Nuclear Location of Mammalian DNA Polymerase Activities*

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Nuclei were isolated from monolayer cultures of mouse and human cells using a nonaqueous procedure of cell fractionation in which lyophilized cells were homogenized and centrifuged in 100% glycerol. In previous work we have shown that the nuclear pellet and cytoplasmic supernatant fraction contained 10% or less of the nucleic acids characteristic of the other cell fraction.

Aqueous extracts made from fresh cultures and from nonaqueous material at each step of the fractionation procedure were assayed for DNA polymerase activity. Activities were normalized to DNA contents of extracted material. Specific activity was preserved quantitatively through freezing and drying the cells, but was found to be unstable in glycerol suspensions with approximate half-lives of 1 h at 23°C and 4 h at 0-4°C. Activities were relatively stable at -25°C, however, so that by homogenizing only 15 min at 4°C and centrifuging at -25°C we preserved approximately 85% of the specific activity of fresh cultures in the nonaqueous nuclear fraction.

Sedimentation analyses showed that the nuclear fraction contained both DNA polymerase-α and -β in approximately the proportions expected if all polymerase activities were confined to the nuclei in living cells. DNA polymerase-α was found to be more unstable in glycerol suspensions than DNA polymerase-β. Nuclear location of both activities was found in exponential cultures and in 3T3 mouse cultures synchronized in the G₁ and S phases of the cell division cycle. We found no evidence for cytoplasmic factors affecting nuclear polymerase activities. We have concluded that the two major DNA polymerases are nuclear although one, DNA polymerase-α, frequently is present as a weakly bound nuclear protein.

Four distinguishable DNA polymerase activities (E.C. 2.7.7.7) have been solubilized from vertebrate tissues and cell cultures. The activities are DNA polymerase-α, -β, -γ, and -mt in the current nomenclature (1) and have been distinguished by different sedimentation coefficients, template preferences, pH preferences, sensitivities to sulfhydryl-blocking reagents, and apparently different locations within the cell. The reader is referred to Refs. 2 and 3 for reviews. Most of the activity extracted from dividing cells is DNA polymerase-α (4-14), although the role of this or of any of the polymerases in replication is not yet clear.

Despite a likely functional association with nuclei, DNA polymerase-α activity has been found predominantly in the postmitochondrial cytoplasmic fraction of broken cells while DNA polymerase-β activity remained with nuclei (5, 7-13). In several instances, however, some (5, 7, 10-13), or nearly all (14-16), of DNA polymerase-α activity was recovered from nuclear fractions from cells broken and centrifuged in media of low ionic strength. Furthermore, a large fraction of activity has been retained in nuclei isolated using Behrens' (17) nonaqueous cell fractionation (18-21), although recoveries of activity were either incomplete or anomalously high (21) and were not resolved into the different species. Also, Lynch et al. (14) have used the nonaqueous fractionation of Kirsch et al. (22) with regenerating rat liver and have extracted the majority of DNA polymerase-α activity from the nuclear fraction, although fractionation was incomplete due to technical difficulties. Finally, Herrick et al. (23) have fractionated mouse L cells without breakage by cytochalasin B nucleation (24) and have found the majority of DNA polymerase-α activity in the nucleus-containing "karyoplast" fraction. The association of a readily soluble DNA polymerase-α activity with nuclei is, therefore, becoming established although questions remain concerning the reliability of fractionation required to demonstrate nuclear location.

We report here further evidence for association of DNA polymerase-α with cell nuclei. To avoid extraction of soluble material during fractionation, we have adapted a nonaqueous method of nuclear isolation (22) to three cell lines in monolayer culture. We have paid particular attention to the recovery of activities and their quantitation through the cell division cycle. Preliminary reports of this work have been published in abstract form (25, 26).

EXPERIMENTAL PROCEDURES

Materials—Spectral grade glycerol was purchased from Mallinckrodt or from Matheson, Coleman, and Bell and was used without...
further purification. Analytical reagent grade glyc erol contained approximately 5% (v/v) water and was not suitable for cell fractionation. Freon-12,1 purchased from refrigeration supply houses, was contained in a cylinder at 0°C and the cylinder was purchased from Flow Laboratories; crystallized trypsin and purified calf thymus DNA from Worthington; chromatographically purified bovine DNAse-I from P-L Biochemicals; soybean trypsin inhibitor, bovine serum albumin, and purified yeast RNA from Sigma; and nucleosides from Schwarz/Mann. 3,5-Diaminobenzoic acid dihydrochloride was purchased from Aldrich and was purified by three or four precipitations in 6 N HCl and filtration of a 2 M solution through activated charcoal.

Cell Culture-The aneuploid mouse fibroblast cell line Balb 3T3, clone 12A3 cl 10 (27), was cultured as monolayers in Dulbecco's modification of Eagle's medium (28) with 36 mM NaHCO3 plus 5% or 20% calf serum in 10-cm plastic Petri dishes in a humid 7% CO2 atmosphere at 37°C. The SV40 virus-transformed diploid mouse line, SVT2 (29), was cultured in the same way, but with 3% or 5% calf serum and the human cell line, KB (30), with 5% or 10% calf serum. By changing medium daily at the higher serum concentration, 3T3 cells could be grown to 0.1 ml of packed cells/culture; SVT2 and KB cells could be grown to 0.25 ml of cells/culture.

The cell lines were examined for contamination with mycoplasmas by making autoradiographs of cultures labeled with tritiated thymidine. We found no cytoplasmic labeling which would have indicated contamination.

Synchronization of 3T3 Cells-Cells were plated at 5 x 104/cm2 (1 x 106/cm2) in medium containing 5% calf serum. The medium was changed (again 5% calf serum) after 3 days and the cells were incubated either 3 or 4 more days. At either time, 6 or 7 days after plating, each culture contained 3 x 105 cells (6 x 105/cm2) in the "contact-inhibited" G0 state (31). Some cultures were then stimulated to synthesize DNA and divide again by replacing the medium with fresh medium containing 20% calf serum. Autoradiographic analysis (32) of cultures before and after serum stimulation showed that G0 cultures had fewer than 0.1% cells synthesizing DNA. The fraction of cells synthesizing DNA began to rise 12 h after stimulation with over 90% synthesizing DNA at 20 h. The fraction dropped again later with all cells completing two or three rounds of replication after stimulation. G0 cultures were harvested either 6 or 7 days after plating and S cultures were harvested 20 h after serum stimulation of G0 cultures.

Nonynchronous Cell Fractionation-A more detailed description of this method will be published elsewhere (33). Monolayer cultures were washed with NaCl/P (5) and trypsinized (100 μg/ml of trypsin in NaCl/P) for 2 to 8 min at 2°C (34). The trypsin solution was decanted, residual trypsin was inactivated by rinsing with 20 μl of inhibitor in NaCl/P, and the tenascously attached monolayers were washed twice more with NaCl/P. The cells were then suspended in NaCl/P, by pipetting and centrifuged (1000 g, 30 s, 2°C). The pellet of cells (<0.5 ml) was suspended in 2 or 3 times its volume in either NaCl/P, or in 10 mM sodium phosphate (pH 7.2) and then frozen by dripping the concentrated cell suspension into melting Freon-121 (m.p. -158°C). The Freon-12 was removed and a test mixture up to 300 pmol of dATP incorporated/mixture of 100 μl. Culture supernatants were then separated at 2°C and stored at -28°C or -76°C.

DNA polymerase-P, 8.3 to 9.3 (no maximum in this interval), and for the species to be assayed. Increasing the salt concentration or time of extraction, and specific activity was enzyme units per μg of cellular DNA.

Extraction and Assay of DNA Polymerase Activity-Whole cells in hypotonic phosphate (1 volume NaCl/P, plus 2 volumes of 10 mM sodium phosphate, pH 7.2) or nonaqueous material in glycerol were extracted for 2 min at 2°C by vigorous shaking with 9 volumes of assay-extraction mixture which contained 100 mM Tris/HCl, 100 mM potassium phosphate2-5 mM MgCl2, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 10 μM concentration each of dCTP, dGTP, and dTTP, 100 μM (3H)dATP at 16 Ci/mole, 12.5% (v/v) ethylene glycol, and 0.375% (v/v) Triton X-100. Total volume was 0.4 ml. The pH of the extraction mixture was either 7.2 or 8.6 at 25°C depending on the enzyme species to be assayed. Increasing the salt concentration or time of extraction did not extract more activity. Triplicate samples of the extraction mixture were removed for measurement of DNA content, as described above, before addition of the activated DNA primer-template.

The extraction mixture was next mixed at 2°C with an equal volume of the solution containing activated DNA primer-template described above. Triplicate samples of 100 μl were incubated for 5 min at 37°C. The reaction was stopped by adding 1 ml of ice-cold 10% (v/v) trichloroacetic acid plus 30 mM Na2PP3, after 5 min at 0°C, precipitated material was collected by suction on Whatman GF/C glass fiber filters. The filters were washed three times at 0°C with 2 ml of 10 mM HCl plus 30 mM Na2PP3, and twice with 95% ethanol. Each filter was then air-dried and extracted for 5 min at 23°C with 0.25 ml of NCS solubilizer (Amersham/Searle) and mixed with 5 ml of toluene-based scintillation fluid (4.6 g of New England Nuclear Omnifluor/kg of toluene). Samples were counted in a Nuclear Chicago scintillation counter at 50% efficiency. In Table I and Fig. 1, 1 unit of enzyme activity was 1 pmol of dATP incorporated into acid precipitable material per h in the linear portion of the time course of incorporation, and specific activity was enzyme units per μg of cellular DNA.

Extracts from cells were fractionated by sucrose gradient sedimentation (described below) into preparations enriched in DNA polymerase-α and DNA polymerase-β. Activities from these preparations were optimized with respect to pH and concentrations of enzyme template, potassium phosphate, magnesium chloride, ethylene glycol, 2-mercaptoethanol, and deoxyribonucleoside triphosphates. Concentrations used in assay were at or near optimum. pH optima for mouse DNA polymerase-α were 7.0 to 7.2, for mouse DNA polymerase-β, 8.3 to 9.3 (no maximum in this interval), and for both human activities, 8.4 to 9.0.

Incorporation of [3H]dATP into acid-insoluble material was linear in time for approximately 8 min with decreasing rates of incorporation thereafter to 30 min. At any time of incubation between 1 and 30 min, incorporation was proportional to the amount of extract in the mixture up to 300 pmol of dATP incorporated/mixture of 100 μl.

Sedimentation Analysis of Polymerase Activities-Samples were extracted for 2 min at 2°C with 2 volumes of sucrose gradient extraction buffer which contained 0.5 M potassium phosphate,3 pH 7.5, 20 mM 2-mercaptoethanol, 1.5% (v/v) Triton X-100, 2 mM EDTA, 50 mM ethylene glycol and were centrifuged (27,000 x g, 20 min, 0°C). Samples of 200 μl of the supernatant extract were mixed with approximately 1 μg each of fluorescent-labeled (38) bovine serum albumin (ss, 4.3) and immunoglobulin G (s, 7.0) and applied to the tops of linear sucrose gradients, which contained 5% to 20% (w/v) sucrose in 50 mM potassium phosphate, pH 7.4, and 1.5% (v/v) 2-mercaptoethanol, formed in 5-ml cellulose nitrate centrifuge tubes. The gradient tubes were centrifuged at 48,000 rpm (188,000 g), for 24 h at 4°C in a

1 Freon-12, dichlorodifluoromethane, is a registered trademark of Union Carbide Corp.
2 The abbreviation used is: NaCl/P, which contained 140 mM NaCl, 2.7 mM KCl, 8 mM NaHPO4, 1.3 mM KH2PO4, 0.9 mM CaCl2, 0.5 mM MgCl2, pH 7.2, at 25°C.
3 Molarity given refers to potassium ion.
4 "Triton X-100," a nonionic detergent, is a registered trademark of Rohm and Haas Corp.
5 Fluorescent-labeled proteins, gifts of Drs. S. Hughes and G. Wahl, University of Utah, were prepared by the methods of Kawamura (38).
Beckman SW 50.1 centrifuge rotor. Thirty fractions of 175 μl were collected from the bottom of each tube.

Gradient fractions were assayed for DNA polymerase activity by mixing 10 μl of each fraction with 200 μl of 50 mm Tris-HCl, pH 7.2 or 8.6, 10 mm KCl, 7.6 mm MgCl₂, 2.5 mm 5-mercaptoethanol, 50 μM concentration of each of the four deoxyribonucleoside triphosphates with [3H]dATP at 650 Ci/mol, 0.5 mg/ml of activated DNA, and 0.25 mg/ml of bovine serum albumin. The potassium concentration of the reaction mixture was 55 mm. The reaction mixtures were incubated at 37°C for 30 min and processed for measurement of acid-insoluble radioactivity as described above. Units of activity in Figs. 2 and 3 were actual picomoles of dATP incorporated into acid-insoluble material during the 30-min incubation.

The remaining material in each sucrose gradient fraction was diluted with 3 ml of water. Fluorescence of the fluoresceinated sedimentation reference proteins was measured at 530 nm with excitation at 490 nm.

RESULTS

Cell Fractionation—As reported elsewhere (33) our nonaqueous procedure yielded nuclear and cytoplasmic cell fractions contaminated with approximately 10% of the RNA species characteristic of the opposite fraction. Cytoplasmic fractions contained 5 to 10% of cellular DNA. Electrophoresis of nuclear and cytoplasmic proteins also showed low levels of cross-contamination of the two cell fractions although the relative amounts were not quantitated (33).

Recovery of Polymerase Activity in Fractionation—Four types of cells, 3T3-G, 3T3-S, SVT2, and KB, were fractionated and the specific activity of DNA polymerases was measured in extracts made at seven steps during fractionation. Results are shown in Table I. Specific activity (polymerase activity divided by DNA content) was retained quantitatively through the course of decay was not exponential. At 3°C virtually all activity was lost by 24 h, but at 4°C a relatively stable residue of 10 to 15% remained after 144 h. At -20°C, glycerol mixtures lost approximately 10% of total activity in 144 h, and at -76°C mixtures lost less than 5% in 144 h.

Because of the temperature-dependent inactivation of activity, we minimized exposure of lyophilized material to glycerol at temperatures above -25°C. Homogenization below 0°C was impossible due to the high viscosity and occasional freezing of supercooled glycerol (m.p. +17°C), but homogenization could be completed in 15 min at 0°C followed by prompt chilling on solid CO₂. After centrifugation (130,000 × g, 12 to 18 h, -25°C), the cytoplasmic supernatant fraction was decanted at a temperature just warm enough to pour, approximately 0°C, and then the separated fractions were stored at -28°C or -76°C. The time of exposure of lyophilized material to glycerol above -25°C was thus held to approximately 30 min. Losses of 50% or greater occurred if centrifugation was performed at higher temperatures (130,000 × g, 2, 0°C). Therefore, a loss of 11 to 17% in specific polymerase activity in isolated nuclei (Table I) could have been due to the temperature-dependent inactivation observed in Fig. 1.

Sedimentation Analysis of Polymerase Activities—Sucrose

Table 1

| Step in fractionation        | 3T3-G, a | 3T3-S a | SVT2 | KB  |
|-----------------------------|----------|---------|------|-----|
| Activity                   | Specific activity | Activity | Specific activity | Specific activity | Activity | Specific activity |
| Fresh whole cells           | 11.1 ± 0.2 | 24.4 ± 0.1 | 38.5 ± 0.7 | 162 ± 7 |
| Frozen cells                | 11.2 ± 0.2 | 24.5 ± 0.5 | 38.6 ± 0.7 | 164 ± 7 |
| Dried cells                 | 11.5 ± 0.5 | 24.0 ± 2.5 | 37.8 ± 3.1 | 161 ± 21 |
| Dried cells plus glycerol   | 10.9 ± 0.3 | 23.6 ± 0.7 | 37.7 ± 1.1 | 154 ± 6 |
| Homogenate                  | 238 ± 4 | 9.2 ± 0.3 | 292 ± 9 | 438 ± 13 | 33.3 ± 1.0 | 971 ± 19 | 144 ± 6 |
| Cytoplasmic supernatant     | 11.1 ± 0.2 | 9.9 ± 0.8 | 21.0 ± 4.2 | 43 ± 3 | 63 ± 31 | 52 ± 3 | 196 ± 41 |
| Nuclear pellet              | 222 ± 11 | 9.7 ± 0.3 | 270 ± 11 | 21.6 ± 0.8 | 393 ± 28 | 32.2 ± 1.1 | 896 ± 53 | 141 ± 6 |

a 3T3 cells were synchronized in the G₀ and S phases ("Experimental Procedures").

Activity given was for a particular centrifuged homogenate. Activity measured within 5 min of mixing at 0°C.

Activity measured after 15 to 20 min homogenization at approximately +4°C.

Fractions obtained after centrifuging homogenate 12 to 18 h at -25°C.
The cellular location of DNA polymerase-α was nuclear in...
contact-inhibited G₁ phase 3T3 cells as well as in serum-stimulated S phase 3T3 cells and in rapidly dividing 3T3, SVT₂, and KB cells (Table I). Nuclear location during all stages of the cell cycle argues against a proposed migration of cytoplasmic polymerases to the nucleus at the beginning of DNA synthesis (42–45). Also, the nearly quantitative recovery of activity in the nuclear fraction argues against a significant contribution to polymerase activity from other cytoplasmic factors. It is possible that recently described factors which can enhance polymerase activity (46) are also weakly bound nuclear components. The example of one weakly bound nuclear protein of some interest, DNA polymerase-α, should promote re-examination of cellular locations of other macromolecules.

Levels of extractable DNA polymerase activity, DNA polymerase-α in particular, have been observed to be higher in proliferating cells than in resting cells (9, 11, 47–49). Baril and Laszlo (41) compared polymerase activities from several rat tissues and observed a rough proportionality between activity and rates of DNA synthesis. These observations supported the contention that the rate of DNA replication is controlled by the activity of DNA polymerase-α. In our work with synchronized 3T3 cells, however, we observed that S cultures contained approximately 1000 times as many cells synthesizing DNA as did G₁ cultures, hence a 1000-fold higher rate, yet the amount of DNA polymerase-α activity in G₁ cultures was lower than that in S cultures by only a factor of 2 to 5. These observations showed that in 3T3 cells the frequency of initiation of S phase and overall rate of DNA synthesis were not simply proportional to the extractable activity of DNA polymerase-α.

Although polymerase activities are nuclear, the elution of DNA polymerase-α activity from nuclei during aqueous cell fractionation might be evidence for an inactive storage form, within the nucleus, for the majority of DNA polymerase-α molecules. Cells engaged in exceptionally rapid DNA synthesis, such as early cleavage embryos (15, 50), regenerating rat liver (10, 14), or mouse cells productively infected with polyoma virus (51), have been reported to have exceptionally high proportions of total DNA polymerase or DNA polymerase-α tightly bound to nuclei after conventional aqueous fractionation. If DNA polymerase-α is directly involved with DNA replication, which is by no means certain, then levels of more tightly bound DNA polymerase-α should be more nearly proportional to rates of DNA synthesis than levels of total DNA polymerase-α. Experiments testing this prediction have not yet been done.

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