with a confocal laser scanning microscope fitted with a double photodetector (grey level images, excitation and emission wavelength as in Fig. 2); bottom: corresponding three-dimensional representation of the relative fluorescence intensities (colour codes as in Fig. 3); left, PCNA distribution; right, BrdUrd distribution. The $HCl^{(-)}$ procedure was used prior to simultaneous immunofluorescent labelling. Final magnification, ×1685.

Fig. 6. Distribution of PCNA within a BrdUrd negative nucleus (arrows) and in a nucleus at the beginning of the S phase (arrowhead). A. PCNA distribution; B, BrdUrd distribution. Top: central optical sections acquired with a confocal laser scanning microscope fitted with a double photodetector (grey level images, excitation and emission wavelength as in Fig. 2); bottom: corresponding three-dimensional representation of the relative fluorescence intensities (colour code as in Fig. 3). The $HCl^{(-)}$ procedure was used for simultaneous immunofluorescent labelling. Final magnification, ×1685.

porated BrdUrd. The S phase temporal progression was assessed by reference to the known ordering of BrdUrd intranuclear distribution (van Dierendonck et al., 1989; Fox et al., 1991; Usson and Humbert, 1992; Humbert and Usson, 1992) and to observations made on parallel synchronized cultures stained for either BrdUrd or PCNA. BrdUrd vs. PCNA intranuclear co-location was estimated from optical sections acquired using a confocal laser scanning microscope. Using this method, we confirmed the published results that strongly suggested the co-location of PCNA and BrdUrd on the basis of comparison of the immunofluorescent labelling in parallel synchronous cell cultures (Bravo and Macdonald-Bravo, 1987; van Dierendonck et al., 1991). However, in this respect our results did not fit exactly with the results obtained by Kill et al. (1991) after double biotin-11-dUTP and PCNA staining. These authors sometimes observed a lack of correspondence between PCNA patterns and DNA replication patterns; the DNA replication patterns resembling the expected preceding patterns of PCNA. It is true that DNA replication labelling shows an integration of events, while PCNA labelling shows an instantaneous event. However, we did not observe such rapid relocation of PCNA to new sites of DNA replication. The lack of correspondence that we observed was minimal and concerned the relative amplitudes of the two fluorescence signals (Fig. 3). The mild acidic hydrolysis that we used should have removed the relocated PCNA during S phase as it does for PCNA staining before S phase. This would explain the discrepancy from the results of Kill et al. (1991). However, using a procedure excluding HCl hydrolysis (the $HCl^{(-)}$ procedure), we still did not observe such relocation of PCNA during S phase. Could the enzymic procedure be responsible for the discrepancy by mediating a partial dissociation of PCNA from DNA? All the PCNA patterns that we observed were consistent with the PCNA patterns observed in untreated controls and we observed PCNA staining before S phase using the $HCl^{(-)}$ procedure, suggesting that this was not the case. On the other hand, it is also unlikely that the enzymic procedure was not effective enough for BrdUrd detection, since all the BrdUrd incorporation patterns that we observed were consistent with those previously described (van Dierendonck et al., 1989; Fox et al., 1991; Usson and Humbert, 1992; Humbert and Usson, 1992). Furthermore, neither HCl hydrolysis nor the enzymic procedure could be responsible for the PCNA staining that we observed in late S phase cells. We suggest that (i) in MCF-7 cells the transition time between successive replication patterns is shorter than in the fibroblastic cells studied by Kill et al. (1991), that (ii) in MCF-7 cells the delay between the assembly of pre-replication complexes and DNA replication is shorter, or that (iii) the dissociation of the DNA replication complexes from the replicated DNA is slower in MCF-7 cells. In order to confirm or negate these hypotheses, a study of the displacement between replication complexes and the replicated DNA, using BrdUrd pulse-chase experiments, would be of particular interest.

Kill et al. (1991) also reported that they did not observe DNA replication patterns corresponding to the first PCNA pattern A (very few foci of PCNA staining). However, this type of DNA replication pattern (very few foci of staining) was observed at the very early stage of DNA replication by Banfalvi et al. (1990) on permeabilized CHO cells pulse labelled for 10 minutes with biotin-11-dUTP. We also observed this type of pattern on normal fibroblastic cells (MRC-5) BrdUrd labelled for 1 hour (Humbert and Usson, 1992). As the cell population was growing exponentially, these cells did not spend the entire labelling hour in S phase. Therefore, the reason for this discrepancy may be due to the duration of labelling and the cell sampling used by Kill et al. (1991). Since the number of discrete DNA replication foci rapidly increases at the beginning of S phase (Banfalvi et al., 1990; Humbert and Usson, 1992), 90 minutes of replication marker incorporation may be too long to visualize the very first DNA replication steps.

One interesting observation comes from comparisons between PCNA and BrdUrd labelling and concerns non-S phase PCNA labelling. While PCNA staining is similar to BrdUrd staining during S phase, some authors previously noted that there was a delay between the appearance of PCNA immunofluorescence and the initiation of DNA replication (Celis and Celis, 1985; Hutchison and Kill, 1989; Nakane et al., 1989). Fixation (Bravo and Macdonald-Bravo, 1987) and antibody specificity (Huff et al., 1990) were shown to be crucial factors for the localization of PCNA. However, the delay between the appearance of PCNA immunostaining and DNA replication initiation could be due to a time-lapse between the assembly of replication complexes and DNA replication initiation, which could be dependent on the cell type and the proliferation rate. Kill et al. (1991) clearly confirmed this hypothesis by in vitro and in vivo experiments: after synchronization, the first cells they observed were PCNA positive (pattern A) and biotin-11-dUTP negative. Using the $HCl^{(-)}$ procedure we also observed PCNA positive cells that were BrdUrd negative. Most of these cells showed a homogeneous PCNA intranuclear distribution, as has been observed in other studies (Celis and Celis, 1985; Hutchison and Kill, 1989; Nakane et al., 1989; van Dierendonck et al., 1991), probably resulting partly from non-specific staining (van Dierendonck et al., 1991). However, a few of these PCNA positive/BrdUrd negative cells showed a PCNA distribution very similar to that of an early S phase nucleus. This is particularly obvious in Fig. 6. The fact that we did not observe such cells following the $HCl^{(+)}$ procedure may be due to loss of PCNA that was not firmly engaged in replication forks. This hypothesis is supported by the data of Landberg and Roos (1991), which showed that after cell permeabilization by 0.1% Triton X-100 detergent, the PC10 anti-PCNA antibody was a specific S phase marker. We therefore agree with Kill et al. (1991) when they conclude that PCNA localization precedes the initiation of DNA replication. We add that

while the BrdUrd method ($HCl^{(+)}$ procedure) is more suitable for the analysis of the displacement between firmly engaged replication complexes and replicated DNA during S phase, the method of biotin-11-dUTP microinjection applied by Kill et al. (1991) is more suitable for the identification of cells just before initiation of DNA replication. Indeed, it should be noted that using the $HCl^{(-)}$ procedure we could not make our observations on dense cell clusters, which were often weakly stained for BrdUrd (low signal to noise ratio). Celis et al. (1986) also observed cells that were both PCNA positive and tritiated thymidine negative, but showed a PCNA staining distribution different from that which was commonly found before S phase. The authors surmised that these cells had withdrawn from the cell cycle. As PCNA does not dissociate from DNA when DNA synthesis is inhibited (Hutchison and Kill, 1989), their interpretation may be valid. However, these cells could be G_1 phase cells reaching S phase by a different path from the other cells, instead of leaving the cell cycle, which would explain the particular staining pattern. This hypothesis is supported by the data of du Manoir et al. (1991), who postulated the existence of two Ki-67 staining pathways, within the 2*C* population, for reaching S phase. BrdUrd/PCNA/Ki-67 or biotin-11-dUTP/PCNA/Ki-67 triple staining could help to elucidate the nature of the cell sub-population described by Celis et al. (1986) and to determine whether or not these cells are proliferating cells.

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