Biochem. J. (1978) **173**, 309–314
Printed in Great Britain

Involvement of Deoxyribonucleic Acid Polymerase β in Nuclear Deoxyribonucleic Acid Synthesis

By TAUSEEF R. BUTT,* WILLIAM M. WOOD,† ERIC L. McKAY and ROGER L. P. ADAMS
Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

(*Received* 4 *November* 1977)

The effects on DNA synthesis *in vitro* in mouse L929-cell nuclei of differential extraction of DNA polymerases α and β were studied. Removal of all measurable DNA polymerase α and 20% of DNA polymerase β leads to a 40% fall in the replicative DNA synthesis. Removal of 70% of DNA polymerase β inhibits replicative synthesis by 80%. In all cases the nuclear DNA synthesis is sensitive to *N*-ethylmaleimide and aCTP (arabinosylcytosine triphosphate), though less so than DNA polymerase α. Addition of deoxyribonuclease I to the nuclear incubation leads to synthesis of high-molecular-weight DNA in a repair reaction. This occurs equally in nuclei from non-growing or S-phase cells. The former nuclei lack DNA polymerase α and the reaction reflects the sensitivity of DNA polymerase β to inhibition by *N*-ethylmaleimide and aCTP.

Mammalian cells contain two major DNA-dependent DNA polymerases (Lindsay *et al.*, 1970; Weissbach *et al.*, 1971; Sedwick *et al.*, 1972; Adams *et al.*, 1973; Chang *et al.*, 1973; Bollum, 1975; Weissbach, 1977). DNA polymerase β (the smaller enzyme) is found exclusively or predominantly in nuclei and readily aggregates (Adams *et al.*, 1973; Srivastava, 1974; G. Brun, L. Assairi & R. Wicker, unpublished work; de Recondo & Abadiedebat, 1976). The higher-molecular-weight enzyme, DNA polymerase α, which also exists in several aggregate forms (Srivastava, 1974; Holmes *et al.*, 1974, 1976; Craig & Keir, 1975*a*), is found largely in the post-microsomal supernatant fractions after centrifugation of cell homogenates (Lindsay *et al.*, 1970; Adams *et al.*, 1973; Craig & Keir, 1975*a*; Furlong *et al.*, 1973), but is believed to be located within the nucleus *in vivo* (Herrick *et al.*, 1976; Foster & Gurney, 1976). Although the activity of DNA polymerase β is relatively independent of the state of growth of the cells the total and nuclear activities of DNA polymerase α are markedly increased in rapidly growing cells (Lindsay *et al.*, 1970; Adams *et al.*, 1973; Furlong *et al.*, 1973; Chang *et al.*, 1973; Craig *et al.*, 1975; Claycomb, 1975). Such results appear to implicate DNA polymerase α in DNA replication and relegate DNA polymerase β to a role in repair of DNA. To obtain direct proof of such roles we have studied DNA synthesis in isolated nuclei in an attempt to correlate such activity with the presence of a particular enzyme. A preliminary communication of these results has already been published (Butt *et al.*, 1976).

Abbreviations used: aCTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; DNAase, deoxyribonuclease.

* Present address: Department of Biochemistry, Schools of Medicine and Dentistry, Georgetown University, Washington, DC 20007, U.S.A.

† Present address: Department of Biochemistry, University of Pittsburgh, Pittsburgh, PA 15261, U.S.A.

Experimental

Incubation of nuclei

Cells (mouse L929) were grown and nuclei prepared as previously described (Lindsay *et al.*, 1970) by homogenization in 0.25M-sucrose buffered with 20mM-Tris/HCl (pH7.5)/5mM-mercaptoethanol. To measure their endogenous DNA-synthetic capacity about 10^7 nuclei were incubated in 0.2ml under the conditions described by Hershey *et al.* (1973), in the presence of 40mM-Tris/HCl (pH7.8)/100mM-NaCl/10mM-$MgCl_2$/0.5mM-EDTA/4mM-mercaptoethanol/5mM-ATP, containing 0.1mM each of dATP, dGTP, dCTP and [*Me*-^{3}H]dTTP (50μCi/μmol or 2mCi/μmol; The Radiochemical Centre, Amersham, Bucks., U.K.). These conditions were shown to be optimal for the synthesis *in vitro* of DNA by L929-cell nuclei. The reaction was terminated by addition of 1ml of 50mM-$Na_4P_2O_7$. The final pellet was dissolved in 0.5M-perchloric acid at 70°C, and samples were taken for determination of radioactivity and, in some cases, DNA (Burton, 1956). In some experiments the cells were prelabelled by overnight incubation in [6-^{3}H]thymidine (0.1μCi/ml), in which case [α-^{32}P]dTTP (The Radiochemical Centre) was used in the incubation mixture in place of [^{3}H]dTTP. When this was the case the reaction was terminated by addition of 1ml of a solution containing sodium

dodecyl sulphate (1%, w/v), EDTA (2mM), *p*-aminosalicylate (3%, w/v), butanol (5%), NaCl (0.25M) and salmon sperm DNA (0.5mg/ml). When all material had dissolved the protein was removed by shaking with an equal volume of phenol (88%, v/v), *m*-cresol (12%, v/v) and 8-hydroxyquinoline (0.1%, w/v) and the DNA precipitated from the aqueous phase with 2 vol. of absolute ethanol. The precipitate was dissolved in 0.3M-NaOH (37°C, 1h) and applied to Whatman 3MM filter-paper discs for acid washing and determination of radioactivity with a Phillips liquid-scintillation spectrometer.

Extraction of DNA polymerases

Nuclei were extracted by suspending them in the homogenization buffer containing the appropriate concentration of KCl. The nuclei were washed three times for each extraction before final resuspension in homogenization buffer.

DNA polymerase activity was assayed as described by Lindsay *et al.* (1970) with 10% activated calf thymus DNA (Aposhian & Kornberg, 1962) as template/primer.

Sucrose gradients (3.2ml of 5–20% sucrose containing 20mM-Tris/HCl, pH7.5, 5mM-mercaptoethanol and 0.4M-KCl) were overlayered with 0.3ml of enzyme solution and centrifuged for 12h at 50000rev./min in the SW56 rotor of the Spinco ultracentrifuge. Samples were collected with an MSE gradient harvester and 20μl samples assayed for DNA polymerase activity at a final KCl concentration of 66mM. The presence of 0.4M-KCl in the gradients was essential to prevent the aggregation of the DNA polymerases, which otherwise obscures their separation.

Alkaline gradients

When the products were to be analysed on alkaline gradients the higher-specific-radioactivity [^{3}H]dTTP was used. The endogenous reaction was stopped by addition of an equal volume of neutral lysis buffer (1% *N*-laurylsarcosine, 10mM-EDTA and 20mM-Tris/HCl, pH7.5). Lysis was left to continue for 1h on ice. For sucrose-gradient centrifugation lysates were incubated for 5h at 37°C with self-digested Pronase (2mg/ml) and the DNA was precipitated overnight at −20°C with 3 vol. of ethanol. The DNA was redissolved in 1% laurylsarcosine containing 10mM-EDTA and 0.4M-NaOH. For equilibrium centrifugation the lysed nuclear suspension was deproteinized twice with equal volumes of phenol (88%), *m*-cresol (12%), 8-hydroxyquinoline (0.1%) and 0.25% chloroform. The DNA from the final aqueous layer was precipitated overnight with ethanol and redissolved in 0.015M-NaCl containing 1.5mM-sodium citrate (0.1×SSC buffer) (Krokan *et al.*, 1975).

Alkaline sucrose gradients (3.2ml of 5–20% sucrose containing 0.1M-NaCl, 0.4M-NaOH, 0.1% *N*-laurylsarcosine and 10mM-EDTA) were overlayered with an 0.3ml sample of DNA and centrifuged for 4h at 4°C and 50000rev./min in the SW56 rotor of the Spinco ultracentrifuge. Virus SV40 DNA (form II) was used as a size marker. Samples were collected by upward displacement directly on to Whatman 3MM filter paper for acid washing and subsequent determination of radioactivity.

For the alkaline caesium sulphate equilibrium centrifugation 2g of Cs_2SO_4 was added to 2.8ml of 0.1×SSC buffer containing 80–100μg of DNA at a final pH of 12.5. Centrifugation was for 48h at 20°C in the Spinco SW56 rotor at 35000rev./min. Fractions were collected by tube puncture and samples taken for determination of refractive index, absorbance and radioactivity.

Results and Discussion

Nuclear DNA synthesis

Apart from preparations from cells infected with viruses (Reichard *et al.*, 1974; de Pamphilis & Berg, 1975) only a limited amount of DNA synthesis occurs in nuclei isolated from animal cells, and such synthesis has been shown to largely represent the elongation of short chains that were already growing *in vivo* (Lynch *et al.*, 1970; Hershey *et al.*, 1973). The system described here is similar in the following aspects.

(1) The ability of nuclei isolated from mouse L929 cells to synthesize DNA reflects the rate of DNA synthesis *in vivo* (Fig. 1). Unless otherwise stated the

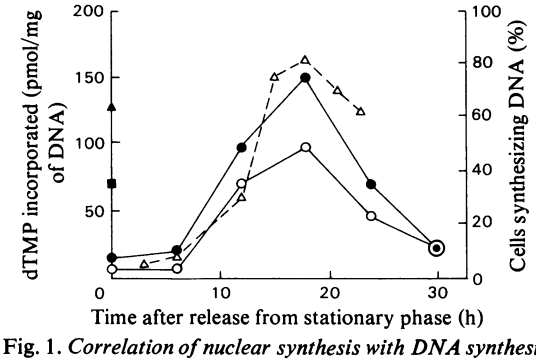

Fig. 1. *Correlation of nuclear synthesis with DNA synthesis in vivo*

L929 cells were released from stationary phase by subculture and nuclei isolated therefrom used in the assay *in vitro*. Incorporation is shown after an incubation of 5min (○) and 30min (●). △, Result of a radioautographic analysis of the proportion of cells incorporating [^{3}H]thymidine *in vivo*; ■ and ▲, incorporation by nuclei from stationary-phase cells when DNAase I (25μg/ml) was added to the incubation mixture for 5 and 30min respectively.

experiments to be reported were all carried out on S-phase cells, i.e. cells harvested 18–20h after subculture of stationary cells. (2) After 20s incubation the DNA made by isolated nuclei is in small pieces and little ligation occurs on continued incubation (Figs. 2*a* and 2*b*). The small size of the product is not the result of nuclease activity, as shown by the lack of marked degradation of the DNA originally present. (3) When nuclei are isolated from cells prelabelled with bromodeoxy[^{14}C]uridine the DNA made *in vitro* forms a broad band of density intermediate between the ^{14}C and the absorbance peak when centrifuged to equilibrium in alkaline caesium sulphate gradients (results not shown). (4) The rate of incorporation of nucleotides by isolated nuclei decreases rapidly on incubation (Figs. 1 and 3). Over the first 5min the rate *in vitro* is only about 2% of the rate *in vivo* calculated on the basis that a cell has to double its DNA content (10pg for a G_1-phase L929 cell) in 6h. If, when incorporation has begun to reach a plateau after 15min incubation, a second sample of nuclei or activated DNA is added this

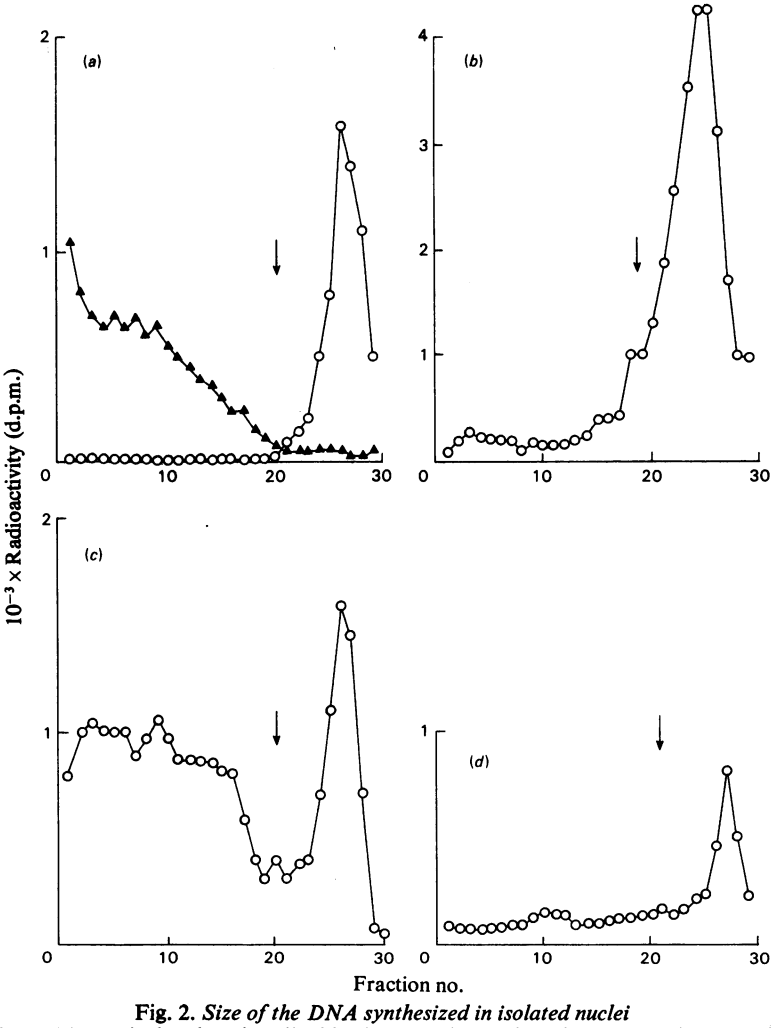

Fig. 2. *Size of the DNA synthesized in isolated nuclei*

After incubation the DNA was isolated as described in the Experimental section. It was then centrifuged on alkaline sucrose gradients containing *N*-laurylsarcosine and EDTA: (*a*) 20s incubation of S-phase nuclei (▲, position of DNA labelled *in vivo* with [^{14}C]thymidine); (*b*) 5min incubation of S-phase nuclei; (*c*) 20s incubation of S-phase nuclei in the presence of pancreatic DNAase (25μg/ml); (*d*) 20s incubation of S-phase nuclei that had been extracted with 0.15M-KCl to remove DNA polymerase α. Sedimentation is from right to left and the arrow indicates the position of a virus SV40 form-II marker (16–18S).

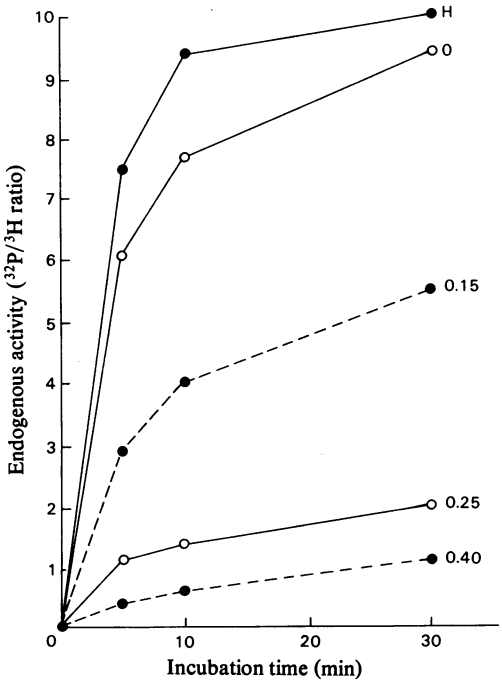

Fig. 3. *Effect of salt extraction on nuclear DNA synthesis* Mouse L929 cells were labelled overnight with [6-^{3}H]thymidine before assay of nuclear DNA synthesis *in vitro* with [^{32}P]dTTP. The results are expressed in arbitrary units as $^{32}P/^{3}H$ so as to take account of losses in nuclear material on salt extraction. H, initial homogenate; 0, nuclei prepared as described in the Experimental section; 0.15, nuclei extracted three times with 0.15M-KCl in homogenization buffer; 0.25, nuclei further extracted three times with 0.25M-KCl in homogenization buffer; 0.4, nuclei further extracted three times with 0.4M-KCl in homogenization buffer. Before assay nuclei were resuspended and assayed in a final salt concentration of 100mM-NaCl.

leads to reinitiation of DNA synthesis, indicating that primer/template is the limiting factor in the incubation.

It is this nuclear reaction that we show below to be relatively unaffected by the removal of DNA polymerase α from nuclei; yet sensitive to the removal of DNA polymerase β.

Nuclear DNA polymerases

We have previously shown that nuclei isolated from mouse L929 cells contain both DNA polymerase α and DNA polymerase β (Adams *et al.*, 1973) and that the activity of DNA polymerase α (but not DNA polymerase β) depends on the state of growth of the cells. This fact appears to implicate DNA polymerase α in DNA synthesis. Both enzymic activities can be completely removed by exhaustively extracting nuclei with 0.4M-KCl or 0.2M-potassium phosphate treatments, which abolish nuclear DNA synthesis (Fig. 3). However, extraction of nuclei with intermediate salt concentrations leads to a selective removal of DNA polymerase α activity. At 0.15M-KCl most, if not all, the DNA polymerase α is removed from the nuclei together with about 20% of DNA polymerase β (Fig. 4). This treatment decreases the ability of nuclei to make DNA by 20–40% (Fig. 3). Extraction with 0.25M-KCl removed about 70% of the DNA polymerase β and the endogenous activity of nuclei to make DNA is decreased in proportion (Figs. 3 and 4). The size of the product is similar to that made by unextracted nuclei (Fig. 2*d*). These new findings appear to indicate a role for DNA polymerase β in the reaction studied in isolated nuclei.

It is indeed hard to counter the argument that a small amount of DNA polymerase α remains in these extracted nuclei. This enzyme may remain attached to the growing chain and thereby catalyse the elongation of the 'Okazaki' piece without ever becoming available to added template/primer. This argument holds despite the observation that the mechanism of action of DNA polymerase α *in vitro* is distributive (Chang, 1975). As part of a replication complex *in vivo*, it may well be able to act progressively.

In an attempt to circumvent this argument we have investigated the sensitivity of nuclear DNA synthesis to various inhibitors.

Inhibitor studies

N-Ethylmaleimide inhibits a wide variety of enzymes that rely on a free SH group for activity. DNA polymerase α is one such enzyme, but DNA polymerase β is not (Table 1). The insensitivity of DNA polymerase β to *N*-ethylmaleimide is most apparent when assayed in fractions taken from a sucrose-gradient separation. In contrast, some crude preparations of DNA polymerase β (e.g. extracts of S-phase nuclei from which DNA polymerase α has previously been removed) show up to 60% inhibition by 2mM-*N*-ethylmaleimide. Some other β-polymerases show sensitivity to SH-blocking reagents and this has been associated with the presence of auxiliary proteins (Mosbaugh *et al.*, 1976, 1977; de Recondo & Abadiedebat, 1976). DNA synthesis occurring in nuclei isolated from S-phase cells shows a similar intermediate sensitivity to *N*-ethylmaleimide (Table 1). The effect of *N*-ethylmaleimide is similar whether or not the nuclei have been extracted with 0.15M-KCl to remove DNA polymerase α (Table 1), and it seems possible that a complex of DNA polymerase β

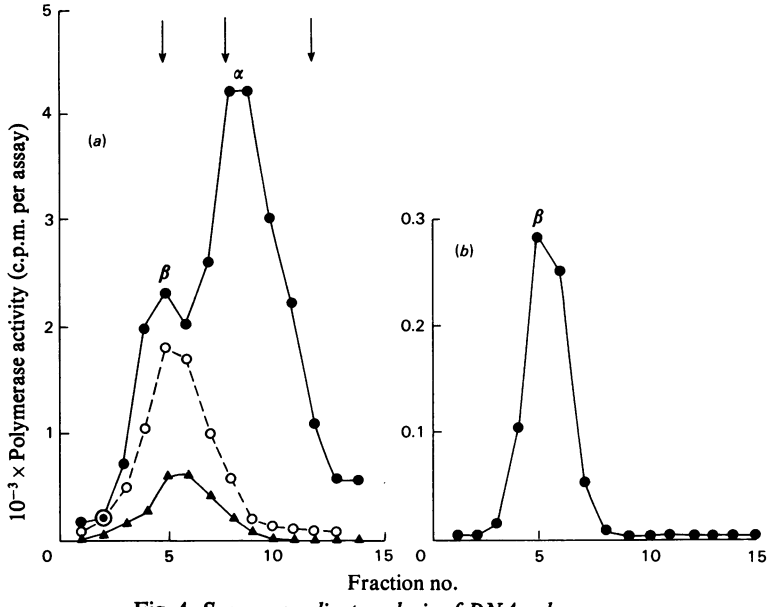

Fig. 4. *Sucrose-gradient analysis of DNA polymerases*

Analyses were performed of nuclear extracts of (*a*) S-phase and (*b*) stationary-phase cells. Nuclei were prepared as described in the Experimental section and washed three times in homogenization buffer containing: no KCl (●); 15mM-KCl (○); or 0.25M-KCl (▲). The DNA polymerase activity remaining was extracted with 0.4M-KCl in homogenization buffer and layered on 5–20% neutral sucrose gradients containing 0.4M-KCl. Sedimentation is from left to right and the arrows indicate the positions of haemoglobin (4.1S) human γ-globulin (7S) and catalase (11.4S).

Table 1. *Effect of inhibitors*

Preparation	Activity (% of control) +2mM-*N*-Ethylmaleimide	+200μM-aCTP
DNA polymerase α	8	39
DNA polymerase β	73	99
Nuclei (S-phase)	24	43
Nuclei (S-phase)*	32	57
Nuclei (stationary+ DNAase)	65	103

* These S-phase nuclei were extracted with 0.15M-KCl before incubation. Enzyme preparations are from tubes corresponding to the peak of a sucrose gradient such as the one shown in Fig. 4.

together with auxiliary proteins is responsible for extending 'Okazaki' pieces in these nuclei.

DNA polymerase β is not highly sensitive to omission of one deoxyribonucleoside triphosphate from the incubation (Lindsay *et al.*, 1970; Craig & Keir, 1975*b*). It is also insensitive to the presence of aCTP, in contrast with the sensitivity of DNA polymerase α and nuclear DNA synthesis (Table 1). aCTP probably inhibits DNA synthesis by decreasing the rate of chain extension after its misincorporation (Craig & Keir, 1975*b*; Cozzarelli, 1977). If very short chains are being synthesized aCTP will have little effect; it will have a more profound inhibitory effect the longer the DNA chain being synthesized. This inhibition may therefore reflect the nature of the reaction rather than the enzyme catalysing that reaction and thus gives little further evidence with regard to the enzyme involved.

Bolden *et al.* (1975) have studied the effect of phosphonoacetic acid on the nuclear synthesis and the activity of DNA polymerases of HeLa cells. At 20μg/ml this compound inhibits nuclear DNA synthesis *in vitro* by about 33%. The same concentration inhibits the activity of DNA polymerase α by 73% and DNA polymerase β by 9%.

Perhaps a more satisfactory approach is that of assessing the inhibition of nuclear DNA synthesis by antibodies raised against the two DNA polymerases, and in a study by G. Brun, L. Assairi & R. Wicker (unpublished work) antibodies to both polymerases α and β produced some inhibition of the nuclear DNA synthesis.

Repair synthesis

Addition of small amounts of pancreatic deoxyribonuclease I to the reaction mixture leads to

increased incorporation of deoxyribonucleoside triphosphates into DNA by nuclei from either S-phase or stationary-phase cells (Figs. 1 and 2*c*). The latter may be stimulated up to 10-fold. Under these conditions a considerable proportion of the product is of high molecular weight (Fig. 2*c*). Similar results have previously been reported by Lynch *et al.* (1970). This reaction is presumed to result from the repair of small gaps or nicks introduced into the DNA by the pancreatic DNAase. It can also be simulated by addition of nicked native DNA to intact stationary-phase nuclei (Lindsay *et al.*, 1970).

As nuclei isolated from stationary cells contain very low activities of DNA polymerase α (Fig. 4) it is presumed that this 'repair' reaction is catalysed by DNA polymerase β. However, the reaction differs from the replicative reaction typical of S-phase nuclei in that it is insensitive to aCTP and *N*-ethylmaleimide (Table 1). We interpret this to mean that the repair reaction involves the growth of short chains in a reaction catalysed by DNA polymerase β unaided by *N*-ethylmaleimide-sensitive auxiliary proteins.

Conclusions

It is difficult to reach any firm conclusion as to which DNA polymerase is involved in DNA replication. One possibility is that a complex form of DNA polymerase β is involved in the limited extension of 'Okazaki' pieces studied in isolated nuclei. Whether, when present, DNA polymerase α can also perform this function is unclear, but it is assumed that, because of its ability to extend an RNA primer (Chang & Bollum, 1972), DNA polymerase α will initiate the synthesis of 'Okazaki' pieces. The change-over from DNA polymerase α to DNA polymerase β may depend largely on the availability of the two enzymes or enzyme complexes.

It seems clear, however, that DNA polymerase β has a function in the repair of DNA. This reaction occurs in all growth phases, and when studied in isolated nuclei the product sediments with bulk DNA. DNA polymerase β shows a similar *N*-ethylmaleimide-sensitivity to the repair reaction, and the lack of sensitivity to aCTP probably reflects the short lengths of DNA synthesized when repairing small gaps.

We thank Professor R. M. S. Smellie for interest and for providing the necessary facilities and the Medical Research Council for funds including the salary of T. R. B. W. M. W. was in receipt of an M.R.C. studentship. Thanks are also due to Miss Christine McClure for skilled technical assistance. We also thank Dr. R. R. Meyer and Dr. G. Brun for helpful discussion and for preprints of some publications.

References

Adams, R. L. P., Henderson, M. A. L., Wood, W. M. & Lindsay, J. G. (1973) *Biochem. J.* **131**, 237–246
Aposhian, H. V. & Kornberg, A. (1962) *J. Biol. Chem.* **237**, 519–525.
Bolden, A., Auker, J. & Weissbach, A. (1975) *J. Virol.* **16**, 1584–1592
Bollum, F. J. (1975) *Prog. Nucleic Acid Res. Mol. Biol.* **15**, 109–144
Brun, G., Rougeon, F., Lauber, M. & Chapeville, F. (1974) *Eur. J. Biochem.* **41**, 241–251
Burton, K. (1956) *Biochem. J.* **62**, 315–323
Butt, T. R., Wood, W. M. & Adams, R. L. P. (1976) *Biochem. Soc. Trans.* **4**, 807–810
Chang, L. M. S. (1975) *J. Mol. Biol.* **93**, 219–235
Chang, L. M. S. & Bollum, F. J. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1354–1360
Chang, L. M. S., Brown, M. & Bollum, F. J. (1973) *J. Mol. Biol.* **74**, 1–8
Claycomb, W. C. (1975) *J. Biol. Chem.* **250**, 3229–3235
Cozzarelli, N. R. (1977) *Annu. Rev. Biochem.* **46**, 641–668
Craig, R. K. & Keir, H. M. (1975*a*) *Biochem. J.* **145**, 225–232
Craig, R. K. & Keir, H. M. (1975*b*) *Biochem. J.* **145**, 215–224
Craig, R. K., Costello, P. A. & Keir, H. M. (1975) *Biochem. J.* **145**, 233–240
de Pamphilis, M. L. & Berg, P. (1975) *J. Biol. Chem.* **250**, 4348–4354
de Recondo, A. M. & Abadiedebat, J. (1976) *Nucleic Acids Res.* **3**, 1823–1837
Foster, D. N. & Gurney, T. (1976) *J. Biol. Chem.* **251**, 7893–7898
Furlong, N. B., Novak, W. B. & Stubblefield, T. E. (1973) *Cell Tissue Kinetics* **6**, 303–313
Herrick, G., Spear, B. B. & Veomath, G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1136–1139
Hershey, H. V., Stieber, J. F. & Mueller, G. C. (1973) *Eur. J. Biochem.* **34**, 383–394
Holmes, A. M., Hesslewood, I. P. & Johnston, I. R. (1974) *Eur. J. Biochem.* **43**, 487–499
Holmes, A. M., Hesslewood, I. P. & Johnston, I. R. (1976) *Eur. J. Biochem.* **62**, 229–235
Krokan, H., Birklid E. & Prydz, H. (1975) *Biochemistry* **14**, 4227–4232
Lindsay, J. G., Berryman, S. & Adams, R. L. P. (1970) *Biochem. J.* **119**, 839–848
Lynch, W. E., Brown, R. F., Umeda, T., Langreth, S. G. & Lieberman, I. (1970) *J. Biol. Chem.* **245**, 3911–3916
Mosbaugh, D. W., Kunkel, T. A., Stalker, D. M., Tcheng, J. E. & Meyer, R. R. (1976) *Nucleic Acids Res.* **3**, 2341–2352
Mosbaugh, D. W., Stalker, D. M., Probst, G. S. & Meyer, R. R. (1977) *Biochemistry* **16**, 1512–1518
Reichard, P., Eliasson, R. & Soderman, G. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4901–4905
Sedwick, D. W., Wang, T. S. F. & Korn, D. (1972) *J. Biol. Chem.* **247**, 5026–5033
Srivastava, B. I. S. (1974) *Cancer Res.* **34**, 1015–1026
Weissbach, A. (1977) *Annu. Rev. Biochem.* **46**, 25–47
Weissbach, A., Schlabach, A., Fridlender, B. & Bolden, A. (1971) *Nature (London) New Biol.* **231**, 167–170