Deoxyribonucleic Acid Polymerases of BHK-21/C13 cells

RELATIONSHIP TO THE PHYSIOLOGICAL STATE OF THE CELLS, AND TO SYNCHRONOUS INDUCTION OF SYNTHESIS OF DEOXYRIBONUCLEIC ACID

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BHK-21/C13 cells were grown in culture under conditions that provided exponentially growing cells and quiescent cells, by modifying the concentration of serum in the growth medium. The high-molecular-weight DNA polymerase (DNA polymerase I) from exponentially growing cells accounted for 90% of the total polymerase activity; the low-molecular-weight DNA polymerase (DNA polymerase II) accounted for the remaining 10%. In quiescent cells, DNA polymerase I contributed only 39% of the total polymerase activity and DNA polymerase II 61%. The total amount of DNA polymerase I in exponentially growing cells was 11.3-fold greater than that in quiescent cells, whereas the amount of DNA polymerase II appeared to be relatively independent of the physiological state of the cells. In an extension of these experiments, cells in a quiescent state (G_0 cells) were stimulated by the 'serum-step-up' method of Bürk (1970) to grow and to enter a synchronous wave of DNA synthesis (S-phase cells), 87% of the cells synthesizing DNA at 20h after the 'serum step-up'. During the synchrony experiment, the total cytoplasmic and total nuclear DNA polymerase activities each increased about 4-fold in parallel with the increase in the rate of DNA synthesis. Cytoplasmic polymerase activity was always greater than nuclear polymerase activity. The increases observed were maximal at 20h after 'serum step-up'. By 26h, there was a decrease in enzyme activity (8% for cytoplasmic polymerase and 16% for nuclear polymerase, both relative to the maximum at 20h), but the rate of DNA synthesis had declined by 37% relative to the maximum at 20h. In G_0 cells, DNA polymerase II (mol.wt. 46000 ± 4000) was the predominant species, there being twice as much of it as of the total DNA polymerase I. In these cells there was little DNA polymerase IC and ID; the amounts of IA (mol.wt. 900×10^3 – 1100×10^3) and IB (mol.wt. 460×10^3 – 560×10^3) were about equal but small.

The evidence for the presence of multiple forms of replicative DNA-dependent DNA polymerases (DNA nucleotidyltransferase; EC 2.7.7.7.; hereafter referred to as DNA polymerase) in eukaryotic cells, their possible role in the biosynthesis of DNA, and their apparent subcellular distribution has been reviewed by Craig & Keir (1974). All actively replicating eukaryotic cells and tissues examined contain two major species of DNA polymerase distributed between the cytoplasm and nucleus. These enzymes, from a wide variety of cell types, have been separated on the basis of molecular weight by both centrifugation on sucrose density gradients and gel filtration. For the sake of simplicity they will be referred to here as DNA polymerase I and DNA poly-

merase II; respectively, they possess sedimentation coefficients of 6–8S and 3.3S as assessed by density-gradient centrifugation in sucrose or glycerol.

Circumstantial evidence implicating DNA polymerase I activity in the DNA replication process has been adduced from a variety of experimental systems. For example, quantitative analyses of DNA polymerases in rat brain during the various stages of development (Chiu & Sung, 1972*a,b,c*), in normal and regenerating rat liver (Chang & Bollum, 1972), and in mouse L-cells in the stationary phase of growth and after stimulation to proliferate (Chang *et al.*, 1973), all revealed a direct correlation between the maximum rate of DNA synthesis *in vivo* and the maximum amounts of DNA polymerase I. Conversely, when DNA synthesis was at a minimum the amounts of this DNA polymerase decreased markedly. In cells stimulated to proliferate, relatively little difference in the amount of DNA polymerase II was observed

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when comparison was made with the corresponding quiescent cells.

Although this substantial evidence implies a direct correlation between activity of DNA polymerase I and the rate of replication of DNA *in vivo*, there is little evidence to support its direct association with the replication machinery of the cell nucleus. Earlier work with cell cultures synchronized with respect to the cell cycle by the use of metabolic inhibitors (Littlefield *et al.*, 1963; Gold & Helleiner, 1964; Lindsay *et al.*, 1970) or mitotic selection (Madreiter *et al.*, 1971) revealed an increase in the total DNA-polymerizing activity associated with the nucleus in S phase. However, this work was not definitive; in no case was the DNA-polymerizing activity identified.

Exponentially growing BHK-21/C13 cells contain both DNA polymerase I and DNA polymerase II activities (Craig & Keir, 1975a). In the present paper we demonstrate (i) a correlation of the activities of these enzymes with the physiological conditions of the cells by comparison of cells growing in the exponential phase with cells maintained in a minimal medium (quiescent cells); and (ii) the fluctuation of total DNA polymerase activities in cytoplasmic and nuclear fractions during the cell cycle, and the identification of the individual species involved.

Experimental

Materials

These were as described by Craig & Keir (1975a,b).

Buffers and growth media

Buffer A was 10mM-Tris-HCl (pH 7.5 at 4°C)–5mM-2-mercaptoethanol. Buffer B was 0.2M-potassium phosphate (pH 7.5)–5mM-2-mercaptoethanol. TD buffer was 25mM-Tris-HCl (pH 7.5 at 4°C)–0.14M-NaCl–0.05mM-KCl–0.2mM-Na₂HPO₄. ETC₁₀ medium was Eagle's medium (Glasgow modification) (Craig & Keir, 1975a) made with 10% (v/v) with calf serum and 10% (v/v) with tryptose phosphate broth. ETC_{0.5} medium was ETC₁₀ except with calf serum at 0.5% (v/v). EC₁₀ medium was Eagle's medium (Glasgow modification) containing bovine calf serum at 10% (v/v). Eg_{SOH}C₁₀ medium was Eagle's medium [Bürk (1970) modification] containing bovine calf serum at 10% (v/v). Eg_{SOH}C_{0.25} medium was as for Eg_{SOH}C₁₀ medium but containing bovine calf serum at 0.25% (v/v). Trypsin–Tris–saline was TD buffer containing trypsin (0.25% w/v), dextrose (0.1%, w/v), Phenol Red (0.0015% w/v), streptomycin (0.01%, w/v), and penicillin (10⁵ units); pH was adjusted to 7.7 at 18°C with

1M-HCl. Formal–saline was 0.085M-NaCl–0.1M-Na₂SO₄–1.33M-formaldehyde.

Methods

Production of exponentially growing and quiescent cell cultures. BHK-21/C13 (baby hamster-kidney) cells were grown in cylindrical glass roller-bottles in monolayer culture as described by Craig & Keir (1975a). Bottles were seeded with 5 × 10⁷ cells in ETC₁₀ medium in a final volume of 200 ml and rotated at 1 rev./3 min at 37°C; 36 h after seeding, the cells were in the exponential phase of growth. At this stage they were either harvested for immediate enzyme extraction, or incubated for a further 3–4 days in ETC_{0.5} medium before being harvested for enzyme extraction. During maintenance in ETC_{0.5} medium, metabolic activities of the cells decline to a basal level after 3–4 days (Bürk, 1970; Rolton & Keir, 1974). The cells are then said to be quiescent.

Harvesting of cells and preparation of cell extracts. To harvest cells, the medium was removed and the cell sheet rinsed with trypsin–Tris–saline (20 ml/bottle) at room temperature. Excess of trypsin was drained off, and the bottles were left for 1–2 min. Ensuing operations were at 4°C. Ice-cold EC₁₀ medium (20 ml/bottle) was added, the cells were shaken off the glass, and collected by centrifugation at 200g_{av} for 5 min. Cells were again washed in EC₁₀ medium as above, followed by resuspension in TD buffer (10 ml/bottle of cells). The cell number was determined by using an Improved Neubauer haemocytometer. The cells were again sedimented, and finally resuspended in the hypo-osmotic buffer A (5 ml/bottle of cells) and allowed to swell for 5 min at 4°C. They were disrupted as described by Craig & Keir (1975a) in a glass homogenizer tube with a Teflon pestle. After centrifugation at 800g_{av} for 10 min, the resulting supernatant fraction ('cytoplasm') was retained, and the sediment (nuclei and cell debris) suspended in buffer B (2.5 ml/bottle of cells) and left for 1 h.

Both the 'cytoplasmic' and 'nuclear' fractions were then processed as described by Craig & Keir (1975a). Briefly, the procedure includes high-speed centrifugation which yielded supernatant fractions; these were concentrated by precipitation with (NH₄)₂SO₄. The precipitates were dissolved in buffer B and the 'cytoplasmic' and 'nuclear' extracts thus obtained were combined. This preparative method ensured removal of insoluble cell debris, microsomal fraction, ribosomes and mitochondria before exposure of the 'soluble cytoplasmic' and 'nuclear' fractions to the hyperosmotic buffer B and high concentrations of (NH₄)₂SO₄. Solubilization of mitochondrial proteins, including mitochondrial DNA polymerase, and of extraneous proteins in other cell debris was thereby avoided.

Cell growth and establishment of synchrony. BHK-21/C13 cells (baby hamster kidney; Macpherson & Stoker, 1962) were grown in monolayer culture in cylindrical glass roller-bottles as described by Craig & Keir (1975a) and harvested by treatment with trypsin (Craig & Keir, 1975b).

The cells were washed twice by suspension in EC₁₀ medium (20ml/2.5 × 10⁸ cells) at 4°C and centrifugation at 200g_{av.} for 5 min, and finally resuspended in Eg_{soH}C_{0.25} medium to give a final monolayer density of 5 × 10⁴ cells/cm² in either 5 cm or 9 cm diam. vented plastic Petri dishes. This represented 3 × 10⁶ cells/10 ml in the 9 cm dishes and 1 × 10⁶ cell/5 ml in the 5 cm dishes. All 5 cm dishes contained in addition two 13 mm² glass coverslips. Immediately after seeding with cells, the Petri dishes were transferred to gas-tight humidified incubators at 37°C in an atmosphere of CO₂+air (5:95). After 24 h the medium which usually contained some dead cells, was poured off and replaced with fresh Eg_{soH}C_{0.25} medium. The Petri dishes were then reincubated at 37°C in CO₂+air (5:95) for a further 72–84 h after which time the cells were in a quiescent state (the G₀ state). A synchronous wave of cell growth was then initiated at 84 h by replacing the medium with Eg_{soH}C₁₀ medium.

Pulse-labelling and radioautography. At selected times, the medium in two 5 cm Petri dishes was replaced with the same medium (5 ml/dish) at 37°C containing [6-³H]thymidine (5 μCi/ml; The Radiochemical Centre, Amersham, Bucks., U.K.). The Petri dishes were reincubated for 1 h at 27°C in CO₂+air (5:95), and the pulse of radioactive precursor was terminated by washing the cell monolayers twice in TD buffer at 4°C. The cells were then fixed in formal-saline.

The formal-saline was aspirated off, and the cell monolayers were washed *in situ* three times with 10 ml portions of 5% (w/v) trichloroacetic acid at intervals of 10 min. The monolayers were then rinsed once with water, twice with ethanol, and dried at room temperature for 24 h. Each coverslip was then removed, placed in a glass vial containing 10 ml of scintillation fluid, and measured for radioactivity in the Intertechnique model SL-30 liquid-scintillation spectrometer. The scintillation fluid was the toluene-based solution described by Craig & Keir (1975a).

For radioautography, the coverslips were removed from the scintillation fluid, rinsed once with ethanol, and mounted (cell monolayer uppermost) with XAM (Gurr; Searle Diagnostics Ltd., High Wycombe, Bucks., U.K.) on glass microscope slides. The XAM was dried (48 h at room temperature), and finally coated with Ilford L4 photographic emulsion. After 4–6 weeks the slides were developed in Kodak D-19 developer, and for every time-point, five random fields, each containing about 2000 cells,

were photographed during phase-contrast microscopy. The labelling indices were then determined (see the Results section).

Harvesting of cells. At each time-point monitored by [³H]thymidine incorporation, the cells from four 9 cm Petri dishes were exposed to trypsin at room temperature for 1 min (Craig & Keir, 1975b), the cell sheet was then rinsed off with EC₁₀ medium at 4°C, and the cells were collected by centrifugation at 200g_{av.} for 5 min. The sedimented cells were washed once more in EC₁₀ medium and finally resuspended in 5 ml of TD buffer, all at 4°C. The cell number was then determined by using an Improved Neubauer Haemocytometer. The cells were again sedimented, and stored at –70°C in readiness for subcellular fractionation for enzyme studies.

Subcellular fractionation. Cells stored at –70°C were thawed, resuspended in 2 ml of the hypo-osmotic buffer A and left for 5 min at 4°C. They were then disrupted as described by Craig & Keir (1975a). The homogenate was processed at 4°C. Centrifugation at 800g_{av.} for 10 min yielded a supernatant termed the 'low-speed cytoplasmic fraction' and a sediment (nuclei and cell debris). The latter was washed by a single suspension and centrifugation in buffer A containing 0.32 M-sucrose to remove most of the cytoplasmic contamination. The final sediment was suspended in 1.5 ml of buffer A and termed the 'low-speed nuclear fraction'. Portions of each fraction were assayed for DNA polymerase I by using an 'activated' DNA from salmon sperm as primer-template (Loeb, 1969) and the conditions optimal for that species of polymerase (Craig & Keir, 1975a).

Total cell extracts of synchronized cultures. Cells from thirty 9 cm Petri dishes were harvested by exposure to trypsin (Craig & Keir, 1975b), and total cell extracts prepared and analysed for DNA polymerase activity after gel filtration through Sepharose 6B or centrifugation in sucrose density gradients (Craig & Keir, 1975a,b).

Miscellaneous. Sucrose-density-gradient centrifugation and gel filtration on a calibrated column of Sepharose 6B, estimation of molecular weights and the optimum conditions of assay for DNA polymerases and deoxyribonucleases were as described by Craig & Keir (1975a,b).

Results

Comparison of the chromatography profiles on Sepharose 6B of the extract from exponentially growing cells (Fig. 1) and the extract from quiescent cells (Fig. 2), revealed not only a considerable degree of heterogeneity of BHK-21/C13 cell DNA polymerases, but also marked differences in the total DNA polymerase activity present, and in its distribution between polymerase species I and II. The

Fig. 1. Gel filtration of the DNA polymerases from exponentially growing cells

A total cell extract (see the Experimental section) was analysed on the calibrated column of Sepharose 6B equilibrated with buffer B. DNA polymerase was assayed under conditions optimal for polymerase I.

Fig. 2. Gel filtration of the DNA polymerases from quiescent cells

A total cell extract (see the Experimental section) was analysed on the calibrated column of Sepharose 6B equilibrated with buffer B. DNA polymerase was assayed under conditions optimal for polymerase I.

extract from exponentially growing cells produced a profile showing a major but skewed peak of enzyme activity (DNA polymerase I) and a distinct minor peak (DNA polymerase II), the peaks contributing 90 and 10% respectively of the recovered polymerase activity. Molecular-weight estimations covered a wide range; the major activity of DNA polymerase I, IA, was eluted before urease, with an apparent molecular weight of 900×10^3 – 1100×10^3 . A minor shoulder, DNA polymerase ID, corresponded to an apparent molecular weight of 140×10^3 – 200×10^3 . DNA polymerase II was eluted just before ovalbumin giving a molecular-weight estimate of 46000 ± 4000 .

A similar analysis of the quiescent cell extract clearly showed heterogeneity, but in sharp contrast, DNA polymerase II contributed 61% of the total recovered activity, whereas the various components

Table 1. Comparison of the activities of DNA polymerases from exponentially growing and quiescent cells

The data are derived from the experiments described in Figs. 1 and 2. Activities are expressed as pmol of dTMP residues incorporated/30 min per 10^6 cells at 37°C.

	Exponential cells	Quiescent cells	Ratio exponential/quiescent
DNA polymerase I	333.0	29.5	11.30
DNA polymerase II	30.6	45.4	0.67
Ratio I/II	10.9	0.65	

of DNA polymerase I contributed only 39%. The distribution of activities within the DNA polymerase I region also differed from that shown by the exponential cell extract. Quiescent cells were found to contain little DNA polymerase IA or ID, the bulk of the DNA-polymerizing activity being distributed between DNA polymerases IB and IC of apparent molecular weights 460×10^3 – 560×10^3 and 270×10^3 – 320×10^3 respectively.

Knowledge of the initial cell numbers before extraction allowed a calculation to be made about the relative specific activities of DNA polymerases I and II in both solutions. The data in Table 1 show that the amount of DNA polymerase II increased by 33% in cells taken from the exponential to the quiescent state, whereas DNA polymerase I activity in exponentially growing cells was 11-fold greater when compared with the activity in quiescent cells.

In order to gain an insight into the possible direct involvement of DNA polymerase I with the replication machinery of the cell nucleus, experiments were also carried out to investigate the intracellular location of DNA polymerases I and II in cells synchronized with respect to the cell cycle.

A major effect of seeding exponentially growing BHK-21/C13 cells into $Eg_{SOH}C_{0.25}$ medium is that the number of cells actively synthesizing DNA falls to 3–4% after 3–4 days, as assessed by radioautography (Fig. 3a). Concurrent analysis of the total DNA polymerase activity associated with the 'low-speed cytoplasmic' and 'low-speed nuclear' fractions likewise revealed a decrease in total DNA polymerase activity in both fractions over a similar period (Fig. 3b). The decrease was particularly marked in the 'low-speed cytoplasmic' extract.

When the $Eg_{SOH}C_{0.25}$ medium in the now quiescent BHK-21/C13 cell culture was replaced by $Eg_{SOH}C_{10}$ medium, there was a lag phase of 12 h followed by entry of the cells into a synchronous wave of DNA synthesis (S phase), maximum incorporation of [^3H]thymidine occurring after 20–21 h. Radioautographic analysis (Fig. 4a) showed that 87% of the nuclei at 20–21 h had incorporated [^3H]thymidine

Fig. 3. Synthesis of DNA and activity of DNA polymerase

The rate of synthesis of DNA was monitored by measurement of incorporation of [³H]thymidine and by radioautography (see the Experimental section). The activities of nuclear and cytoplasmic DNA polymerase were measured under assay conditions optimal for DNA polymerase I. (a) The cross-hatched columns and the open circles respectively represent incorporation of [³H]thymidine and the percentage of the cell nuclei labelled, after a 1 h pulse delivered at the times indicated. (b) ○, Cytoplasmic DNA polymerase; ●, nuclear DNA polymerase.

and that this value had dropped to 55% by 25–26 h. Cells synchronized in this manner grew to confluence, and could be subcultured thereafter in the normal manner.

Analysis of total DNA polymerase activity in the 'low-speed nuclear' and 'low-speed cytoplasmic' fractions throughout the cell cycle (Fig. 4b), revealed an increase in the total DNA polymerase activity of both fractions; initially the increase closely paralleled the increase in [³H]thymidine incorporation. However, neither fraction showed the extent of decrease after 20 h that was shown by the pattern of

Fig. 4. Synthesis of DNA and activity of DNA polymerase during synchrony of growth established by 'serum step-up'

The experimental conditions were the same as those described in Fig. 1 except for the 'serum step-up' (see the Experimental section). (a) The cross-hatched area and open circles respectively represent incorporation of [³H]thymidine and the percentage of the cell nuclei labelled, after 1 h pulse delivered at the times indicated. (b) ●, Cytoplasmic DNA polymerase; ○, nuclear DNA polymerase.

[³H]thymidine incorporation. If anything, the decrease was small for both enzyme activities.

At all times throughout the cell cycle the proportion of total DNA polymerase activity, when calculated as pmol of dTMP residues incorporated per 10⁶ cells, was greater in the 'cytoplasmic' than in the 'nuclear' fraction. In both fractions, maximum DNA polymerase activity corresponded to a 4-fold increase over that measured in G₀ cells.

Acid and alkaline deoxyribonuclease activities in the 'cytoplasmic' and 'nuclear' fractions were also measured, on both native and denatured DNA substrates. However, no clearly reproducible trends could be observed during cell cycle.

In order to identify the nature of the DNA polymerase activities in the G₀ and S-phase cells, total cell extracts were prepared from G₀ and late S-phase

Fig. 5. Gel filtration of DNA polymerases from cells in the G_0 state

The cells (5×10^7) were harvested after 84 h in $Eg_{SOH}C_{0.25}$ medium and processed as previously described (Craig & Keir, 1975a,b), resulting in a 'total' cell extract (1.0 ml) in buffer B; 0.8 ml of this was analysed by gel filtration on the calibrated column of Sepharose 6B equilibrated with buffer B. Fractions were assayed for DNA polymerase under conditions optimal for DNA polymerase I.

Fig. 6. Gel filtration of DNA polymerases from cells in S phase

Cells were placed in synchrony of growth by 'serum step-up' (see the Experimental section) 22–23 h after addition of $Eg_{SOH}C_{10}$ medium, that is during S phase (Fig. 2), and the cells (9.2×10^7) were harvested and processed as previously described (Craig & Keir, 1975a,b). Before pooling the concentrated 'cytoplasmic' (1.25 ml) and 'nuclear' (0.72 ml) extracts, 200 μ l of the cytoplasmic extract was taken for analysis by centrifugation on sucrose density gradients (Fig. 5). The fractions were then pooled resulting in essentially a 'total' cell extract (1.77 ml) in buffer B. 1.5 ml of this was analysed on the calibrated column of Sepharose 6B equilibrated with buffer B. Fractions were assayed for DNA polymerase under conditions optimal for DNA polymerase I.

cell cultures, the latter being harvested 22–33 h after the addition of $Eg_{SOH}C_{10}$ medium.

Analysis of the extracts by gel filtration on Sepharose 6B demonstrated unequivocally that the increase in DNA polymerase activity that accompanied the onset of DNA synthesis was attributable to the induction of the heterogeneous group of DNA

Table 2. Activities of DNA polymerases I and II in G_0 cells and S-phase cells

The data are derived from the experiments described in Figs. 5 and 6. Enzyme activities are expressed as pmol of dTMP residues incorporated/60 min per 10^6 cells at 37°C.

	S-phase cells	G_0 cells	S/ G_0
DNA polymerase I	112.4	16.5	6.8
DNA polymerase II	16.4	33.4	0.48
Ratio I/II	6.85	0.494	

Fig. 7. Sedimentation analysis of cytoplasmic DNA polymerases from cells in S phase

Cells were placed in synchrony of growth by 'serum step-up'. Cytoplasmic and nuclear fractions were prepared 22–23 h after addition of $Eg_{SOH}C_{10}$ medium, and 200 μ l of the cytoplasmic fraction (see the legend to Fig. 6) was taken for analysis by centrifugation for 15 h on sucrose density gradients prepared in buffer B. Fractions from the gradients were assayed for DNA polymerase under conditions optimal for DNA polymerase I, and also a marker enzyme, lactate dehydrogenase (LDH). Sedimentation was from left to right.

polymerase activities making up DNA polymerase I (Craig & Keir, 1975b). The total extract of G_0 cells revealed (Fig. 5) a single major peak of activity representing 67% of the total and being eluted at the position of DNA polymerase II (mol.wt. 46000 ± 4000). The remaining activity consisted of small amounts of DNA polymerase IA and IB (mol.wt. 900000–1100000 and 460000–560000 respectively). In contrast, in late S-phase cells DNA polymerase II contributed only 13% of the activity recovered (Fig. 6). The remainder was accounted for by a skewed peak of activity corresponding to DNA

polymerase IA and IB, with a small peak being eluted later and corresponding to DNA polymerase ID (mol.wt. 140000–200000).

Knowledge of the initial cell number before cell extraction allowed a calculation to be made about the relative specific activities of DNA polymerases I and II in the G₀ and late S-phase cells. The results (Table 2) show that whereas the overall activity of DNA polymerase II apparently decreased by 50% when the cells entered the S phase from the G₀ state, the total activities associated with DNA polymerase I increased nearly 7-fold.

In order to facilitate comparison of these data with other similar published results, a portion of the concentrated 'cytoplasmic' fraction obtained from the late S-phase cells was analysed by sucrose-density-gradient centrifugation. The resulting profile (Fig. 7) again revealed the heterogeneous nature of the enzymes, sedimentation coefficients ranging from 5–9S. Little significant activity was evident sedimenting at 3–4S.

Discussion

The observations following fractionation of total cell extracts from exponential and quiescent cell cultures and from G₀ and S-phase cell cultures by gel filtration on Sepharose 6B, clearly demonstrate that (a) a direct correlation exists between the activity of DNA polymerase I and the rate of DNA synthesis *in vivo*, (b) DNA polymerase II and DNA synthesis *in vivo* appear not to be related, and (c) polymerases I and II correspond respectively to the 6–8S and 3.3S DNA polymerases identified in regenerating rat liver (Chang & Bollum, 1972) and serum-stimulated mouse L-cells (Chang *et al.*, 1973).

The results also confirm our earlier observations (Craig & Keir, 1975b) that DNA polymerase I shows marked heterogeneity. The possibility exists that all of the DNA polymerase I activities are attributable to one identical enzyme subunit, complexed in varying degrees to other protein subunits which may or may not be enzymes or proteins involved in the DNA replication process.

Such a concept is supported by the following observations. In BHK-21/C13 cells, DNA polymerase IA is the major activity only in truly exponentially growing cell cultures (Fig. 1) or in cells in S phase (Fig. 6), but is almost totally absent from quiescent cells (Fig. 2) and G₀ cells (Fig. 5). One interpretation of this train of events, is that the apparent decrease in DNA polymerase IA is the consequence of the loss from it of subunit(s) which may or may not effect the polymerization *in vitro* of deoxyribonucleotides, the magnitude of such dissociation being reflected in a decline of both apparent molecular weight and

enzyme activity, in parallel with decreasing cell proliferation.

Such a postulate is supported by Hecht & Davidson (1973) who observed an apparent interconversion of DNA polymerases from mouse testis and by Chang *et al.* (1973) who detected a DNA-polymerizing activity with a sedimentation coefficient of 5S derived from the 6–8S species of mouse L-cells. Similar observations have been made on DNA polymerases from calf thymus (Chang *et al.*, 1973; Holmes *et al.*, 1974) and rat spleen and liver (Holmes *et al.*, 1974).

The data arising from experiments conducted after subcellular fractionation of BHK-21/C13 cells at various stages of the cell cycle also raise the interesting possibilities (a) that DNA polymerase I is released into the cytoplasm as a consequence of DNA replication and (b) that the degree of association of DNA polymerase I with the cell nucleus appears to parallel the rate of DNA synthesis *in vivo*, being maximal at the peak of S phase and thereafter decreasing with the rate of DNA synthesis *in vivo*.

The anomalous subcellular distribution of DNA polymerase I has already been discussed (Craig & Keir, 1975b); it is clear that the increase in DNA polymerase activity associated with the 'nuclear' fraction, must be a direct result of the presence of DNA polymerase I, as the overall amounts of DNA polymerase II do not change much in response to DNA replication. It must be emphasized that as the rate of DNA synthesis decreases towards the end of S phase, the total DNA polymerase activity associated with the nuclei also decreases, the cytoplasmic activities remaining relatively constant at the S-phase value. We have occasionally observed that the 'cytoplasmic' DNA polymerase activities reached a maximum only after the total activity associated with the nuclei had started to decrease. Thus a reasonable interpretation of the results might be that the polymerase I was released into the cytoplasm as a consequence of replication.

A similar concept has been proposed by Chang *et al.* (1973), and earlier work on subcultured L-cells (Lindsay *et al.*, 1970) also monitored a similar increase in a cytoplasmic enzyme at the end of S phase. This enzyme activity has since been demonstrated to be due to a 6–8S-type DNA polymerase I (Adams *et al.*, 1973). An association, apparently reversible, of a DNA polymerase activity with the nucleus has also been reported by Fansler & Loeb (1973) who used the naturally synchronous sea-urchin embryo system; in this case the DNA polymerase activity is apparently highest in the nucleus at the beginning of S phase, but declines as replication approaches completion, with a concomitant increase in the cytoplasmic DNA polymerase activity. Unfortunately, the enzyme that associates with the

nucleus in this temporal fashion has yet to be identified unambiguously as that which appears in the cytoplasmic fractions, by ion-exchange chromatography or gel filtration.

In the experiments with BHK-21/C13 cells a DNA polymerase I species with molecular weight greater than polymerase IA has on occasion been observed (see Figs. 5 and 6) by gel filtration through Sepharose 6B. However, the amount of polymerase activity involved has always been small in these cases.

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