Changes in Cyclin/Proliferating Cell Nuclear Antigen Distribution During DNA Repair Synthesis

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Abstract. UV irradiation of quiescent human fibroblasts immediately triggers the appearance of the nuclear protein cyclin/proliferating cell nuclear antigen (PCNA) as detected by indirect immunofluorescent staining after methanol fixation. This was found to be independent of new synthesis of cyclin/PCNA by two-dimensional gel analysis and cycloheximide treatment. The intensity of the immunofluorescent staining of cyclin/PCNA observed in UV-irradiated cells corresponded with the UV dose used and with the DNA repair synthesis detected by autoradiography. The nuclear staining remains as long as DNA repair activity is detected in the cells. By extracting the UV-irradiated quiescent cells with Triton X-100 and fixing with formaldehyde, it was possible to demonstrate by indirect immunofluorescence rapid changes in the cyclin/PCNA population after irradiation, a small proportion (5–10%) of which is tightly associated to the nucleus as determined by high salt extraction. By incubating at low temperature and depleting the ATP pools of the cells before UV irradiation, we have demonstrated that the changes in cyclin/PCNA distribution observed involve at least two different nuclear associations.

THE proliferating cell nuclear antigen (PCNA; 18, 24, 25, 27)[1] and cyclin (reviewed in references 1, 10) have been shown to be identical (17). This protein is present in variable amounts in normal proliferating and transformed cells from a variety of vertebrates (1, 10). The synthesis of cyclin/PCNA is regulated during the cell cycle, increasing during the DNA replication period (2). In quiescent cells, where cyclin/PCNA expression is very low, mitogens induce a coordinate synthesis of this protein and DNA (3).

Immunofluorescence studies of cyclin/PCNA have shown that its nuclear distribution changes dramatically through the S-phase and that it is tightly associated with DNA replication sites (4, 5, 10, 18).

The observations that cyclin/PCNA is required for SV-40 DNA replication in vitro (20, 21) and that it is identical to the auxiliary protein of DNA polymerase δ (6, 22), a protein that enhances the ability of the polymerase to use templates which contain long single-stranded regions (26), are the most direct proofs that this protein plays a role in DNA synthesis.

DNA polymerase δ is the most recently discovered of the four DNA polymerases known at present (for review see reference 13). It possesses a 3' to 5' exonuclease activity (7) which may contribute to the fidelity of DNA replication (11, 15, 16). This suggests that both polymerases, α and δ, could function jointly during DNA replication. Moreover, recent evidence has shown that DNA polymerase δ plays an impor-

tant role during DNA replication and DNA repair synthesis (11, 12, 15, 19). Being cyclin/PCNA, a putative regulatory subunit of DNA polymerase δ, we found it important to study the effect of UV irradiation in the synthesis and distribution of this protein in quiescent human fibroblasts. Here we present evidence that cyclin/PCNA appearance in UV-irradiated quiescent cells correlates with DNA repair synthesis.

Materials and Methods

Cells

Human skin fibroblasts were grown in DME supplemented with 10% FCS and antibiotics (10 U/ml of penicillin, 50 µg/ml of streptomycin). Quiescent cells were obtained by transferring confluent cultures to serum-free medium for 72 h.

Indirect Immunofluorescence and Autoradiography

Cells grown on glass coverslips (9 × 9 mm) were washed twice with PBS before fixation. Methanol fixation was carried out at −20°C for 5 min. Formaldehyde treatment was done at 4°C for 20 min using 4% formaldehyde in PBS. Cells were permeated with 0.1% Triton X-100 for 1 min at room temperature. After washing extensively with PBS, the coverslips were covered with 10 µl of human anti–PCNA autoantibody (1:50 in PBS) and incubated for 1 h at 37°C in a humid environment. Coverslips were washed with PBS and covered with 10 µl of rhodamine-conjugated rabbit anti–human immunoglobulin (1:50 in PBS, Dakopatts a/s, Glostrup, Denmark). After 1 h of incubation at 37°C, the coverslips were washed extensively with PBS and mounted on Moviol 4-88 (Hoechst AG, Frankfurt, Federal Republic of Germany [FRG]).

For detection of DNA repair synthesis, cells were labeled with 10 µCi/ml [methyl-³H]thymidine and processed for autoradiography. Radiographs were exposed for 15 d.

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1. *Abbreviation used in this paper:* PCNA, proliferating cell nuclear antigen.

Ultraviolet Light Irradiation

Cells were grown on glass coverslips in 24-well plates. For UV irradiation, the medium was removed and replaced with PBS (0.5 ml/well). Cultures were irradiated with a 30-W germicidal lamp (254 nm; Osram Mannheim, FRG). The UV doses were monitored with a UV intensity meter (UV-Messgerät, 87-3964; Vetter GmbH, Wiesloch, FRG). After irradiation, the PBS was replaced with serum-free DME (1 ml/well).

Results

Induction of Immunofluorescent Staining of Cyclin/PCNA by UV Irradiation in Quiescent Cells

Previous studies have demonstrated that cells quiescent for 24–48 h contained a nuclear population of cyclin/PCNA that was not detected by immunofluorescent staining after methanol fixation (5). In methanol-fixed cells, this antigen presented strong staining during S-phase only when associated to the DNA replication sites (5). These observations prompted us to investigate possible changes of cyclin/PCNA staining during DNA repair synthesis in quiescent human skin fibroblasts. Cells were irradiated with UV light (40 J/m²) and labeled for 4 h with [³H]thymidine to detect DNA repair activity by autoradiography or fixed with methanol for immunofluorescent analysis. Quiescent cells normally negative for cyclin/PCNA staining (Fig. 1 *A*) presented a nuclear punctuated distribution of the antigen after irradiation (Fig. 1 *B*). Autoradiographs showed that in >95% of the cells DNA repair activity was induced after UV irradiation (Fig. 1 *C*). Control quiescent cells presented ∼1% of labeled nuclei (not shown). Incubation of UV-irradiated cells for different periods of time up to 24 h revealed that cyclin/ PCNA staining was still present at that time in cells with active DNA repair synthesis, demonstrating that the appearance of antigen staining was not transient and that it was associated with DNA repair.

The homogenously punctuated staining pattern of cyclin/ PCNA observed during DNA repair synthesis in quiescent cells was different to that in S-phase cells where its pattern is heterogenous and varies throughout this period (4, 8). In contrast, the punctuated staining of cyclin/PCNA did not significantly change during DNA repair synthesis.

A correlation between the intensity of cyclin/PCNA staining and the UV doses used was observed (Fig. 2). A significant amount of immunofluorescent staining was found when quiescent cells were UV irradiated with 5 J/m² (Fig. 2 *B*). The intensity of the response increased up to UV doses of 40 J/m². Identical results were obtained when DNA repair activity was determined by autoradiography (not shown). At all the UV doses the nuclear localization of cyclin/PCNA was identical, punctuated, and uniformly distributed.

Appearance of Cyclin/PCNA Staining After UV Irradiation Is Independent of Protein Synthesis

To determine if UV-induced DNA repair synthesis triggered the synthesis of cyclin/PCNA, quiescent cells were labeled for 1 h with [³⁵S]methionine at different times after UV treatment. Two-dimensional gel analysis of the labeled proteins demonstrated that cyclin/PCNA synthesis was not induced for at least the first 6 h after irradiation (not shown), suggesting that the protein present in quiescent cells was responsible for the immunofluorescent staining detected dur-
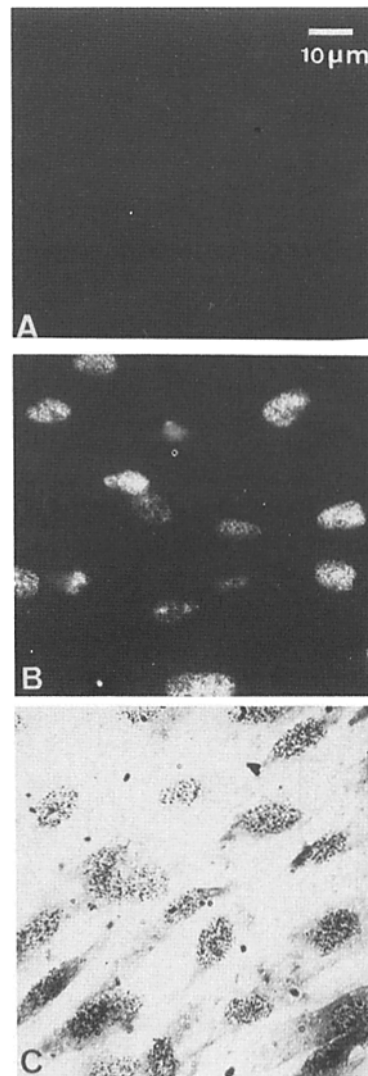

Figure 1. UV-induced cyclin/PCNA staining in quiescent cells. Immunofluorescence staining of cyclin after methanol fixation of quiescent cells (*A*) or cells after 4-h UV irradiation with 40 J/m² (*B*). Autoradiograph of cells labeled for 4 h with [³H]thymidine (10 µCi/ml) after irradiation (*C*).

ing DNA repair synthesis. It was possible that induction of cyclin/PCNA staining could be due to the synthesis of protein(s) necessary for the association of cyclin/PCNA to a specific nuclear complex. To study this suggestion quiescent cells pretreated with cycloheximide for 2 h were UV irradiated and analyzed for the presence of cyclin/PCNA 4 h later. Appearance of cyclin/PCNA staining was unaffected by a dose of cycloheximide (10 µg/ml) that inhibited >95% of the protein synthesis (Fig. 3). These results demonstrated that all molecules necessary for triggering cyclin/PCNA changes are present in the nucleus of quiescent cells.

Cyclin/PCNA Staining Induced by UV Irradiation Is Independent of DNA Synthesis

The appearance of cyclin/PCNA staining after UV irradiation of quiescent cells could depend on DNA repair activity
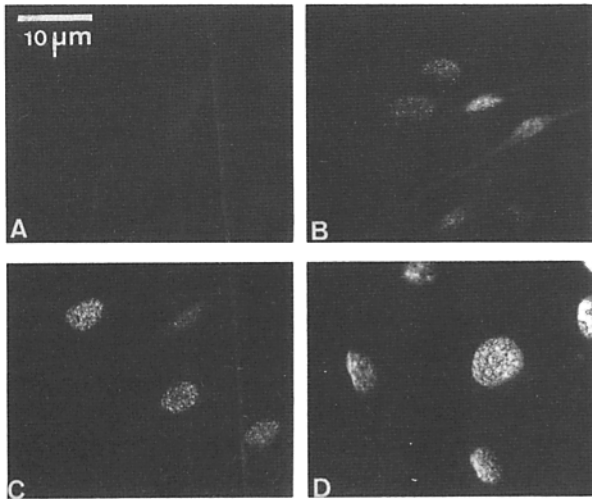
Figure 2. Correlation between cyclin/PCNA staining and UV doses. Immunofluorescent staining of cyclin after methanol fixation of quiescent cells (*A*) or cells 1 h after irradiation with 5 J/m² (*B*), 10 J/m² (*C*), and 40 J/m² (*D*) of UV dose.
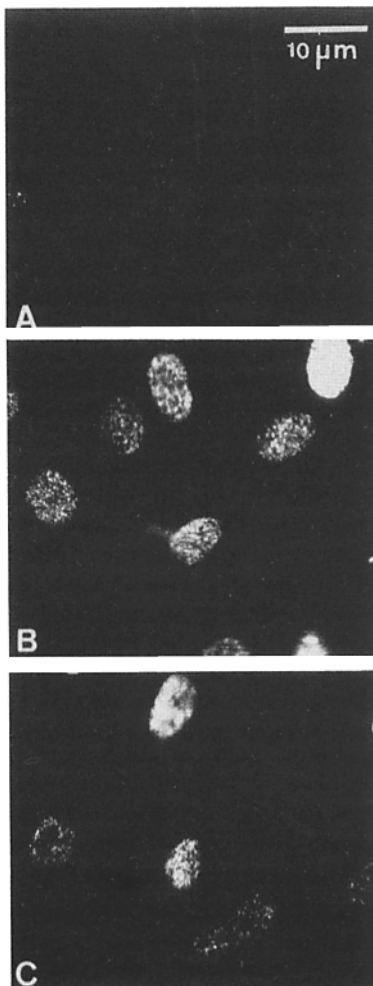

Figure 3. Effect of cycloheximide on irradiation-induced cyclin/PCNA staining. (*A*) Quiescent cells; cells UV-irradiated in the presence (*B*) or absence (*C*) of cycloheximide. Cells were methanol fixed 2 h after irradiation. UV dose, 40 J/m².
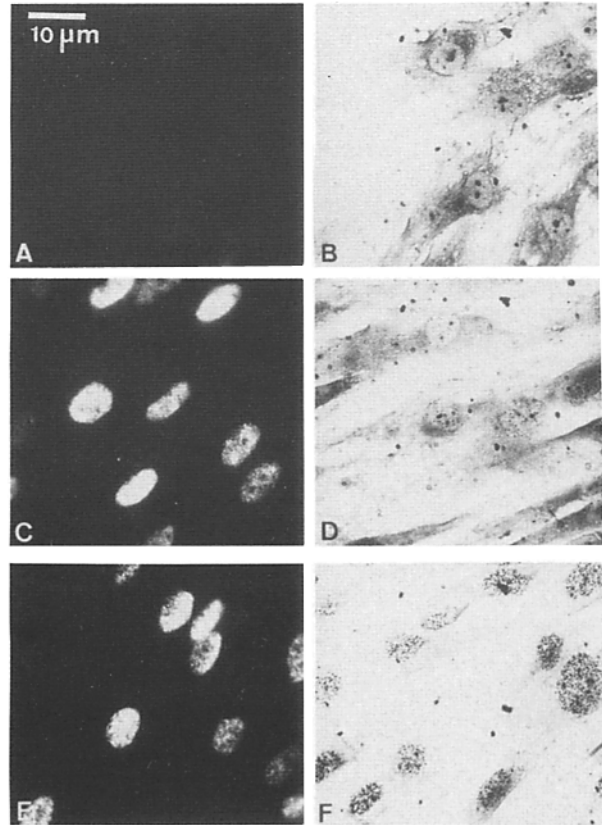

Figure 4. Effect of aphidicolin in cyclin/PCNA staining of UV-irradiated cells. (*A*) Quiescent cells; (*C*) cells irradiated in the presence of aphidicolin 10 µg/ml; (*E*) Control irradiated cells. (*B*, *D*, and *F*) Their respective autoradiographs. Cells were methanol fixed or processed for autoradiography after 4 h irradiation. UV dose, 40 J/m².

itself. To study this, cells were pretreated with aphidicolin, an inhibitor of DNA polymerases α and δ (14), for 2 h before UV irradiation. Cells were then labeled with [³H]thymidine for 4 h in the presence of the drug and processed for autoradiography or immunofluorescence. The results showed that cyclin/PCNA staining after UV irradiation was not affected in cells presenting >95% inhibition in DNA repair activity (Fig. 4). As it was possible that DNA synthesis inhibition was only slowing down the appearance of cyclin/PCNA staining after UV irradiation, a detailed kinetics of the early appearance of the antigen was done in the absence or presence of aphidicolin. As shown in Fig. 5, as early as 15 s after irradiation nuclear cyclin/PCNA staining could be observed. The intensity of the staining increased up to 5 min. Aphidicolin did not affect the kinetics of appearance of the nuclear staining nor its intensity. This suggests that the relocation of cyclin/PCNA is not triggered by DNA repair synthesis itself but possibly precedes it.

Changes in Cyclin/PCNA Population During DNA Repair Synthesis

The population of cyclin/PCNA present in cells quiescent for 24–48 h has been demonstrated to be detectable by immunofluorescence analysis only after formaldehyde fixation (5).
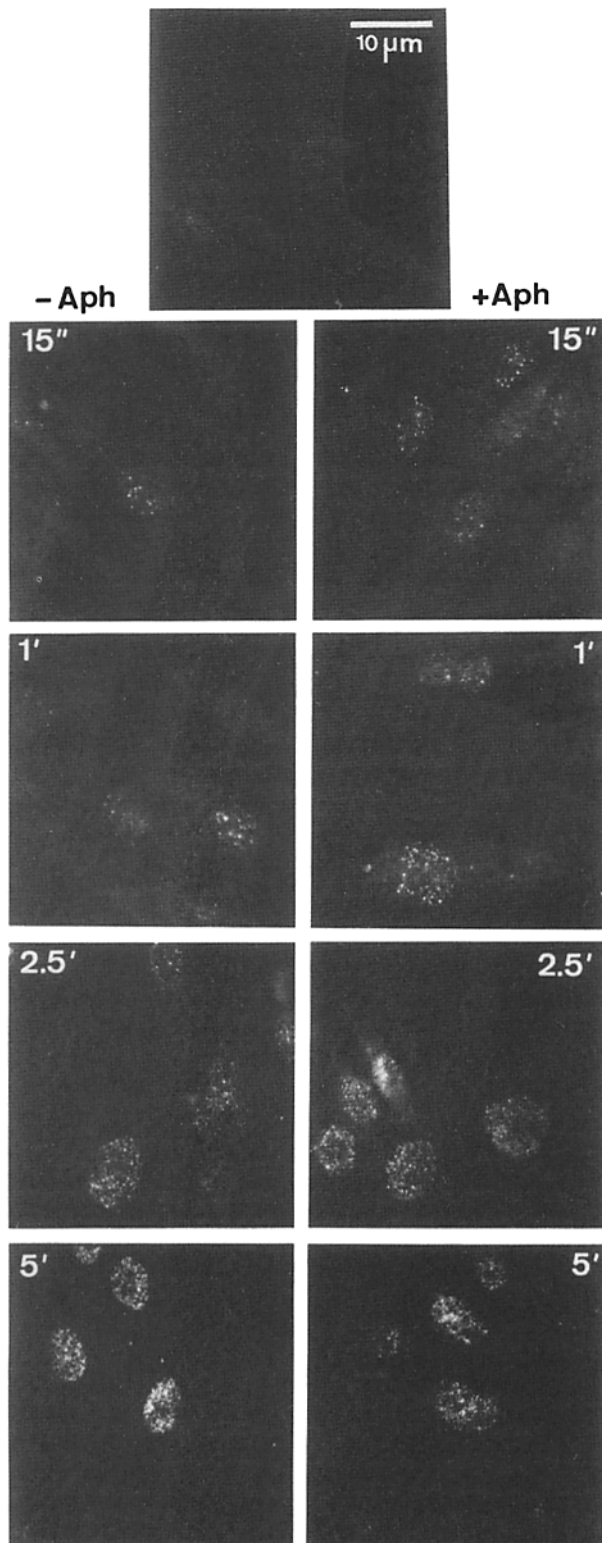
Figure 5. Rapid induction of cyclin/PCNA staining by UV irradiation is independent of DNA synthesis. Quiescent cells were irradiated with a UV dose of 40 J/m² and methanol fixed at the indicated times. Cells were incubated for 2 h with 10 μg/ml aphidicolin before irradiation (+Aph). Control cells (−Aph).

This cyclin population was also shown to be extractable by Triton X-100, suggesting that it was loosely attached to nuclear components (5). To determine if changes in this population could be observed during DNA repair synthesis, UV-irradiated quiescent cells were extracted with Triton X-100 before formaldehyde fixation and processed for immunofluorescence. Quiescent nonirradiated cells fixed with formaldehyde showed a strong nuclear staining for cyclin/PCNA (Fig. 6 A), however if the cells were extracted with detergent before formaldehyde fixation no antigen could be detected (Fig. 6 B). In contrast, UV-irradiated cells extracted with Triton X-100 before formaldehyde fixation presented a strong cyclin/PCNA staining (Fig. 6 C). A small proportion (5–10%) of cyclin/PCNA remained associated to the nucleus after high salt extraction as determined by two-dimensional gel analysis (not shown) and immunofluorescent staining (Fig. 6 D).

Appearance of Two Cyclin Populations After UV Irradiation

The different fixation procedures used allowed us to detect by immunofluorescent staining different populations of cyclin. Methanol fixation restricted the detection of the antigen to that present only in the replication sites. Formaldehyde fixation allowed the detection of the total antigen, independent of its nuclear localization. This prompted us to investigate if the change in cyclin/PCNA distribution detected in methanol- or Triton-formaldehyde-fixed cells after UV irradiation could be dissociated from each other. For this reason, we studied the effect of temperature and ATP synthesis inhibitors on cyclin/PCNA appearance after UV irradiation. Quiescent cells incubated on ice for 15 min were irradiated and fixed at different times with methanol or Triton-formaldehyde. No differences were observed in the appearance of the cyclin/PCNA population detected after Triton-formaldehyde fixation (Fig. 7, B, D, and F), however the cyclin/PCNA population detected after methanol fixation (present only in DNA replication sites) was completely absent (Fig. 7, A, C, and E). DNA repair activity as determined by autoradiography was completely inhibited under these conditions (not shown). This last observation is in agreement with the immunofluorescence analysis after methanol fixation which detected antigen present only in a replicative complex.

To decrease the cellular ATP pools, inhibitors of oxidative phosphorylation and glycolysis were used. Cultures were incubated for 30 min in glucose-free medium in the presence of 10 mM sodium arsenate, 10 mM sodium azide, and 12 mM 2-deoxyglucose (23) before irradiation. Cells were kept in this medium for different times and processed for immunofluorescence or autoradiography. The presence of ATP synthesis inhibitors did not affect the appearance of the detergent-resistant population of cyclin/PCNA (Fig. 8 D), however, this treatment completely blocked the appearance of cyclin/PCNA detected after methanol fixation. These results suggest the presence of a complex to which cyclin/PCNA immediately associates after DNA damage, even at low temperature and in the absence of energy.

Discussion

We have demonstrated that UV irradiation of quiescent cells induces a rapid increase in the nuclear staining of cyclin/PCNA in methanol-fixed cells. A similar effect has been also
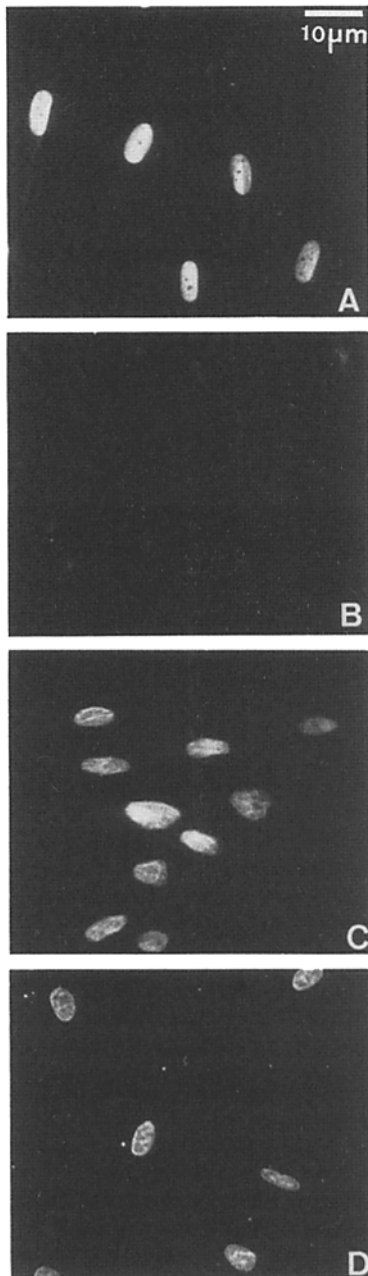
Figure 6. Changes in cyclin/PCNA populations after UV irradiation. Immunofluorescent staining of (*A*) quiescent cells; (*B*) quiescent cells extracted with Triton X-100; (*C*) UV-irradiated quiescent cells extracted with Triton X-100 and (*D*) followed by 2-min incubation in PBS containing 0.5 M NaCl. Cells were fixed with formaldehyde.

Figure 7. Effect of temperature on cyclin/PCNA appearance in UV-irradiated cells. Quiescent human fibroblasts preincubated on ice for 30 min were UV irradiated at 40 J/m^2 and methanol fixed after (*A*) 15 min, (*C*) 30 min, and (*E*) 2 h; or Triton extracted and fixed in formaldehyde after (*B*) 15 min, (*D*) 30 min, and (*F*) 2 h before processing for immunofluorescent analysis.

observed in transformed non S-phase cells (9). The punctuated immunofluorescent pattern observed was homogeneously distributed and remained during the time in which the cells exhibited active DNA repair synthesis. The appearance of staining was independent of cyclin/PCNA synthesis as determined by two-dimensional gel electrophoresis and cycloheximide treatment, showing that cyclin/PCNA and all other components required to initiate DNA repair synthesis are still present in cells qui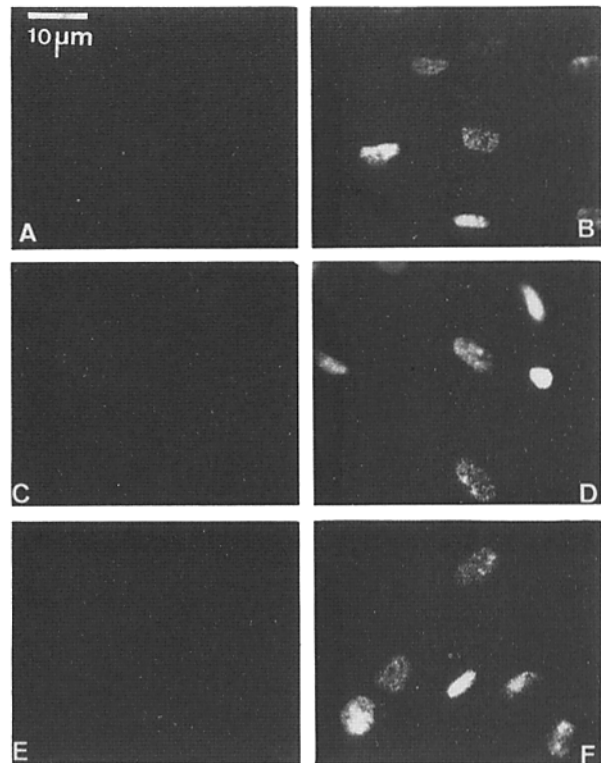escent for 24–48 h. The intensity of cyclin/PCNA staining depended on the dose of UV irradiation used and was in agreement with the activity in DNA repair synthesis as determined by autoradiography. Inhibition of DNA repair synthesis by aphidicolin did not affect the intensity and kinetics of appearance of cyclin/PCNA staining, demonstrating that DNA repair itself was not responsible for the changes observed.

Cyclin/PCNA present in quiescent cells can only be detected by immunofluorescent staining after formaldehyde fixation. This antigen is immediately released from the nucleus when cells are permeated with Triton X-100, demonstrating that it is loosely attached in the nucleus. UV irradiation immediately altered the behaviour of cyclin/PCNA in quiescent cells. The protein became resistant to detergent extraction and a small fraction of it (5–10%) was tightly associated to the nucleus as determined by its resistance to high salt extraction.

The changes in cyclin/PCNA distribution observed after methanol or Triton–formaldehyde treatment proved to involve at least two different transitions as shown by the studies using low temperature and ATP synthesis inhibitors. These results suggest that after UV irradiation, the free population of cyclin/PCNA present in quiescent cells rapidly associates to a nuclear component, possibly a presynthesis complex, becoming resistant to detergent extraction; this is independent of ATP and temperature. The transition of this population of cyclin/PCNA to that detected by methanol fixation
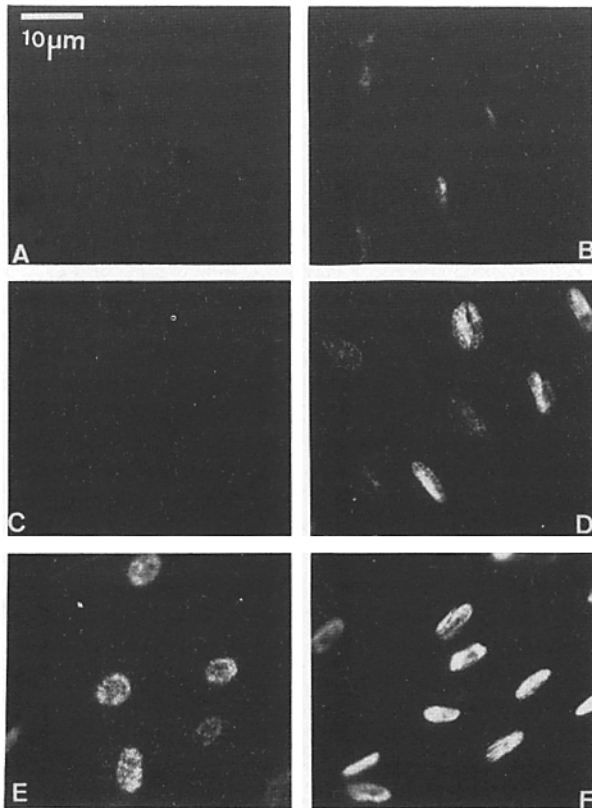
Figure 8. Effect of ATP depletion on cyclin/PCNA staining in UV-irradiated cells. Nonirradiated quiescent cells (*A* and *B*). Irradiated quiescent cells deprived of ATP (*C* and *D*). Irradiated quiescent cells (*E* and *F*). Methanol-fixed cells (*A*, *C*, and *E*). Triton-extracted formaldehyde-fixed cells (*B*, *D*, and *F*). UV dose used was 40 J/m².

requires ATP and is temperature sensitive. The latter association possibly being the replicative complex as its nuclear localization is identical to that of the DNA replicative sites.

From the observations presented here, it is possible to infer that cyclin/PCNA can exist in the nucleus as three different populations, one that is free in the nucleoplasm, a second that is possibly present in the presynthetic complex, and a third population belonging to the replicative complex.

The results presented here together with the recent demonstration that DNA polymerase δ is involved in DNA repair (12, 19), and with the previous observations that cyclin/PCNA is identical to the auxiliary protein of DNA polymerase δ (6, 22, 26), and that it is required for SV-40 DNA replication in vitro (20, 21) strongly suggest that cyclin/PCNA also plays a role during DNA repair.

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