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Effect of Drugs on Deoxyribonucleic Acid Synthesis in Isolated Mammalian Cell Nuclei

COMPARISON WITH PARTIALLY PURIFIED DEOXYRIBONUCLEIC ACID POLYMERASES

By JULIAN F. BURKE,* PATRICIA M. DUFF and COLIN K. PEARSON†
Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, Scotland, U.K.

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In order to ascertain the identity of the DNA-dependent DNA polymerase responsible for the observed DNA synthesis in nuclei isolated from baby-hamster kidney (BHK-21/C13) cells a comparative study was carried out on the effects of some drugs, reported to influence DNA synthesis, on DNA synthesis catalysed by these nuclei and by partially purified DNA polymerase-α and -β. In all cases DNA synthesis by isolated nuclei and polymerase-α was inhibited to similar extents by *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, novobiocin, heparin and phosphonoacetic acid; polymerase-β was much less affected by these compounds. Ethidium bromide inhibited all DNA synthesis to similar extents, although at low concentrations (about 2μg/ml) synthesis in isolated nuclei was stimulated. The results are discussed in relation to the proposal that DNA polymerase-α catalyses the covalent extension of Okazaki fragments that these nuclei carry out *in vitro*.

Our studies with nuclei isolated from BHK-21/C13 cells show that synthesis *in vitro* is limited to a covalent elongation of Okazaki pieces previously initiated *in vivo* (Burke & Pearson, 1979). These DNA fragments were shown to be about 100–200 nucleotides long by sucrose-density-gradient and gel-electrophoretic techniques, and we did not detect any ligation of these into higher-molecular-weight DNA.

Since this DNA synthesis, although limited, is well defined it provides an opportunity to determine the identity of the enzyme that catalyses this elongation process. We have established that these isolated nuclei contain two DNA-dependent DNA polymerases (DNA nucleotidyltransferase, EC 2.7.7.7), and this paper describes our attempts to determine which of these catalyses the observed DNA synthesis *in vitro*, with a view to assigning a putative role for this enzyme *in vivo*. This was carried out by examining the effects of various drugs on DNA synthesis catalysed by the endogenous enzyme(s) in isolated nuclei, and comparing this with their effects on the activities of the partially purified DNA polymerase-α and -β.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

* Present address: Department of Medical Viral Oncology, Roswell Park Memorial Institute, Buffalo, NY 14263, U.S.A.

† To whom reprint requests should be addressed.

Experimental

Materials

Nucleotides were purchased from P-L Biochemicals, Milwaukee, WI, U.S.A., and ethidium bromide, 2-mercaptoethanol, *N*-ethylmaleimide and *p*-hydroxymercuribenzoate from Sigma (London) Chemical Co., Poole, Dorset, U.K. Heparin was from Evans Medical, Speke, Liverpool, U.K., and phosphonoacetic acid was from ICN Pharmaceuticals, Plainsview, NY, U.S.A. [*methyl*-^{3}H]Thymidine 5′-triphosphate (15–30Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Cell culture

Baby-hamster kidney (BHK-21/C13) cells were used throughout this work (Macpherson & Stoker, 1962). Cells were grown routinely in monolayer culture at 37°C in an atmosphere of CO_2/air (1:19) in 2.24-litre bottles (House & Wildy, 1965) with 200ml of Eagle's medium, Glasgow modification, containing 10% (w/v) tryptose–phosphate broth and 10% (v/v) calf serum. Cells were routinely checked for mycoplasma contamination.

Isolation of nuclei and partially purified DNA-dependent DNA polymerases

Nuclei were isolated from late-exponentially growing cells (about 10^9 cells) by procedures described by

Craig & Keir (1975*b*). They were extracted with 6ml of 0.2M-potassium phosphate buffer, pH7.5, for 1h at 4°C and the lysate was subsequently centrifuged for 1½h and 4°C at 100000$g_{av.}$ in an MSE 6×5.5ml swing-out rotor. The supernatant was diluted with 0.1M-potassium phosphate buffer, pH7.5, to 12ml and loaded on a phosphocellulose column (1.7cm× 9.0cm) equilibrated with the phosphate buffer (Craig & Keir, 1975*a*). DNA polymerases were eluted from the column with 60ml of 0.3M-potassium phosphate, pH7.5, and fractions containing enzyme activity were pooled and dialysed for 4h in the cold against 10× 100vol. changes of 10mM-Hepes buffer, pH7.4, containing 50mM-NaCl. The dialysed sample (16ml) was loaded at 2ml/h on a 2% single-stranded DNA–agarose column (3.0cm×1.0cm) (Schaller *et al.*, 1972), which was then washed with 10ml of the Hepes buffer solution. Finally, 40ml of a linear gradient of 0.05–0.8M-NaCl was passed through the column at 3ml/h and 1ml fractions were collected. DNA polymerase-α was eluted at 0.15M-NaCl and polymerase-β at 0.5M-NaCl (for nomenclature of polymerases, see Weissbach *et al.*, 1975). Polymerase-α was purified 150-fold and polymerase-β 250-fold by these procedures.

Mercaptoethanol at 5mM was normally present in all buffers used during the isolation of nuclei and polymerases, except in those experiments involving *N*-ethylmaleimide and *p*-hydroxymercuribenzoate.

Assay for DNA synthesis in isolated nuclei

In addition to the required drug (see below) the assay medium contained 10mM-ATP, 0.5mM each of dATP, dCTP and dGTP, 1.2μM-[^{3}H]dTTP (958mCi/mmol; obtained by mixing non-radioactive dTTP with the radioactive molecules), 10mM-$MgCl_2$ and 50mM-Hepes buffer, pH7.5. Nuclear suspension (50μl), containing 1×10^6 nuclei in 0.32M-sucrose buffered in 50mM-Hepes, was added to start the reaction in a final volume of 200μl. After 30min at 37°C DNA synthesis was stopped by adding an equal volume of ice-cold 10%(w/v) trichloroacetic acid, the precipitates were collected on GF/C filter discs (2.5cm diam.) and, after washing and drying, these were counted for radioactivity (Pearson *et al.*, 1976). Counts were recorded over 10min at an efficiency of 33%.

Assay for DNA synthesis by partially purified polymerases

Conditions for measuring polymerase activities were those that gave maximum synthesis by isolated nuclei; they were therefore identical with those described above except that partially purified enzymes were present instead of nuclei and the assays contained 100μg of 'activated' salmon sperm DNA (Loeb, 1969)/ml or 'activated' calf thymus DNA (100μg/ml) as template. These conditions differ from our standard BHK-cell DNA polymerase-α assay because ATP is present at 10mM, and they differ from the DNA polymerase-β assay because ATP is present and because the pH is at 7.5, whereas pH8.9 is optimum for BHK-cell DNA polymerase-β (Craig & Keir, 1975*a*).

Identical conditions throughout were chosen because variations in pH and metal ion concentration would affect the activities of these DNA-synthesizing reactions (Byrnes *et al.*, 1973) and this could influence the response of these systems to the various drugs used. For example, the inhibition by heparin of a DNA-dependent DNA polymerase isolated from *Chlamydomonas reinhardii* is affected by changes in ionic strength of the medium (Ross & Harris, 1978).

Results

Effect of various drugs on DNA synthesis

Having established the presence of both DNA polymerase-α and -β in the isolated nuclei (see the Experimental section), we attempted to determine

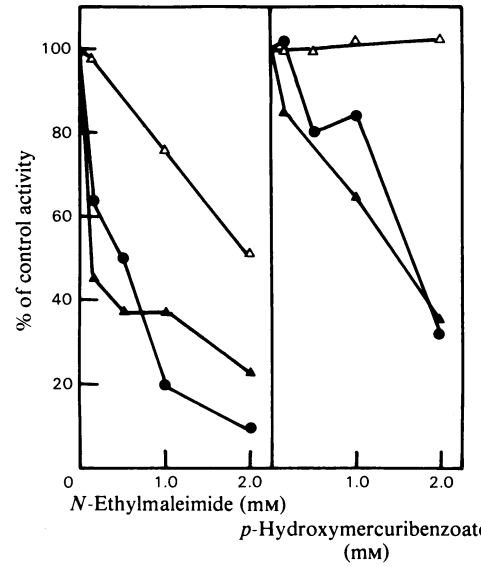

Fig. 1. *Effect of thiol-group-blocking agents on DNA synthesis in vitro during a 30min incubation at 37°C of isolated nuclei, and partially purified DNA polymerase-α and -β in the presence of [^{3}H]dTTP*

For other details see the Experimental section. Control radioactivities (incubation without inhibitor) were 173c.p.m. for isolated nuclei (●), 602c.p.m. for DNA polymerase-α (▲) and 515c.p.m. for DNA polymerase-β (△); 100c.p.m. represents approx. 0.2pmol of dTMP incorporated.

which of these enzymes catalysed the synthesis of the DNA observed in the nuclei under our assay conditions.

The effects of the two thiol-group-blocking agents *N*-ethylmaleimide and *p*-hydroxymercuribenzoate are shown in Fig. 1. Inhibition of endogenous DNA synthesis by isolated nuclei and DNA polymerase-α was very similar, and at low concentrations of either inhibitor this was different from the response of the polymerase-β. Thus at 0.2mM-*N*-ethylmaleimide endogenous nuclear DNA synthesis was diminished by 38% and that catalysed by polymerase-α was decreased by 56%, whereas the activity of the β-enzyme was unaffected. This latter enzyme was also unaffected by *p*-hydroxymercuribenzoate at any of the concentrations used.

Novobiocin inhibited both the endogenous DNA synthesis by the isolated nuclei and that catalysed by polymerase-α to very similar extents; for example 75–82% inhibition occurred with 2.0mM-novobiocin, whereas polymerase-β was inhibited by only 30% even with 5mM-novobiocin (Fig. 2).

Ethidium bromide, an intercalating dye for DNA and known inhibitor of DNA polymerase-α (Byrnes *et al.*, 1975), was found to inhibit DNA synthesis catalysed by the nuclei and the polymerases to similar extents (Fig. 2). However, DNA synthesis in the nuclei was stimulated (130% of controls) by ethidium bromide at 5μg/ml. Although this stimulatory effect of low concentrations of ethidium bromide was repeatable, the degree of stimulation varied considerably between experiments (up to 300% of control activity).

Work by Lazarus & Kitron (1974) showed that DNA polymerase-α from BHK cells is inhibited by the anionic mucopolysaccharide heparin, unlike polymerase-β. Our work confirms this and also shows that the endogenous nuclear DNA synthesis was inhibited to similar extents as the α-enzyme by comparable concentrations of heparin (Fig. 2).

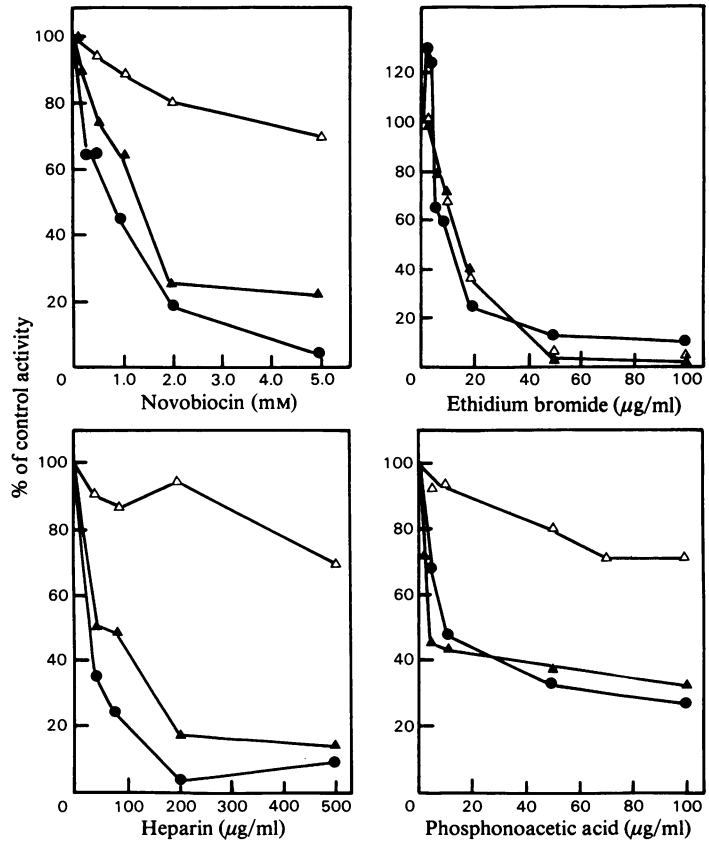

Fig. 2. *Effect of various drugs on DNA synthesis in vitro*
For details see the legend to Fig. 1. Control radioactivities were 416c.p.m. for isolated nuclei (●), 1384c.p.m. for DNA polymerase-α (▲) and 897c.p.m. for polymerase-β (△).

Phosphonoacetic acid has been shown to inhibit DNA synthesis in isolated HeLa-cell nuclei, and DNA synthesis catalysed by partially purified HeLa-cell DNA polymerase-α and -γ (Bolden *et al.*, 1975); DNA polymerase-β from these cells was unaffected. Our results with BHK-cell nuclei and partially purified polymerases shows that the polymerase-β was virtually unaffected by low concentrations of phosphonoacetic acid (5–10μg/ml), which were sufficient to inhibit the activities of both DNA polymerase-α and nuclear DNA synthesis by about 52–57%. Even with phosphonoacetic acid at 100μg/ml polymerase-β activity was diminished by only 28%, although at this concentration the inhibition was presumably not specific.

Dissociation of the replication complex by NaCl

It is well established that DNA polymerase-β is tightly bound to chromatin and that polymerase-α can more readily be dissociated from it with salt. Butt *et al.* (1976, 1978) reported that exposure of mouse L929-cell nuclei to 0.2M-KCl removed all assayable DNA polymerase-α from them, but had little effect on their ability to synthesize DNA, and thus reasonably concluded that the remaining DNA polymerase-β was responsible for the observed DNA synthesis *in vitro*. When our BHK-cell nuclei were suspended in 0.2M-NaCl for 20min at 4°C their subsequent ability to synthesize DNA varied only from 0 to 50% of controls, despite the removal of considerable DNA polymerase-α activity into the medium (assayed with an activated DNA template). In all experiments, however, the residual DNA-synthetic activities of the nuclei were inhibited (over 90%) by heparin at 200μg/ml, a concentration that has virtually no effect on DNA polymerase-β. It seems likely therefore that the remaining chromatin-bound polymerase-α molecules catalysed the observed nuclear DNA synthesis (see the Discussion section).

Discussion

We have confirmed previous observations from this laboratory (Craig & Keir, 1975*a*,*b*) and others (Lazarus & Kitron, 1975) that both DNA polymerase-α and polymerase-β are present in nuclei isolated from BHK-21/C13 cells. Polymerase-α represented 33% of the total activity and polymerase-β 67%; we did not assay for the presence of polymerase-γ. This relative proportion of the two polymerases is similar to that found in nuclei from HeLa cells (Weissbach *et al.*, 1971) and from regenerating rat liver (Lynch *et al.*, 1975). Having demonstrated the presence of these two enzymes in our isolated nuclei we then attempted to determine which enzyme catalysed the DNA synthesis carried out *in vitro*.

N-Ethylmaleimide and *p*-hydroxymercuribenzoate are two well-established thiol-group-blocking agents which inhibit DNA polymerase-α (Weissbach *et al.*, 1971; Baril *et al.*, 1973), and their use has been suggested by Bollum (1975) as being the best criterion for distinguishing between the polymerase-α and -β enzymes, although it has been reported that DNA polymerase-β from Novikoff hepatoma cells is inhibited by *N*-ethylmaleimide (Mosbaugh *et al.*, 1976). Our studies with BHK-cell polymerases show that DNA polymerase-α is more sensitive than polymerase-β to these agents, and the inhibition of endogenous nuclear DNA synthesis closely resembles that of the α-enzyme, suggesting that it may be catalysed by this enzyme. It is unlikely that the nuclear DNA synthesis is catalysed by DNA polymerase-γ, which is also inhibited by *N*-ethylmaleimide, since other properties of the nuclear DNA-synthesizing system, such as pH and salt optima, are different from those of the γ-enzyme.

DNA synthesis in other systems *in vitro* has also been shown to be inhibited by these thiol-group-blocking agents, for example HeLa-cell nuclei (Friedman & Mueller, 1968; Friedman, 1974) and permealysed mouse L-cells (cells rendered permeable to nucleoside triphosphates by a cold shock with near-iso-osmotic buffer; Berger & Johnson, 1976).

Although the mechanism by which novobiocin inhibits DNA synthesis is unknown, it has been shown to inhibit the activity of DNA polymerase-α from rat liver nuclei (Lynch *et al.*, 1976) and polymerase A, one of two DNA polymerases from rat brain nuclei (Sung, 1974). From the elution profiles of these brain enzymes on DEAE-cellulose the polymerase A (Sung's nomenclature) has properties characteristic of DNA polymerase-α, and polymerase-B resembles polymerase-β in its properties. We found that both the BHK-cell endogenous nuclear DNA synthesis and DNA polymerase-α were more sensitive than polymerase-β to novobiocin.

The stimulatory effect of low concentrations of ethidium bromide on DNA synthesis by isolated nuclei is presumably a consequence of a change in the conformation of DNA caused by this intercalating dye (Paoletti, 1978). A similar stimulation of DNA and RNA synthesis *in vitro* has been reported (Meyer *et al.*, 1972; R. R. Meyer, personal communication), and ethidium bromide *in vivo* at 0.1–0.5μg/ml was shown to stimulate BHK-cell nuclear DNA synthesis by 250% (Zunino *et al.*, 1975). We did not detect a clear stimulation of DNA synthesis by using the partially purified polymerases and 'activated' DNA as template, nor was there a difference in the response of polymerase-α and -β to ethidium bromide, unlike a previous report showing the α-enzyme to be more sensitive to this drug than the β-enzyme (Zunino *et al.*, 1975).

The anionic mucopolysaccharide heparin was shown by Lazarus & Kitron (1974) to inhibit DNA

polymerase-α but not polymerase-β from BHK-21/C13 cells. Our work confirms this and shows that DNA synthesis by the isolated nuclei is also inhibited by heparin with a sensitivity almost identical with that of the α-enzyme.

Phosphonoacetic acid, once thought to be a specific inhibitor of herpes-simplex-virus-induced DNA-dependent DNA polymerase, is now known to inhibit host-cell DNA polymerases too, although the effect seems to depend on the type of cells. Bolden *et al.* (1975) found that HeLa-cell DNA polymerase-α activity was inhibited by 48 and 90% by phosphonoacetic acid at 5 and 100μg/ml respectively, whereas polymerase-β was virtually unaffected at these concentrations. Phosphonoacetate also inhibits DNA polymerase-α from duck embryo fibroblasts, but not the β-polymerase (Leinbach *et al.*, 1976), whereas in WI-38 cells neither the α- nor the β-enzyme was inhibited by this compound (Mao *et al.*, 1975). We found that phosphonoacetic acid inhibited DNA synthesis by isolated BHK-cell nuclei and by DNA polymerase-α to similar extents (50–60% inhibition at 10μg of phosphonoacetate/ml), whereas the β-enzyme was much less sensitive (5–10% inhibition) at this concentration.

Other comparisons between nuclear DNA synthesis and the partially purified polymerases include the following: (1) the optimum pH for nuclear DNA synthesis and for polymerase-α activity is 7.5–7.8, whereas the optimum pH for polymerase-β is 8.5–8.9; (2) the detergent Brij 58 at 2% (w/v) irreversibly inhibited both nuclear DNA synthesis and the activity of the polymerase-α by 80–90%, whereas polymerase-β was slightly activated under these conditions (120% of controls); (3) the apparent K_m values for dTTP for the isolated nuclei and polymerase-α were similar (0.5–1.0 and 0.85–2.0μM respectively) and, although we have not determined this for the polymerase-β under our experimental conditions, the results of Craig & Keir (1975*a*) from this laboratory suggest that the value for this enzyme would be substantially different.

The results described are consistent with the proposal that DNA polymerase-α catalyses the observed DNA synthesis by the isolated nuclei, that is, the covalent elongation *in vitro* of Okazaki pieces initiated *in vivo*. It is difficult to reconcile the discrepancy between our conclusion and that of Butt *et al.* (1976, 1978), who consider from their work with mouse L929-cell nuclei that polymerase-β, in association with other proteins, catalyses the observed DNA synthesis. We consider it unlikely that the properties of the β-enzyme within such a complex would be so altered that its properties would then resemble closely those of polymerase-α in each of the many different ways described in this present manuscript for the BHK-cell system. Butt *et al.* (1976, 1978) do point out that there may be residual polymerase-α in their salt-extracted nuclei, which could be responsible for their observed nuclear DNA synthesis, these particular enzyme molecules remaining attached to the growing DNA chain thereby catalysing the extension of Okazaki pieces. It is clear that further work is required before unambiguous roles can be assigned to these two enzymes.

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