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Cell Cycle-Specific Expression and Nuclear Binding of DNA Polymerase α

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The expression and distribution of DNA polymerase α was measured by flow cytometry and confocal laser scanning microscopy. Expression was proportional to DNA content in proliferating cells, while only S-phase cells retained DNA polymerase α after detergent extraction. Nuclear DNA polymerase α binding may be one of the key events of S-phase entry.

The DNA polymerases α (pol α) and δ (pol δ) are involved in replicative DNA synthesis in mammalian cells (15, 23, 29, 41). It has been suggested (10, 33) and experimentally verified (32) that pol α and pol δ cooperate in a replication complex in which the polymerases carry out lagging- and leading-strand synthesis, respectively. The regulation of pol α synthesis and the kinetics of its accumulation during the cell cycle as well as the intracellular localization of the enzyme have been matters of discussion. Most of the pol α is extracted with the cytoplasmic fraction in exponentially growing cultures, despite the obvious function of the enzyme in the nucleus (11, 25, 42). On the other hand, after paraformaldehyde fixation, pol α is localized to the nucleus of all interphase cells in exponentially growing cultures (1, 18). Other studies indicate that a fraction of pol α is bound to the nuclear matrix (8, 9, 12, 27, 28, 36, 44); the association seems to be S-phase specific (8, 12, 27). In this study, we have used flow cytometry and confocal laser scanning microscopy to determine the expression, nuclear binding, and localization of pol α in resting and proliferating cells.

The expression of pol α in resting versus proliferating cells was studied in normal peripheral human B lymphocytes stimulated with anti-μ and low-molecular-weight B-cell growth factor (13, 30). Two million cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at 0°C, permeabilized in 0.1% Triton X-100–PBS, and stained with a three-layer procedure for pol α or c-Myc, as follows: Cells were resuspended in 100 μl of either (i) a 17-μg/ml concentration of purified SJK 132-20 monoclonal antibody (MAb) or supernatants from SJK 132-20 or SJK 287-38 hybridoma cultures (31; anti-pol α MAb; American Type Culture Collection), (ii) anti-c-Myc MAb 3C7 (kindly provided by G. Evan) diluted 1/100, or (iii) PBS-hybridoma growth medium (controls). After incubation with the first layer for 30 min, cells were washed with PBS, incubated in 100 μl of biotinylated horse anti-mouse immunoglobulin G (Vector) diluted 1/50 in PBS for 30 min, washed, incubated in 100 μl of streptavidin-fluorescein isothiocyanate (FITC) (Amersham) diluted 1/50 in PBS for 30 min, and washed. The pellets were resuspended in 500 μl of PBS containing 0.1% Triton X-100, 200 μg of RNase A per ml, and 12.5 μg of propidium iodide (Calbiochem) per ml for flow cytometry. The green (≤540 nm) and red (≥640 nm) fluorescence as well as small-angle light scattering of cells stained with FITC for nuclear antigens and propidium iodide for DNA were measured in an Argus flow cytometer with 470- to 490-nm excitation (Skatron).

Resting B lymphocytes were negative; i.e., they showed a fluorescence intensity similar to that of cells incubated without primary antibody (Fig. 1A and H; data not shown for SJK 287-38). After mitogenic stimulation for 60 h, a fraction of the cells fluoresced much more intensely, representing cells in the S and G_2 phases and a fraction of the cells in the G_0/G_1 phase (Fig. 1C). It was estimated from the width of the distributions that the specific fluorescence of resting cells was less than 5% of that observed in stimulated B lymphocytes in the G_1 phase. Pol α was barely expressed after 24 h (Fig. 1B). In contrast, the c-Myc protein was maximally induced after 6 h of stimulation (Fig. 1E to G). Hence, pol α synthesis is a late event during mitogenic activation of B lymphocytes, which enter S phase around 36 h after stimulation (26). The negative cell population in Fig. 1C probably corresponded to nonresponding cells, which are always present in stimulated cultures (30). Downregulation of pol α levels seem to be associated with more permanent shutdown of DNA replication in fibroblasts (18, 40) and lymphocytes (2, 18; Fig. 1).

The cell cycle distribution of pol α in continuously proliferating cells was investigated in cell lines U698 (20), Reh (24), and NHIK 3025 (21) in the same way as for normal B lymphocytes. The cells were grown in RPMI 1640 (GIBCO; U698 and Reh) or minimal essential medium (GIBCO; NHIK 3025) supplemented with 10% fetal calf serum. The cellular contents of pol α increased steadily through the cell cycle for all three cell lines; cells in the G_2/M phase contained approximately twice as much pol α as did cells in the G_1 phase (Fig. 2A and B; data not shown for Reh and NHIK 3025 cells). To determine the absolute number of epitopes, about 10^5 beads of diameter 8.4 μm covered with 110,000 determinants for mouse antibodies (Flow Cytometry Standards Corp.) were added to the cell pellets before addition of the first layer. The mixing of cells and beads ensured identical staining conditions and kinetics during the incubations with the second and third layers. Saturating amounts of the primary antibody were used. The 8.4-μm beads have no red fluorescence and scatter more light than do the cells; the median FITC fluorescence intensities of beads and cells in the mixture could therefore be estimated separately by gating on red fluorescence and light scatter. Specific FITC

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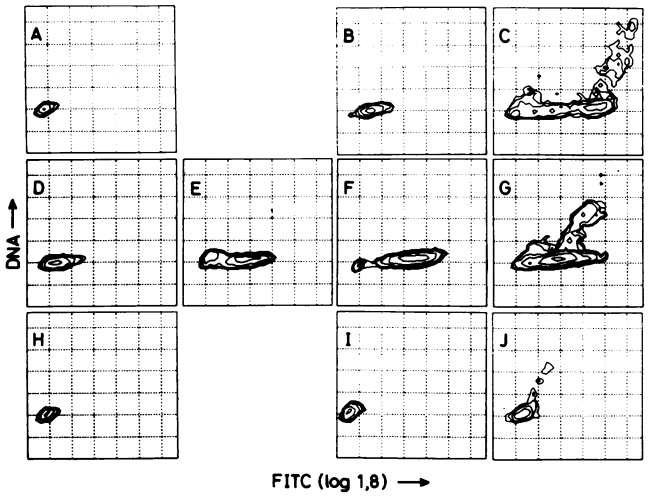

FIG. 1. Quantitation of pol α and c-Myc in stimulated human B lymphocytes. B cells were stimulated with anti-μ and low-molecular-weight B-cell growth factor for 0 (A, D, and H), 6 (E), 24 (B, F, and I), or 60 (C, G, and J) h. Whole cells were paraformaldehyde fixed, permeabilized, and stained with either purified SJK 132-20 anti-pol α MAb (A to C), 3C7 anti-c-Myc (D to G), or PBS (control; H to J) and FITC-labeled secondary reagents. Cells were stained for DNA with propidium iodide, and green (FITC) versus red (propidium iodide) fluorescence was measured by flow cytometry. The singlet peaks of 1.33-μm calibration beads were gated away in these figures but were positioned in the same channels as shown in Fig. 2C to E.

fluorescence intensities of the anti-pol α stained cells and beads were obtained by subtracting the corresponding intensities in the control sample. Cells in S phase expressed about 50% more pol α than did cells in the G_1 phase (Fig. 2A and B; Table 1). Labeling was confined to the nuclei of all interphase cells and to the cytoplasm of mitotic cells. The

TABLE 1. Numbers of pol α molecules in cells in the G_1 and S phases of the cell cycle

Cell type (no. of expts)	No. of molecules (mean ± SD)[a]		
	G_1	S	S, normalized[b]
Reh (3)	15,300 ± 4,700	22,100 ± 7,000	14,700 ± 4,700
NHIK 3025 (2)	37,800 ± 10,900	47,000 ± 8,000	31,300 ± 5,300
NHIK 3025, normalized[c]	15,100 ± 4,400	18,800 ± 3,200	12,500 ± 2,100

[a] The number of epitopes (i.e., number of monomeric molecules for monoclonal antibodies) was calculated by multiplying the fractional specific FITC fluorescence of cells relative to beads with 110,000.

[b] Calculated by dividing the figures in the S column by 1.5, which was the average DNA content of S-phase cells relative to G_1 cells.

[c] Calculated by dividing the figures in the NHIK 3025 row by 2.5 (the G_1 DNA content of 3025 cells is 5n.)

number of pol α molecules per unit of DNA was not significantly different in the G_1 and S phases of Reh and NHIK 3025 cells (Table 1). An average of 14,500 (standard deviation [SD] = 4,000; standard error of the mean [SEM] = 1,300; 10 experiments) pol α molecules per diploid cell was obtained from the values in Table 1. Hence, we find no cyclic variations in the amount of pol α relative to total DNA content during the cell cycle of continuously proliferating cells, in agreement with the results of Wahl et al. (40). The same lack of cyclic variations seems to valid for the proliferating cell nuclear antigen (PCNA) (17, 43), a cofactor of pol δ (23). The expression of pol α in Reh, U698, and stimulated B cells was compared pairwise in three experiments. The relative amounts of pol α in the G_1 phase were 1.13 for Reh/U698, 1.05 for B cells/U698, and 0.60 for B cells/Reh. Hence, the expression of pol α per unit of DNA was similar in normal proliferating diploid B cells, G_1 Reh, G_1 U698, and G_1 NHIK 3025. These results indicate that an increased level of pol α is not a common consequence of neoplastic transformation. Resting B cells expressed less

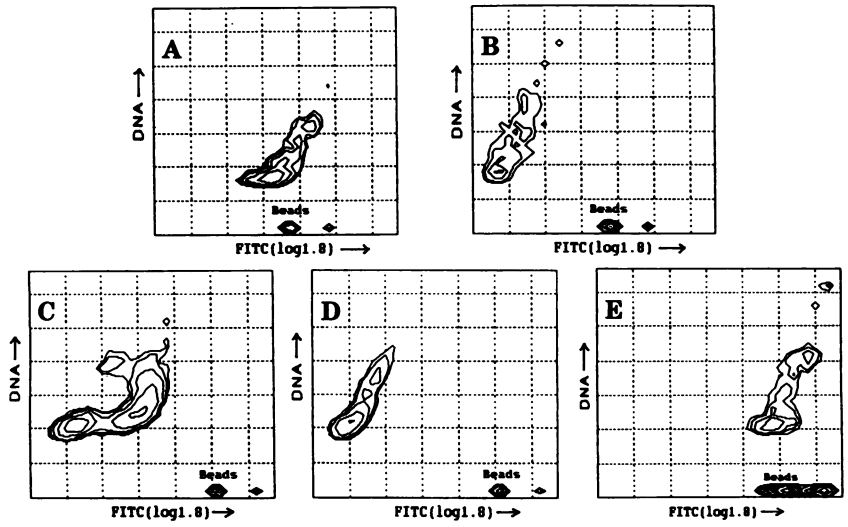

FIG. 2. Quantitation of pol α in U698 cells and nuclei. Whole cells (A and B) and isolated nuclei (C to E) of exponentially growing U698 cells were paraformaldehyde fixed, permeabilized, and stained with either purified SJK 132-20 anti-pol α MAb (A and C), PBS (control; B and D), or DCM 905 anti-c-Myc MAb (E) and FITC-labeled secondary reagents. Cells were stained for DNA with propidium iodide, and green (FITC) versus red (propidium iodide) fluorescence was measured by flow cytometry. The peak labeled "beads" represents 1.33-μm fluorescent microbeads.

than 5% of the pol α found in proliferating diploid B cells, corresponding to less than 800 molecules per cell.

When the three cell lines were extracted with 0.1% Nonidet P-40–10 mM NaCl–5 mM $MgCl_2$–0.1 mM phenylmethylsulfonyl fluoride–10 mM phosphate buffer (pH 7.4) for 5 min at 0°C to isolate nuclei prior to fixation and staining as for whole cells, fluorescence microscopy revealed that only a fraction of the nuclei were positive for pol α. Detergent-extracted mitotic cells were negative. Correlated flow cytometric measurements of the DNA and pol α content of U698 nuclei showed that the pol α-positive subpopulation consisted of the S-phase nuclei and possibly minor fractions of the nuclei with G_1 and G_2/M content (Fig. 2C and D). Since resolution of the DNA measurement was not optimal under these conditions of fixation and staining, it was impossible to accurately calculate the fractions of pol α-positive cells with G_1 and G_2/M DNA content. However, these fractions were small, and they probably represent cells prepared for S-phase entry (late G_1) and cells that have just terminated DNA replication (early G_2). Similar results were obtained with Reh and NHIK 3025 nuclei and with the SJK 287-38 antibody. As controls, cell cycle-resolved expression of the nuclear protein c-Myc in isolated U698 nuclei (Fig. 2E; 1/250 dilution of DCM 905 antibody from Cambridge Research Biochemicals) and of the Ki67, NO38/nucleophosmin/B23, p82, and p125 antigens in isolated Reh nuclei (data not shown; antibodies from DAKO [Ki67 diluted 1/50] and our laboratory [anti-NO38, anti-p82, and anti-p125]) was measured in the same way as for pol α. The expression of all of these antigens in nuclei increased steadily through the cell cycle. We conclude that mainly S-phase nuclei have accessible epitopes for the two pol α antibodies SJK 132-20 and SJK 287-38, most likely due to extraction of all pol α from most non-S-phase nuclei during the isolation procedure.

The specific immunofluorescence intensities of isolated S-phase nuclei were considerably lower than that of whole S-phase cells (Fig. 2). The proportions of pol α epitopes in detergent-extracted S-phase nuclei relative to the total amount in S-phase cells were 27% (SD = 10%; six experiments) for Reh cells, 29% (SD = 3%; three experiments) for U698 cells, and 22% (SD = 8%; three experiments) for NHIK 3025 cells. Consequently, there were no significant differences between the cell lines in this regard. The mean proportion of the total amount of pol α retained in S-phase nuclei for all cell lines was 26% (SD = 8%; SEM = 2.3%; 12 experiments). This corresponds to 5,700 (SEM = 700) pol α epitopes in an average S-phase nucleus with a DNA content between 2n and 4n. The numbers of pol α molecules in nuclei in early, mid- and late S phase were determined. The level of pol α epitopes was slightly higher in mid-S-phase nuclei than in early-S-phase nuclei for the three cell lines, but the differences were not significant (Table 2). The amounts of bound pol α did not display any local maxima or minima through S phase (Fig. 2C; other histograms not shown). DNA content was resolvable to a coefficient of variation of less than 5%, demonstrating that the S phase could have been subdivided into 20 fractions without producing any local maxima or minima. Hence, the amount of bound pol α was essentially constant through S phase, suggesting a fairly constant rate of DNA replication through S phase. This finding is not in agreement with a local minimum of bound pol α in mid-S phase (8, 12) and not consistent with a local minimum in the DNA replication rate in mid-S phase (7, 12). Assuming that the pol α molecules replicate the lagging strand and an equal number of pol δ molecules replicate the leading strand (10, 32, 33), we calculated a chain elongation rate per pol α/δ pair of 0.37 μm/min (5,700 active pol α molecules, an equal number of active pol δ molecules, $6 \cdot 10^9$ base pairs/diploid cell = $2.0 \cdot 10^6$ μm of DNA, and 8-h S-phase duration). This elongation rate is in good agreement with the range determined by Vogelstein et al. (0.25 to 0.5 μm/min [39]).

TABLE 2. Relative numbers of pol α epitopes in nuclei at different stages in S phase

Cell type (no. of expts)	Relative no. of epitopes (mean ± SD)[a]		
	Early S	Mid-S	Late S
Reh (6)	0.95 ± 0.05	1.08 ± 0.08	0.97 ± 0.09
U698 (2)	0.94 ± 0.10	1.03 ± 0.03	1.03 ± 0.08
NHIK 3025 (1)	0.93	1.09	0.98
Total (9)	0.94 ± 0.06	1.07 ± 0.07	0.99 ± 0.08

[a] Relative to the average for S-phase nuclei. Early S, mid-S, and late S represent nuclei with DNA contents of 1.1 to 1.3, 1.4 to 1.6, and 1.7 to 1.9, respectively, relative to the G_1 DNA content.

The subnuclear distribution of the bound pol α molecules in S-phase nuclei and the total pol α in whole cells was investigated in a Zeiss LSM 10 laser scanning microscope equipped with a Plan-Apochromat objective (63×; numerical aperture = 1.4). The staining was as for flow cytometry except that the cells were resuspended in PBS–50% glycerol for analysis. FITC fluorescence was excited with the 488-nm argon laser line and observed through a ≥520-nm long-pass interference filter. The distribution patterns in pol α-positive Reh nuclei varied from a nearly continuous distribution of many small spots (Fig. 3A) to a few large spots (Fig. 3B). Small spots were found neither in nucleoli nor in the heterochromatic regions close to the nuclear lamina. Large spots were in some cases localized to the nuclear lamina. Nuclear volumes were calculated as $V = 4/3 \cdot \pi \cdot (a \cdot b)^{1.5}$, where a and b were the short and long semi-axes of the ellipsoid near-median nuclear sections, respectively. The average volume of positive Reh nuclei with spots smaller than 0.7 μm was 725 μm^3 (SD = 214 μm^3), while the average volume of positive nuclei with spots larger than 0.7 μm was 1,015 μm^3 (SD = 83 μm^3). These values suggest that the nuclei with small spots were in early S phase and that the nuclei with large spots were in late S phase.

Figure 3C shows pol α distributions observed in the nuclei of whole fixed Reh cells. Diffuse staining was evident in the interchromatin region in all nuclei of fixed whole cells. Some nuclei of fixed whole cells displayed a granular pattern in addition to the diffuse staining in the interchromatin region.

Similar fractions of pol α (26%; this work) and PCNA (20 to 30% [5]; 35% [17]) are selectively retained in all S-phase nuclei, and the spatial distribution patterns of bound pol α (Fig. 3A and B) and PCNA (3–6) are reminiscent of the bromodeoxyuridine/biotin-UTP pulse-labeling patterns observed at different times during S phase (16, 19, 35). Both nuclear pol α binding (data not shown) and PCNA binding (3) occur in the presence of aphidicolin; the binding is therefore independent of active DNA replication. We suggest that immobilization of pol α, PCNA, and other components of the replication machinery is necessary for and may be one of the key events of S-phase entry.

What regulates the nuclear binding of DNA replication enzymes at the end of the G_1 phase? It has been argued that the level of pol α could not be the factor regulating initiation of S phase since no large cyclic variations were observed (40). However, if nuclear pol α binding is cooperative, a

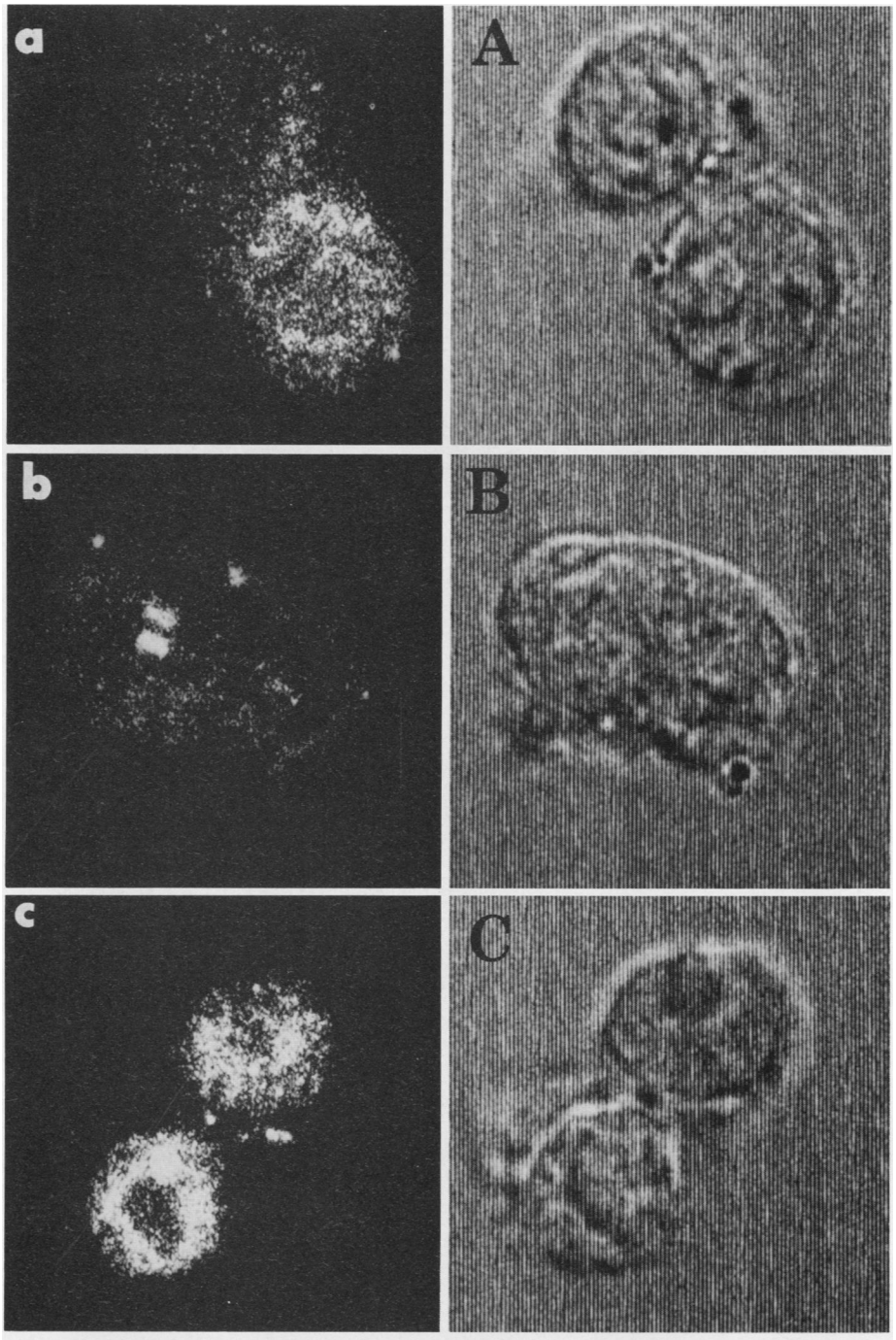

FIG. 3. Localization of pol α in isolated Reh nuclei and whole cells. Isolated nuclei (A and B) and whole cells (C) of exponentially growing Reh cells were paraformaldehyde fixed and stained with supernatants from SJK 287-38 anti-pol α MAb producing hybridoma cells and FITC-labeled secondary reagents. Cells were analyzed by laser scanning microscopy for FITC fluorescence (a to c; confocal) and transmitted light (A to C; nonconfocal). The fields shown are 23 by 23 μm. Note the presence of a positive S-phase cell (lower right) as well as a smaller negative, probably G_1 cell (upper left) in panel A. A positive S-phase cell is shown in panel B. Whole cells were all positive under these conditions, and the two cells shown in panel C were representative of the whole population (except for mitotic cells).

small increase in enzyme levels could trigger binding if the amount of free pol α was considerably higher than the amount bound during S phase. The replication of approximately 25 replicons is coordinated in a replication cluster (14, 22). Furthermore, it has been reported that the number of small biotin-dUTP spots found in early-S-phase nuclei is 2 to 300 (19). This is in excellent agreement with the number of pol α molecules bound during the S phase of diploid cells, i.e., 5,700 (this work). The replication enzymes also seem to be physically associated during S phase (34, 37, 38). Assembly of replication proteins in the nucleus could therefore be cooperative. Our unpublished calculations have shown that

the pol α concentration increase required to proceed from zero to complete nuclear binding is larger than that observed during the whole G_1 phase whatever the degree of cooperativity. We conclude that the levels of pol α cannot be the factor regulating entry into S phase. Also, the distributions of total pol α in G_1- and S-phase cells (Fig. 2A) overlap considerably, indicating that S-phase entry is not strictly controlled by the levels of pol α. Some other mechanism, such as a change in the conformation of the binding site or of the replication enzymes or occupation of the binding site by other molecules, may specifically regulate initiation of S phase.

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