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Intracellular localization of DNA polymerase α

(cytochalasin B/enucleation/cytoplasmic DNA polymerase/DNA cytochemistry)

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ABSTRACT **Immunoglobulin (IgG) and the F(ab')$_2$ fragment of IgG were prepared from serum of a rabbit immunized with purified calf thymus DNA polymerase α (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7). An indirect immunofluorescent method based on these reagents was used to detect the intracellular localization of DNA polymerase α in primary fetal bovine fibroblasts. The results show that the bulk of DNA polymerase α is located in the perinuclear region of the cytoplasm. Immunofluorescent staining of cytoplast and Ficoll-Paque gradient-purified karyoplast fragments resulting from cytochalasin enucleation show the presence of DNA polymerase α in cytoplasts and the virtual absence of the enzyme in the nucleus of the karyoplast itself. The implication of this unusual intracellular location for DNA polymerase α is discussed.**

The original description of DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) in eukaryotic systems (1, 2) noted the presence of the major amount of its activity in the cytoplasmic fraction of buffered sucrose homogenates, with a minor amount of activity in the nucleus. This finding has been confirmed by other early investigators (3). A report suggesting transport of activity to the nucleus during S phase has been published also (4). Subsequent work with nonaqueous solvents (5, 6) has suggested a nuclear localization. Use of cytochalasin B to produce cytoplasts and karyoplasts also has been interpreted to prove a nuclear localization of DNA polymerase α (7). Biochemical studies routinely use a cytoplasmic fraction for isolation of DNA polymerase α. DNA polymerase β has been found to be nuclear and cytoplasmic (8, 9) and DNA polymerase γ has been localized in mitochondria (10, 11).

Because all of these studies have required breakage of cells, the arguments have centered on how the cells were disrupted and the quantitative analysis of the result. This matter could be settled by demonstrating localization of the enzyme in whole-cell preparations. We have now performed such studies on fixed whole cells and can demonstrate the cytoplasmic localization of DNA polymerase α.

MATERIALS AND METHODS

Cells. Primary fetal fibroblasts from bovine kidney (BOS cells in the 3rd and 6th passages, received from F. Bach, University of Wisconsin) and fetal bovine spleen cells (in the 7th to 10th passages, received from E. H. Stephenson, Walter Reed Army Institute of Research, Washington DC) were grown as monolayers in Ham's F-10 medium containing 15% (vol/vol) fetal calf serum.

Antibody to DNA Polymerase α. The antibody used for this study was a monospecific antibody against DNA polymerase α, prepared by immunizing a rabbit with calf thymus DNA polymerase α. Immunoglobulin (IgG) and F(ab')$_2$ fragment of IgG were prepared from the immune serum. The preparation and characterization of this antibody have been described (12).

Immunofluorescence. An indirect immunofluorescent technique was used to test for the presence of DNA polymerase α. Cells growing on cover slips, cytocentrifuge preparations of purified karyoplasts, and re-adhered cytoplasts were washed three times in medium F-10 without serum, fixed in absolute methanol for 10 min at 4°C, and air dried. The IgG or F(ab')$_2$ fragment of rabbit anti-calf thymus DNA polymerase α or preimmune IgG (diluted to 100–400 μg/ml in phosphate-buffered saline) was used in the primary incubation for 30 min at room temperature. The secondary antibody was the fluorescein isothiocyanate-conjugated F(ab')$_2$ fragment of goat anti-rabbit IgG and was applied at a concentration of 100 μg/ml for 30 min. The cover slips and slides were washed in phosphate-buffered saline after each incubation. Samples were mounted in buffered glycerol and examined for immunofluorescence with a Zeiss photomicroscope III. Photography was by spot integration with Professional Ektachrome ASA 200. Exposures were made at 800 ASA and the film was push-processed to 800 ASA in development.

Enucleation of Cells with Cytochalasin B. The kinetic study on the enucleation process was carried out on bovine kidney cells grown on cover slips as described by Prescott *et al.* (13), except that centrifugation was carried out for 15 min, 30 min, and 45 min at 5000 rpm in the HB-4 rotor in a Sorvall RC-5 centrifuge.

To obtain enough cells to carry out the purification of karyoplasts, cells were grown on the sides of 150-ml Sorvall glass centrifuge bottles (Corex 157) in a roller apparatus. Subconfluent monolayers were spun at 10,000 × *g* for 10 min in the Sorvall centrifuge at 37°C to remove loosely attached cells. The growth medium was then replaced with fresh medium containing serum and 10 μg of cytochalasin B (Aldrich) per ml. After a 15-min incubation at 37°C, the monolayers were centrifuged for 45 min at 10,000 × *g* to pellet crude karyoplasts. The pellet (containing karyoplasts, cytoplasmic fragments, and contaminating whole cells) was incubated for 1 hr in growth medium in a tissue culture flask to allow whole cells and cytoplasts to reattach to the substrate. The crude karyoplast suspension was then purified on 1–6% Ficoll-Paque gradients as described by Lucas *et al.* (14). Two bands were obtained from the Ficoll-Paque gradient. The upper band containing cytoplasmic fragments and the lower band containing purified karyoplasts were carefully removed from the gradient. Cytocentrifuge preparations of these two fractions were prepared and processed for DNA polymerase α immunofluorescence. The gradient-purified karyoplasts were 87% viable as determined by trypan blue exclusion.

Cytoplasts remaining on the Sorvall bottle walls after the enucleation procedure were removed by trypsinization and al-

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lowed to adhere to cover slips in growth medium for 2 hr in a Leighton tube. The re-adhered cytoplasts were prepared for DNA polymerase α immunofluorescence as described.

RESULTS

Specificity of Anti-DNA Polymerase α. (*i*) The antibody preparation used for this study is highly specific for bovine DNA polymerase α (12). About 0.15 μg of IgG effected 50% neutralization of 1 unit of bovine enzyme, whereas more than 10 times as much IgG was required to give 50% neutralization of an equivalent amount of mouse L cell, HeLa, or chicken embryo DNA polymerase α. No neutralization of bovine DNA polymerase β or terminal transferase was found.

(*ii*) The bovine cell line that was used did not stain with preimmune rabbit IgG or with the fluorescein isothiocyanate–conjugated [$F(ab')_2$] fragment of goat anti-rabbit IgG used as the secondary antibody. Staining of equal intensity and quality was achieved with either IgG of rabbit anti-calf thymus DNA polymerase α or the $F(ab')_2$ fragment prepared from that IgG. No staining of unfixed cells could be demonstrated.

(*iii*) Immunoelectrophoresis and immunodiffusion of purified DNA polymerase α showed a single sharp precipitin arc (12). Immunoelectrophoresis of the crude extracts of calf thymus or bovine kidney cells showed no precipitin line at all. When the extracts were concentrated 10-fold by ultrafiltration, a single faint band could be detected in the extracts. This faint band showed an antigen mobility identical to that found with the purified DNA polymerase α.

(*iv*) Adsorption of antibody with total bovine-muscle soluble protein coupled to Sepharose 4B, acetone powder of bovine liver, or purified rat brain tubulin did not change the quality or intensity of fluorescence elicited with rabbit anti-calf thymus DNA polymerase α IgG. Adsorption of the IgG with purified calf thymus DNA polymerase α, on the other hand, completely eliminated the fluorescence.

Immunofluorescence of Whole Cells. Bovine fetal spleen cells (Fig. 1 *A* and *B*) and bovine kidney cells (Fig. 1 *C* and *D*) stained with the anti-DNA polymerase α IgG exhibited intense cytoplasmic staining localized in the perinuclear region. The fluorescence was rather granular in appearance and did not encompass the entire cytoplasm (compare phase contrasts in Fig. 1 *A* and *C*). The fluorescent region was not always symmetrically displaced around the nucleus and appeared to be associated with other structural elements in the perinuclear region.

The nucleus appeared dark in most cells, with occasional cells having a suggestion of hazy fluorescence over the nucleus. These areas could be photographed by using "spot integration" over the nucleus, but in such photographs the cytoplasm was then grossly overexposed (Fig. 1 *E* and *F*). The apparent nuclear fluorescence might be an artifact of cell geometry, but in any case, it was relatively dim compared to cytoplasmic fluorescence.

All cells appeared to have cytoplasmic fluorescence. In cells rounding up in preparation for division, the localization could not be visualized. With a 38-hr generation time (for bovine kidney cells) and an estimated 12-hr S phase, one expected around 30% of bovine fibroblasts to be in S phase, and this was confirmed by [^{3}H]thymidine pulses and autoradiography. The number of cells showing some nuclear fluorescence was always less than 5%.

Immunofluorescence in Cell Fragments from Cytochalasin B-Treated Cells. The rather striking results obtained by the direct visualization of DNA polymerase α in unbroken cells prompted us to reexamine the cytochalasin effect in primary bovine fibroblasts. Cytochalasin B treatment does indeed enucleate cells in the manner previously described. Fig. 2 *A* and *B* show the phase and fluorescence of bovine kidney cells after only a 15-min centrifugation at 5000 rpm in the presence of cytochalasin *B*, a condition insufficient to enucleate. Enucleation was complete after 45 min of centrifugation. Examination of the kinetics of the enucleation process showed that the nucleus migrates to the top of a stalk-like projection with the margins of the cell still attached to the glass slide. In the stalk there is often a bulge, and this bulge contains the granular structures that we have ascribed to the perinuclear regions and others (15) have called the "centrosphere" because of the presence of centrioles. This centrosphere retained much of the immunofluorescence.

When the "stalk" was ruptured by centrifugation, two types of particles were found in the pellet (crude karyoplast fraction). About 50% of the particles appeared to be nuclei by Giemsa

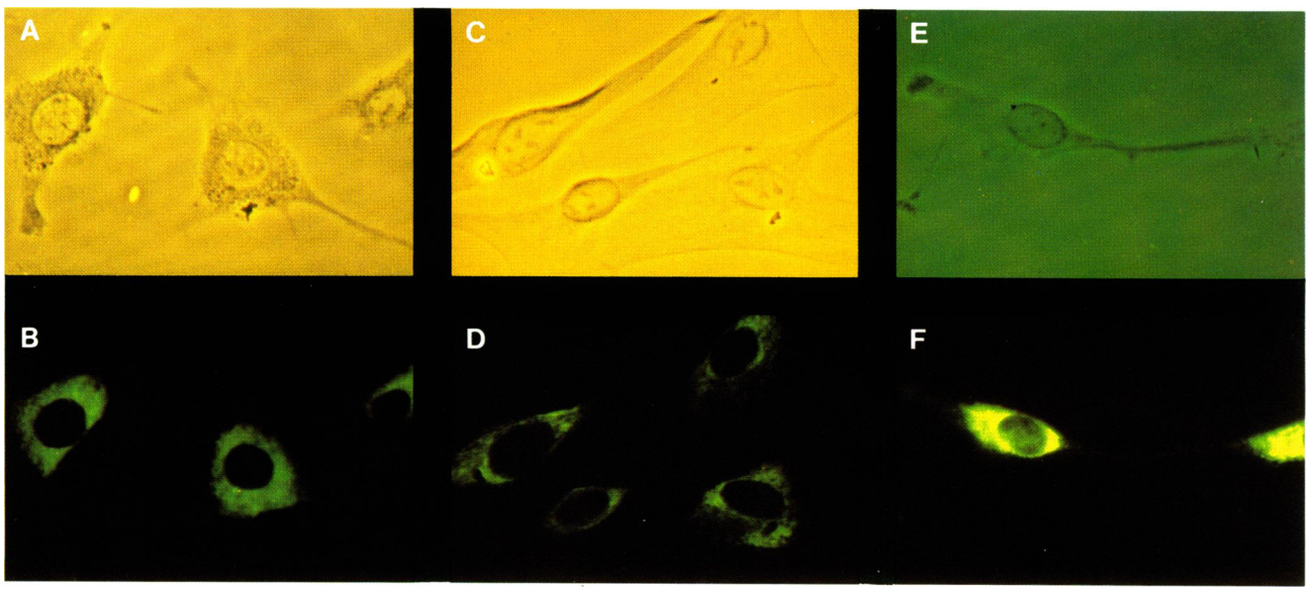

FIG. 1. Immunofluorescent staining of DNA polymerase α of bovine fibroblasts. Phase contrast (*A*) and immunofluorescence (*B*) of fetal bovine spleen cells. Phase contrast (*C* and *E*) and immunofluorescence (*D* and *F*) of bovine kidney cells.

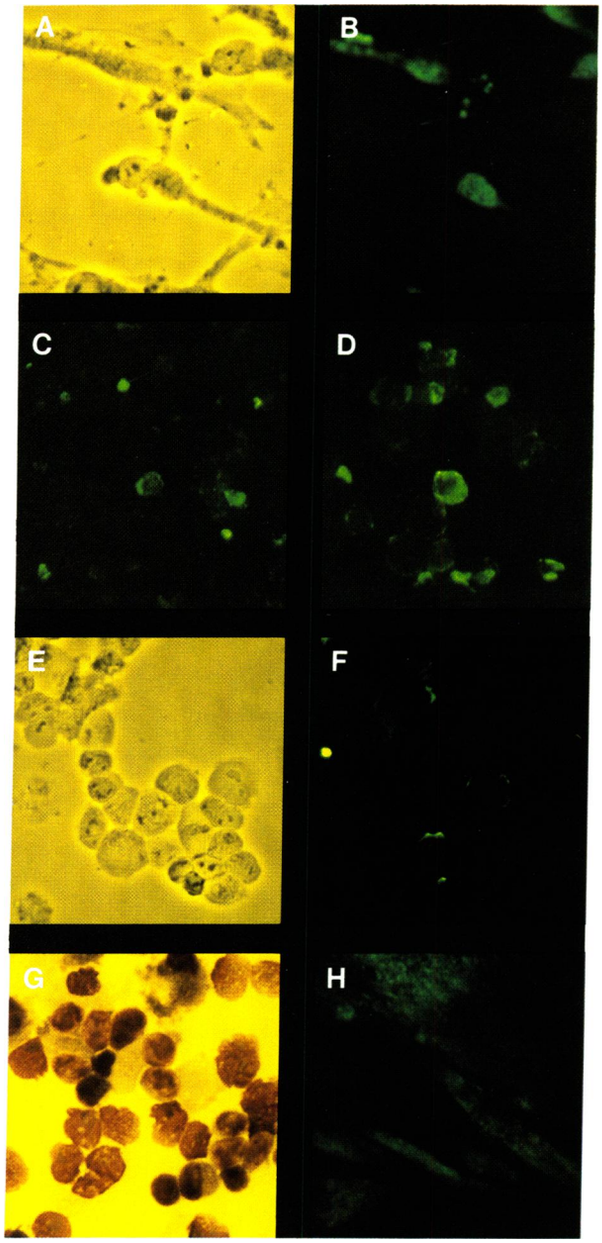

FIG. 2. Cytochalasin-treated bovine kidney cells and cell fragments. (*A* and *B*) Phase contrast and immunofluorescence, respectively, of cytochalasin B-treated bovine kidney cells after a 15-min centrifugation at 5000 rpm, a condition insufficient to enucleate the cells. (*C*) Immunofluorescent staining of the upper band of the Ficoll-Paque gradient after fractionation of the crude karyoplasts obtained by the enucleation procedure. (*D*) Immunofluorescence of a typical field of gradient-purified karyoplasts. (*E* and *F*) Phase contrast and immunofluorescence, respectively, of a clean field of gradient-purified karyoplasts. (*G*) Giemsa stain of a typical gradient-purified karyoplast preparation. (*H*) Immunofluorescence of re-adhered cytoplasts.

stain and the other 50% did not give the magenta stain produced by DNA. Most particles that contained nuclei showed a rim of fluorescence about the nucleus, and some particles showed fluorescent tabs (possibly the centrosphere) associated with nuclei. The nonnuclear particles stained intensely with the anti-DNA polymerase-α antibody. No significant amount of fluorescence could be detected in the nucleus proper. When the crude karyoplast fraction was purified on a Ficoll-Paque gradient, most of the nonnuclear particles appeared in the top band on the gradient and could be shown to be immunofluorescent (Fig. 2*C*).

The lower band on the Ficoll-Paque gradient contained the karyoplasts. Immunofluorescent staining for DNA polymerase-α in the gradient-purified karyoplasts showed fluorescent rims and tabs on some karyoplasts and some nonnuclear particles in a typical field on the microscope slide (Fig. 2*D*). Fig. 2 *E* and *F* show the phase contrast and fluorescence of one of the cleanest fields found on the slide made from the gradient-purified karyoplasts. Intense fluorescent rims around the dark nucleus can still be seen. Giemsa-stained preparations of typical Ficoll-Paque gradient-purified karyoplasts show the same results (Fig. 2*G*). Hence, the so-called karyoplast fraction is always contaminated with cytoplasm and often contains part of the perinuclear structure.

The cytoplast fraction remaining on the glass was isolated and stained for DNA polymerase α (Fig. 2*H*). Some of the cytoplasmic fragments appeared to be negative for immunoreactive material. Most cells did show a weakly fluorescent area relocalized to the center of the cytoplasmic fragment, and all were devoid of a nucleus.

DISCUSSION

The combined cytological and immunological methods used in this study to examine the intracellular localization of DNA polymerase α clearly demonstrate that the bulk of DNA polymerase α is localized in the perinuclear region of the cell. We are unable to detect DNA polymerase α in the nucleus. The conclusion drawn in this study differs quantitatively from that of Herrick *et al.* (7). These investigators showed that 88% of total DNA polymerase α was present in their karyoplast fraction. The discrepancy between these two studies can be explained by the extent of cytoplasmic contamination in the karyoplasts used. Lucas *et al.* (14) showed that karyoplasts obtained by a simple enucleation procedure are grossly contaminated with cytoplasmic fragments. By examining the distribution of DNA polymerase α in fractions derived from cytochalasin enucleation of mammalian cells, we show that DNA polymerase α present in the crude karyoplast fraction is perinuclear in origin. Assaying for DNA polymerase α activity in extracts made from karyoplasts after simple enucleation procedures (7, 16) cannot distinguish between a perinuclear and an intranuclear location of the enzyme.

The correlation between the levels of DNA polymerase α with the rates of DNA synthesis suggests a replicative role for this enzyme (9, 17). It is possible that the level of DNA polymerase α in the cell greatly exceeds the number of enzyme molecules required for DNA synthesis. The method used for this study is not sufficiently sensitive to detect a small amount of the enzyme (<5% of total enzyme) that might be present in the nucleus. The perinuclear location of DNA polymerase α is consistent with the hypothesis that a small amount of the enzyme may be transported into the nucleus during DNA synthesis (4). An alternative explanation for the failure to detect DNA polymerase α in the nucleus is that those enzyme molecules involved in the process of DNA replication may be complexed with other replication proteins and DNA, thereby masking antigenic determinants.

The apparent perinuclear localization of the bulk of DNA polymerase α in the cell is an interesting finding, but the significance is not clear at present. It is possible that the perinuclear region of the cell contains the "... multienzyme complex for metabolic channeling in mammalian DNA replication ..." described by veer Reddy and Pardee (16) and that these multienzyme complexes detach from the karyoplast during the purification on the Ficoll-Paque gradient. It would be of considerable interest to examine the level of the multienzyme complex for DNA replication in gradient-purified karyoplast fractions.

All previous arguments against a cytoplasmic localization of DNA polymerase have been based on broken-cell preparations. We feel that prejudicial errors creep into the interpretation of results obtained from these preparations. There are now two publications demonstrating cytoplasmic localization of DNA polymerase in whole-cell preparations. The first demonstration was published more than 15 years ago (18) and involved pinocytosis of DNA by Amoeba, followed by demonstration of thymidine labeling in the cytoplasm. The present experiments involve direct detection of DNA polymerase α molecules by specific antibodies applied to fixed whole-cell preparations. There can be little doubt now that a major fraction of DNA polymerase α is cytoplasmic in normal mammalian cells. Inquiry into the regulatory aspects of the transport phenomena implied by these findings seems most appropriate.

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1. Bollum, F. J. (1958) *J. Am. Chem. Soc.* **80,** 1766.
2. Bollum, F. J. & Potter, V. R. (1958) *J. Biol. Chem.* **233,** 478–482.
3. Keir, H. M. (1965) *Prog. Nucleic Acid Res. Mol. Biol.* **4,** 81–128.
4. Littlefield, J. W., McGovern, A. P. & Margeson, K. B. (1963) *Proc. Natl. Acad. Sci. USA* **49,** 102–107.
5. Keir, H. M., Smellie, R. M. S. & Siebert, G. (1962) *Nature (London)* **196,** 752–754.
6. Foster, D. N. & Gurney, T. (1976) *J. Biol. Chem.* **251,** 7893–7898.
7. Herrick, G., Spear, B. B. & Veomett, G. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1136–1139.
8. Chang, L. M. S. & Bollum, F. J. (1972) *Biochemistry* **11,** 1264–1272.
9. Chang, L. M. S. & Bollum, F. J. (1972) *J. Biol. Chem.* **247,** 7948–7950.
10. Bertazzoni, U., Scovassi, A. I. & Brun, G. (1977) *Eur. J. Biochem.* **81,** 237–248.
11. Hübscher, U., Kuenzle, C. C. & Spadari, S. (1977) *Eur. J. Biochem.* **81,** 249–258.
12. Chang, L. M. S. & Bollum, F. J. (1981) *J. Biol. Chem.* **256,** 494–498.
13. Prescott, D. M., Myerson, D. & Wallace, J. (1972) *Exp. Cell Res.* **71,** 480–483.
14. Lucas, J. J., Szekely, E. & Kates, J. R. (1976) *Cell* **7,** 115–122.
15. Shay, J. W., Porter, K. R. & Prescott, D. M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 3059–3963.
16. veer Reddy, G. P. & Pardee, A. G. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3312–3316.
17. Chang, L. M. S., Brown, M. & Bollum, F. J. (1973) *J. Mol. Biol.* **74,** 1–8.
18. Prescott, D. M., Bollum, F. J. & Kluss, B. C. (1962) *J. Cell Biol.* **13,** 172–174.