DNA Repair Synthesis in Human Fibroblasts Requires DNA Polymerase δ*

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When UV-irradiated cultured diploid human fibroblasts were permeabilized with Brij-58 then separated from soluble material by centrifugation, conservative DNA repair synthesis could be restored by a soluble factor obtained from the supernatant of similarly treated HeLa cells. Extensive purification of this factor yielded a 10.2 S, 220,000-dalton polypeptide with the DNA polymerase and 3'- to 5'-exonuclease activities reported for DNA polymerase δ II (Crutè, J. J., Wahl, A. F., and Bambara, R. A. (1986) Biochemistry 25, 26–36). Monoclonal antibody to KB cell DNA polymerase α, while binding to HeLa DNA polymerase α, did not bind to the HeLa DNA polymerase δ. Moreover, at micromolar concentrations N2(p-n-butylnaphthyl)-2'-deoxyguanosine 5'-triphosphate (BuPdGTP) and 2-(p-n-butylanilino)-2'-deoxyadenosine 5'-triphosphate (BuAdATP) were potent inhibitors of DNA polymerase α, but did not inhibit the DNA polymerase δ. Neither purified DNA polymerase α nor β could promote repair DNA synthesis in the permeabilized cells. Furthermore, under conditions which inhibited purified DNA polymerase α by greater than 90%, neither monoclonal antibodies to DNA polymerase α, BuPdGTP, nor BuAdATP was able to inhibit significantly the DNA repair synthesis mediated by the DNA polymerase δ. Thus, it appears that a major portion of DNA repair synthesis induced by UV irradiation might be catalyzed by DNA polymerase δ. When xeroderma pigmentosum human diploid fibroblasts were utilized, DNA repair synthesis dependent upon ultraviolet light could be restored by addition of both T4 endonuclease V and DNA polymerase δ, but not by addition of either one alone. This result suggests that cytosol-depleted permeabilized DNA repair-defective human fibroblasts and HeLa DNA polymerase δ might be exploited to provide a functional assay for purifying active DNA repair factors from DNA repair-proficient cells without a preknowledge of their function.

Several laboratories have utilized "permeabilized" cultured human cells to study repair DNA synthesis (Ciarrocchi and Linn, 1978; Ciarrocchi et al., 1979; Dresler et al., 1982; Roberts and Lieberman, 1979) and replicative DNA synthesis (Cattellot et al., 1979; Enomoto et al., 1983; Fox et al., 1977; Narkhammar and Hand, 1985; Reinhard et al., 1979). Such systems exhibit conservative DNA repair synthesis that is dependent upon a DNA damaging treatment either before or after permeabilization. This response is specifically lacking if ultraviolet (UV) irradiation is utilized as the damaging agent with xeroderma pigmentosum cells (Dresler et al., 1982; Roberts and Lieberman, 1979; Ciarrocchi and Linn, 1978). Based on the observation that permeabilized cells are able to take up active T4 UV endo- and exonucleases (Ciarrocchi et al., 1979; Tanaka et al., 1975) as well as pancreatic DNAse (Ciarrocchi and Linn, 1978), our laboratory has attempted to exploit such a system in order to have a functional assay for purifying from normal cells activities which are lacking in DNA repair-defective cells. It became apparent, however, that a soluble DNA repair factor was diluted out during such attempts. We have detected this factor in soluble extracts from HeLa cells and, utilizing cytosol-depleted permeabilized normal human fibroblasts for a functional assay of its activity, have purified it extensively. We report here that this factor, which is required for DNA repair synthesis, appears to be a 220,000-dalton polypeptide which copurifies with the 10.2 S DNA polymerase and 3'- to 5'-exonuclease activities recently described for calf thymus DNA polymerase δ II (Crutè et al., 1986). Furthermore, we believe that this is the first isolation and characterization of DNA polymerase δ from Hela cells.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were grown in suspension in Joklik's modified Eagle's medium containing 5% calf serum, supplemented with penicillin, streptomycin, and L-glutamine. The F65 (normal) human fibroblast strain was obtained from the Naval Biomedical Research Laboratory (Oakland, CA) and xeroderma pigmentosum fibroblast strain GM2990 (complementation group A) was from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ). Cells were grown in a humidified 5% CO2 incubator at 37°C. Subculturing of fibroblasts from 150 cm2 tissue culture flasks to tissue culture dishes of 10-cm diameter followed tryptic treatment (0.05%) and suspension in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Upon attaining a confluent state, cultures of F65 fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. Xeroderma pigmentosum fibroblasts were grown and maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum.

DNA Repair Assay—Postconfluent cultures (i.e. cultures in a confluent state for at least 3 days but not exceeding 15 days) were utilized.

DNA Repair Assay—Postconfluent fibroblast cultures in tissue culture were washed twice with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 6.46 mM Na2HPO4, 1.47 mM KH2PO4), and the liquid was drained off before the cells were UV- or mock-irradiated. Irradiation was with a General Electric germicidal lamp G 15T8 (15 watt) at an incident dose rate of 2.0 J/m2/s. Forty J/m2 was the dose used unless otherwise noted. One-half ml of 0.05% trypsin was added to each dish. After 5 min at 37°C, 2.5 ml of phosphate-buffered saline were added, and the cells were transferred to a 50-ml polypropylene conical tube and pelleted in a clinical centrifuge at room temperature. The cells were resuspended at 0°C in permeabilization buffer (36 mM
HELa DNA Polymerase δ and DNA Repair

HELa DNA polymerase δ assay—DNA polymerase activity was assayed with activated salmon sperm DNA template under conditions described previously (Linn et al., 1976), except that KCl was omitted and 50 mM Tris-HCl (pH 7.5) was used. One unit of DNA polymerase activity catalyzes the incorporation of 1 n mole of total nucleotide/h at 37 °C. DNA polymerase activity was assayed using poly(dA) primed with (dT)10 (Midland Certified Reagent Co., Midland, TX) with an average interprimer distance of 150 nucleotides as described by Crute et al. (1986), except that ATP was omitted, and the reaction was stopped by the addition of 0.2 mM Na3P2O7 followed by 0.30 M Tris-HCl, pH 7.5, and 0.5 mM EDTA. Samples were filtered through Whatman GF/C filters presoaked for at least 1 h in 0.1 M Na2P2O7, then the filters were washed twice with 1 M KCl containing 0.1 M Na3P2O7, once with 50% ethanol, and dried. Radioactivity was determined by liquid scintillation counting. One unit of DNA repair factor activity stimulates the incorporation of 1 pmol of [3H]dTMP/h/106 cytosome-depleted permeabilized fibroblasts at 30°C.

RESULTS

Preliminary Characterization of the Assay System—To prepare permeabilized fibroblasts, postconfluent cultures exhibiting little semiconservative DNA replication were exposed to Brij-58 by a modification of the procedure of Reinhard et al. (1979). Preliminary experiments showed that UV-irradiated cells treated with buffer containing 0.01% Brij-58 and no KCl then washed by centrifugation exhibited DNA repair synthesis similar to that of unfractonated permeabilized cells prepared by a hypotonic treatment (Ciarrocchi and Linn, 1978). This response was lost, however, when 0.65% Brij-58 and 250 mM KCl were utilized, but could be restored by addition of a soluble factor from either human fibroblasts or HeLa cells

<table>
<thead>
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<th>Table I</th>
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<tr>
<td>HeLa cell extract</td>
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<td>HeLa cell extract</td>
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<td>HeLa cell extract</td>
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The abbreviations used are: HEPES, N-2-hydroxyethylpipercyan-N'-2-ethanesulfonic acid; DTT, dithiothreitol; EGTA, [ethylendiamine(oxyethylene)nitritro]tetraacetic acid; BuPdGTP, N2-(p-n-butylphenyl) -deoxyguanosine 5'-triphosphate; BuADTTP, 2-(p-n-butylamino)-5'-deoxyadenosine 5'-triphosphate; BrUrd, bromodeoxyuridine; BrUTP, bromodeoxyuridine 5'-triphosphate; SDS, sodium dodecyl sulfate.

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prepared by Dounce homogenization and high-speed centrifugation.

As with the nonreconstituted systems (Ciaccio and Linn, 1978; Dresler et al., 1982; Roberts and Lierman, 1979), repair DNA synthesis mediated by this factor became maximal near a dose of 5 J/m² and was constant to above 100 J/m². Moreover, permeabilized postconfluent fibroblast cultures exhibited higher levels of DNA repair synthesis than subconfluent ones. On the other hand, cell extract prepared from HeLa cells in stationary phase contained the same level of factor as that prepared from cells in logarithmic phase. Synthesis was linearly dependent upon the amount of HeLa cell factor and, when incubated at 30 °C, it continued for at least 120 min.

Heating the soluble HeLa extract to 45 or 50 °C resulted in preferential loss of DNA synthesis which was not dependent upon UV irradiation, while heating for 10 min at 70 °C inactivated all activity. Since the former treatment resulted in DNA synthesis which could be totally UV-dependent (depending upon the state of the cells used for permeabilization), it was utilized during purification and before characterization of the factor.

The UV-dependent DNA synthesis brought about by the heated HeLa cell extract required that some process(es) occur in intact fibroblasts following UV irradiation but prior to permeabilization (Table I). Thus, when irradiated cells were kept at 0 or 37 °C prior to permeabilization, then permeabilized and incubated at 30 °C either alone or with heat-treated HeLa cell extract, the DNA synthesis mediated by the extract occurred only when the intact fibroblasts had been incubated at 37 °C (Table I). This result indicates that the heated HeLa cell extract activity might continue a DNA repair process which begins in the intact cells after exposure to UV light. These initial steps appear to be completed within 15 min, at least to an extent which does not limit subsequent factor-dependent DNA synthesis for 60 min at 30 °C.

When aphidicolin, a specific inhibitor of DNA polymerases α and δ (Goscin and Byrnes, 1982), was present at a concentration of 10–20 μg/ml, UV-dependent DNA synthesis was inhibited to less than 30% suggesting the involvement of one or both of these enzymes in the DNA repair synthesis. This result also agrees with observations made with the nonreconstituted system (Dresler et al., 1982).

Heated Cell Extract Mediates Repair DNA Synthesis, Not Semiconservative DNA Replication—To verify that DNA synthesis stimulated by the HeLa cell extract was conservative, intact postconfluent fibroblasts with DNA prelabeled with [3H]dTTP were preincubated in medium containing BrdUrd, UV-irradiated, reincubated further for 15 min in medium containing BrdUrd, permeabilized, and finally incubated in the presence of [3H]dTTP, BrdUTP, and heat-treated extract. The DNA was then partially purified and centrifuged to equilibrium in alkaline CsCl gradients (Fig. 1). Indeed, as expected for repair DNA synthesis, the [3H]dTMP which was incorporated banded with the light [3H]DNA and not the heavy BrdUMP strand. (A proper analogous analysis with replicating cells showed semiconservative replication to have occurred in that case. See legend to Fig. 1).

**TABLE II**

Purification of soluble repair factor from HeLa cells

Approximately 6.2 × 10⁶ HeLa cells from 84 liters of culture were processed as described under “Experimental Procedures” and in Figs. 2, 3, and 6. Fraction 36 from hydroxylapatite chromatography was termed Fraction VIα and purified further by glycerol gradient ultracentrifugation (Fig. 6). Values were corrected to reflect purification of the entire preparation.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
<td>units/mg</td>
</tr>
<tr>
<td>I. Soluble extract</td>
<td>667</td>
<td>4940</td>
<td>2510</td>
<td>0.508</td>
</tr>
<tr>
<td>II. 30-50% (NH₄)₂SO₄ precipitate</td>
<td>60.5</td>
<td>1670</td>
<td>1470</td>
<td>0.880</td>
</tr>
<tr>
<td>III. Heat treatment (45 °C, 12 min)</td>
<td>174</td>
<td>1670</td>
<td>1210</td>
<td>0.725</td>
</tr>
<tr>
<td>IV. DEAE-Sephacel</td>
<td>52.5</td>
<td>427</td>
<td>526</td>
<td>1.23</td>
</tr>
<tr>
<td>V. Phosphocellulose</td>
<td>31.4</td>
<td>15.9</td>
<td>463</td>
<td>29.1</td>
</tr>
<tr>
<td>VI. Hydroxylapatite</td>
<td>21.1</td>
<td>3.20</td>
<td>372</td>
<td>116</td>
</tr>
<tr>
<td>VIα. Hydroxylapatite Fraction 36</td>
<td>2.35</td>
<td>0.228</td>
<td>43.0</td>
<td>189</td>
</tr>
<tr>
<td>VIIα. Glycerol gradient ultracentrifugation</td>
<td>5.88</td>
<td>0.047</td>
<td>48.6</td>
<td>1000</td>
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Purification of Soluble Factor—To purify the activity (Table II), HeLa extract was fractionated by ammonium sulfate precipitation, heat-treated, and passed through DEAE-Sephas- 
cel where the factor eluted as two broad peaks. One peak that eluted at 120 mM NaCl was highly labile (half-life of less than 1 day at -80 °C) and not further characterized. The second peak that eluted at 180 mM NaCl was stable when stored at -80 °C or over liquid nitrogen, and was further purified on phosphocellulose (Fig. 2) where the activity eluted as a single sharp peak at 220 mM NaCl (designated Fraction V).

Following dilution, Fraction V was passed through hydrox- 
yapatite (Fig. 3) where the activity eluted as a broad peak at 140 mM potassium phosphate, ahead of the bulk of the activated DNA-directed DNA polymerase activity which was present in Fraction V. This later polymerase activity was sensitive to aphidicolin and N-ethylmaleimide and insensitive to the inhibitor of DNA polymerase β and γ, dideoxyTTP, so that it was presumably a DNA polymerase α.

Preliminary Characterization of Repair Factor Activity—Fracti-
on V lacked the following activities that could be implicated in DNA repair: endonuclease on undamaged, UV-irradiated or apurinic duplex or single-stranded DNAs; exonu-
clase on duplex or denatured Escherichia coli DNA; DNA-
dependent or independent ATPase; native DNA binding ac-
HeLa DNA Polymerase δ and DNA Repair

FIG. 4. Cosedimentation of soluble repair factor with δ DNA polymerase and exonuclease. A sample of Fraction V was diluted with TDEG buffer to reduce the NaCl concentration to 0.2 M, and concentrated by applying to a 2-ml column of phosphocellulose pre-equilibrated with TDEG buffer containing 0.2 M NaCl, then eluted with TDEG buffer containing 0.5 M NaCl. A 0.2-ml aliquot was layered onto a 5-ml glycerol gradient (20–40%) in TDEG buffer containing 0.2 M NaCl and centrifuged in a Beckman SW50.1 rotor at 49,000 rpm for 20 h at 4°C. Fifteen-drop fractions were collected from the bottom. Molecular weight markers (catalase (11.3 S, 232,000) and hemoglobin (4.3 S, 68,000)) were layered onto a parallel gradient. W, DNA repair activity; +, DNA polymerase activity with poly(dA)-oligo(dT); 0, nuclease activity with [3H](dT)70. Nuclease activity was assayed as described by Lee et al. (1980) in a final reaction volume of 60 μl using [3H](dT)70 prepared as described by Crute et al. (1986). One unit of nuclease catalyzes the release of 1 nmol of dTMP/h at 37°C. Repair activity values are the averages of duplicate assays. Inset, the ratio of repair activity to nuclease activity. Sedimentation is from right to left.

Coseedimentation of Repair Factor with δ DNA Polymerase and Nuclease Activities—A concentrated sample of Fraction V from a separate purification was sedimented through a glycerol gradient (Fig. 4) in which activity sedimented at 10.2 S. When a synthetic DNA polymerase substrate, poly(dA) primed with oligo(dT), was used, a DNA polymerase profile was observed that coseedimented exactly with the DNA repair activity (Fig. 4). This synthetic polymer is an active template for DNA polymerase δII (Crute et al., 1986), a DNA polymerase with the striking property of having an intrinsic 3'– to 5'-exonuclease activity (Byrnes et al., 1976). When exonuclease activity with [3H](dT)70 (a preferred substrate for the δ-exonuclease) was assayed across the glycerol gradients, a sharp peak of exonuclease was found also to coseediment exactly with the DNA repair activity (Fig. 4). No exonuclease was detected with native or denatured E. coli DNA.

To verify that the repair factor exonuclease, like that of δ-polymerase, acts in the 3' to 5' direction, the degradation of (dT)30 labeled at the 5' end with [32P]04 and at the 3' end with [3H]dTMP, was monitored (Fig. 5). The more rapid release of 3H than 32P from the substrate verifies 3' to 5' directionality. With 5.0 mM AMP present, the initial rate of the exonuclease was inhibited by 45%, also in agreement with observations made with δ-polymerase (Byrnes et al., 1977; Crute et al., 1986; Lee and Toomey, 1987).

Copurification of repair activity and δ-polymerase activity was also observed during phosphocellulose (Fig. 2) and hydroxylapatite (Fig. 3) chromatography although the column profile of the latter showed differences in trailing patterns (see below). Finally, when the repair activity from the hydrox-
Fig. 6. Glycerol gradient ultracentrifugation and SDS-polyacrylamide gel electrophoresis of DNA polymerase δ/soluble repair factor. Upper, Fraction VIa (0.2 ml) was sedimented through a glycerol gradient (25–45%) in 130 mM potassium phosphate (pH 7.5), and 5 mM DTT as described in Fig. 4, except that centrifugation was for 32 h. Nine-drop fractions were collected from the bottom. ■, DNA repair activity; ◗, DNA polymerase activity with poly(dA)-oligo(dT); ○, DNA polymerase activity with activated salmon sperm DNA template. Repair activity values are the averages of duplicate assays. Sedimentation is from right to left. Lower, samples (11.5 µl) from fractions of the gradient shown in panel A were subjected to electrophoresis in a SDS-polyacrylamide slab gel (1.5 mm thick; 3% stacking, 5% separating gels) and proteins detected by silver-staining. Lane 15 contained approximately 1 µg of protein and lane M, molecular weight markers (myosin, β-galactosidase, phosphorylase b, and bovine serum albumin). The band present in all lanes corresponding to 54 kDa derives from 0-mercaptoethanol and was present when only SDS and β-mercaptoethanol were put into the well.

Hydroxylapatite column (fraction 36, Fig. 3) was sedimented through a glycerol gradient, it cosedimented with the DNA polymerase activity and with a peptide that had a molecular size of 220,000 daltons on an SDS-polyacrylamide gel (Fig. 6). This peptide was the only one detected in the peak fraction. The sedimentation coefficient and gel band location are consistent with repair factor being a monomeric form of DNA polymerase δ II with a molecular weight of approximately 220,000.

Purified α- and δ-Polymerase Preparations Do Not Substitute for Repair Factor—Because of the presence of a 9.6 S, 175-kDa α-polymerase catalytic subunit in the crude factor preparations, it was of interest to test whether purified HeLa α-polymerase could serve as a repair factor. In numerous trials with different preparations of HeLa α-polymerase, including the multiprotein complex of DNA polymerase αs, at various stages of purity (Krauss and Linn, 1986; Vishwanatha et al., 1986), we consistently failed to detect any repair factor activity. Additionally, δ-polymerase activity cannot be detected in repair factor fractions obtained after the DEAE-chromatography step and, conversely, purified HeLa β-polymerase (Mosbaugh and Linn, 1983) lacks repair factor activity.

Interestingly, however, the complex of HeLa DNA polymerase αs, when added with the purified repair factor, stimulated UV-dependent DNA synthesis in the reconstituted assay by 22%, suggesting that α-polymerase might be able to take part in a DNA repair process which δ-polymerase might be regulating. This may explain the unusual profile of repair activity upon hydroxylapatite with respect to δ-polymerase activity (Fig. 3). The large and highly active peak of α-polymerase, situated over the trailing edge of the δ-polymerase activity peak, likely contributes to the apparent stimulation of repair activity in those trailing fractions.

Effect of Specific Inhibitors of DNA Polymerase α on the DNA Polymerase Activities Resolved by Hydroxylapatite—Hydroxylapatite chromatography, routinely used to separate DNA polymerases α and δ (Lee et al., 1980), yielded a column profile in which poly(dA)-oligo(dT)-directed DNA polymerase activity was resolved from the bulk of the activated DNA-directed DNA polymerase activity (Fig. 3). Yet a small amount of polymerase activity utilizing the activated DNA substrate
FIG. 7. Effect of monoclonal antibody directed against DNA polymerase $\alpha$ on the DNA polymerase activities resolved by hydroxyapatite. Standard binding assays of DNA polymerase $\alpha$-specific antibody SJK 237-71 or control antibody P3 were performed as described by Tanaka et al. (1982), except that a suspension of protein A covalently linked to Sepharose CL-4B that had been preadsorbed with goat anti-mouse IgG was used. Panels A and B show the effect of monoclonal SJK 237-71 nonneutralizing antibody on Fractions 35 and 45, respectively, eluting from hydroxyapatite (see Fig. 3). Panels C and D show the effect of control monoclonal P3 antibody on the same two fractions. This nonimmune IgG is the monoclonal IgG produced by myeloma cell line P3, the parent of the fusion partner used to produce the DNA polymerase $\alpha$-specific hybridomas (Tanaka et al., 1982). Following binding to antibody and removal of Sepharose-bound immune complexes by centrifugation, DNA polymerase activities on activated salmon sperm DNA template (0) or on poly(dA).oligo(dT) template (+) remaining in the supernatant solution were determined by the standard assays. 100% activity values were determined in parallel binding assays omitting the monoclonal antibody but including the anti-mouse IgG antibody/protein A/Sepharose beads and represented 0.38 and 0.58 unit of activated DNA-directed and poly(dA).oligo(dT)-directed DNA polymerase activity, respectively, in panels A and C; and 4.2 and 0.13 units of activated DNA-directed and poly(dA).oligo(dT)-directed DNA polymerase activity, respectively, in panels B and D.

FIG. 8. Effect of BuPdGTP and BuAdATP on the DNA polymerase activities resolved by hydroxyapatite. Standard DNA polymerase assays were carried out as described under "Experimental Procedures." Panels A and B show the effect of BuPdGTP and BuAdATP, respectively, on Fractions 37 (0) and 47 (+) eluting from hydroxyapatite (see Fig. 3) when activated salmon sperm DNA was the template primer. Panels C and D show the effect of BuPdGTP and BuAdATP, respectively, on the same two fractions when poly(dA).oligo(dT) was the template primer. 100% activity values were determined in parallel assays omitting the inhibitors and represented 0.80 and 7.8 units of activated DNA-directed DNA polymerase activity, respectively, for Fractions 37 and 47; and 0.86 and 0.37 unit of poly(dA).oligo(dT)-directed DNA polymerase activity, respectively, for Fractions 37 and 47.

(seen in Fig. 3 as a leading shoulder of activity) persisted in the peak $\delta$-polymerase fractions. When Fraction 36 was sedimented through a glycerol gradient both polymerase activities cosedimented, together with repair activity and the solitary 220-kDa band seen on SDS-polyacrylamide gels (Fig. 6). The absence of a characteristic ~175-kDa $\alpha$-polymerase band in gradient fractions containing activated DNA-directed DNA polymerase activity suggested that the shoulder of activated DNA-directed DNA polymerase observed in the hydroxyapatite fractions was activity derived from the $\delta$-polymerase polypeptide and not from a contaminating $\alpha$-polymerase. To demonstrate this, mouse monoclonal antibody SJK 237-71 (nonneutralizing), which was developed against human KB cell $\alpha$-polymerase by Tanaka et al. (1982), was bound to...
protein A-Sepharose beads via goat antiamouse IgG, then mixed with hydroxylapatite fraction 35 and the supernatant following centrifugation assayed for DNA polymerase on either activated DNA or poly(dA)-oligo(dT). Both polymerase activities were unaffected by control monoclonal IgG (P3) and by concentrations of SJK 237-71 that remove HeLa α-polymerase from the supernatant by 63% (Fig. 7, A and C). When the experiment was repeated with hydroxylapatite fraction 45, the poly(dA)-oligo(dT)-directed DNA polymerase activity was again unaffected by both control monoclonal IgG and SJK 237-71, but the activated DNA-directed DNA polymerase activity was removed from the supernatant by SJK 237-71 (Fig. 7, B and D). We thus conclude that the small peak of polymerase activity which acts on activated DNA, and is represented by Fractions 35-37, is derived from the δ-polymerase polypeptide. On the other hand, the bulk of the very large peak of activated DNA-directed DNA polymerase activity centered about Fraction 45 is derived from α-polymerase. Finally, the poly(dA)-oligo(dT)-directed DNA polymerase activities of both Fractions 35 and 45 were unaffected by SJK 237-71 which suggests that δ-polymerase is indeed present in all hydroxylapatite fractions containing repair activity. A form of DNA polymerase δ that has significant activity on natural DNA substrates as well as poly(dA)-oligo(dT) has been reported from calf thymus (Crute et al., 1986) and from rabbit bone marrow (Byrnes and Black, 1978).

The effects of BuPdGTP and BuAdATP on the DNA polymerases resolved by hydroxylapatite chromatography were examined in the standard assay systems using activated salmon sperm DNA or poly(dA)-oligo(dT) as template primers. BuPdGTP and BuAdATP are potent inhibitors of DNA polymerase α, whereas DNA polymerase δ is relatively insensitive (Lee et al., 1985). These inhibitors exhibited similar effects when activated salmon sperm DNA was used as template primer (Fig. 8, A and B). DNA polymerase δ (hydroxylapatite fraction 37, Fig. 3) was inhibited only by BuPdGTP and BuAdATP concentrations which were approximately 4 orders of magnitude greater than concentrations required to inhibit α-polymerase (hydroxylapatite fraction 47). When poly(dA)-oligo(dT) was used as template primer, the polymerase activities of both Fraction 37 and 47 were inhibited as though they were polymerase δ (Fig. 8, C and D), indicating once again that δ-polymerase is present in all hydroxylapatite fractions containing repair activity.

**Effect of Inhibitors on the Repair Activity**—Since DNA polymerase α may be present in the permeabilized cells, the question remained whether it might play a role in UV-induced DNA repair synthesis. To explore this possibility, monoclonal anti-DNA polymerase α IgG or the DNA polymerase α-specific inhibitors BuPdGTP and BuAdATP were added to the standard DNA repair assay, together with DNA polymerase δ (Table III). At levels of antibody that inhibited HeLa DNA polymerase α greater than 90% repair activity was not affected with respect to the nonimmune control IgG. Concentrations of BuPdGTP and BuAdATP that inhibited HeLa DNA polymerase α greater than 95% (Fig. 8, A and B) affect repair activity only minimally, consistent with results reported for permeabilized human fibroblasts (Dresler and Fratini, 1986). The inhibitory potency of BuPdGTP is not affected by incubation with permeabilized cells, nor does it appear likely that there is a barrier to entry of BuPdGTP into permeabilized cells (Dresler and Fratini, 1986). Moreover, the existence of a permeability barrier which limits entry of antibodies into the nuclei of permeabilized cells seems unlikely apriori because the nuclei are permeable to DNA polymerase δ, a significantly larger molecule at 220,000 daltons. Overall, these findings are most consistent with the involvement of DNA polymerase δ in UV-induced DNA repair synthesis, with DNA polymerase α serving a secondary role at best.

To probe the possibility that the exonuclease activity of DNA polymerase δ is required for DNA repair synthesis, 5′-AMP was added to the standard DNA repair assay (Table
purified T4 endonuclease V was added to cytosol-depleted, permeabilized xeroderma pigmentosum, complementation permeabilized DNA repair-defective cells (xeroderma pigmentosum endonuclease V. Cells made permeable by Sendai virus or pair system is revealed by the response of cytosol-depleted, this may suggest a role for the exonuclease in DNA repair, an polymerase activity in the permeabilized cells.

Studies with Xeroderma Pigmentosum Fibroblasts and Exogenous Protein—The potential application of this DNA repair system is revealed by the response of cytokine-depleted, permeabilized DNA repair-defective cells (xeroderma pigmentosum, complementation group A to exogenous phase T4 endonuclease V. Cells made permeable by Sendai virus or osmotic disruption can take up T4 endonuclease V, and the inability of xeroderma pigmentosum cells to repair UV-induced DNA damage can be overcome by this treatment (Tanaka et al., 1975; Ciarrocchi and Linn, 1978). However, when purified T4 endonuclease V was added to cytokine-depleted, permeabilized xeroderma pigmentosum, complementation group A fibroblasts, no UV-dependent dTMP incorporation was observed, unless HeLa DNA polymerase δ was also added (Table IV). This result not only reaffirms that δ-polymerase is required for repair DNA synthesis, but also suggests the application of this system to an in vitro complementation assay composed of DNA repair-defective cells, factors obtained from repair-proficient cells, and purified DNA polymerase δ. (Preliminary observations support this belief: HeLa soluble extract and purified δ-polymerase together restore UV-dependent DNA repair synthesis in cytokine-depleted, permeabilized xeroderma pigmentosum, complementation groups A and B, fibroblasts). When the same experiment was carried out with normal fibroblasts, dTMP incorporation was again strongly stimulated by added T4 endonuclease V plus δ-polymerase with UV-irradiated, but not with untreated, cells (Table IV). This effect by T4 endonuclease V with normal cells has been previously reported (Ciarrocchi and Linn, 1973).

Different Template Activity—Table V shows the relative activities of HeLa DNA polymerase δ on various templates. The preferred template is poly(dA)·(dT)₁₅, but significant activity was also observed using activated salmon sperm DNA. However, the alternating dA-dT polymer, utilized well by the δ-polymerases of rabbit bone marrow (Byrnes et al., 1976; 1977) and human placenta (Lee et al., 1985; Lee and Toomey, 1987). The most distinctive feature is the association of the polymerase with 3'- to 5'-exonuclease, and like the nuclease of two activities characteristic of DNA polymerase δ: namely, DNA polymerase activity with poly(dA)·oligo(dT) and 3'- to 5'-exonuclease activity; it is clearly separable from other activities known to be implicated in DNA repair. This conclusion is also supported by recent reports that concluded that DNA replication and repair may be at least partly dependent upon DNA polymerase δ (Dresler and Frattini, 1986, 1987; Dresler and Kimbro, 1987; Decker et al., 1987). In addition, Prelich et al., (1987a) have reported that proliferating cell nuclear antigen is required for SV40 DNA replication in vitro and that proliferating cell nuclear antigen is identical with a DNA polymerase δ accessory protein (Prelich et al., 1987b; Bravo et al., 1987) that is required by DNA polymerase δ for its polymerase activity with templates having low primer/template ratios (Tan et al., 1986). Based on these observations Prelich et al. (1987, a and b) suggest a function for DNA polymerase δ in replicative DNA synthesis.

It is not merely DNA polymerase activity per se that is required for DNA repair synthesis in the cytokine-depleted cells, because the polymerase activity of δ-polymerase cannot be fulfilled by either DNA polymerase α or δ or DNA polymerase I large (Klenow) fragment.² Possibly a critical role for δ-polymerase could be in the initiation of repair synthesis at the gap left by incision/excision events, or by excision of damage from the 3'-terminus by its DNase activity so that a proper primer is formed. In fact, while inactive in mediating DNA repair alone, α-polymerase increases DNA repair synthesis by 20-25% when added with δ-polymerase, suggesting that α-polymerase might be able to catalyze some repair synthesis subsequent to or in conjunction with the reaction of δ-polymerase. Thus, at least one function for DNA polymerase δ appears to exist that is distinct from that of DNA polymerase α.

HeLa DNA polymerase δ shares many of the distinctive properties previously reported for δ-polymerase from rabbit bone marrow (Byrnes et al., 1976; 1977; Goscin and Byrnes, 1982), calf thymus (Crute et al., 1986; Wahl et al., 1986) and human placenta (Lee et al., 1985; Lee and Toomey, 1987). The most distinctive feature is the association of the polymerase with 3'- to 5'-exonuclease, and like the nuclease of all previously isolated δ-polymerases, this nuclease is inhibited by 5'-AMP (Byrnes et al., 1977; Crute et al., 1986; Lee and Toomey, 1987). HeLa DNA polymerase δ shows a characteristic preference for the synthetic DNA template poly(dA) primed with (dT)₁₅ although it has activity with activated natural DNA. DNA polymerase δ activity with either template-primer is relatively resistant to inhibition by BuPdGTP and BuAdATP; >250 μM inhibitor is required to give 50% inhibition of activity, a result consistent with those obtained from the δ-polymerases of calf thymus (δ II; Wahl et al., 1986) and human placenta (Lee et al., 1985).

The potential for an in vitro complementation assay to be able to identify mammalian DNA repair factors has engendered the development of a variety of systems utilizing isolated nuclei (Smith and Hanawalt, 1978), cell-free extracts obtained by osmotic disruption (Ciarrocchi and Linn, 1978) or sonication (Mortelmans et al., 1976), permeabilized cells (Dresler et al., 1982; Roberts and Lieberman, 1979), or viable cells microinjected with cell extracts (de Jenge et al., 1983). To date none of these has been entirely successful. We have made preliminary observations of a positive response of xeroderma pigmentosum cytokine-depleted permeabilized fibroblasts to exogenously supplied crude cell extract provided that

² C. Nishida and S. W. Krauss, unpublished observations.
purified δ-polymerase was also added, suggesting a system for identifying DNA repair factors which are absent from cells which are DNA repair-defective. The strength of such a system, if successful, would be its providing a means to purify such factors in an active state without having to presuppose the nature of such activities or to design and prepare complex substrates.

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