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DNA synthesis on discontinuous templates by human DNA polymerases: implications for non-homologous DNA recombination

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ABSTRACT

DNA polymerases catalyze the synthesis of DNA using a continuous uninterrupted template strand. However, it has been shown that a 3′→5′ exonuclease-deficient form of the Klenow fragment of *Escherichia coli* DNA polymerase I as well as DNA polymerase of *Thermus aquaticus* can synthesize DNA across two unlinked DNA templates. In this study, we used an oligonucleotide-based assay to show that discontinuous DNA synthesis was present in HeLa cell extracts. DNA synthesis inhibitor studies as well as fractionation of the extracts revealed that most of the discontinuous DNA synthesis was attributable to DNA polymerase α. Additionally, discontinuous DNA synthesis could be eliminated by incubation with an antibody that specifically neutralized DNA polymerase α activity. To test the relative efficiency of each nuclear DNA polymerase for discontinuous synthesis, equal amounts (as measured by DNA polymerase activity) of DNA polymerases α, β, δ (± PCNA) and ε (± PCNA) were used in the discontinuous DNA synthesis assay. DNA polymerase α showed the most discontinuous DNA synthesis activity, although small but detectable levels were seen for DNA polymerases δ (+PCNA) and ε (–PCNA). Klenow fragment and DNA polymerase β showed no discontinuous DNA synthesis, although at much higher amounts of each enzyme, discontinuous synthesis was seen for both. Discontinuous DNA synthesis by DNA polymerase α was seen with substrates containing 3 and 4 bp single-strand stretches of complementarity; however, little synthesis was seen with blunt substrates or with 1 bp stretches. The products formed from these experiments are structurally similar to that seen *in vivo* for non-homologous end joining in eukaryotic cells. These data suggest that DNA polymerase α may be able to rejoin double-strand breaks *in vivo* during replication.

INTRODUCTION

DNA polymerases catalyze the synthesis of DNA using a continuous uninterrupted template strand. However, *in vitro* evidence suggests that, at least for the exonuclease-free large fragment of *Escherichia coli* DNA polymerase I (exo⁻ KF), DNA synthesis can occur across a template with a discontinuous phosphodiester backbone (1,2). Clark (1) demonstrated that exo⁻ KF joined a blunt radiolabeled double-strand oligonucleotide to an unlinked single-strand homopolymer of various lengths or a heteropolymer and that DNA synthesis proceeded to the end of the template strand. Interestingly, this reaction appeared to involve as an intermediate the addition, in a non-templated manner, of a single nucleotide by the DNA polymerase to the 3′-end of the labeled strand of the duplex substrate. Discontinuous DNA synthesis was dependent on complementarity between the 1 nt 3′-protruding single strand (3′-PSS) and the nucleotide at the 3′-terminus of the unlinked oligonucleotide. It was earlier shown that in addition to exo⁻ KF, a number of eukaryotic DNA polymerases could carry out non-templated addition to the end of a blunt substrate (3,4). Our laboratory has shown that in addition to blunt-ended substrates, exo⁻ KF as well as a DNA polymerase from *Thermus aquaticus* (Taq) can perform discontinuous DNA synthesis using substrates that have short complementary stretches (2). The substrates are joined together, resulting in an overlap of between 1 and 4 nt depending on the length of the complementarity at the termini. DNA polymerase can initiate synthesis across the aligned overlap. Furthermore, the rate and extent of overlap formation is dependent on the length of the complementarity as well as the presence of any mismatches. In fact, the efficiency of discontinuous DNA synthesis is much higher for overlap substrates than for blunt-end substrates (5). At early time points, the amount of discontinuous synthesis seen with substrates containing 3 or 4 bp overlaps is at least 10-fold greater than that for blunt-end substrates or substrates with 1 or 2 bp overlaps.

The ability of some DNA polymerases to join unlinked template strands has important biological implications with respect to single- and double-strand breaks encountered during

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the course of replication and following the induction of DNA damage.

Ionizing radiation and radiomimetic agents cause various DNA lesions, including base damage, single-strand breaks and double-strand breaks (6–9). Of these lesions, double-strand breaks have potentially the most deleterious consequences for mammalian cells, because if not repaired faithfully, they result in point mutations, small and large deletions and large chromosomal rearrangements (10). Therefore, all organisms have evolved mechanisms for processing double-strand breaks. In *Escherichia coli* and *Saccharomyces cerevisiae*, homologous recombination accounts for nearly all of the repair of double-strand breaks (11). In higher eukaryotes, particularly mammalian cells, homologous recombination occurs infrequently compared with non-homologous end joining (12). Unlike homologous recombination, non-homologous end joining (illegitimate or non-homologous recombination) depends less on sequence homology and usually involves the gain or loss of DNA (12–14). Therefore, although the structural integrity of the DNA is restored, non-homologous recombination is not a true repair mechanism, because the informational integrity of the DNA is seldom restored. A number of studies have demonstrated that non-homologous end joining can involve the pairing of complementary DNA ends of 1–5 nt (15,16). Generally, these stretches are too short to stabilize the two ends thermodynamically, implying that there must be an alignment factor(s) to help rejoin and stabilize the ends. Furthermore, the types of recombined junctions during non-homologous end joining in mammalian cells are consistent with those seen for DNA synthesis by exonuclease-free prokaryotic DNA polymerases on discontinuous templates.

As a first step in determining whether a subset of non-homologous events in mammalian cells results from discontinuous DNA synthesis by endogenous DNA polymerases, we tested crude extracts from mammalian cells and purified human DNA polymerases for their ability to join unlinked DNA templates and initiate DNA synthesis.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). T4 polynucleotide kinase was obtained from Gibco BRL (Gaithersburg, MD). Exonuclease-free *E.coli* DNA polymerase I Klenow fragment was obtained from either New England BioLabs (Beverly, MA) or US Biochemical (Cleveland, OH). Restriction endonucleases and mung bean nuclease were purchased from New England BioLabs. Oligonucleotides were either synthesized using an Applied Biosystems (Foster City, CA) 391 DNA synthesizer and purified by HPLC or purchased from Operon Technologies Inc. (Alameda, CA), already HPLC purified, or from Genosys Biotechnologies (The Woodlands, TX), already polyacrylamide gel electrophoresis purified. Poly(dA)$_{3000–5000}$ and oligo(dT)$_{12–18}$ were purchased from Midland Certified Reagent Co. (Midland, TX). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO).

Tissue culture

Chinese hamster ovary K1 (CHO-6), xrs-5 and V3 cells were grown as monolayer cultures in McCoy's medium supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA), glutamine and penicillin/streptomycin (Gibco BRL). HeLa cells were obtained from the Tissue Culture Facility at the University of California, Berkeley.

Preparation of hamster extracts

Nuclear extracts were prepared by the method of Dignam (17,18). Briefly, monolayer cultures were scraped from 850 cm^2 roller bottles and washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). HeLa cells were centrifuged and washed in PBS. The cells were swollen in hypotonic solution (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT) and Dounce homogenized (~30 strokes, tight pestle). Cells were centrifuged, the nuclei resuspended in hypertonic solution (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM PMSF, 0.5 mM DTT) and the soluble protein fraction was dialyzed against 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT. Aliquots were quick-frozen in liquid nitrogen and stored at –80°C until used in these studies.

DNA polymerases and assays

Purified human recombinant DNA polymerase β was obtained from S.Wilson (National Institute of Environmental Health Sciences) and D.Wilson (Lawrence Livermore National Laboratory). DNA polymerase δ was a generous gift of A.So (University of Miami). To purify DNA polymerases α and ε and proliferating cell nuclear antigen (PCNA), crude cellular extracts were prepared following the procedure of Nishida *et al.* (19), except that the crude extracts were dialyzed against 25 mM KPO_4, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% glycerol. A 20–50% ammonium sulfate precipitation was made from the crude extracts and dialyzed against 50 mM Tris–HCl, pH 8.0, 1 mM DTT, 1 mM EDTA, 10% glycerol (TDEG) and 50 mM NaCl (fraction III). The final protein concentration was 7–15 mg/ml. DNA polymerases α and ε and PCNA were purified according to previously published protocols (20) except that the order of the hydroxyapatite and monoS columns was transposed (see Table 1). All chromatography was performed using a Pharmacia FPLC system. Briefly, fraction III was loaded onto a 26 × 180 mm DEAE–Sephacel column pre-equilibrated with TDEG + 50 mM NaCl, washed with 2 column volumes and eluted with a 10 column volume gradient of 50–500 mM NaCl in TDEG. Thirteen milliliter fractions were collected. DNA polymerase assays were performed as previously described (20,21). DNA polymerase α and ε activity co-eluted at ~210 mM NaCl (19). DNA polymerase δ (measured as PCNA-dependent DNA synthesis) eluted in the flow-through as well as eluting early in the gradient (~70 mM NaCl), whereas most of the DNA polymerase β activity (DNA synthesis in the presence of 150 mM NaCl) appeared in the 50–70% ammonium sulfate precipitation (data not shown). Polymerase α/ε activity was pooled (fraction IV), diluted to 180 mM NaCl and loaded onto a 16 × 130 mm P-11 phosphocellulose column. After a 2 column volume wash, the column was eluted with a 10 column volume gradient of 180–500 mM NaCl in TDEG. Co-eluted DNA polymerase α and ε activities were pooled (fraction V), ammonium sulfate precipitated, resuspended and dialyzed against TDEG + 50 mM NaCl. Fraction V was then loaded on a 1 ml monoS (HR 5/5; Pharmacia, Uppsala, Sweden)

Table 1. Purification of DNA polymerase α

		Volume (ml)	Total Protein[a] (mg)	Activity (U/ml)	Specific Activity (U/mg)	Fold Purification
	crude	166	1122	45.3[c]	6.70	-
III.	20%-50% $(NH_4)_2SO_4$ cut	27.0	1013	123.7[c]	3.29	-
IV.	DEAE pool	75.0	69.2	78.9[d]	85.5	26
V.	phosphocellulose pool	1.50	0.27	95.9[d]	539	164
VI.	monoS pool	1.00	0.13	191.8	1500	455
VII.	HAP fraction 40	0.30	0.003[b]	94.8	9480	2880

[a]Bradford assays were used to measure protein amounts, except where noted.
[b]Absorbance at 280 nm was used to estimate the protein amount in fraction VII.
CActivity reflects the presense of various DNA polymerases.
[d]Activity reflects the presense of DNA polymerase ε.

column and eluted with a 50–500 mM NaCl gradient in TDEG. The vast majority of DNA polymerase ε did not bind to the column, although a small amount eluted early in the gradient. DNA polymerase ε was pooled and frozen for later use in these studies. DNA polymerase α eluted at ~215 mM NaCl. Polymerase α activity was pooled (fraction VI) and dialyzed against 20 mM potassium phosphate, pH 8.0, 5 mM DTT, 1 mM EDTA and 20% glycerol (PDEG). It was then loaded onto a 1 ml hydroxyapatite column and eluted with a 20–500 mM potassium phosphate gradient. The peak of DNA polymerase α activity (fraction VII) eluted at 220 mM potassium phosphate.

PCNA eluted from the DEAE–Sephacel column at 285 mM NaCl. Pooled PCNA was dialyzed against PDEG and loaded onto a hydroxyapatite column. The flow-through was then loaded on a monoQ column and eluted with a TDEG plus 50–600 mM NaCl gradient. PCNA eluted at ~445 mM NaCl. PCNA was detected by western blotting with a mouse monoclonal antibody (PC10) from Santa Cruz Biotechnologies (Santa Cruz, CA).

Discontinuous DNA synthesis assay

A schematic of the assay conditions used in this study is shown in Figure 1. The assay relies on the fact that when the radiolabeled double-stranded substrate (L:C, labeled:complemetary) joins with the single-stranded template primer (T), DNA polymerase can use the 3′-OH group on the labeled strand (L) to synthesize DNA across the joined DNA molecules, using the T strand as template. For most experiments, the double-stranded substrate contained a 3 nt 3′-PSS complementing the 3′-most nucleotides on the T strand (although for the experiments described in Table 3, other ends were also used). This small complementary region is used by the DNA polymerase complex to facilitate end joining.

Using the protocol supplied by the manufacturer, we 5′-end-labeled the L strand with [γ-^{32}P]ATP and separated it from unincorporated nucleotides by gel filtration on a 1 ml Sephadex G25 fine spin column (Pharmacia, Uppsala, Sweden) and a Microcon 3 (Amicon, Beverly, MA) microconcentrator. The L strand was annealed to its complementary strand (C) by heating to 90°C for 2 min and slowly cooling to ambient temperature.

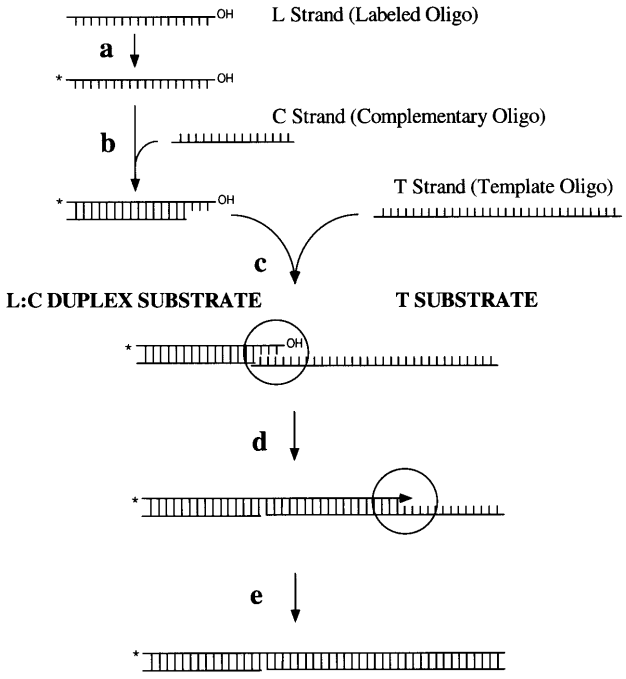

Figure 1. Schematic of the discontinuous DNA synthesis assay. (**a**) An oligonucleotide (L) is end-labeled (asterisk) with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and (**b**) annealed to a complementary oligonucleotide C. (**c**) The L:C duplex and the template oligonucleotide T are incubated at 25°C with extracts in 25 mM Tris–HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$ and 1 mM each dNTP, dGMP, TMP and DTT for various times. For more highly purified DNA polymerase α fractions, the reaction conditions were changed to pH 7.0, 20 mM KCl and incubation at 37°C; BSA (0.2 mg/ml) was also added. All other reactants remained the same. (**d**) If DNA end joining occurs, DNA polymerase (represented as a circle) will initiate synthesis from the 3′-OH group of the L strand and extend synthesis, using the T strand as template. (**e**) Full-length extension products are denatured and resolved on 12% denaturing polyacrylamide gels. The L strand of the duplex is an 18mer, with a 3 nt 3′-PSS. The discontinuous DNA synthesis reaction lengthens the L strand from 18 nt to a longer species, depending on the length of the T strand. Using a 31 nt single-stranded T strand with 3 nt complementing the 3′-PSS of the L:C duplex results in a 46 nt product.

The L:C duplex and the T strand were added to the DNA end joining reaction mixture at a molar ratio of 1:10 (83 pmol:830 pmol/ 25 µl reaction), although the reaction proceeded at all molar ratios tested (1:5, 1:20 and 1:40; data not shown). The reaction mixture also contained 25 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 1 mM each deoxynucleotide triphosphate (dNTP), dGMP, TMP and DTT and 2–30 µg protein extract. In crude cell extracts, the reactions were carried out at 25°C to minimize non-specific nuclease activity. Further along in the purification, the temperature was increased to 37°C. As a positive control, reactions with exo⁻ KF were run in parallel (2,5).

Electrophoresis

Denaturing electrophoresis loading dye (80% v/v formamide, 10 mM NaOH, 1 mM EDTA, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol, 0.1% w/v orange G) was added at each time point in a reaction. The double-stranded DNA products denatured after being incubated at 95°C for 5 min and were then quick-chilled on ice for several minutes.

Samples were applied to a 12% denaturing polyacrylamide (19:1 acrylamide:bisacrylamide) sequencing gel and subjected to electrophoresis at a voltage of 40 V/cm (60 W). After electrophoresis, the gels were dried and exposed to XAR5 film (Kodak, Rochester, NY) and a Molecular Dynamics (Sunnyvale, CA) PhosphorImager SE screen. Data were quantified using the ImageQuant software supplied with the phosphorimager.

Restriction mapping and junction sequencing

For restriction mapping, T substrates containing an *Eco*RI site within 21 nt of the expected rejoin junction or an *Eco*RV site at the expected rejoin junction were used in the discontinuous DNA synthesis reaction. After incubation for either 4 or 24 h, the products were phenol/chloroform extracted. The products were split and treated with either *Eco*RI or *Eco*RV or left untreated for 1 h at 37°C.

Sequencing across the junction was performed by thermal cycle sequencing, using a primer complementary to the newly synthesized DNA in the discontinuous DNA synthesis reaction. The discontinuous DNA synthesis reactions were phenol/chloroform extracted, ethanol precipitated and treated with mung bean nuclease at 37°C for 1 h to remove any remaining single-stranded T DNA strands. The reactions were phenol/chloroform extracted again to remove the mung bean nuclease, ethanol precipitated and used in the thermal cycle sequencing reactions. The sequencing reactions were performed with either a ^{32}P-labeled primer or by direct incorporation of [α-^{32}P]dCTP using Taq DNA polymerase (Perkin-Elmer, Branchburg, NJ) (22).

DNA polymerase inhibitors

All inhibitors were added at the start of the reactions. Aphidicolin was dissolved in dimethyl sulfoxide (DMSO) and added to the reaction at a final concentration equivalent to that of dCTP (1 mM, although various concentrations were tested; data not shown). Stock solutions of NaCl and ddNTPs were made up in water; ddNTPs were used at a concentration identical to that of the dNTP concentration, since, like aphidicolin, they are competitive inhibitors relative to dNTPs. DMSO was used at a final concentration of 10% (v/v).

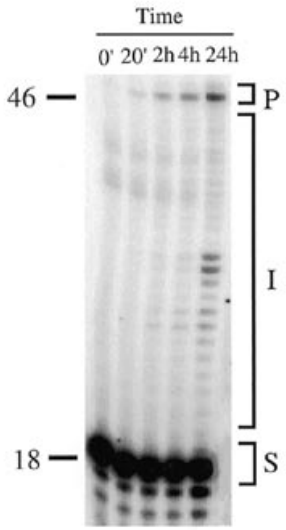

Figure 2. Discontinuous DNA synthesis in HeLa cell extracts. Joined products (P) and extension/degradation intermediates (I) are visible after incubation of HeLa cell extracts under discontinuous DNA synthesis conditions for various times. The L substrate (S) is also visible. The length of the products is consistent with that seen when only purified exo⁻ KF is used (run on the same gel; data not shown).

RESULTS

Discontinuous DNA synthesis in HeLa cell extracts

Purified bacterial DNA polymerases lacking 3′→5′ exonuclease activity can bridge unlinked template strands and initiate DNA synthesis across the joined strands, using double-stranded and single-stranded oligonucleotides as model substrates for double-strand breaks (1,2). To determine whether this occurred in eukaryotic cells, we initially used cellular extracts from HeLa cells in a discontinuous DNA synthesis assay (Fig. 1). Discontinuous DNA synthesis can occur if a DNA polymerase or some other factor in the cell extract aligns the double-strand oligonucleotide (L:C) to the single-strand template (T) strand. The DNA polymerase can then initiate synthesis from the 3′-OH group on the labeled (L) strand of the duplex, cross the joined overlap (usually a 3 bp overlap) and continue DNA synthesis using the T strand as template. After incubation, extracts from HeLa cells (Fig. 2) produced a full-length product on 12% denaturing polyacrylamide gels, demonstrating that human cells can perform discontinuous DNA synthesis across the two substrates. The lengths of the products are consistent with the expected values for the given substrates and with the exo⁻ KF positive controls. We observed degradation/intermediate products in the extracts that we attribute mainly to endogenous 3′→5′ exonuclease activity as well as incomplete or distributive synthesis.

The amount of discontinuous DNA synthesis correlated directly with the amount of extract used in the reactions (data not shown). Furthermore, no discontinuous DNA synthesis was observed when the extracts were heat denatured (65°C, 15 min), treated with SDS (0.5%), incubated without the L:C, T or both substrates or replaced with bovine serum albumin (data not shown).

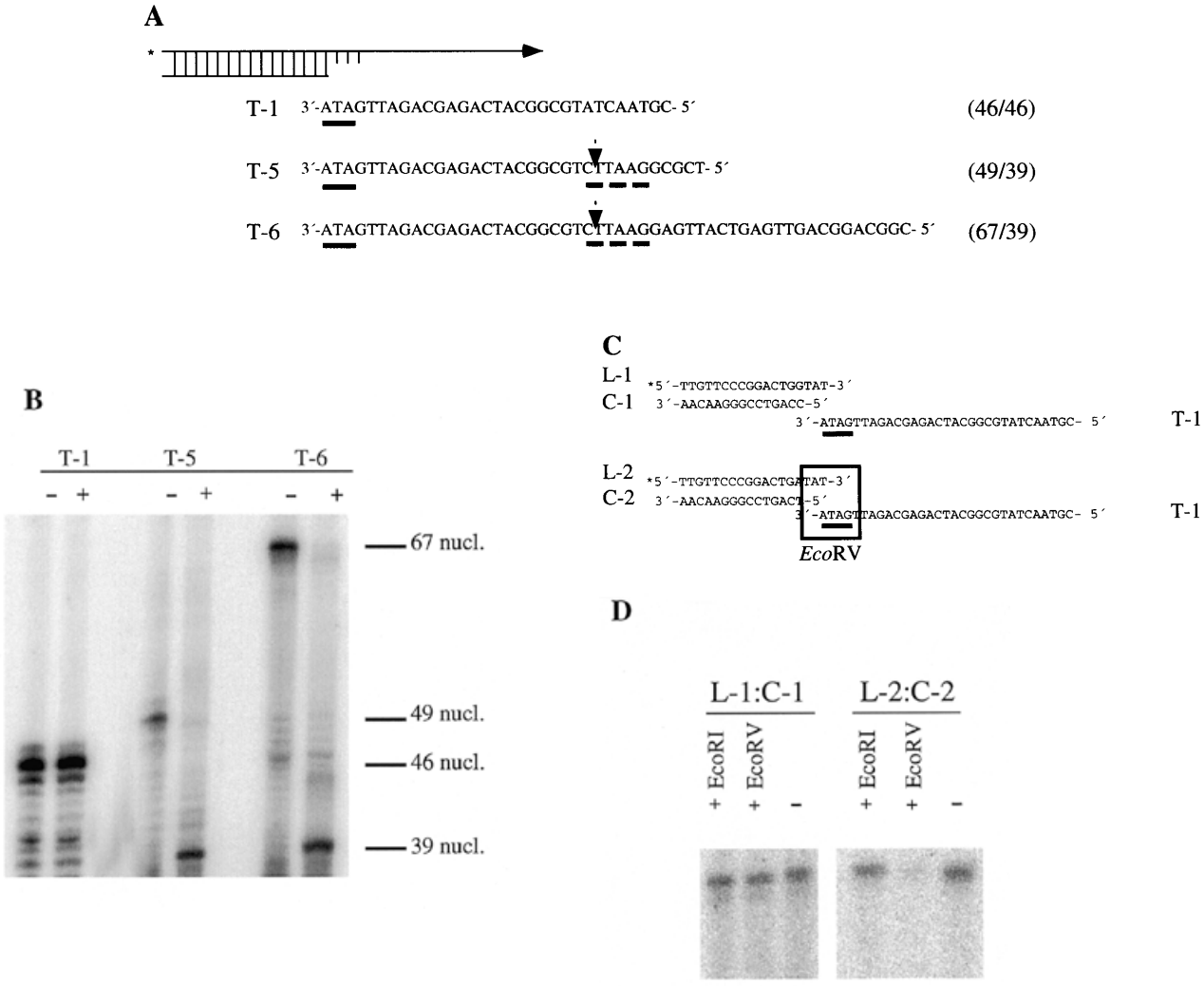

Figure 3. Restriction mapping. (**A**) The sequence of the T strands used for restriction mapping and location of the *Eco*RI sites (dotted underline) are shown (T-1 has no *Eco*RI site). The size of the full-length product before and after *Eco*RI digestion is shown in parentheses. The location of the *Eco*RI break on the extended L strand is shown as an arrow above the T-5 and T-6 strands. The 3 nt site on the T strand, complementary to the 3′-PSS of the L strand, is underlined. (**B**) The gel shows migration of the discontinuous DNA synthesis products before (–) and after (+) *Eco*RI digestion for the various T strands. (**C**) The sequence of the L-1:C-1 and L-2:C-2 duplexes is shown. If discontinuous DNA synthesis occurs with no nucleolytic processing and with annealing of the L:C duplex and T strand at the 3 nt site of complementarity (underline), an *Eco*RV site is generated in the L-2:C-2 reaction product but not in the L-1:C-1 reaction product. (**D**) The gel shows nearly complete elimination of the full-length product after *Eco*RV treatment in the L-2:C-2 reaction but not in the L-1:C-1 reaction. An *Eco*RI/*Eco*RV negative control digestion (–) is shown. All lengths were measured by comparing migration of the bands against known standards run on the same gel.

Characterization of the joined products

To determine whether the newly synthesized DNA resulted from rejoining of the T strand to the L:C duplex, we used T strands of various lengths: our standard T-1 strand (31mer); T-5 (34mer); T-6 (52mer). All three strands contained a 3 nt region of complementarity to the L strand at their 3′-ends. Additionally, T-5 contained an *Eco*RI site 4 nt from the 5′-end and T-6 contained an *Eco*RI site near the middle of the strand (Fig. 3A). All reactions with these strands gave full-length joined products consistent with their expected lengths (Fig. 3B). Furthermore, the lengths of the products were consistent with those seen in the exo$^-$ KF positive controls (data not shown).

When the reaction products were restriction digested with *Eco*RI, the full-length products decreased in size for T-5 and T-6, but not for T-1, because T-1 has no *Eco*RI site (Fig. 3B). For T-5, the full-length product decreased from 49 to 39 nt; for T-6, the full-length product decreased from 67 to 39 nt. Both products were reduced in size to a position consistent with the location of the *Eco*RI site in their respective T strands. Because there was no other *Eco*RI site in any other substrate, these data indicate that the T strand joined with the L:C duplex and that DNA synthesis extended the L strand, using the T strand as template.

To characterize the sequence at the site of the junction, we changed the G residue immediately 5′ of the 3′-PSS in the standard L strand (L-1) to an A residue (L-2; Fig. 3C). If end joining occurs between the L-2:C-2 duplex and the T-1 strand, without any nucleolytic processing, an *Eco*RV site is generated. When the reaction products were digested with *Eco*RV, nearly all of the full-length products decreased in size for the L-2:C-2

Table 2. Effect of DNA polymerase inhibitors on discontinuous synthesis

Inhibitor	% End Joining
None	100
Aphidicolin, 1mM	20
NaCl, 150 mM	20
Dimethyl sulfoxide, 10%	125
ddNTPs, 1mM	75

[a]Data are normalized to the negative control at 2 h.

duplex, suggesting that an *Eco*RV site was formed at the junction (Fig. 3D). Full-length products from the L-1:C-1 duplex showed no *Eco*RV digestion.

To further confirm that the sequence we saw in the joined products was due to the two substrates, we sequenced the products by thermal cycle sequencing. The sequence confirmed the restriction mapping results; the full-length products showed no gain or loss of nucleotides at the site of the junction and the T strand was used as template for the newly synthesized DNA (data not shown).

DNA polymerase inhibitors in HeLa cell extracts

To determine which of the four nuclear DNA polymerases were involved in the discontinuous DNA synthesis reaction in HeLa cell extracts, we carried out a series of experiments to test whether various DNA polymerase inhibitors could inhibit the formation of full-length joined products (Table 2). The reaction was strongly inhibited by the presence of NaCl (150 mM) and aphidicolin, both known inhibitors of replicative DNA polymerases (α, δ and ε) (23,24). Conversely, the reaction was only weakly inhibited by ddNTPs, suggesting that DNA polymerase β, which is highly sensitive to ddNTPs (23,24), was not required for the discontinuous DNA synthesis seen in HeLa cell extracts. Furthermore, DMSO (10%) had a stimulatory effect in discontinuous DNA synthesis activity. DMSO is known to stimulate DNA polymerases α and δ but not the 220 kDa subunit of DNA polymerase ε (21,25).

Fractionation of HeLa cell extracts

Since the inhibitor experiments suggested that one or more of the replicative DNA polymerases was responsible for discontinuous DNA synthesis in crude cell extracts, we fractionated the extracts to determine which DNA polymerase(s) was responsible for the activity. Ammonium sulfate precipitations of 0–20, 20–50 and 50–70% were made. Although each fraction showed some discontinuous synthesis, most of the activity was concentrated in the 20–50% cut (fraction III). Most of the DNA polymerase β activity was found in the 50–70% cut (data not shown). Fraction III was loaded onto a DEAE–Sephacel column (see Materials and Methods) and eluted with a 50–500 mM NaCl gradient (Fig. 4A). Discontinuous DNA synthesis co-eluted with the peaks of DNA polymerase α and ε activities (fractions 45–49). No discontinuous DNA synthesis was observed in the flow-through or early in the gradient, where DNA polymerase δ eluted.

Immuno-neutralizing DNA polymerase α activity eliminated discontinuous DNA synthesis

To determine whether DNA polymerase α or ε was responsible for the discontinuous DNA synthesis, the anti-DNA polymerase α monoclonal antibody SJK 132-20 (a generous gift of T.S.-F.Wang, Stanford University) was added to the reaction. SJK 132-20 neutralizes DNA polymerase α activity (26). Incubation of SJK 132-20 (~1.0 μg/ml) with the peak DNA polymerase α fraction from the DEAE–Sephacel column resulted in a reduction in DNA synthesis activity to that of 15% of the control (data not shown). Furthermore, SJK 132-20 does not diminish DNA polymerase ε activity; indeed, it may actually stimulate activity (27). When SJK 132-20 was added to aliquots of fractions across the DEAE–Sephacel elution, all discontinuous DNA synthesis was abrogated (Fig. 4B), suggesting that DNA polymerase α is responsible for all of the discontinuous DNA synthesis seen in HeLa extracts.

Purification of DNA polymerase α

The pooled DNA polymerase α/ε activities from the DEAE–Sephacel column (fraction IV) were concentrated and loaded onto a P-11 phosphocellulose column. DNA polymerase α/ε activities co-eluted from the column (fraction V) and were loaded onto a monoS column (HR 5/5; Pharmacia), which separated the two activities. Most of the DNA polymerase ε activity did not bind the column, while a small fraction eluted early in the gradient. DNA polymerase α eluted at 225 mM NaCl (fraction VI). The absence of any cross-contamination between the two DNA polymerases was confirmed by western blotting with anti-DNA polymerase α polyclonal antisera (3-10α, a generous gift of T.S.-F.Wang) and with an anti-DNA polymerase ε monoclonal antibody (3C5.1, kindly provided by S.Linn). Discontinuous DNA synthesis activity co-eluted with DNA polymerase α activity (data not shown). Furthermore, no discontinuous synthesis was observed in those fractions containing DNA polymerase ε activity. DNA polymerase α was further purified on an hydroxyapatite column (Table 1).

The relative efficiency of DNA polymerases in discontinuous synthesis

DNA polymerase α is responsible for the majority of DNA polymerase activity in exponentially growing cultured cells. To test whether the discontinuous DNA synthesis seen with DNA polymerase α was due to the large amount of enzyme activity present or whether DNA polymerase α can perform discontinuous DNA synthesis more efficiently than other polymerases, we measured the level of discontinuous synthesis for various DNA polymerases normalized to DNA synthesis activity as measured on activated salmon sperm DNA (Fig. 5). Approximately 0.2 U DNA polymerases α, β, δ or ε or exo$^-$ KF were used in the discontinuous DNA synthesis assay. DNA polymerase α still showed more discontinuous synthesis than any other DNA polymerase tested. Although at this concentration DNA polymerase β and exo$^-$ KF showed little or no discontinuous synthesis (Fig. 5, lanes 1 and 4), increasing the amount of each enzyme resulted in the appearance of a joined replication product (data not

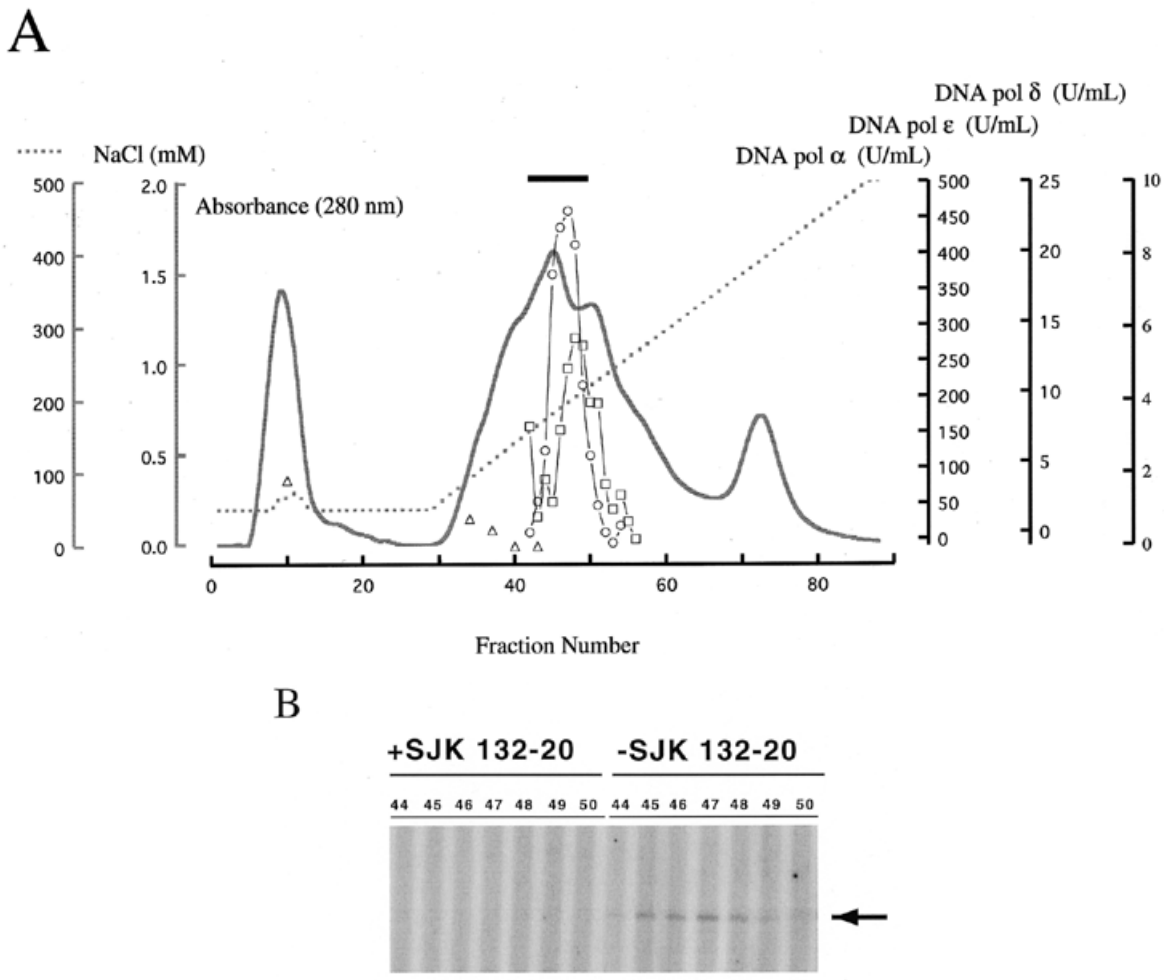

Figure 4. (**A**) Chromatogram of the DEAE–Sephacel column (see Materials and Methods). DNA polymerase α (open circles) and ε (open squares) assays were performed according to Chui and Linn (20). DNA polymerase δ (open triangles) assays were performed similarly to that of DNA polymerase ε except that ~5 µg partially purified PCNA was added to the reaction. Since under these assay conditions DNA polymerase ε is also detected, any PCNA-independent DNA polymerase activity was subtracted to yield an approximate indication of DNA polymerase δ activity. The bar above fractions 45–49 indicates the position where discontinuous DNA synthesis was observed. (**B**) Discontinuous DNA synthesis from the DEAE–Sephacel fractions was completely eliminated by an anti-DNA polymerase α neutralizing antibody. SJK 132-20 was incubated in an aliquot of each fraction at 4°C at a final concentration of ~1 µg/ml. Two microliter aliquots were then used in the discontinuous DNA synthesis reactions. Control samples (–SJK 132-20) were diluted with PBS and incubated at 4°C as well.

shown). Furthermore, DNA polymerase δ in the presence of PCNA and DNA polymerase ε in the absence of PCNA showed faint but detectable levels of discontinuous DNA synthesis (Fig. 5, lanes 6 and 7).

Substrate analysis

To test the efficiency of DNA polymerase α on various discontinuous substrates we used oligonucleotide sets containing the standard 3 bp 3′-PSS, oligonucleotides containing a 1–4 bp 3′-PSS, a blunt substrate, two single-strand oligonucleotides containing a 3 bp 3′-PSS and a small trinucleotide (TAT) complementary to the terminus of the T strand (Table 3).

Good levels of discontinuous DNA synthesis were observed with the 3 and 4 bp 3′-PSS substrates, with the 3 bp 3′-PSS substrate showing a greater amount of products. The 2 bp 3′-PSS substrate showed reduced levels of discontinuous DNA synthesis as compared with the 3 bp overlap substrate, as did the 2 bp 3′-PSS substrate in combination with a T strand containing a non-complementary A residue at the terminus (see 2 bp*). In contrast, little product formation was seen with the blunt substrate or the 1 bp 3′-PSS substrate.

To examine whether the discontinuous synthesis seen with the standard 3 bp overlap substrate was dependent on double-strand DNA, a reaction was performed with only single-strand DNA (the L:C substrate lacked the C oligonucleotide). Very small amounts of full-length products were seen with this substrate, suggesting that discontinuous DNA synthesis is dependent on a double-strand substrate. Furthermore, no products were seen when a trinucleotide (TAT) oligonucleotide was substituted for the duplex substrate in the reaction.

Role of Ku80 and DNA-PK$_{cs}$

To test whether other factors implicated in non-homologous end joining would affect the observed discontinuous DNA synthesis reaction, we prepared nuclear extracts from hamster cell lines xrs-5 (defective in Ku80) and V3 (defective in DNA-PK$_{cs}$) as

Table 3. Discontinuous DNA synthesis by DNA polymerase α with various substrates (fmol product/reaction)

Time	3 bp	4 bp	2 bp	2 bp*	1 bp	blunt	3 bp ss	TAT
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20 min	422.2	231.9	67.8	17.5	0.0	0.0	1.5	0.0
2 hr	5719.4	1377.3	537.6	125.5	10.5	5.1	8.5	0.1

3 bp	*5′-TTGTTCCCGGACTGGTAT 3′-AACAAGGGCCTGACC ATAGTTAGACGAGACTACGGCGTATCAATGC- 5′
4 bp	*5′-TTGTTCCCGGACTGGTAT 3′-AACAAGGGCCTGAC CATAGTTAGACGAGACTACGGCGTATCAATGC- 5′
2 bp	*5′-TTGTTCCCGGACTGGTAT 3′-AACAAGGGCCTGACCA TAGTTAGACGAGACTACGGCGTATCAATGC- 5′
2 bp*	*5′-TTGTTCCCGGACTGGTAT 3′-AACAAGGGCCTGACCA ATAGTTAGACGAGACTACGGCGTATCAATGC- 5′
1 bp	*5′-TTGTTCCCGGACTGGTAT 3′-AACAAGGGCCTGACCAT ATAGTTAGACGAGACTACGGCGTATCAATGC- 5′
blunt	*5′-TTGTTCCCGGACTGGTAT 3′-AACAAGGGCCTGACCATA ATAGTTAGACGAGACTACGGCGTATCAATGC- 5′
3 bp ss	*5′-TTGTTCCCGGACTGGTAT ATAGTTAGACGAGACTACGGCGTATCAATGC- 5′
TAT	*5′-TAT ATAGTTAGACGAGACTACGGCGTATCAATGC- 5′

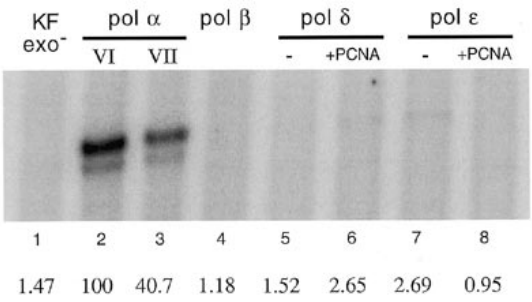

Figure 5. Discontinuous synthesis was measured on various DNA polymerase preparations, all normalized to DNA polymerase activity as measured on an activated salmon sperm DNA substrate. DNA polymerase α from the monoS (fraction VI) and hydroxyapatite (fraction VII) columns was tested. Partially purified PCNA was added to a final concentration of 1.0 µg/ml. The numbers below each lane number are the relative values of discontinuous DNA synthesis (100 = fraction VI).

well as from a normal hamster cell line (CHO-6). The xrs-5 and V3 cell lines have increased ionizing radiation sensitivity as well as defects in V(D)J recombination (28,29). Nuclear extracts made from these cell lines showed no significant difference in their discontinuous DNA synthesis activities relative to CHO-6 cells, which show a normal radiation response (Fig. 6).

DISCUSSION

We show in this study that human cell extracts are capable of performing DNA synthesis across discontinuous DNA template strands. Furthermore, nearly all of the discontinuous DNA synthesis activity co-eluted with DNA polymerase α activity.

Although most of the discontinuous DNA synthesis is attributable to DNA polymerase α, our results also demonstrate that DNA polymerase δ in the presence of PCNA and DNA polymerase ε in the absence of PCNA can also perform discontinuous DNA synthesis at a much reduced efficiency as compared with DNA polymerase α (Fig. 5). PCNA in the absence of any DNA polymerase activity showed no discontinuous DNA synthesis activity (data not shown). Although contamination by DNA polymerase α of our DNA polymerase δ and ε samples cannot be entirely ruled out, the PCNA-dependent discontinuous DNA synthesis seen for DNA polymerase δ and the PCNA-independent synthesis seen for ε tends to eliminate this possibility, since no difference in discontinuous DNA synthesis was seen with DNA polymerase α in the presence of PCNA (data not shown). When normalized for DNA polymerase activity, DNA polymerase α showed much greater (20- to 50-fold) discontinuous synthesis activity than that seen for exo⁻ KF (Fig. 5; 1,2,5), suggesting that DNA polymerase α can bind and align two discontinuous templates more efficiently than exo⁻ KF. The results seen for DNA polymerase δ and ε are surprising, since both contain 3′→5′ exonuclease activities (23,24) and only bacterial DNA polymerases lacking 3′→5′ exonuclease activity

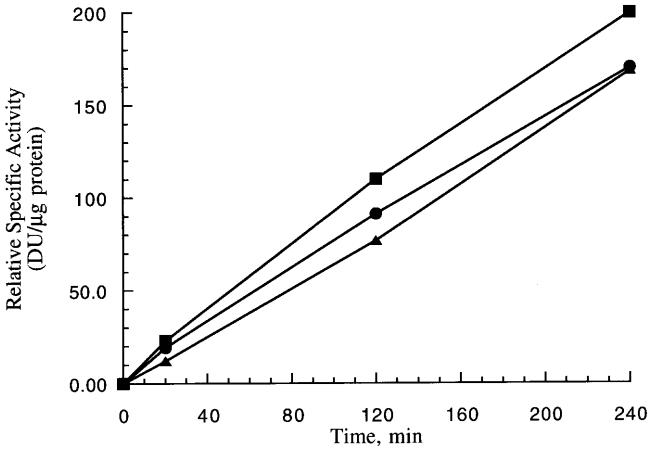

Figure 6. Discontinuous DNA synthesis in extracts from ionizing radiation-sensitive hamster cells. Average DNA end joining activity as a function of time from two biologically independent experiments is shown for one normal (CHO-6) (circles) and two ionizing radiation-sensitive hamster cell lines. Xrs-5 cells (squares) are defective in the 80 kDa subunit of the Ku autoantigen (Ku80); V3 cells (triangles), phenotypically similar to mouse *scid* cells, are defective in DNA-PK_{cs}. DU, arbitrary densitometer units.

have been previously shown to carry out discontinuous DNA synthesis, although if dNMPs are added to the reaction to inhibit 3′→5′ exonuclease activity, KF will carry out discontinuous synthesis (J.S.King, unpublished results). An argument can be made that since DNA polymerase δ and ε have 3′→5′ exonuclease activity, they degrade any discontinuous DNA synthesis products of these enzymes. However, we see no detectable increase in degradation of the substrate; also no significant difference in discontinuous DNA synthesis is seen when dNMPs, inhibitors of 3′→5′ exonuclease activity, are added to the DNA polymerase δ and ε reactions (unpublished). At levels of DNA polymerase activity comparable with that of DNA polymerase α and ε purified from HeLa cells, exo⁻ KF and recombinant human DNA polymerase β showed little or no discontinuous DNA synthesis activity. However, if greater amounts of exo⁻ KF and DNA polymerase β are used in the reaction, discontinuous DNA synthesis for both enzymes is clearly seen (unpublished, but for exo⁻ KF see 2,5). Therefore, all the DNA polymerases tested here demonstrated discontinuous DNA synthesis, however, DNA polymerase α showed the greatest specific activity.

How discontinuous DNA synthesis occurs mechanistically is unclear. Clearly, the two ends of unlinked template molecules must be aligned close enough together for DNA polymerase to synthesize across the rejoined gap. The hypothesis is that DNA polymerase itself has the capacity to bind two different strands of template DNA. Although the DNA polymerase α used in these studies was purified >2800-fold, we cannot completely rule out that some other factor is responsible for binding and aligning the two ends of the DNA template. However, there are two pieces of evidence to suggest that DNA polymerases alone are capable of binding two unlinked DNA molecules. Firstly, discontinuous DNA synthesis is observed with highly purified exo⁻ KF (1,2,5). Secondly, DNA synthesis by KF is blocked by addition of an epoxy-AMP derivative to the 3′-terminus of the elongating chain without release of the epoxy-AMP-terminated template primer (30). However, the 3′→5′ exonuclease activity remains intact, acting not on the epoxy-terminated strand but rather on another duplex substrate, strongly suggesting that another unlinked duplex DNA substrate is able to bind the DNA–KF complex. Furthermore, it appears that DNA polymerases are not unique in being able to bind and link two discontinuous templates. Nudler *et al.* (31) were able to show that histidine-tagged *E.coli* RNA polymerase linked to Ni^{2+} beads was able to transcribe to the end of one DNA template and, while still remaining in a ternary complex, switch to another completely unlinked DNA molecule and continue synthesis, resulting in a hybrid transcript. This observation strongly suggests that some RNA polymerases, like some DNA polymerases, can bind to two separate DNA molecules. The biochemical property of discontinuous synthesis by a polymerase has important biological implications when the enzyme encounters a double-strand break *in vivo*.

DNA polymerase α may in the course of replication encounter double-strand breaks produced either spontaneously or as a result of DNA damaging agents such as ionizing radiation. Such a break would necessarily be a block to replication. However, when DNA polymerase α binds the two ends of the break, synthesis continues across the rejoined gap. The efficiency of the reaction may clearly depend on accessory factors, DNA sequence around the site of the break, chromatin conformation and the presence (or absence) of double-strand break repair proteins.

Our results demonstrate that discontinuous DNA synthesis is facilitated by the presence of a short 3 bp stretch of complementarity at the termini of the substrates. How this short overlap facilitates discontinuous synthesis is still unclear, however, since the T_m of the 3 bp overlap is of the order of 6°C, well below the reaction temperature, thermodynamic stabilization of the two substrates is not likely. Furthermore, neither a 3 bp overlap with two single-strand substrates nor a short trinucleotide primer was sufficient, suggesting that discontinuous DNA synthesis requires at least one double-strand substrate. One possibility is that DNA polymerases recognize double-strand DNA with a 3′-PSS as a substrate for discontinuous DNA synthesis. The fact that in these studies the 3 bp substrate showed greater levels of discontinuous synthesis than the 4 bp substrate and much greater levels than that of the 1 and 2 bp overlap substrates, suggests that there may be an optimal length for the 3′-PSS in the reaction. Little discontinuous DNA synthesis was seen with the blunt-end substrate, also suggesting that discontinuous synthesis is dependent on a 3′-PSS. Although with bacterial DNA polymerases discontinuous synthesis was first observed with blunt substrates (1,2), the reaction involved the non-templated addition of 1 or at most 2 nt, creating a 3′-PSS intermediate which could then be used for discontinuous synthesis. Moreover, the use of substrates with a 3 or 4 bp 3′-PSS greatly increased the extent of discontinuous DNA synthesis by exo⁻ KF at early time points as compared with blunt substrates (5), also suggesting that a 3′-PSS duplex is the preferred substrate. It should also be noted that we (unpublished results) and Clark (4) have observed non-templated addition of 1 (or at most 2) nt to the end of a blunt substrate, suggesting that blunt substrates, if not protected, may be subject to non-templated addition not only by enzymes such as terminal deoxynucleotidyl transferase and REV3 homologs, but also by DNA polymerase α. How biologically relevant are 3′-PSS versus blunt-end DNA *in vivo*? Although the question is difficult to answer, there is growing evidence for extensive 5′→3′ degradation at the site of double-strand breaks in mammalian cells. For example, DNA integration studies show 5′→3′ exonuclease processing at the site of

double-strand breaks prior to integration of exogenous DNA during non-homologous recombination (32). This 5′→3′ exonuclease activity in combination with other factors could lead to the formation of 3′-PSS substrates for discontinuous DNA synthesis. Clearly, other factors, such as Ku/DNA-PK, may compete with this 5′→3′ exonuclease activity for double-strand breaks, although it is interesting that discontinuous DNA synthesis was unaffected in hamster ionizing radiation-sensitive cell lines (Fig. 6). It is possible that in addition to the major Ku/DNA-PK-dependent pathway for processing of double-strand breaks, DNA polymerase α may rejoin double-strand breaks during replication in a manner mechanistically equivalent to discontinuous DNA synthesis, perhaps on a sub-class of breaks where a small amount of exonucleolytic activity was sufficient to generate 3′-PSS ends or where non-templated addition to the end of a break is generated/stabilized in association with other factors.

Previous work has shown that products of *in vitro* discontinuous DNA synthesis by exo⁻ KF on substrates that resemble double-strand breaks or intermediates in double-strand-break rejoining (1,2,5) resemble the *in vivo* junction products seen in eukaryotic double-strand break rejoining (12–14,33). In this study we show that discontinuous synthesis activity exists in human and mammalian cell extracts, that DNA polymerase α is responsible for that activity and that the rejoined products of overlap substrates used in these experiments are formally equivalent to that seen for rejoined overlap substrates *in vivo*.

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