

DNA Polymerase δ Is Required for Human Mismatch Repair *in Vitro**

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HeLa nuclear extract was resolved into a depleted fraction incapable of supporting mismatch repair *in vitro*, and repair activity was restored upon the addition of a purified fraction isolated from HeLa cells by *in vitro* complementation assay. The highly enriched complementing activity copurified with a DNA polymerase, and the most pure fraction contained DNA polymerase δ but was free of detectable DNA polymerases α and ϵ . Calf thymus DNA polymerase δ also fully restored mismatch repair to the depleted extract, indicating DNA polymerase δ is required for mismatch repair in human cells. However, due to the presence of DNA polymerases α and ϵ in the depleted extract, potential involvement of one or both of these activities in the reaction cannot be excluded.

Mismatch repair ensures genetic stability by correcting DNA biosynthetic errors and by blocking recombination between diverged DNA sequences (reviewed in Refs. 1–3). Defects in human MutS α or MutL α result in genetic destabilization (4–8), are the genetic basis of tumor predisposition in hereditary nonpolyposis colorectal cancer kindreds (9–13), and have been implicated in a subset of sporadic cancers (5, 14–17). In addition to its ability to recognize mismatched base pairs (16, 18), MutS α also recognizes certain classes of DNA damage (19, 20), and MutS α or MutL α defects are associated with resistance to certain DNA-damaging agents, including several that are used in anticancer chemotherapy (4, 21–26).

The *Escherichia coli* mismatch repair reaction has been reconstituted *in vitro* (27, 28). Briefly, MutS recognizes a mismatch and MutL binds to this complex. This activates a latent, MutH-associated endonuclease that incises the unmethylated strand of a hemimethylated d(GATC) sequence, which may be located either 5' or 3' to the mispair. The nick serves as the primary signal that directs repair to the unmethylated strand, and in the presence of DNA helicase II, exonucleolytic excision initiates at the nick and proceeds toward the mismatch through the action of either RecJ or exonuclease VII when incision is 5' to the mismatch, or exonuclease I when the nick is 3' to the mispair (28, 29). The resulting single-strand gap is then filled by DNA polymerase III holoenzyme, and covalent continuity is restored to the repaired strand by DNA ligase. Single-stranded DNA-binding protein is also a required com-

ponent of the reaction.

Four activities have been implicated in the human mismatch repair reaction. MutS α (a heterodimer of MSH2 and MSH6) and MutL α (a heterodimer of MLH1 and PMS2) were isolated by virtue of their ability to restore strand-specific mismatch correction to nuclear extracts of repair-deficient tumor cell lines (16, 18, 30). In addition to its subunit function in MutS α , MSH2 forms a molecular complex with the MutS homolog MSH3 (31, 32). This MutS β complex binds to insertion/deletion mismatches and presumably plays a role in their processing. Proliferating cell nuclear antigen (PCNA)¹ has also been implicated in the human mismatch repair reaction (33). However, the activities involved in the excision stage of the reaction and the DNA biosynthetic activity responsible for repair synthesis remain to be identified. To this end we have used biochemical methods to deplete nuclear extracts of a required repair activity and have used depleted extracts as the basis for an *in vitro* complementation assay to isolate additional components of the system. We describe here experiments implicating DNA polymerase δ in the human mismatch repair reaction.

EXPERIMENTAL PROCEDURES

Preparation of Nuclear Extracts and a Depleted Extract Deficient in Repair—Human HeLa S₃ cells were cultured and nuclear extracts prepared as described previously (16, 34). All fractionation steps were performed at 0–4 °C. Samples of repair-proficient HeLa nuclear extract were resolved independently on heparin-Sepharose 4B (35) and the immobilized dye resin Reactive Brown 10 (Sigma) to generate mismatch repair deficient fractions. HeLa nuclear extract (93 mg) was diluted with 0.025 M HEPES-KOH (pH 7.6), 0.1 mM EDTA, 2 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride (PMSF, concentration relative to a 23 °C saturated solution in isopropanol), and 1 μ g/ml leupeptin (buffer A) to make the conductivity equivalent to buffer A containing 0.1 M KCl, centrifuged briefly to remove insoluble material, and applied to a 5.5 cm \times 1.5-cm² column of heparin-Sepharose 4B equilibrated in buffer A containing 0.1 M KCl. The material that flowed through the column, representing 80% of the starting material, was briefly dialyzed against buffer A containing 0.1 M KCl, frozen in small aliquots with liquid nitrogen, and stored at –80 °C (Fraction H1). Nuclear extract (70 mg) was resolved in a similar manner on a 5.1 cm \times 0.8-cm² column of Reactive Brown 10 equilibrated in 0.025 M Tris-HCl (pH 7.6), 0.1 mM EDTA, 2 mM dithiothreitol, 0.1% (saturated) PMSF, 1 μ g/ml leupeptin, and 0.05 M KCl. The material that did not bind the resin, representing 60% of the starting material, was treated as above (Fraction R1).

Mismatch Repair and DNA Polymerase Assays—*In vitro* mismatch repair assays with nuclear extracts (50 μ g) and 100 ng (24 fmol) of heteroduplex f1MR DNA were performed as described previously (5, 16, 34). Briefly, this method uses a 6.4-kilobase pair circular heteroduplex that contains a site-specific, strand-specific incision to provide strand direction to the reaction. The presence of the mismatch within overlapping sites for two restriction endonucleases serves to block cleavage by either activity, with repair conferring sensitivity to one endonuclease or the other depending on which strand is corrected. Assay for comple-

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¹ The abbreviations used are: PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; HRP, horseradish peroxidase.

mentation of depleted nuclear extract was performed in a similar manner, except reactions (20 μ l) contained 35 μ g of Fraction H1 and 27 μ g of Fraction R1 (referred to below as H1/R1) in place of nuclear extract, and KCl was maintained at a final concentration of 105–110 mM taking into account salt contributed by depleted extract and column fractions. Reactions were terminated by the addition of 5 μ l of 0.5 M EDTA and repair scored as described previously (5, 34).

DNA polymerase activity was determined in reactions (50 μ l) containing 0.02 M Tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10 μ g of acetylated bovine serum albumin, 0.01 M MgCl₂, 50 μ M each dATP, dGTP, and dTTP, 50 μ M [³²P]dCTP (600–1900 cpm/pmol), and 40 μ g of sonicated, DNase I-activated salmon sperm DNA. Trichloroacetic acid-insoluble radioactivity was determined after incubation at 37 °C for 60 min. When indicated, DNA polymerase activity was also determined in reactions (50 μ l) containing 0.04 M Tris-HCl (pH 6.8), 2 mM 2-mercaptoethanol, 10 μ g of acetylated bovine serum albumin, 1.5 mM MgCl₂, 50 μ M dATP, 50 μ M [³²P]dTTP (600–800 cpm/pmol), and 1 μ g of either poly(dA-dT) or poly(dA)-oligo(dT)_{12–18}, (20:1 nucleotide ratio). Acid-insoluble radioactivity was determined following incubation at 37 °C for 30 min.

The polymerase inhibitor butylphenyl-dGTP was a gift from George Wright (University of Massachusetts, Worcester, MA), and aphidicolin was from Sigma.

Isolation of Activity That Complements Depleted HeLa Nuclear Extract—HeLa nuclear extract (34) derived from 200 liters of culture (Fraction I, 100 ml, 23 mg/ml) was thawed on wet ice, diluted in buffer A to yield a conductivity equivalent to buffer A containing 0.02 M KCl, centrifuged at 12,000 \times *g* to remove insoluble material, and applied at 100 ml/h to a phosphocellulose column (Whatman P-11, 18 cm \times 12.6 cm²) equilibrated with buffer A containing 0.02 M KCl. The column was washed with 450 ml of equilibration buffer and developed with a linear gradient of KCl (2900 ml, 0.02–0.9 M) in buffer A. Fractions that restored repair of a 5'-G-T heteroduplex to depleted H1/R1 nuclear extract eluted at ~0.39 M KCl (Fraction II, 250 ml). Fraction II was dialyzed against buffer A containing 0.05 M KCl until the conductivity was equivalent to buffer A containing 0.085 M KCl, clarified as above, and applied at 25 ml/h to a heparin-Sepharose 4B column (14.6 cm \times 1.75 cm²) equilibrated in buffer A containing 0.085 M KCl. The column was washed with 50 ml of equilibration buffer and developed with a 260-ml linear gradient of KCl (0.085–0.5 M) in buffer A. Fractions complementing depleted H1/R1 extracts eluted as a broad peak centered at ~0.2 M KCl (Fraction III, 84 ml). After freezing in liquid nitrogen and storage at –80 °C, Fraction III was later thawed on wet ice and dialyzed against 0.025 M Tris-HCl (pH 7.6), 0.1 mM EDTA, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A (buffer B) containing 0.085 M KCl until the conductivity was equivalent to buffer B containing 0.1 M KCl. After clarification by centrifugation, the dialysate was applied at 0.7 ml/min onto a 1.0-ml HR 5/5 Mono Q (Pharmacia) FPLC column equilibrated in buffer B containing 0.1 M KCl. The column was washed with 2 ml of equilibration buffer and eluted with a 20-ml linear gradient of KCl (0.1–0.5 M) in buffer B. Fractions were supplemented with dithiothreitol to 1 mM and PMSF to 0.1% by the addition of concentrated stock solutions. Fractions complementing depleted H1/R1 extracts eluted at ~0.31 M KCl (Fraction IV, 3.5 ml). Fraction IV was dialyzed against 0.025 M potassium phosphate (pH 7.0), 0.1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A (buffer C) also containing 0.085 M KCl until the conductivity was approximately equivalent to buffer C containing 0.085 M KCl. The dialysate was applied at 0.7 ml/min onto a 1.0-ml HR 5/5 Mono S (Pharmacia) FPLC column equilibrated in buffer C containing 0.085 M KCl. The column was washed with 3 ml of equilibration buffer, eluted with a 15-ml linear gradient of KCl (0.085–0.5 M) in buffer C, and fractions adjusted to 1 mM dithiothreitol and 0.1% PMSF as before. Fractions complementing depleted H1/R1 extracts eluted at ~0.3 M KCl, and individual fractions were either used directly or frozen in small aliquots with liquid nitrogen and stored at –80 °C. Fraction V in Table I represents the column fractions (17–19, 2.4 ml) containing the peak of repair complementing activity.

Immunological Methods—Polyclonal antibody ID α , raised in chicken against the recombinant p180 subunit of human DNA polymerase α , was kindly provided by Teresa Wang (Stanford University, Stanford, CA) (36). Antiserum to DNA polymerase δ was raised in a New Zealand White rabbit against the synthetic multiple antigenic peptide LYQKEVSHLSALEERFSRLW, corresponding to residues 1036–1055 of the catalytic subunit of calf thymus DNA polymerase δ (37). Antibodies to human DNA polymerase ϵ were generously provided by Stuart Linn (University of California, Berkeley). Monoclonal antibodies raised against recombinant rat PCNA were obtained from Boehringer Mann-

heim. Mouse monoclonal antibodies against human single-stranded DNA-binding protein (RPA) and rabbit antibodies raised against the DNA polymerase accessory factor RF-C were gifts from Jerard Hurwitz (Sloan-Kettering Cancer Center, New York). Rabbit anti-chicken IgG cross-linked to horseradish peroxidase (HRP), rabbit anti-mouse IgG cross-linked to HRP, and goat anti-rabbit IgG cross-linked to HRP were from Sigma. Sheep anti-mouse IgG cross-linked to HRP was purchased from Amersham, and ¹²⁵I-labeled goat anti-rabbit IgG was from ICN.

For immunological blot analysis, proteins were loaded onto 8% SDS-polyacrylamide gels and electrophoresed at 200 V in a mini-gel apparatus (Bio-Rad) until bromphenol blue present in the loading dye had run off the gel. Proteins were transferred to a nylon membrane (Bio-trans, ICN), which was probed with primary antibodies as indicated, and immune complexes visualized using an ECL chemiluminescence kit (Amersham) in the case of peroxidase linked secondary antibodies, or by autoradiography in the case of ¹²⁵I-labeled secondary antibodies.

DNA Polymerases and PCNA—Immunopurified human KB cell DNA polymerase α was a gift of William Copeland (NIEHS, Research Triangle Park, NC). One unit of DNA polymerase α activity represents the amount of enzyme required to incorporate 1 nmol of labeled dNMP into activated salmon sperm DNA in 1 h at 37 °C (38). Purified calf thymus DNA polymerase δ was a generous gift of Antero So (University of Miami, Miami, FL). One unit of DNA polymerase δ activity is defined as the incorporation of 1 nmol of dNMP into poly(dA)-oligo(dT) in 1 h at 37 °C in the presence of 2–4 μ g/ml PCNA (39). The plasmid pT7hPCNA, encoding human PCNA under the control of a T7 promoter, was a gift of Bruce Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Human PCNA was expressed in *E. coli* BL21(DE3) and purified to apparent homogeneity by a modification of the method of Fein and Stillman (40).

RESULTS

In an effort to isolate unidentified activities involved in human mismatch repair, including those for which mutations might render cell lines inviable, we sought to generate biochemically deficient nuclear subfractions that were defective in mismatch repair *in vitro*. HeLa nuclear extract was resolved into crude bound and unbound fractions on both heparin-Sepharose 4B and Reactive Brown 10 resins (see “Experimental Procedures”). None of the individual subfractions obtained in this manner were able to support mismatch repair *in vitro*, but reactions containing both unbound fractions (this mixture was designated H1/R1) converted DNA heteroduplexes into a new species with an electrophoretic mobility consistent with that expected for a gapped DNA molecule (data not shown). This suggested that H1/R1 was lacking one or more components required for repair DNA synthesis. This depleted fraction proved to be deficient in a single required activity, a DNA polymerase required for the repair synthesis reaction.

HeLa nuclear extract was resolved by chromatography on phosphocellulose, and column fractions were assayed for their ability to restore mismatch repair activity to depleted H1/R1 extract. This column yielded a single, symmetrical peak of complementing activity, which was further purified as described under “Experimental Procedures” and summarized in Table I. The most highly purified fraction (Fraction V) was enriched about 2,800-fold relative to nuclear extract.

H1/R1 complementing activity was purified by virtue its ability to restore G-T heteroduplex repair. In addition to its inability to support G-T heteroduplex repair, H1/R1 extract was also defective in repair of a two-nucleotide insertion/deletion mismatch. As shown in Fig. 1, repair of both types of mismatch was fully restored by addition of Fraction V. Since strand directionality of the human mismatch repair reaction can be provided by a nick located either 3' or 5' to the mismatch (41), the ability of Fraction V to restore repair with both heteroduplex orientations was also tested. Fig. 1 demonstrates that the activity of Fraction V is independent of mismatch \rightarrow nick orientation.

As noted above, although mismatch rectification did not occur in H1/R1 extract, heteroduplex processing was observed,

TABLE I
Purification of H1/R1 complementing activity from 200 liters of HeLa cells

Purification was performed as described under "Experimental Procedures." Protein was determined by the method of Bradford, utilizing bovine serum albumin as a standard (50).

Fraction	Total protein	Specific activity	Yield
	mg	pmol/mg	%
I. Nuclear extract	2270	0.20 ^a	100
II. Phosphocellulose	78	5.70	100
III. Heparin-Sepharose	3.4	98	75
IV. Mono Q	0.22	100	5.0 ^b
V. Mono S	0.036 ^c	560	4.5

^a Since nuclear extracts are repair proficient, the specific activity of Fraction I was extrapolated from the value for Fraction II, assuming 100% recovery of activity.

^b A low protein concentration and a single freeze-thaw cycle resulted in significant loss of activity.

^c Due to low abundance, the protein content of Fraction V was estimated by integration of the A₂₈₀ tracing generated during FPLC.

H1/R1	+	+	+	+	+	+	+	+
Fraction V	-	-	-	-	+	+	+	+
Nick	5'	5'	3'	3'	5'	5'	3'	3'
Mismatch	G•T	/CA ₁	G•T	/CA ₁	G•T	/CA ₁	G•T	/CA ₁

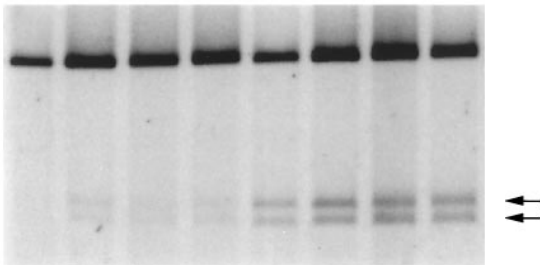


FIG. 1. Activity of the H1/R1 complementing factor is independent of the nature of the mismatch and heteroduplex orientation. Heteroduplex substrates contained a G-T mismatch or a d(CA) dinucleotide insertion/deletion mispair. The strand break directing repair was located either 5' or 3' to the mismatch, as indicated. The upper band corresponds to unrepaired substrate, and arrowheads indicate repair products.

with repair blocked at a late stage in the reaction. This suggested that the depleted extract might be deficient in a DNA polymerase required for repair synthesis, and polymerase activity was found to co-purify with complementing activity (Fig. 2), prompting further test of this possibility by immunological blot analyses. Whereas DNA polymerases α , δ , and ϵ were all present in HeLa nuclear extract, depleted H1/R1 extract contained DNA polymerase α and reduced levels of pol ϵ (~5%), but pol δ was undetectable in this fraction (Fig. 3). On the other hand, pol δ was the only DNA polymerase detected in Fraction V of complementing activity, and levels of this polymerase estimated by immunological blot analysis correlated well with H1/R1 complementing activity during purification, strongly suggesting that DNA polymerase δ was the required activity lacking in the depleted extract. Although PCNA and the human single-stranded DNA-binding protein RPA were resolved from complementing activity during phosphocellulose chromatography, both were present in depleted extract, and this fraction also contained the polymerase accessory factor RF-C (Fig. 3 and data not shown).

The participation of DNA polymerase δ in mismatch repair is also supported by chemical inhibition of repair and DNA polymerase activities. Mismatch repair in HeLa nuclear extracts is blocked by aphidicolin, indicating involvement of DNA polymerases α , δ , and/or ϵ in the reaction (34). We have confirmed this observation with the finding that 100 μ M aphidicolin completely inhibited repair when depleted H1/R1 extract was com-

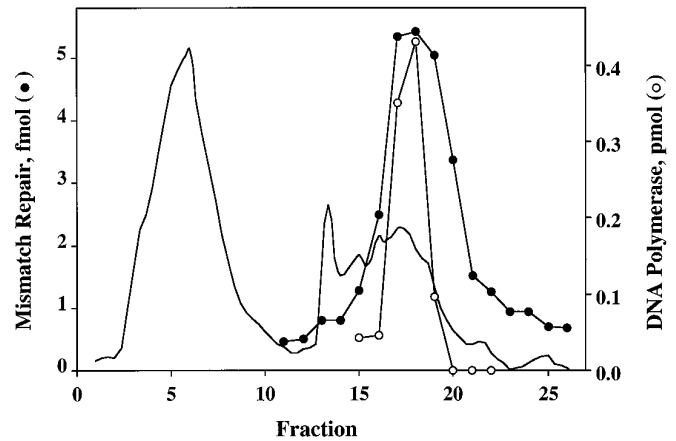


FIG. 2. Mono S chromatography of H1/R1-complementing activity. Complementing activity (Fraction IV) was resolved on a Mono S column as described under "Experimental Procedures." Samples (1 μ l) of each fraction were assayed for the ability to restore repair of a 5'-G-T heteroduplex to depleted H1/R1 extracts (●). DNA polymerase activity was determined with 5- μ l samples using activated salmon sperm DNA as substrate (○). Relative protein abundance is indicated by A₂₈₀ (solid line with no symbols).

plemented with Fraction IV of the purified activity, and DNA synthesis on activated salmon sperm DNA supported by Fraction V was reduced approximately 2-fold by 100 μ M aphidicolin. Furthermore, neither Fraction IV DNA polymerase activity nor repair activity in complemented H1/R1 extract was inhibited by 10 μ M butylphenyl-dGTP (data not shown), suggesting that DNA polymerase α does not have a critical role in mismatch repair (42).

While the foregoing experiments suggested that depletion of pol δ accounted for the repair deficiency of H1/R1 extract, H1/R1 complementing activity was only partially purified, with Fraction V containing about 50 distinct protein species as judged by silver-stained SDS-polyacrylamide gels. To test the sufficiency of pol δ in restoration of repair to H1/R1 extract, the complementing activity of *bona fide* pol δ was tested. As shown in Fig. 4, mismatch repair in the depleted extract was restored to a similar degree by either calf thymus pol δ or Fraction V of the complementing activity isolated here. By contrast, human pol α did not complement the depleted extract. Furthermore, repair activity restored by either pol δ or Fraction V was further enhanced by about 40% upon supplementation of H1/R1 extract with additional exogenous PCNA. This finding is consistent with previous experiments implicating PCNA in repair (33), although in our case the presence of PCNA in the depleted extract (Fig. 3D) prevents a more quantitative assessment of the role of this activity in the reaction. It is noteworthy that PCNA (150–200 ng) was found to stimulate DNA polymerase activity of isolated complementing activity about 2-fold on activated salmon sperm DNA or poly(dA-dT) template-primers, but by more than 10-fold with poly(dA)-oligo(dT) (data not shown). Stimulation of DNA polymerase activity by PCNA on this template-primer is a hallmark of pol δ (43, 44) and provides further evidence that this activity is the major DNA biosynthetic activity present in the fractions isolated here.

DISCUSSION

This work presents direct evidence for involvement of DNA polymerase δ in human mismatch repair. We have shown that pol δ is required for repair of base-base mismatches and small insertion/deletion heterologies, and that the requirement for this activity is evident when the single strand break that directs the reaction is located either 5' or 3' to the mispair. These findings indicate that pol δ plays a general role in the reaction.

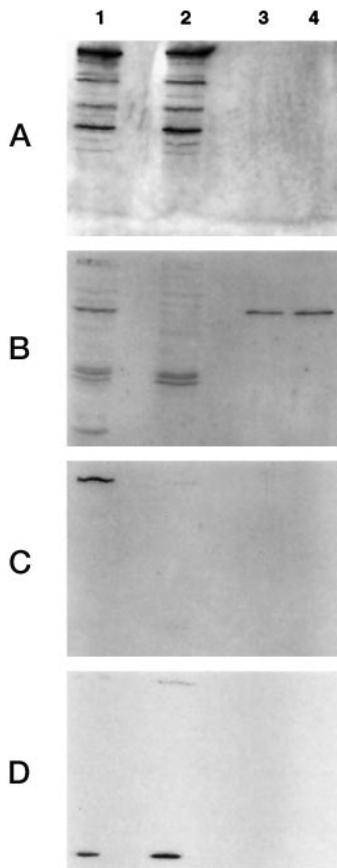


FIG. 3. Isolated complementing activity contains DNA polymerase δ , but is free of detectable polymerase α , polymerase ϵ , and PCNA. Immunological blot analysis was performed as described under "Experimental Procedures." Panel A, DNA polymerase α antibody. Panel B, DNA polymerase δ antibody. Panel C, DNA polymerase ϵ antibody. Panel D, PCNA antibody. In each case lane 1 is complete HeLa nuclear extract (50 μ g), lane 2 is depleted H1/R1 nuclear extract (50 μ g), lane 3 is fraction 16 from the Mono S column (Fig. 2, 440 ng), and lane 4 is fraction 19 (290 ng). Two faint bands with the approximate mobility of the pol δ catalytic subunit are evident in the H1/R1 fraction (lane 2 of panel B). However, analysis of several gels of this sample has indicated that full-length pol δ migrates between these two bands. Hence, H1/R1 appears to be essentially free of detectable pol δ , although we cannot rule out presence of degradation products derived from this polypeptide.

While we cannot exclude participation of DNA polymerases α or ϵ in mismatch repair due to the presence of significant levels of both activities in depleted H1/R1 extract, the observed insensitivity of repair to butylphenyl-dGTP renders pol α an unlikely candidate. Furthermore, while the residual level of pol ϵ in repair-deficient, depleted extract ($\sim 5\%$ of that present in fully active HeLa extracts, Fig. 3) was insufficient to support repair, isolated complementing activity was devoid of detectable levels of this polymerase. Thus, either the human mismatch repair reaction requires both pol δ and pol ϵ , or pol δ is sufficient to meet the polymerase requirement in the reaction. We tend to favor the latter possibility. Results obtained with *Saccharomyces cerevisiae* DNA polymerase mutants may also bear on this point. Strand *et al.* (45) demonstrated that inactivation of the proofreading function of yeast pol δ results in a 5–10-fold increase in mutation rate of d(CA)_n repeat sequences, but proofreading mutations in pol ϵ do not. It is difficult to reconcile the different phenotypes based solely on the functional roles of the two polymerases in chromosome replication (46), but differential participation of the two enzymes in mismatch repair might account for this difference since d(CA)_n mutability correlates strongly with mismatch repair defects (45).

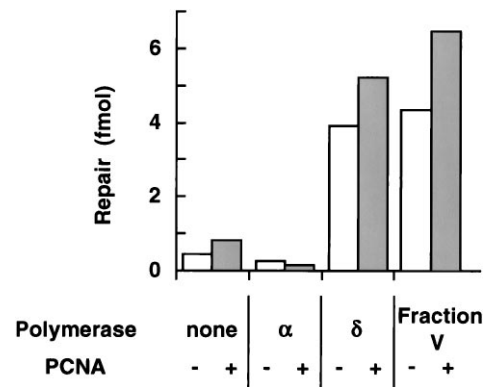


FIG. 4. DNA polymerase δ , but not α restores mismatch repair to H1/R1 nuclear extract. Mismatch repair assays (20 μ l, see "Experimental Procedures") contained depleted H1/R1 nuclear extract, and as indicated, KB cell DNA polymerase α (0.4 units, assayed on activated salmon sperm DNA; Ref. 38), calf thymus DNA polymerase δ (2.5 units, assayed on poly(dA)-oligo(dT) in the presence of PCNA; Ref. 39), 38 ng of Fraction V, and 100 ng of PCNA.

Umar *et al.* (33) have recently found that dinucleotide repeats are unstable in yeast PCNA mutants and, as judged by two hybrid analysis, that PCNA interacts with yeast MutL homologs PMS1 and MLH1 (human PMS2 is a homolog of yeast PMS1). They also demonstrated that mismatch repair in human cell extracts is blocked prior to the excision step by p21^{WAF1} or by a p21 peptide, which binds to PCNA and inhibits PCNA-dependent DNA replication (47). While PCNA involvement would be consistent with a repair role for pol δ since the two proteins are known to interact (44, 48), a definitive argument cannot be based on this observation because PCNA probably also interacts with pol ϵ (49).

Available information on the nature of the human mismatch repair reaction has indicated striking similarities to the bacterial pathway, including conservation of specificity and mechanism, and involvement of homologs of MutS and MutL at early steps of the reaction (reviewed in Ref. 2). The work described here extends these similarities to the DNA polymerase requirement. Repair DNA synthesis in *E. coli* methyl-directed mismatch correction is catalyzed by DNA polymerase III holoenzyme, the enzyme that is also responsible for chromosome replication. Like DNA polymerase III, pol δ plays a major role in the synthesis of new DNA strands at the replication fork, a function it probably shares with pol ϵ (46).

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