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Comparative Analysis of the Intracellular Localization of c-Myc, c-Fos, and Replicative Proteins during Cell Cycle Progression

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In eukaryotic cells, nucleus-cytoplasm exchanges play an important role in genomic regulation. We have analyzed the localization of four nuclear antigens in different growth conditions: two replicative proteins, DNA polymerase α and proliferating cell nuclear antigen (PCNA), and two oncogenic regulatory proteins, c-Myc and c-Fos. A kinetic study of subcellular localization of these proteins has been done. In cultures in which cells were sparse, these proteins were detected in the nucleus. When proliferation was stopped by the high density of culture cells or by serum starvation, these proteins left the nucleus for the cytoplasm with different kinetics. DNA polymerase α is the first protein to leave the nucleus, with the PCNA protein, c-Fos, and c-Myc leaving the nucleus later. In contrast, during serum stimulation c-Fos and c-Myc relocalize into the nucleus before the replicative proteins. We also noticed that in sparse cell cultures, 10% of the cells exhibit a perinuclear staining for the DNA polymerase α, PCNA, and c-Myc proteins but not for c-Fos. This peculiar staining was also observed as an initial step to nuclear localization after serum stimulation and in vivo in *Xenopus* embryos when the G_1 phase is reintroduced in the embryonic cell cycle at the mid-blastula stage. We suggest that such staining could reflect specific structures involved in the initiation of the S phase.

In eukaryotic cells there is continuous exchange of macromolecules between the nucleoplasm and the cytoplasm, and one possible way to regulate the activity of a nuclear protein is to store it in the cytoplasm and control its access to the nucleus. This provides a means for relaying signals originating from the plasma membrane to the nucleus while also offering the advantage of a rapid response time (7, 22, 24).

Nuclear localization is regulated for several proteins involved in gene expression and development, as well as for proteins involved in the cell cycle (see reference 12 for a review). For example, the activity of the dorsal morphogen is directly regulated by nuclear transport in selected regions of the *Drosophila* embryo (26, 28, 34). For the c-Fos protein, nuclear localization is dependent on continuous stimulation of cells by serum factors during cell growth, and in the absence of serum the protein is kept in the cytoplasm (27).

In this study, the localization of four nuclear proteins which respond to the stimulation of cell growth was reanalyzed. c-*myc* and c-*fos* are two proto-oncogenes which are involved in regulatory events leading to the G_1-to-S transition and which are synthesized shortly after serum stimulation (see references 15, 17, and 25 for recent reviews). DNA polymerase α and proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase δ, are two proteins involved in the mechanism of eukaryotic DNA replication (see reference 39 for review). They are coordinately expressed with cell proliferation as a secondary event of serum stimulation (18, 38). All four proteins have been found in the nuclei of growing cells in culture at a time that they are likely to be active.

We report here the localization of these four proteins under different growth conditions: in exponential-phase cell cultures and when cells are induced to a nonproliferating state by serum starvation or by cell contact inhibition. We have also monitored the fate of the proteins in cells passing from a quiescent state to a proliferating state. In situ localization using specific antibodies against these proteins was performed under the same growth conditions for the four proteins. Cellular fractionation followed by Western blot (immunoblot) analysis was also performed in parallel. Both *Xenopus* cells and mouse NIH 3T3 cells were used in this study. *Xenopus* cells in culture were used, since the localization of c-Myc and PCNA was subject to significant variation during early *Xenopus* development. c-Myc is stored in large amounts in the cytoplasm of the nondividing oocyte and is translocated into the nuclei only after fertilization (10), while the nuclear localization of PCNA appears cyclical during early *Xenopus* development (16). NIH 3T3 cells were also employed, as they have been widely employed as an experimental system for subcellular localization experiments.

Our data show that these proteins are found in the cytoplasm at particular stages of cell growth and that their localization varies according to the proliferation state of the cells. We also observed for c-Myc, DNA polymerase α, and PCNA a perinuclear localization which might correlate with an early-G_1 event. The biological significance of this peculiar localization is discussed in conjunction with similar results obtained with mid-blastula-stage *Xenopus* embryos.

MATERIALS AND METHODS

Cells and embryos. Mouse NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics (100 U of penicillin per ml and 50 mg of streptomycin per ml). This cell line was grown as a monolayer culture. Serum starvation was induced after extensive washing in serum-free DMEM and

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culture in DMEM without serum. The NIH 3T3 cells were seeded at low density to have sparse cells (3,500 cells per cm^2). After 36 h the cells were briefly washed in phosphate-buffered saline (PBS) and incubated in DMEM without serum. The *Xenopus laevis* kidney cell line A6 was cultured at 22°C in 75% L15 Leibovitz medium (GIBCO) supplemented with gentamicin and 10% fetal calf serum. *Xenopus* embryos were obtained as described previously (36).

Antibodies and immunodetection. Five different primary antibodies were used. PCNA antibodies were a gift from R. Bravo. α-HLKY is a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the 15 carboxy-terminal amino acids of the human PCNA. It recognizes specifically PCNA from *Xenopus* and mouse cells, and its specificity was confirmed by abolition of the fluorescent signal after preincubation with the corresponding antigenic peptide (16). α-GSIL is a rabbit polyclonal antibody raised against a synthetic peptide corresponding to 15 amino acids of the human PCNA (positions 9 to 22), and its specificity has been shown previously (16). α-c-Fos antibody was a gift from B. Verrier and J. M. Blanchard. It is directed against the region of c-Fos protein spanning amino acids 151 to 292, and its specificity has been tested previously (37). α-M5FB was raised against a synthetic peptide corresponding to 12 amino acids of the human c-Myc protein (APSEDIWK-KFEL) and was a gift from G. Evan (19). The serum was further purified by affinity chromatography on the peptide grafted to a Sepharose column. The antibody against DNA polymerase α was mouse monoclonal antibody SJK 132-20 (35) and was a gift from U. Ubscher. The antibody against the hsp72 heat shock protein is a monoclonal antibody against the human hsp72 protein and was obtained from Amersham.

Cells grown on glass coverslips were washed with PBS, pH 7.4, before fixation. Formaldehyde fixation was carried out at 4°C for 8 min with 1% Triton X-100. After dehydration, cells were conserved in methanol at −20°C. After rehydration, the coverslips were extensively washed with PBS and incubated with the first antibody (antibodies were diluted in PBS with 1% bovine serum albumin) for 1 h at room temperature in a humid chamber. Coverslips were rinsed in PBS before the addition of the second antiserum (fluorescein isothiocyanate [FITC]-conjugated goat anti-rabbit or FITC-conjugated rabbit anti-mouse antiserum). After 1 h of incubation at room temperature, coverslips were treated in PBS containing 50 ng of 4′,-6-diamidino-2-phenylindole (DAPI) per ml for 10 min and extensively washed with PBS. Finally, coverslips were mounted on glass slides in Citifluor (Citifluor Ltd, London, United Kingdom). In each experiment photographs were taken under the same exposure conditions for comparative analysis.

Whole-mount immunocytochemistry was performed according to the method of Leibovici et al. (16) following a protocol described by Dent et al. (6).

Subcellular fractionation. Approximately 5×10^6 cells were collected at 8, 23, or 47 h after the beginning of serum starvation. Cells were fractionated by a method similar to that described by Kaufmann et al. (14). Briefly, cells were washed in PBS, scraped with a rubber policeman, pelleted, and resuspended in STM buffer (0.25 M sucrose, 50 mM Tris [pH 7.4], 5 mM $MgCl_2$, 1 mM dithiothreitol) containing 2 μg of aprotinin per ml and 1 mM phenylmethylsulfonyl fluoride. After 10 min at 4°C, cells were lysed by 30 strokes in a glass Dounce homogenizer and centrifuged at low speed (1,500 × *g*) to pellet nuclei. The supernatant represented the cytoplasmic fraction. Nuclei were washed three times with STM buffer, counted, and centrifuged through a 0.8 M sucrose cushion at 6,000 × *g* for 15 min. The pellet was resuspended in STM buffer, and the nuclei were counted again. Nuclei were then pelleted again, resuspended in STM buffer containing 0.5% Nonidet P-40 and 0.1% deoxycholate, and incubated for 15 min at 4°C. After centrifugation at 3,000 × *g* for 8 min, the resulting supernatant was collected and represented the nucleoplasmic fraction. The detergent-washed nuclei and the cytoplasmic and the nucleoplasmic fractions were adjusted in Laemmli buffer, boiled, centrifuged at 10,000 × *g* to pellet debris, and loaded on a sodium dodecyl sulfate–10% polyacrylamide gel. They were then analyzed by immunoblotting (30).

RESULTS

Localization of DNA polymerase α, PCNA, c-Myc, and c-Fos in asynchronous cells in culture. *Xenopus* cells in culture were used in order to correlate some of our observations with those previously done during *Xenopus* embryonic development (10, 16). Antibodies that had been characterized previously and shown to be specific were used (see Materials and Methods). We also controlled conditions so that we had no FITC immunofluorescence when the first antibody was omitted (Fig. 1 and data not shown). Figure 1 shows indirect immunofluorescence staining of asynchronous *Xenopus* A6 cells incubated with three specific antibodies against DNA polymerase α, PCNA, or c-Myc. Two different cell populations, either in exponential-phase culture (sparse cells) or resuming proliferation (dense cells), were analyzed. Immunolocalizations were performed with samples from the same cultures to minimize experimental variations (detailed in Materials and Methods). In sparse cell cultures (Fig. 1A), DNA polymerase α, PCNA, and the c-Myc proteins were localized in the nucleus, in agreement with most previous observations concerning these proteins. However, for around 10% of the cells, the immunofluorescence staining was localized mainly at the nuclear periphery (Fig. 1B). In dense cell cultures, both c-Myc and PCNA appear localized mainly in the cytoplasm (Fig. 1C). Significant DNA polymerase α staining is also observed in the cytoplasm, although nuclear staining was also present (Fig. 1C). The localization of c-Fos was nuclear in sparse cells, while no signal was detected in dense cell cultures (Fig. 2 and data not shown).

We concluded that the subcellular localization of these proteins varies according to the growth stage of these cells. Actively growing cells exhibit a unique nuclear localization of these proteins, whereas a different behavior was observed in dense cell cultures. Localization at the nuclear periphery was reproducibly observed for DNA polymerase α, PCNA, and c-Myc in a small proportion of growing cells. As asynchronous cell populations were used, such peculiar localization could be because cells were at a specific stage of the cell cycle. We therefore monitored the fate of these proteins in cells induced to enter the G_0 state by serum starvation or reentering the cell cycle after readdition of serum to the culture medium.

Delocalization of DNA polymerase α, PCNA, c-Myc, and c-Fos is observed in response to serum starvation. Synchronization of the cell cycle by serum deprivation of NIH 3T3 cells in culture has been a valuable experimental approach to clarify cellular events linked to quiescence or entry into the cell cycle. In their exponential-growth stage NIH 3T3 cells have all four analyzed proteins localized in the nucleus (Fig. 2, $t = 0$). The behavior of these proteins was monitored by

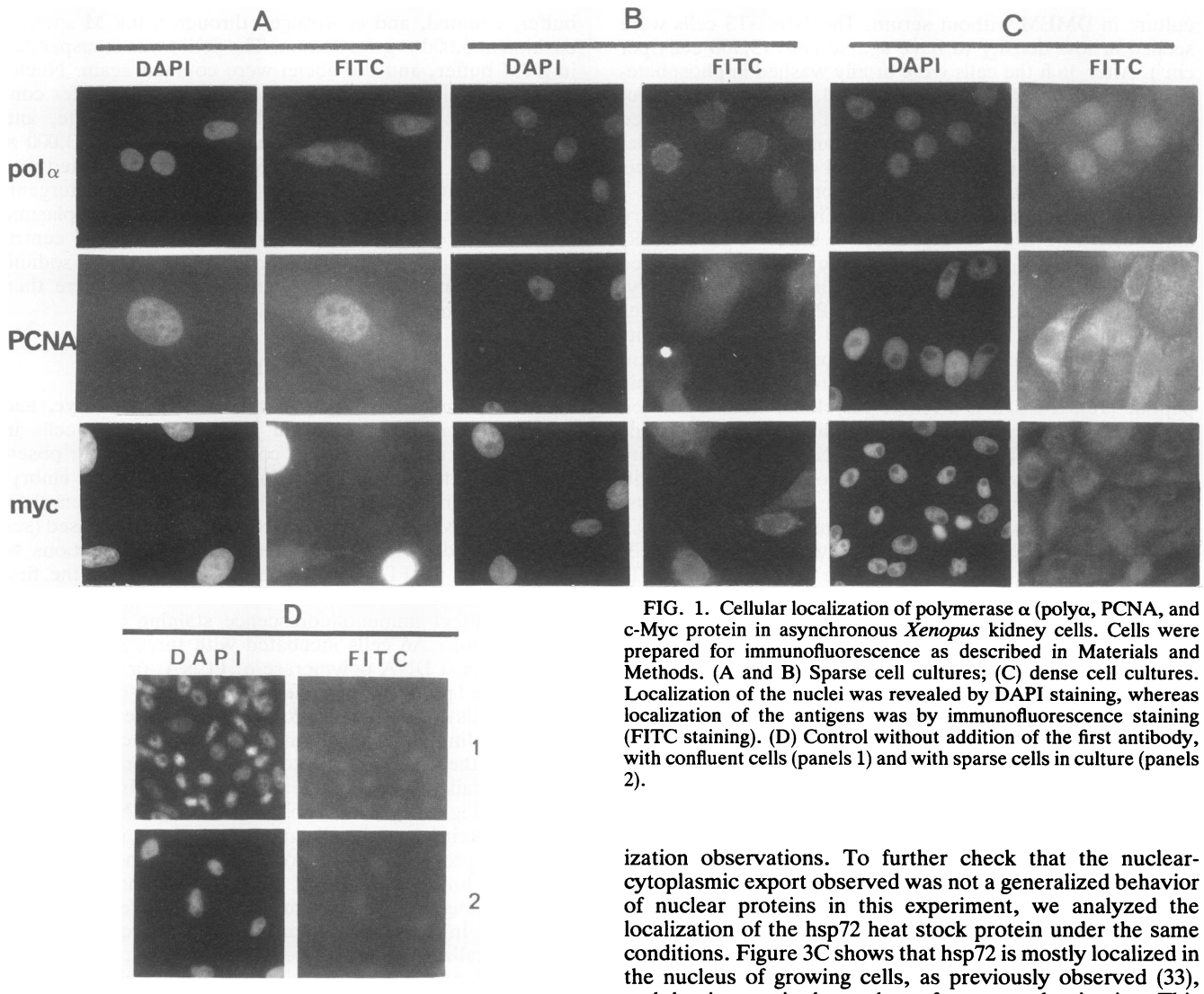

FIG. 1. Cellular localization of polymerase α (polyα, PCNA, and c-Myc protein in asynchronous *Xenopus* kidney cells. Cells were prepared for immunofluorescence as described in Materials and Methods. (A and B) Sparse cell cultures; (C) dense cell cultures. Localization of the nuclei was revealed by DAPI staining, whereas localization of the antigens was by immunofluorescence staining (FITC staining). (D) Control without addition of the first antibody, with confluent cells (panels 1) and with sparse cells in culture (panels 2).

taking different cell samples from the same cell cultures at different times after serum deprivation. Protein staining revealed a nucleus-to-cytoplasm migration with different kinetics. The first delocalization was observed 8 h after serum starvation with DNA polymerase α. PCNA, c-Myc, or c-Fos localization in the cytoplasm was detected at 23 h, and from 47 h no or weak staining was detected for the four proteins. Thus, the cytoplasmic localization of nuclear antigen can be observed either in dense culture cells or in cells deprived of serum and is likely to correspond to cells entering the G_0 state. Since PCNA and DNA polymerase α have relatively long half-lives (3, 38), their cytoplasmic localization might be due to nucleus-to-cytoplasm transport. However, this is less obvious for c-Myc and c-Fos, which have half-lives of less than 20 min (5, 11, 25), and in this case this localization may represent new synthesis of these proteins without nuclear migration. In all cases, cytoplasmic location of these proteins strongly correlates with withdrawal from the cell cycle.

In order to control for the cytoplasmic immunofluorescence observed in situ, we also performed an immunoblotting analysis of subcellular fractions at different times after serum starvation. Figure 3 confirms the in situ immunolocalization observations. To further check that the nuclear-cytoplasmic export observed was not a generalized behavior of nuclear proteins in this experiment, we analyzed the localization of the hsp72 heat stock protein under the same conditions. Figure 3C shows that hsp72 is mostly localized in the nucleus of growing cells, as previously observed (33), and that it stays in the nucleus after serum deprivation. This result is also in agreement with the data observed with the mouse heat stock protein hsp110 (31).

Figure 3 shows that the cytoplasmic localization did not correlate with protein degradation, as full-length proteins were observed. However, this observation does not signify that the proteins will not be degraded when present in the cytoplasm. Indeed, the decrease in the signal observed both by in situ localization and Western blot analysis probably reflected a progressive degradation process during cell progression in G_0. Taken together, these observations indicate that the nucleus-to-cytoplasm export is not a direct consequence of the degradation of these proteins and that such a process takes place only after a significant period of time outside the nucleus. PCNA is recovered in the cytoplasmic fraction before c-Myc, as observed in Fig. 2.

Reentry in the cell nucleus after serum stimulation of starved cells. In order to analyze nuclear events linked to reentry into the cell cycle, serum was put back into the medium of the same cell population 59 h after serum starvation. Two different patterns of localization were observed, one typical of DNA polymerase α and PCNA and one typical for c-Myc and c-Fos. Results obtained with PCNA and c-Fos are illustrated in Fig. 4. Relatively shortly after serum addition (3 h), a positive signal which correlated with nuclear

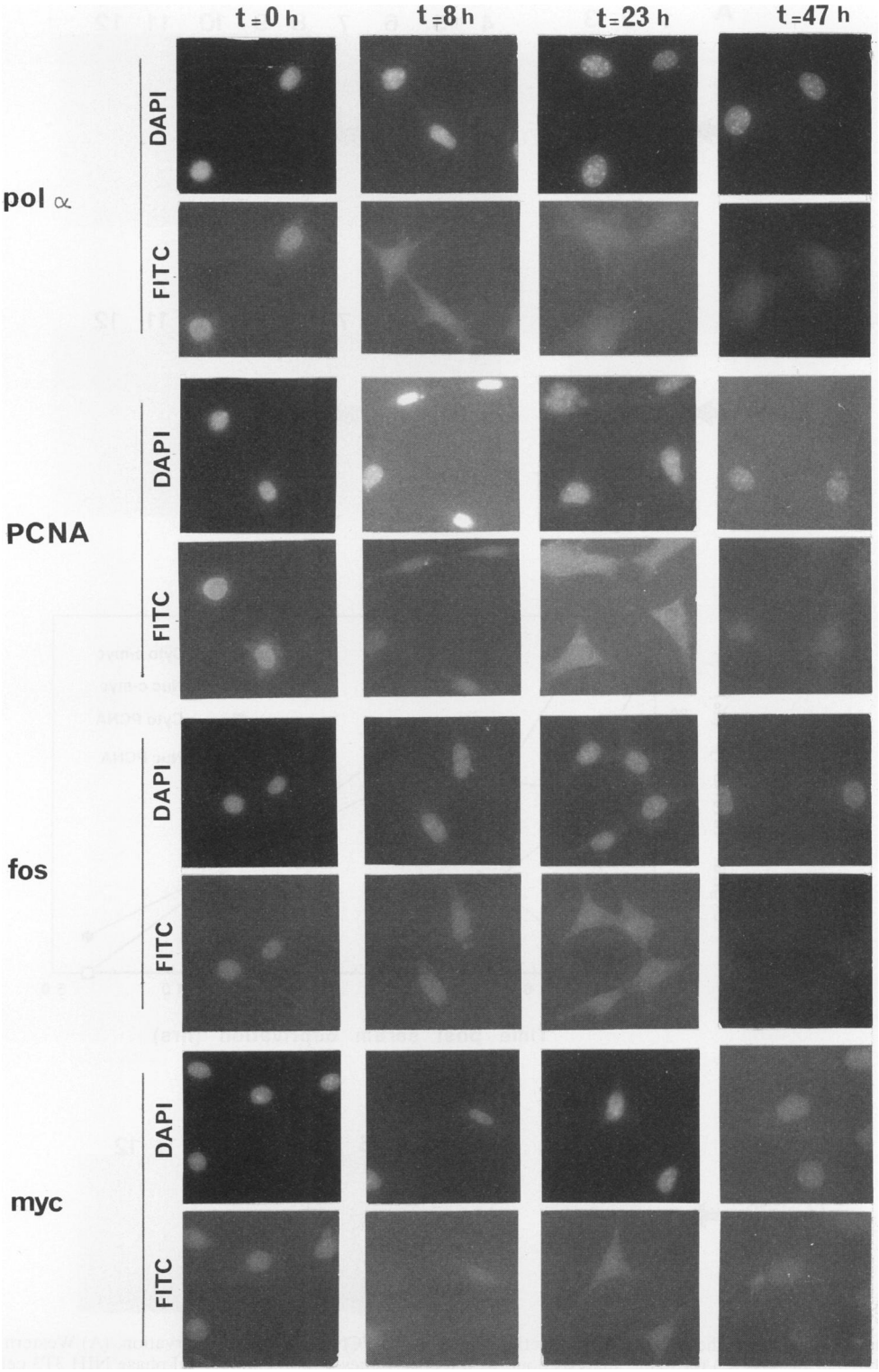

FIG. 2. Kinetics of delocalization of the four nuclear antigens after serum starvation. Exponential-phase NIH 3T3 cell cultures were washed twice in DMEM without serum ($t = 0$) and then put in serum-free DMEM containing antibiotics. Coverslips were fixed and prepared for immunofluorescence as described in Materials and Methods at 8, 23, and 47 h. For each experiment the DNA DAPI staining and FITC staining are shown. polα, polymerase α.

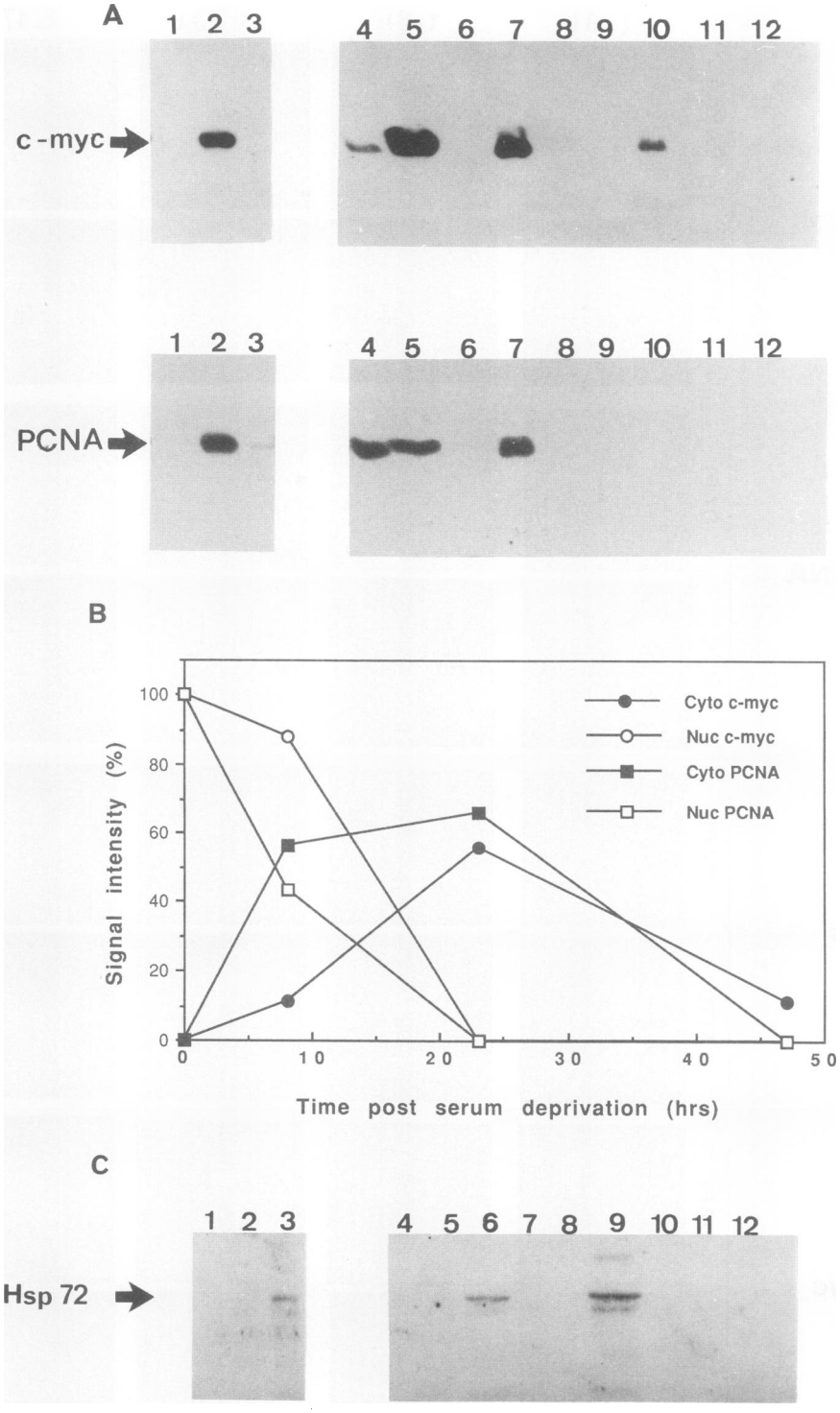

FIG. 3. Biochemical analysis for the delocalization kinetics of c-Myc and PCNA after serum starvation. (A) Western blot analysis of the localization of c-Myc and PCNA; (C) the same analysis done with hsp72. Lanes 1 to 3, exponential-phase NIH 3T3 cell culture in DMEM with 10% calf serum (lane 1, cytoplasmic fraction; lane 2, nucleoplasmic fraction; lane 3, nuclear fraction); lanes 4 to 12, NIH 3T3 cell culture after serum starvation (lanes 4 to 6, 8 h; lanes 7 to 9, 23 h; lanes 10 to 12, 47 h; lanes 4, 7, and 10, cytoplasmic fraction; lanes 5, 8, and 11, nucleoplasmic fraction; lanes 6, 9, and 12, nuclear fraction). (B) Graphic representation of delocalization kinetics. Cyto, cytoplasmic; Nuc, nuclear.

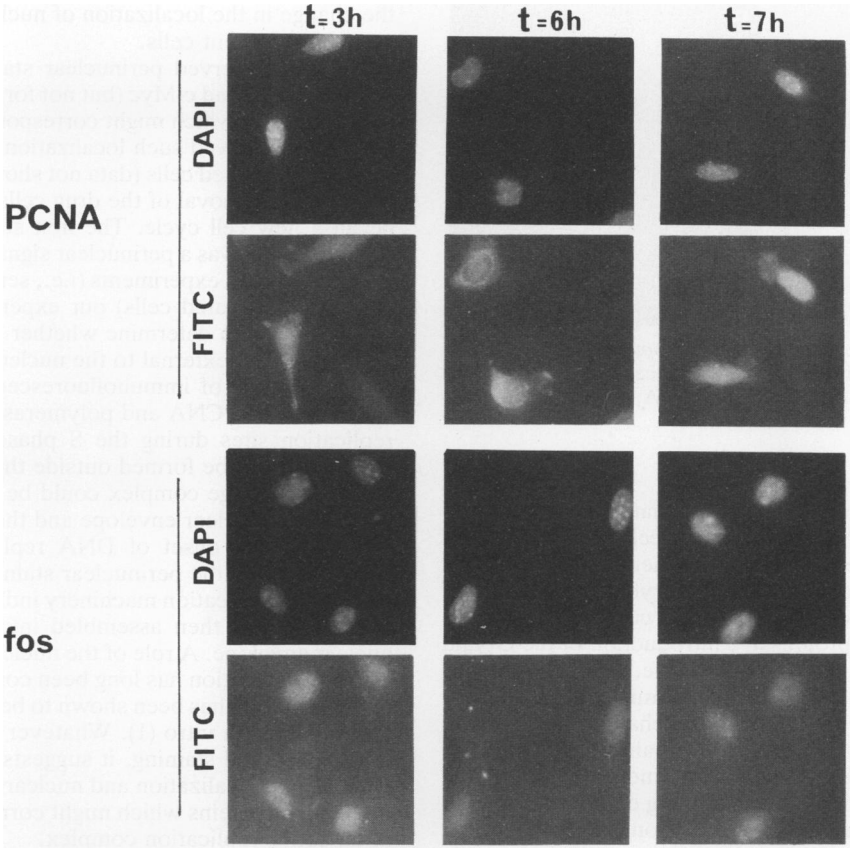

FIG. 4. Kinetics of relocalization of PCNA and c-Fos protein in serum-stimulated quiescent NIH 3T3 cells. Immunofluorescence staining of PCNA and c-Fos after 3, 6, and 7 h of serum stimulation and DNA DAPI staining of the same preparations are shown.

localization was again detected for c-Fos (Fig. 4). At that time PCNA protein was observed as a diffuse signal, and 6 h after serum stimulation, the staining was concentrated at the periphery of the nucleus. This is before the S phase, which occurs mainly 12 h after serum addition in NIH 3T3 cells (23), and is thus likely to represent the G_1 period. The signal became entirely nuclear 7 h after stimulation. In these experiments the cells used for the detection of the four proteins were cultured in the same plate. We therefore considered their different behaviors to be significant.

The results concerning the localization of the four antigens after serum starvation and serum stimulation are summarized in Table 1. After serum stimulation, the two proto-oncogene proteins were first detected in the nucleus while replication proteins reached this localization later.

TABLE 1. Cellular distribution of polymerase α, PCNA, c-Myc, and c-Fos in NIH 3T3 cells after serum starvation or serum stimulation

Protein	Location of protein[a]							
	After serum starvation for:				After serum stimulation for:			In dense cultures
	0 h	8 h	23 h	47 h	3 h	6 h	7 h	
Polymerase α	N, P	C, N	C, N	—	N, C	N, P	N	C, N
PCNA	N, P	N	C	—	C	P	N	C
c-Fos	N	N	C, N	—	N	N	N	—
c-Myc	N, P	N	C, N	—	N	N	N	C

[a] C, cytoplasmic; N, nuclear; P, perinuclear; —, no signal. The same cells were serum starved and then serum stimulated.

The localization of the PCNA protein appears the most sensitive to cell culture variations. No posttranslational modification is known for this protein (2, 29), and therefore the nuclear-cytoplasmic localization may be important for its regulation. Three antigens showed perinuclear staining: c-Myc, DNA polymerase α, and PCNA. We also observed such a pattern for PCNA during the early embryonic development of *X. laevis*.

Detection of PCNA at the nuclear periphery in vivo during early development. We observed perinuclear staining for DNA polymerase α, c-Myc, and PCNA proteins in 10% of fibroblasts in sparse cell cultures (Fig. 1B) and after serum stimulation for the replication proteins PCNA and DNA polymerase α. This specific localization was not unique to cells in culture, as we also observed it during the early development of *X. laevis* by in toto immunochemistry. During early *Xenopus* development, PCNA staining of the nuclei is homogeneous and correlates with the S phase (16). However, at the mid-blastula-stage transition, when G_1 and G_2 phases are again introduced into the cell cycle, perinuclear staining of cells becomes apparent. This pattern coexists in the same embryo with cells exhibiting homogeneous nuclear staining and with unstained cells. Figure 5a shows perinuclear staining in a group of embryonic cells, as compared with a similar observation in cells in culture (Fig. 5b). The similarity between the two independent observations obtained by using different materials and a different experi-

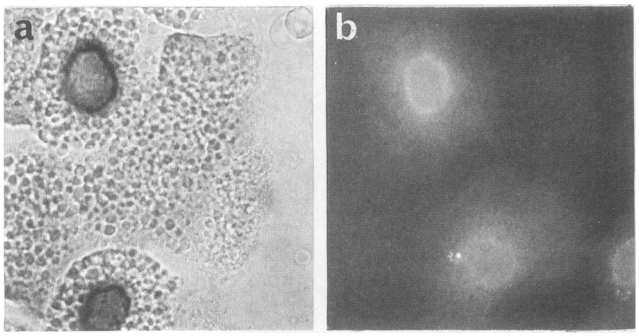

FIG. 5. PCNA localization in sparse *Xenopus* cells and in *Xenopus* mid-blastula-stage embryos. (a) Histological section of *Xenopus* embryos stained with DAPI to detect PCNA; (b) immunofluorescence staining of PCNA in A6 cells.

mental procedure strongly suggests that such staining is characteristic of a short period of the cell cycle. The mid-blastula stage is marked by several biochemical and morphological modifications affecting the embryonic cells. One of these changes is the lengthening of the cell cycle, which is probably caused by a progressive introduction of the G_1 and G_2 phases within the embryonic cell cycle. Thus, the appearance of the perinuclear staining in cell nuclei at this developmental stage could be related to the changes occurring in the embryonic cell cycle. The data obtained with in vitro-cultured cells combined with those obtained with embryonic cells suggest that the perinuclear staining could be related to the G_1 phase of the cell cycle progression.

DISCUSSION

We have shown here that the localization of four nuclear proteins involved in gene expression or replication is subject to significant variation, which is dependent on the physiological state of the cell.

In serum-starved fibroblasts, cytoplasmic accumulation of the proteins is detected, whereas serum stimulation triggers the rapid accumulation of the proteins in the nucleus. Delocalization or relocalization of the four proteins does not follow the same kinetics. The replication proteins PCNA and DNA polymerase α leave the nucleus earlier than c-Fos and c-Myc during serum starvation. In contrast, after serum stimulation c-Myc and c-Fos are rapidly localized in the nucleus (before 3 h), in agreement with previous data (4, 20), and before PCNA and DNA polymerase α. This observation might reflect the function of these proteins, as PCNA and DNA polymerase α are directly involved in the mechanism of DNA replication whereas c-Fos and c-Myc are proteins involved in regulatory events.

The cellular localization of the polymerase α, PCNA, c-Fos, and c-Myc proteins is cell growth dependent. Communications between the nuclear and the cytoplasmic compartments of the cell are mediated by the nuclear pore complex, a large proteinaceous structure that spans the nuclear envelope (9). Changes in pore composition and/or structure of the envelope occur at different growth stages. It was shown that the properties of the nuclear envelope are different between quiescent and dividing cells: nuclei from dividing NIH 3T3 cells take up nucleoplasmin-coated gold particules at a rate seven times higher than that of growth-arrested cells, and the maximum diameter of the transport channels also changes (8). Such modifications could explain the change in the localization of nuclear proteins in proliferating or quiescent cells.

We also observed perinuclear staining for PCNA, DNA polymerase α, and c-Myc (but not for c-Fos) at specific stages of cell growth, which might correspond to the G_1 period. We have also observed such localization in demecolcine (Colcemid)-synchronized cells (data not shown). In this latter experiment, after removal of the drug cells completed mitosis and began a new cell cycle. The first staining observed for the PCNA protein was a perinuclear signal, followed by a nuclear staining. In both experiments (i.e., serum-stimulated cells and demecolcine-treated cells) our experimental procedures did not permit us to determine whether the perinuclear staining was internal or external to the nucleus.

On the basis of immunofluorescence studies which indicated that the PCNA and polymerase α were colocalized at replication sites during the S phase (13), a prereplicative complex might be formed outside the nucleus in the late G_1 phase. This large complex could be assembled and docked close to the nuclear envelope and then translocated into the nucleus at the onset of DNA replication. An alternative explanation for the perinuclear staining is that each component of the replication machinery individually migrates to the nucleus and is then assembled into a complex inside the nuclear envelope. A role of the nuclear envelope in initiation of DNA replication has long been considered, and formation of the envelope has been shown to be necessary for initiation of replication in vitro (1). Whatever the precise localization of the observed staining, it suggests a time period between cytoplasmic localization and nuclear localization for the two replicative proteins which might correspond to the assembly of an active replication complex.

The perinuclear staining observed for DNA polymerase α and PCNA proteins in serum-stimulated fibroblasts occurs long before the S phase, which takes place 12 h after serum addition in NIH 3T3 cells (23). This observation suggests that this staining might be characteristic of a G_1 phase. The G_1 phase of the cell cycle is a regulatory period during which extracellular factors determine whether a quiescent cell will begin to proliferate and whether a normal proliferating cell will continue to cycle or will revert to a quiescent state. The analysis of cell proliferation in eukaryotic systems suggests that the major point of control is in G_1 (23). G_1 events require many hours, and they appear to occur sequentially. Our experiments permitted three sequential steps to be distinguished during this period: (i) synthesis and accumulation in the nucleus of two proteins (c-Fos and c-Myc) activated earlier by serum stimulation, (ii) synthesis and accumulation around the nucleus of two replicative proteins (DNA polymerase α and PCNA), and (iii) nuclear import and accumulation of replication proteins (DNA polymerase α and PCNA). The second step was characterized by perinuclear staining for the polymerase α and PCNA proteins.

We also observed a peripheral staining for PCNA in *Xenopus* embryos at the mid-blastula-stage transition. Before this stage cell cycles are biphasic, consisting of successive S and M phases. During this period PCNA staining was detected only during the S phase and was never observed at the periphery of the nucleus. The mid-blastula-stage transition is marked by a series of structural and functional events, which correspond to the switch from the early embryonic cell cycle to a somatic cell cycle (21, 32). The appearance of perinuclear staining of PCNA protein at the mid-blastula-stage transition might correspond to the introduction of the G_1 phase during development and the acquisition of a somatic cell cycle. Both our in vitro analysis in cell culture

and in vivo observations of early embryos indicate that the nuclear antigens analyzed in this study might be good markers for the analysis of nuclear structures involved in the preparation of the cell for DNA replication. Further analysis of in vitro egg extracts, which are able to reconstruct the nuclear envelope and initiate DNA replication, might give more insight into the nature of these structures as well as the nuclear transport of such proteins during the cell cycle.

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