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Mutations in Yeast Proliferating Cell Nuclear Antigen Define Distinct Sites for Interaction with DNA Polymerase δ and DNA Polymerase ε

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The importance of the interdomain connector loop and of the carboxy-terminal domain of *Saccharomyces cerevisiae* proliferating cell nuclear antigen (PCNA) for functional interaction with DNA polymerases δ (Polδ) and ε (Polε) was investigated by site-directed mutagenesis. Two alleles, *pol30-79* (IL126,128AA) in the interdomain connector loop and *pol30-90* (PK252,253AA) near the carboxy terminus, caused growth defects and elevated sensitivity to DNA-damaging agents. These two mutants also had elevated rates of spontaneous mutations. The mutator phenotype of *pol30-90* was due to partially defective mismatch repair in the mutant. In vitro, the mutant PCNAs showed defects in DNA synthesis. Interestingly, the *pol30-79* mutant PCNA (pcna-79) was most defective in replication with Polδ, whereas pcna-90 was defective in replication with Polε. Protein-protein interaction studies showed that pcna-79 and pcna-90 failed to interact with Polδ and Polε, respectively. In addition, pcna-90 was defective in interaction with the FEN-1 endo-exonuclease (*RTH1* product). A loss of interaction between pcna-79 and the smallest subunit of Polδ, the *POL32* gene product, implicates this interaction in the observed defect with the polymerase. Neither PCNA mutant showed a defect in the interaction with replication factor C or in loading by this complex. Processivity of DNA synthesis by the mutant holoenzyme containing pcna-79 was unaffected on poly(dA) · oligo(dT) but was dramatically reduced on a natural template with secondary structure. A stem-loop structure with a 20-bp stem formed a virtually complete block for the holoenzyme containing pcna-79 but posed only a minor pause site for wild-type holoenzyme, indicating a function of the *POL32* gene product in allowing replication past structural blocks.

The proliferating cell nuclear antigen (PCNA) is the eukaryotic replication clamp required for DNA replication and for various DNA repair processes. These include nucleotide excision repair, postreplication DNA repair, and mismatch repair (19, 36, 45, 46, 51, 53). In mammalian cells, PCNA is a target for the cell-cycle-regulatory protein p21 (Cip1 or WAF1) (11, 31, 37, 54). In vitro, p21 binds to PCNA and inhibits primarily its interaction with DNA polymerase δ (Polδ) (11, 41, 54), although replication factor C (RF-C)-mediated loading of PCNA is also partially inhibited by p21 (15, 40). The crystal structure of the complex between a p21 peptide (amino acids [aa] 139 to 160) and human PCNA shows that the major interactions with p21 reside in the interdomain connector loop (aa 119 to 133) and in the carboxy terminus (aa 252 to 259) of PCNA (16). A monoclonal antibody directed to the loop inhibits stimulation of human Polδ by PCNA (45). These observations suggest that Polδ may bind to the interdomain connector loop and/or the carboxy terminus of PCNA and that this interaction is competed by p21 binding.

Human Polδ has two subunits, a 125-kDa subunit which contains both the polymerase and 3′-5′-exonuclease domains and a 48-kDa subunit which is required for effective stimulation of the polymerase by PCNA (29, 58). In contrast to the human enzyme, yeast Polδ consists of three subunits, a 125-kDa subunit, encoded by the *POL3* gene, which is highly homologous to the human catalytic subunit, and two small subunits (13). The largest of those is the 58-kDa subunit, the product of the *POL31* (*HYS2*) gene (14, 49). This subunit shows extensive sequence similarity to the small 48-kDa subunit of human Polδ (57). The *POL31* gene is essential for yeast cell growth, and mutations in this gene confer temperature sensitivity and hypersensitivity to the replication inhibitor hydroxyurea (HU) (49). The gene for the smallest subunit, *POL32*, was cloned by using information derived from peptide sequencing and is unique (14). *POL32* deletion mutants are viable but show a growth defect consistent with a defect in DNA replication and are hypersensitive to HU. In addition, the *POL32* deletion mutant is inviable in combination with a temperature-sensitive mutation in *POL3* or in *POL31* (14). Genetic studies with *Schizosaccharomyces pombe* show an interaction between Cdc1, the homolog of the human 48-kDa subunit and of the *Saccharomyces cerevisiae* 58-kDa subunit (*POL31* product), and Cdc27. The latter gene shows very limited sequence similarity with the *POL32* gene, suggesting similarities in Polδ structure and function in these two fungi (14).

Previous mutational studies of yeast PCNA have identified amino acids important for its trimeric structure and for DNA repair (1–3). Mutational studies of human PCNA have identified protein regions important for its stability and for in vitro DNA synthesis by Polδ holoenzyme (12, 22). In this paper we present a further alanine substitution mutational analysis of the yeast *POL30* gene with a focus on the interdomain connector loop and the carboxy terminus, both of which are proposed regions of interaction with Polδ. Of the 10 mutants investigated genetically, one with a mutation located in the interdomain connector loop, *pol30-79* (IL126,128AA), and one with a mutation located at the carboxy terminus, *pol30-90* (PK252,253AA), were subjected to a detailed biochemical characterization. As anticipated from the p21 inhibition stud-

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype
PY37	*MAT*α *ura3-52 trp1*Δ*901 leu2-3,112 can1 pol30*Δ*1* + [pBL211 (*POL30 URA3*)]
PY39-x	PY37 + [pBL230-x (*TRP1 pol30-x*)] − [pBL211 (*POL30 URA3*)]
PY50	*MAT*α *can1-100 ade2-1 lys2-1 ura3-52 leu2-3,112 his3-*Δ*200 trp1*Δ*901 pol30*Δ*2::hisG* + [pBL211 (*POL30 URA3*)
PY51	PY50 + [pPY123 (*LEU2 SUP4-o*)]
PY52-x	PY50 + [pPY123 (*LEU2 SUP4-o*)] + pBL230-x − pBL211
PY58	*MAT*α *ura3-52 trp1*Δ*901 leu2-3,112 can1 rth1*Δ*::hisG pol30*Δ*1* + [pBL211 (*POL30 URA3*)]
PY60-x	PY58 + [pBL230-x (*TRP1 pol30-x*)] − [pBL211 (*POL30 URA3*)]
PY67	*MAT***a** *ura3-52 trp1*Δ*901 leu2-3,112 can1 pol30*Δ*1* + [pBL211 (*POL30 URA3*)]
PY69-x	PY67 *leu2-3,112*::pBL248-x (*pol30-x*, *LEU2*) − pBL211
PY71-x	*MAT*α *can1-100 ade2-1 lys2-1 ura3-52 his3-*Δ*200 trp1-*Δ*901 pol30*Δ*2::hisG leu2-3,112*::pBL248-x (*pol30-x*, *LEU2*)
PY72	PY67, *pms1*Δ*::hisG*
PY73-x	PY72, *leu2-3,112*::pBL248-x (*pol30-x*, *LEU2*) − pBL211
PY105	PY50, *pms1*Δ*::hisG*
PY106	PY105 + [pPY123 (*LEU2 SUP4-o*)]
PY107-x	PY105 + [pPY123 (*LEU2 SUP4-o*)] + pBL230-x − pBL211
PY109-x	PY71-x *pms1*Δ*::hisG*

ies, the *pol30-79* mutant PCNA (pcna-79) showed a defect in the interaction with Polδ. This defect could at least in part be attributed to the interaction with the smallest subunit of Polδ, which is not present in purified human Polδ. In contrast, the carboxy-terminal mutant, pcna-90, was deficient in the interaction with DNA polymerase ε (Polε) and also showed a defect in the interaction with the FEN-1 nuclease, an enzyme required for Okazaki fragment maturation and mismatch repair (18, 21, 44, 47). Therefore, this analysis shows that the two DNA polymerases interact with their replication clamp at different sites.

MATERIALS AND METHODS

Yeast strains and plasmids. The yeast strains used in this study are listed in Table 1. Standard growth media and yeast genetic techniques were used. *PMS1* deletion strains were constructed by the gene replacement method with the integration cassette *pms1*Δ*::hisG-URA3-hisG* contained on plasmid pPM601 (a gift of Louise Prakash, University of Texas), followed by deletion of the *URA3* gene by growth on 5-fluoroorotic acid-containing medium. All site-directed PCNA mutations were made by procedures analogous to those described before (3) and were carried in plasmids pBL230-x (*ARS CEN TRP1 pol30-x*) for plasmid-based complementation in yeast (3), in plasmids pBL248-x (*LEU2 pol30-x*) for integration in yeast in the chromosomal *LEU2* locus with expression of *pol30-x* under control of its own promoter (51), or in plasmids pBL228-x (bacteriophage T7 promoter fused to *pol30-x*) for overproduction of mutant PCNAs in *Escherichia coli* (3). Plasmids pBL248-x were linearized with *Hpa*I inside the *LEU2* gene and transformed into strain PY50, PY67, PY72, or PY105 to obtain targeted integration into the *leu2-3,112* allele. Removal of plasmid pBL211 carrying the wild-type *POL30* allele was accomplished by growth on 5-fluoroorotic acid-containing medium. This yielded strains PY71-x, PY69-x, PY72-x, and PY109-x, respectively. PCNA protein levels expressed from *POL30* integrated into the *LEU2* locus were indistinguishable from those expressed from the normal locus (data not shown).

Enzymes. The three-subunit form of Polδ and the five-subunit form of Polε were purified from a protease-deficient strain of yeast as described previously (4, 56). The purity of Polδ was >90%, and that of Polε was ~70%. RF-C was purified to homogeneity from an overproducing yeast strain (14). *E. coli* single-stranded-DNA-binding protein (SSB) was a gift from T. Lohman (Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine). Yeast FEN-1 was purified from *E. coli* as described previously (18).

DNA damage sensitivity measurements. Sensitivity of the mutants to growth on methyl methanesulfonate (MMS)- or HU-containing medium or to UV light was determined as described previously (3). To obtain MMS survival curves, stationary-phase cultures were spun down, and the cells were washed once with water, resuspended to 2×10^7 cells/ml in 50 mM KH_2PO_4 (pH 7.2), briefly sonicated to disperse clumped cells, and treated with 0.5% MMS at room temperature. Aliquots taken after various times were quenched in an equal volume of 10% cold $Na_2S_2O_3$, and appropriate further dilutions were plated on yeast extract-peptone-dextrose (YPD) plates to determine survival (42).

Measurement of spontaneous-mutation rates. To measure mutation rates, cells were grown in rich or selective medium to saturation and then diluted to about 100 cells/ml in 15 separate cultures for each strain and again grown to saturation in rich or selective medium. Appropriate dilutions were plated on indicator plates, and colonies appearing after 4 to 6 days of growth at 30°C were counted. All mutation rate experiments were carried out in duplicate or triplicate, and the averages of the means from each experiment are reported. Mutation rates were calculated by the method of the median (28).

(i) For measurement of cycloheximide resistance, PY39-x cells were grown in YPD, and 10^8 cells were plated on YPD medium containing 2 μg of cycloheximide per ml. Colonies were counted after 4 days.

(ii) Inactivation of the *FUR1* gene results in resistance to 5-fluorouracil (23). PY69-x cells were grown in YPD, and 2×10^6 cells were plated on minimal medium (SD) containing tryptophan, 25 μg of uridine per ml (to complement the defect caused by the *ura3* mutation), and 1 mM 5-fluorouracil. Because of considerable residual growth on the plates, mutation rates could not be calculated, and frequencies relative to that of the wild type were determined.

(iii) Reversion or suppression of *lys2-1o* was measured in strains PY71-x and PY109-x by plating 5×10^7 YPD-grown cells onto minimal medium lacking lysine but containing adenine. Red colonies after 6 days of growth are considered to be due to locus reversion, and white colonies (which are also Ade^+) are considered to be due to suppression.

(iv) Tract instability in a modified *URA3* gene with an in-frame $(GT)_{16}G$ insertion was detected by the appearance of 5-fluoroorotic acid-resistant cells in strains PY69-x and PY73-x carrying plasmid pSH91 (48). Strains were grown on complete minimal medium lacking tryptophan to ensure plasmid maintenance, and 5×10^5 cells were plated on minimal medium (SD) containing uracil and 1 mg of 5-fluoroorotic acid per ml. Colonies were counted after 4 days.

(v) Rates of inactivation of the *SUP4-o* tRNA gene were measured in strains PY51-x and PY107-x essentially as described previously (3). Cells were grown on complete minimal medium lacking leucine to ensure plasmid maintenance, and 5×10^6 cells/plate were plated on complete minimal medium lacking leucine and arginine and supplemented with 80 μg of canavanine per ml. Red colonies appearing after 5 days of growth at 30°C were considered to be due to *SUP4-o* mutations.

Purification of mutant PCNAs from *E. coli*. Overproduction of pcna-79 and pcna-90 in *E. coli* and purification of the mutant PCNAs was performed essentially as described previously (3). The resulting preparations were estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to be >90% pure and contained no detectable DNA polymerase, exonuclease, or endonuclease activity. A gel filtration analysis showed that the mutants migrated as trimers at 2 μM monomer (the lowest concentration tested) (3). PCNA containing an amino-terminal phosphorylatable tag (Ph-PCNA [MRRASVGS-PCNA]) was expressed in plasmid pMM83 (a gift of Michael McAlear) and purified as wild-type PCNA (40). The replication properties of Ph-PCNA were indistinguishable from those of the wild type (data not shown). Phosphorylation of 1 μg of Ph-PCNA was carried out in a 50-μl reaction mixture containing 20 mM HEPES-NaOH (pH 7.0), 12 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 100 mM NaCl, 50 μCi of [γ-^{32}P]ATP, and 3 U of bovine heart cyclic AMP-dependent protein kinase (catalytic subunit; Sigma) at 30°C for 30 min. The labeled protein was passed through a Sephadex G50 column, equilibrated in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–100 mM NaCl, to remove unincorporated radioactivity.

Enzyme assays. (i) Stimulation of Polδ by PCNA on poly(dA) · oligo(dT). Without salt present in the assay, PCNA alone stimulates the processivity of Polδ, and thereby total DNA synthesis, on poly(dA) · oligo(dT) (4, 50). Addition of 75 mM NaCl virtually completely inhibits the reaction with or without PCNA, and RF-C and ATP are required to load PCNA. The 30-μl reaction mixture contained 20 mM Tris-HCl (pH 7.8), 8 mM magnesium acetate ($MgAc_2$), 0.2 mg of bovine serum albumin per ml, 1 mM DTT, 25 μM [^{3}H]dTTP (400 cpm/pmol), 0.5 mM ATP, 100 ng of poly$(dA)_{500}$ · oligo$(dT)_{22}$ (20:1 nucleotide ratio, 0.7 pmol of primer termini), 850 ng of SSB, 75 mM NaCl, 100 fmol (25 ng) of RF-C, 50

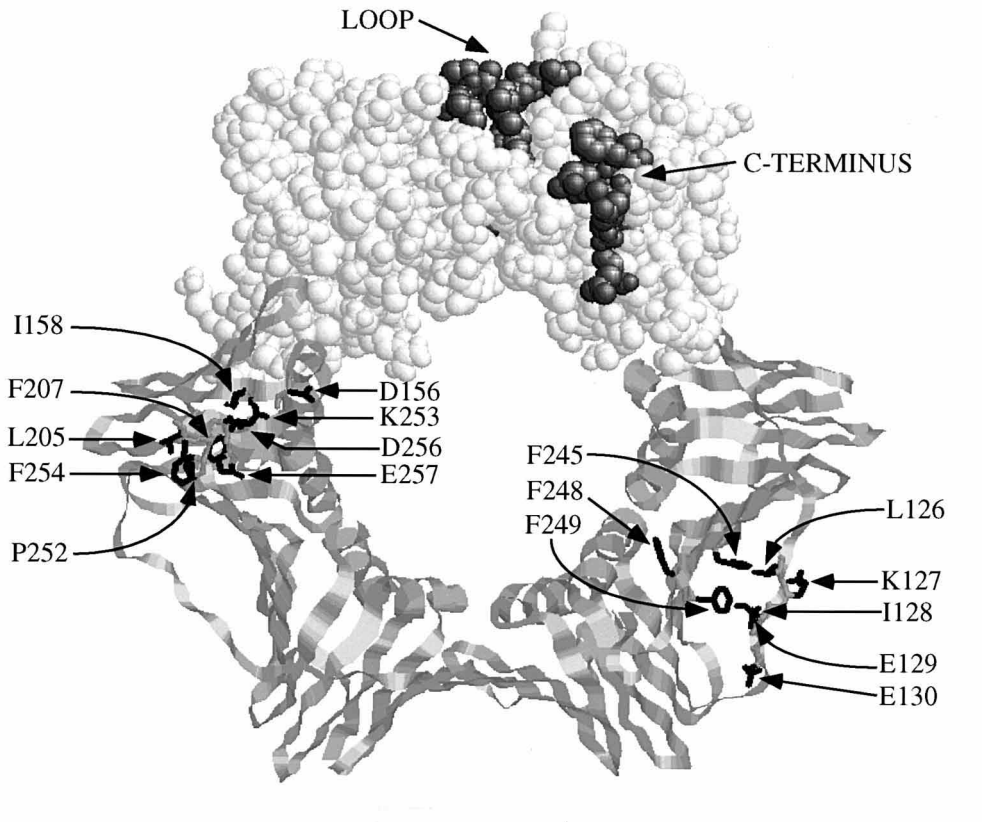

FIG. 1. Locations of the *POL30* mutations. A RasMol rendering of the crystal structure of the homotrimer is shown. The subunit at 12 o'clock is shown as a space-filling model with the mutated amino acids indicated in black. The two other subunits are given as ribbon models, with the side chains of the mutated amino acids near the interdomain connector loop indicated in black in the subunit at 4 o'clock and the side chains of the mutated amino acids near the carboxy terminus indicated in black in the subunit at 8 o'clock.

fmol (10 ng) of Polδ, and various amounts of PCNA. Incubations were for 10 min at 37°C. The reactions were stopped by the addition of 100 μl of 50 mM sodium pyrophosphate–25 mM EDTA–50 μg of calf thymus DNA per ml as carrier, and acid-insoluble radioactivity was determined as described before (56). When an electrophoretic analysis of the replication products was carried out, reaction volumes were reduced to 15 μl, [α-^{32}P]dTTP replaced [^{3}H]dTTP, the reactions were stopped with 10 μl of 95% formamide–20 mM EDTA, and the products were electrophoresed on a 8% denaturing polyacrylamide gel.

(ii) Stimulation of the ATPase activity of RF-C. The 30-μl reaction mixture contained 40 mM Tris-HCl, pH 7.8, 8 mM $MgAc_2$, 0.2 mg of bovine serum albumin per ml, 1 mM DTT, 50 mM NaCl, 40 fmol (100 ng) of multiply primed single-stranded mp18 DNA (average of five primers per mp18 circle), 850 ng of *E. coli* SSB, 50 μM of α-[^{32}P]ATP, and various amounts of PCNA. The mixture was preheated to 37°C, and the reaction was started by the addition of 100 fmol of RF-C. Samples of 10 μl were taken at 4 and 8 min and quenched into 3 μl of 50 mM EDTA–1% SDS–25 mM (each) ADP and ATP as markers for UV detection. The products were separated by polyethylene-cellulose thin-layer chromatography in 0.5 M LiCl–1 M HCOOH. Appropriate regions were cut out, and the radioactivity was counted to determine the percentage of ADP formed.

(iii) Polδ or Polε holoenzyme assay on mp18 DNA. The standard 30-μl reaction mixture contained 40 mM Tris-HCl (pH 7.8), 8 mM $MgAc_2$, 0.2 mg of bovine serum albumin per ml, 1 mM DTT, 100 μM each dATP, dCTP, and dGTP and 25 μM of [^{3}H]dTTP (100 cpm/pmol of deoxynucleoside triphosphate [dNTP]), 0.5 mM ATP, 40 fmol (100 ng) of singly primed single-stranded mp18 DNA (the 36-mer primer is complementary to nucleotides [nt] 6330 to 6295), 850 ng of *E. coli* SSB, 75 mM NaCl, 100 fmol of RF-C, 50 fmol of Polδ or 100 fmol of Polε, and various amounts of PCNA. Incubations were at 37°C for 4 min (Polδ) or 10 min (Polε). The reactions were stopped, and acid-insoluble radioactivity was determined, as described above. When an electrophoretic analysis of the replication products was carried out, [α-^{32}P]dTTP replaced [^{3}H]dTTP. In this case, either the reactions were stopped with 20 μl of 95% formamide–20 mM EDTA and the products were electrophoresed on an 8 or 12% denaturing polyacrylamide gel, or the reactions were stopped with 10 μl of 60% glycerol–50 mM EDTA–1% SDS and the products were electrophoresed on a 1% alkaline agarose gel.

(iv) Inhibition of Polδ and Polε activities by PCNA. The 20-μl reaction mixture contained 40 mM HEPES-NaOH (pH 8.1), 8 mM $MgAc_2$, 0.2 mg of bovine serum albumin per ml, 1 mM DTT, 100 μM each dTTP, dCTP, and dGTP and 12 μM [α-^{32}P]dATP (100 cpm/pmol of dNTP), 40 fmol of singly primed single-stranded mp18 DNA, 850 ng of *E. coli* SSB, 250 fmol of Polδ or 100 fmol of Polε, and various amounts of PCNA. Incubations were at 37°C for 30 min (Polδ) or 10 min (Polε). The reactions were stopped with 10 μl of 60% glycerol–50 mM EDTA–1% SDS, and the products were electrophoresed on a 1% alkaline agarose gel.

(v) PCNA stimulation of FEN-1 activity. The 15-μl assay mixture contained 40 mM Tris-HCl (pH 7.8), 8 mM $MgAc_2$, 0.5 mM ATP, 0.2 mg of bovine serum albumin per ml, 1 mM DTT, 125 mM NaCl, 10 fmol of FLAP substrate, 850 ng of *E. coli* SSB, 100 fmol of yeast FEN-1, 100 fmol of RF-C, and 550 fmol of PCNA (as trimers). After 1, 5, and 15 min at 14°C, 5-μl aliquots were analyzed by 15% denaturing polyacrylamide gel electrophoresis. The FLAP substrate consists of single-stranded mp18 DNA with two oligomers hybridized to it, a 5′-^{32}P-labeled 51-mer (TTTTTTTTTTTTCCTTCCGAAGAGGATCCCCGG GTACCGAGCTCGAATTCG, the FLAP oligonucleotide, with nt 21 to 51 complementary to mp18 nt 6260 to 6230) and a 31-mer (GCCAAGCTTGCATGCC TGCAGGTCGACTCT, the primer oligonucleotide, complementary to mp18 nt 6229 to 6199) (see Fig. 10A).

Biogel A-5m filtration of complexes. Complexes were formed in an 80-μl reaction mixture containing 30 mM Tris-HCl (pH 7.8), 8 mM $MgAc_2$, 100 mg of bovine serum albumin per ml, 1 mM DTT, 800 fmol of singly primed single-stranded mp18 DNA, 17 μg of *E. coli* SSB, 1 mM ATP, 1.6 pmol of RF-C, and 5 pmol of PCNA. Because the enzyme storage buffers generally contained 0.2 to 0.3 M NaCl, the presence of the various proteins at high levels usually added 40 to 50 mM NaCl to the incubation. In reaction mixtures which contained lower levels of NaCl contributed by the enzymes, additional NaCl was added to give a final concentration of 50 mM from all contributions. The mixtures were incubated at room temperature for 1 min and then cooled on ice and immediately filtered through a 1.1-ml Biogel A-5m (Bio-Rad) column equilibrated in 30 mM Tris-HCl (pH 7.5)–5% glycerol–0.1 mM EDTA–8 mM $MgCl_2$–2 mM DTT–100 μg of bovine serum albumin per ml–40 mM NaCl. Filtrations were carried out at 4°C. Three-drop fractions were collected. The DNA eluted predominantly in

TABLE 2. Phenotypes of PCNA mutants

Allele no.	Mutation(s)	Domain	% Identity[a]	Growth[b] at:			% Dumbbell cells[c]	Growth with *RTH1*Δ[d]	Resistance to:			Mutator frequency[g] (*SUP4*)
				14°C	30°C	37°C			UV[e]	HU[f]	MMS[f]	
0	None (wild type)			+++	+++	+++	5	+++	+++	+++	+++	1.0
79	LI126,128AA	Loop	100, 100	++	++	++	31	−	+	+	++	40
14	KE127,129AA	Loop	0, 0	+++	+++	+++	12	+++	+++	+++	+++	0.7
15	EE129,130AA	Loop	0, 100	+++	+++	+++	9	+++	+++	+++	+++	1.1
81	DI156,158AA	C terminus	100, 100	+++	+++	+++	9	+++	++	+++	++	1.0
83	LF205,207AA	C terminus	86, 100	+++	+++	+++	5	+++	+++	+++	+++	0.7
87	F245A	Loop	100	+++	+++	+++	11	+++	+++	+++	++	1.0
89	FF248,249AA	Loop	100, 100	+++	+++	+++	5	+++	+++	+++	+++	1.5
90	PK252,253AA	C terminus	100, 100	+/−	++	++	61	−	++	++	−	7
88	KF253,254AA	C terminus	100, 0	+	+++	+++	47	−	+++	+++	+	4
22	DE256,257AA	C terminus	100, 86	+++	+++	+++	7	+++	+	+++	++	1.0

[a] From a comparison wiht human PCNA and those from *Schizosaccharomyces pombe*, *Xenopus laevis*, *Drosophila melanogaster*, *Plasmodium falciparum*, and rice. These include conserved substitutions F-Y, D-E, and I-V.

[b] Growth of strains PY39-x on YPD. +++, wild-type growth; ++, minor defects; +, more severe defects; +/−, minimal growth; −, no growth.

[c] Cells were grown at 30°C in YPD to 10^6 cells/ml and then for 16 h in YPD at 14°C. The cells were fixed with formaldehyde and sonicated, and a total of about 300 cells were observed under a microscope. Those in which the bud was approximately equal in size to the mother cell (dumbbell) were counted. About 20% of *pol30-79*, 25% of *pol30-88*, and 50% of *pol30-90* dumbbells were enlarged.

[d] Growth of *rth1*Δ *pol30-x* double mutants was determined with strains PY60-x at 14°C on YPD medium. For symbols, see footnote *b*.

[e] Survival in response to a dose of 90 J/m^2: +++, 10 to 15%; ++, 5 to 10%; +, 2 to 5%.

[f] Growth of strains PY39-x on YPD plus 100 mM HU or 0.015% MMS. For symbols, see footnote *b*.

[g] The spontaneous mutator frequencies in the *SUP4-o* tRNA gene were determined with strains PY52-x.

fractions 8 to 10 (void volume), free proteins eluted in fractions 13 to 16, and free nucleotides eluted in fractions 17 to 20. The void-volume fractions of the gel filtration column were pooled, and 30 μl of complex was assayed in a 90-μl reaction mixture containing final concentrations of 40 mM Tris-HCl (pH 7.8), 8 mM $MgAc_2$, 0.2 mg of bovine serum albumin per ml, 100 mM each dGTP, dCTP, and dATP, 10 μM [α-^{32}P]dTTP, and 150 fmol of Polδ. Aliquots were withdrawn at various times, and the products were separated by electrophoresis through an alkaline agarose gel as described above.

Affinity binding to PCNA beads. All steps were carried out at 0 to 4°C. PCNA, pcna-79, or pcna-90 (0, 20, 60, and 180 μg each) together with PCNA beads (2 to 3 μl [about 8 to 12 μg of PCNA]) in 200 μl of binding buffer (50 mM Tris [pH 7.6], 10% glycerol, 0.5 mM EDTA, 3 mM DTT, 1 mg of bovine serum albumin per ml, 0.02% Nonidet P-40, 0.1% ampholyte 3.5-9) plus 150 mM NaCl (BB_{150}) was mixed by continuous rotational inversion with 0.4 μg of Polδ in a thin-walled microcentrifuge tube (PCR tube). After 1 h, the bottom of the tube was pierced with a 27-gauge needle, and the solution was spun through at 20 × *g* for a few seconds, taking care not to let the beads become dry. The beads were resuspended in 25 μl of $BB_{1,000}$, and after 10 min, the solution was spun through and released Polδ was quantitated in a Polδ holoenzyme assay. To quantitate binding of RF-C (0.2 μg) to the beads, the buffer was modified to also contain 8 mM $MgCl_2$ and 1 mM ATP, and the salt level for initial binding was 350 mM NaCl (BB_{350}-MgATP) (14). Release of RF-C from the beads was accomplished with $BB_{1,000}$ containing 2 mM EDTA. RF-C released was quantitated in a Polδ holoenzyme assay.

Protein affinity blot. All steps were carried out at room temperature. Yeast Polδ was electrophoresed in an SDS–10% polyacrylamide gel, and the proteins were transferred electrophoretically to GeneScreen. The membrane was equilibrated in buffer A (40 mM Tris-HCl [pH 7.6], 150 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT) and then incubated for 10 min in 7 M guanidinium hydrochloride in buffer A, followed by sequential twofold dilutions with buffer A for 10 min each until a concentration of 0.4 M guanidinium was reached. The membrane was then immersed for 10 min in buffer A, followed by incubation in 5% nonfat milk in buffer A for 10 min and in 25% fetal calf serum in buffer A for 20 min to block the membrane. The membrane was probed with 100 ng of ^{32}P-labeled Ph-PCNA per ml (1×10^6 to 2×10^6 cpm/ml) with or without competitor PCNA or mutant PCNA in buffer A containing 25% fetal calf serum for 2 h at room temperature and then washed twice in 50 ml of buffer A for 1 to 2 min and processed for autoradiography.

RESULTS

Site-directed mutagenesis of the *S. cerevisiae POL30* gene. In our mutational scheme, we targeted the putative "front" side of PCNA, i.e., the side facing the direction of DNA synthesis, at two regions which may be important for interaction with Polδ: (i) the interdomain connector loop and (ii) the carboxy-terminal domain (Fig. 1). Site-directed alanine replacement mutations were introduced at evolutionarily conserved amino acids. In particular, those conserved hydrophobic amino acids which, from an analysis of the crystal structure of PCNA, showed considerable solvent exposure were targeted for mutagenesis (27). The rationale for this strategy was that evolutionary conservation would signify functional importance and that solvent-exposed hydrophobic side chains would be involved in protein-protein interactions. Leu126, Ile128, and Phe245, which cluster together at the interdomain connector loop, show >35% solvent exposure (Fig. 1). The side chains of Phe248 and Phe249 are almost completely buried inside the structure, but they were chosen because of their conservation in all PCNAs and their proximity to the other residues in the loop. The choice of mutations in the carboxy-terminal regions was based on similar considerations. However, of the hydrophobic amino acids, only Phe254, which is an isoleucine in the PCNAs from all other organisms, was largely solvent exposed. The *pol30-14*, *pol30-15*, and *pol30-22* mutants were from a previous mutational screen but were reinvestigated because of the location of the mutations (3).

Phenotypes of yeast mutants bearing PCNA mutations. The mutations were introduced as the sole source of PCNA in haploid yeast cells by the plasmid shuffle technique (7). The properties of the yeast strains are summarized in Table 2. None of the mutations is lethal, but several mutants showed defects in cell growth, sensitivity to DNA-damaging agents, and increased rates of spontaneous mutations. Slow growth was observed in the *pol30-79*, *pol30-88*, and *pol30-90* mutants (Table 2 and data not shown). The growth defects of the mutants with the carboxy-terminal mutations *pol30-88* and *pol30-90* were more pronounced at 14°C, a phenomenon observed before with other PCNA mutants (1, 3). The large-budded cell phenotype observed in these mutants has also been documented previously for other PCNA mutants with growth defects and is consistent with defects in DNA replication (1, 3). The three mutants which showed poor growth at 14°C were also more severely defective in an *RTH1* deletion background (Table 2). All the double mutants were viable at 30°C, but those combining *rth1*Δ with *pol30-79*, *pol30-88*, or *pol30-90* grew poorly at 23°C and failed to grow at 14°C.

Those mutants which showed the most severe growth defects

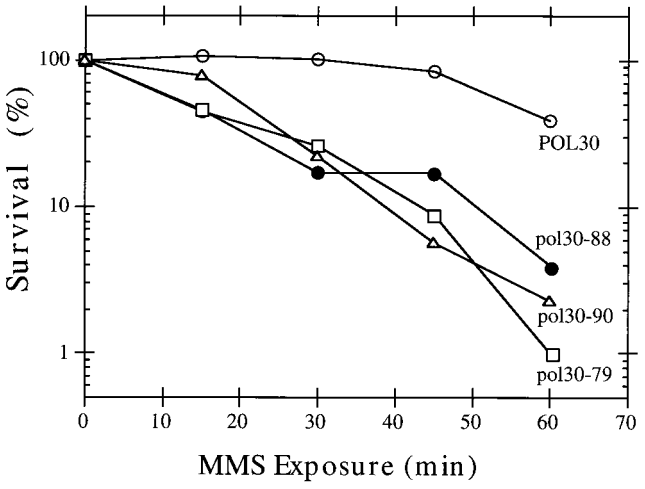

FIG. 2. MMS survival curves for *POL30* mutants. See Materials and Methods for details.

were also most sensitive to the effects of DNA-damaging agents (Table 2). UV sensitivity of the mutants was only marginal and was much lower than observed in previously identified PCNA mutants (3). The *pol30-79*, *pol30-88*, and *pol30-90* mutants were similarly sensitive to transient exposure to MMS (Fig. 2). However, the mutants with carboxy-terminal mutations, *pol30-88* and *pol30-90*, were much more sensitive than the *pol30-79* mutant to growth on MMS-containing medium, perhaps indicating a cell cycle defect, likely in the intra-S-phase checkpoint, in these mutants (38). HU sensitivity was pronounced in the *pol30-79* and *pol30-90* mutants. This is expressed as slow growth (Table 2) and an increased rate of cell death (data not shown).

Mutator phenotype of *pol30-79* and *pol30-90* mutants. Spontaneous mutation rates of the mutants were initially determined in a forward mutation assay which measures the inactivation of the ochre-suppressing *SUP4-o* tRNA gene present on a plasmid. This assay identified the *pol30-79* and *pol30-90* mutants as mutators, and they were subjected to further investigation (Table 2). The *pol30-88* mutant also showed up as a modest mutator in this assay, but as its mutations in PCNA overlap with those of *pol30-90*, it was presumed to have a phenotype similar to that of the *pol30-90* mutant and was not further investigated.

Spontaneous-mutation rates were measured with several different targets which measure either point mutagenesis (cycloheximide resistance and *lys2* reversion or suppression), instability of dinucleotide repeats in a 33-nt $(GT)_{16}G$ tract, or general gene inactivation by any mechanism (5-fluorouracil resistance and *SUP4-o* inactivation). For some targets the rates were compared to those in the respective isogenic *PMS1* deletion mutants, which are defective for mismatch repair (25). The *pol30-90* mutation caused a 4- to 7-fold increase in the rate of point mutations (measured as rates of cycloheximide resistance and reversion or suppression of the *lys2-1o* allele), a 24-fold increase in dinucleotide repeat instability, and 5- and 23-fold increases in *SUP4-o* inactivation and 5-fluorouracil resistance, respectively, indicating that the mutator phenotype in the *pol30-90* mutant is very general (Table 3). If this increased mutation rate resulted from a defect in DNA synthesis, i.e., in insertion fidelity or proofreading efficiency, then the individual rates observed in the *pol30-90* strain and in the mismatch repair-defective *pms1*Δ strain should multiply in the *pms1*Δ *pol30-90* double mutant (35). On the other hand, if the mutator phenotype of the *pol30-90* mutant resulted from a defect in mismatch repair, then the rates in the double mutant should be the same as those in the *pms1*Δ single mutant. The data in Table 3 are consistent with an epistatic relationship between *pol30-90* and *pms1*Δ, indicating that the *pol30-90* mutant is defective for mismatch repair.

TABLE 3. Mutator phenotypes of *pol30-79* and *pol30-90* mutants

Genotype	Mutation rate[a]					
	Fur^{r}[b]	Cyh^{r}	*lys2-1o*: Locus reversion	*lys2-1o*: Suppression	$(GT)_{16}G$ instability	*SUP4-o* mutation
PMS1 POL30	1	1	1	1	1	1
PMS1 pol30-79	1.2	1.7	0.8	1.2	2.2	16
PMS1 pol30-90	23	7	3.6	6.9	24	4.6
*pms1*Δ *POL30*			4.1	7.1	110	8
*pms1*Δ *pol30-79*			3.4	6.7	90	18
*pms1*Δ *pol30-90*			4.8	6.8	130	7.3

[a] See Materials and Methods for details. All rates are relative to those for the wild type (*PMS1 POL30*). Spontaneous-mutation rates for the wild type were 3×10^{-8} (per generation) for cycloheximide resistance (Cyh^{r}), 2×10^{-9} for *lys2-1* locus reversion, 3.8×10^{-8} for *lys2-1* suppression, 3×10^{-5} for $(GT)_{16}G$ tract instability, and 2×10^{-7} for *SUP4-o* mutation.

[b] Frequencies of mutation to 5-fluorouracil resistance (Fur^{r}) were compared.

In sharp contrast, the mutator phenotype in the *pol30-79* mutant is very target specific. The mutation rate for *SUP4-o* was dramatically increased, whereas mutation rates for other targets were not at all or only minimally affected (Table 3). This indicates that, unlike in the *pol30-90* mutant, mismatch repair may be only very minimally affected in the *pol30-79* mutant. In addition, the spontaneous-mutation rate of the *pms1*Δ *pol30-79* double mutant was not higher than that of the single *pol30-79* mutant. The *SUP4* regions of 30 independently derived mutants were PCR amplified with primers bracketing the tRNA gene, and the resulting fragments were analyzed by electrophoresis on a 3% MetaPhor agarose gel. The PCR-amplified products of all but one of the isolates showed the same size as a wild-type control (220 nt). The product from one isolate was about 215 nt in size (data not shown). This indicates that the majority of spontaneous mutations in the tRNA gene occurring in the *pol30-79* mutant result from substitution mutations and/or 1- or 2-nt insertions or deletions (the limit of resolution of the gel), which should serve as substrates for the mismatch repair system (5, 25).

In vitro replication defects of pcna-79 and pcna-90. Because of their elevated mutagen sensitivities and spontaneous mutation rates, we selected the PCNAs encoded by the *pol30-79* allele in the interdomain connector loop and the carboxy-terminal *pol30-90* allele for further biochemical studies. Both mutant PCNAs were purified to near homogeneity from an *E. coli* overexpression system. A gel filtration analysis showed them to have a trimeric structure, an expected result since the mutations do not localize to the subunit interfaces (data not shown). The mutant PCNAs were tested for their abilities to support processive Polδ-mediated (Fig. 3A) and Polε-mediated (Fig. 3B) replication of a singly primed circular template (M13mp18). This assay tests for PCNA interactions with RF-C, the respective DNA polymerase, and DNA. Figure 3A shows that about 1,000-fold-higher levels of pcna-79 were required to achieve even modest levels of DNA synthesis by Polδ. Addition of the macromolecular crowding agent polyethylene glycol stimulated the assay significantly, indicating that replication with pcna-79 is accompanied by frequent disassembly

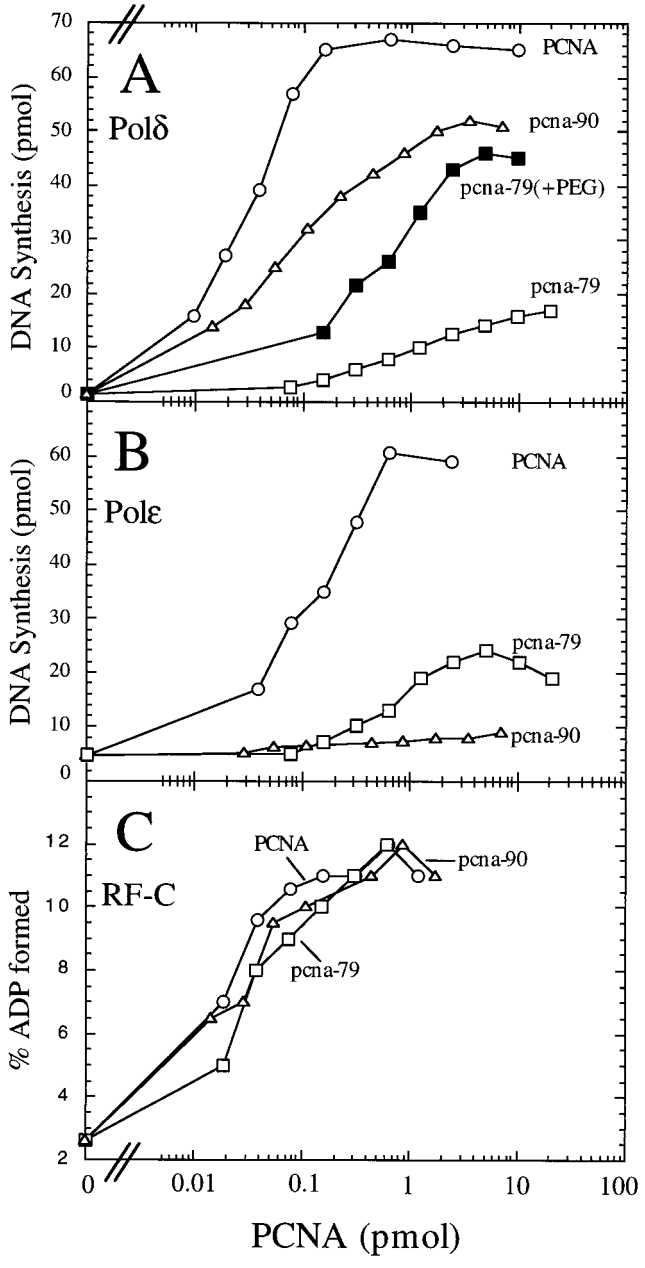

FIG. 3. In vitro replication properties of pcna-79 and pcna-90. (A) Replication of M13mp18 DNA by Polδ holoenzyme. Replication by the pcna-79 holoenzyme is carried out either without (open squares) or with (filled squares) 6% polyethylene glycol 8000 (PEG). (B) Replication of M13mp18 DNA by Polε holoenzyme. (C) ATPase activity of RF-C. The amount of PCNA added per assay is expressed as picomoles of trimers and plotted on a log scale. Each assay mixture contained 0.04 pmol of primed M13mp18 molecules. See Materials and Methods for details.

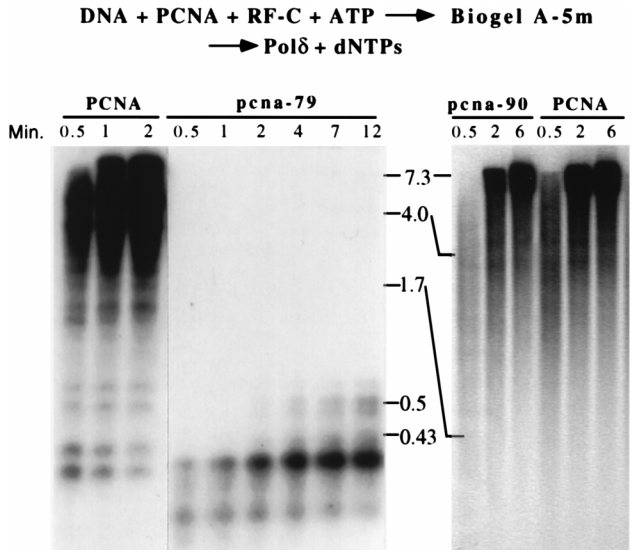

FIG. 4. Processivity of Polδ holoenzyme during replication of single-stranded M13mp18 DNA. A flow diagram of the experiment is given at the top. Wild-type or mutant complexes were made and isolated as described in Materials and Methods. Time points for subsequent replication with Polδ are indicated. The samples were sized in a 1.5% (left panel) or 1% (right panel) alkaline agarose gel and autoradiographed. Migration positions for DNA size markers (in kilobases) are indicated. The experiments in the left and right panels are separate and therefore cannot be directly compared with regard to autoradiographic intensities.

and reassembly of Polδ holoenzyme, with the latter step being stimulated by polyethylene glycol. A minor defect in Polδ holoenzyme activity was observed with pcna-90, which affected the total level of DNA synthesis rather then the response to the concentration of PCNA (Fig. 3A). The opposite situation was observed when Polδ holoenzyme activity was measured, with pcna-90 being completely inactive and pcna-79 having a reduced activity (Fig. 3B).

None of these defects was due to a defect in the loading of the mutant PCNA by RF-C. RF-C loads PCNA onto the primer terminus of primed DNA in an ATP-dependent fashion. Although a detailed biochemical mechanism for this step has not yet been described, it appears that release of RF-C from the ternary PCNA · RF-C · DNA complex is accompanied by hydrolysis of the bound ATP. As a result, the ATPase activity of RF-C is stimulated by PCNA when primed DNA is also present (10, 30, 52, 56). Therefore, the ATPase stimulation assay measures the ability of PCNA to interact with RF-C and with the DNA. The data in Figure 3C indicate a wild-type interaction between RF-C and pcna-79 or pcna-90. In addition, a gel filtration analysis which allows separation of DNA-bound PCNA from free PCNA, followed by Western blotting to quantitate DNA-bound PCNA, showed that pcna-79 and pcna-90 were loaded by RF-C to similar extents as wild-type PCNA (results not shown).

pcna-79-dependent Polδ replication is associated with extensive pausing on a structured template. For further insight into the defect associated with the pcna-79 holoenzyme, the products of Polδ replication on singly primed M13mp18 were visualized by autoradiography. Figure 4 shows that replication with wild-type holoenzyme was highly processive and nearly complete after 1 min at 37°C. A comparable reaction with pcna-79 holoenzyme, in contrast, resulted in the accumulation of small replication intermediates, with no completely replicated molecules visible even after 12 min. Replication intermediates of similar size were also observed in the wild-type holoenzyme reaction, but only as transient pause sites. These data suggest that intrinsic secondary structure in the M13mp18 template may lead to aborted elongation by the mutant Polδ holoenzyme.

Progression of DNA synthesis by the pcna-90 Polδ holoenzyme was very similar to that by the wild-type holoenzyme, with the exception that the total amount of DNA synthesis was

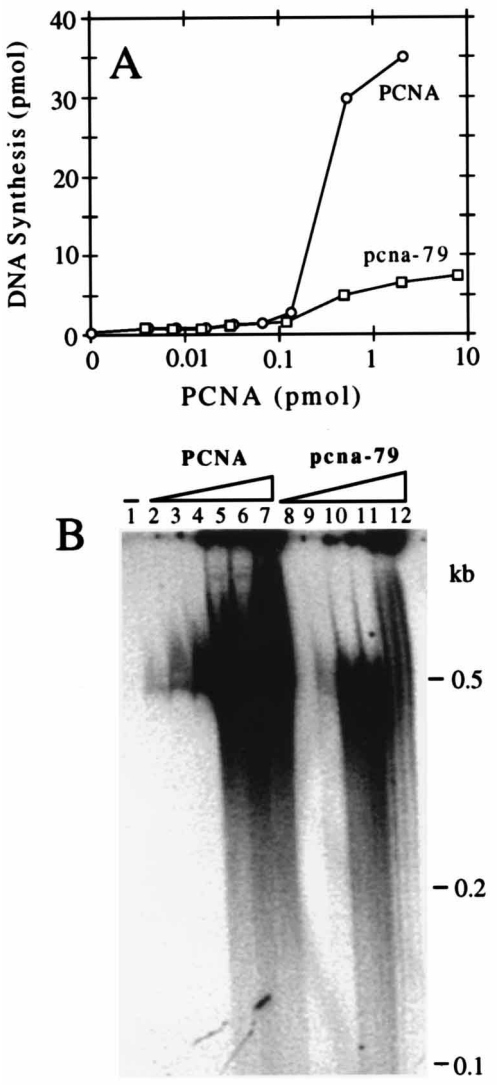

FIG. 5. Replication of $(dT)_{22}$-primed $poly(dA)_{500}$ by Polδ holoenzyme. (A) Total DNA synthesis as a function of PCNA or pcna-79 concentration. (B) Analysis of replication products. DNA was electrophoretically fractionated on an 8% polyacrylamide gel. Lane 1, no PCNA; lanes 2 to 7: 0.04, 0.07, 0.15, 0.6, 2.5, and 10 pmol of PCNA, respectively; lanes 8 to 12: 0.04, 0.15, 0.6, 2.5, and 10 pmol of pcna-79, respectively.

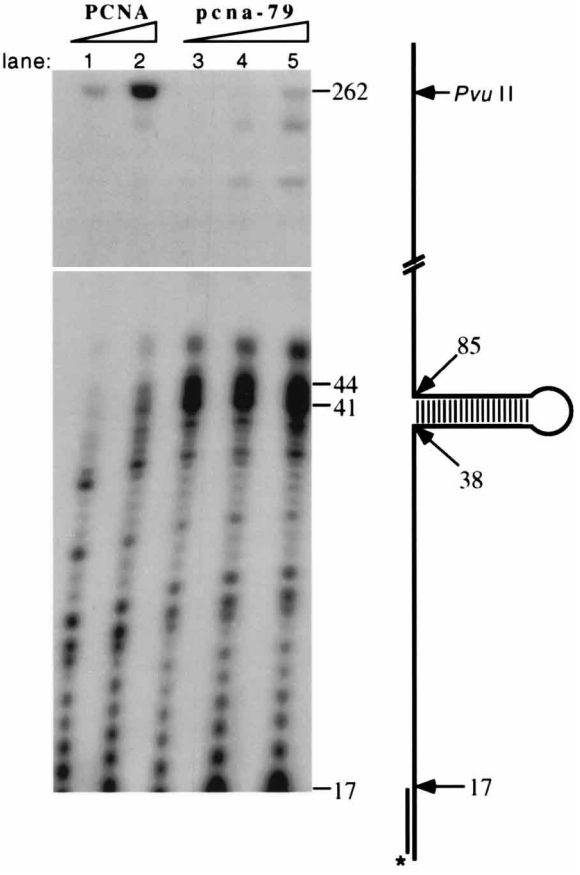

FIG. 6. pcna-79 containing holoenzyme pauses at a DNA hairpin. A ^{32}P-end-labeled 17-mer primer was annealed to single-stranded M13mp7 DNA and used for replication by Polδ as described in Materials and Methods for replication of M13mp18 DNA, except that the assay mixture contained the four dNTPs at 100 μM each, 0.1 pmol of primed circles, and 0.06 pmol (lane 1) or 0.5 pmol (lane 2) of wild-type PCNA or 0.3 pmol (lane 3), 1.2 pmol (lane 4), or 5 pmol (lane 5) of pcna-79. Replication products were digested with *Pvu*II, electrophoresed in an 8% denaturing polyacrylamide gel, and visualized by autoradiography. Marker positions (in nucleotides) are from sequencing lanes with the same primer. The template DNA, with the positions of the primer, the stem-loop, and the site of cutting by *Pvu*II indicated, is depicted beside the autoradiogram. The middle portion of the autoradiogram has been deleted; it contained no radioactivity.

lower (Fig. 4). These data and those in Fig. 3A suggest that RF-C-mediated loading of pcna-90 onto the primed DNA produces a fraction of complexes (about 30 to 50%) which is inactive for replication upon addition of Polδ. Whether this fraction of DNA-bound pcna-90 fails to bind Polδ or whether it fails to replicate when bound in a complex with Polδ is not clear from these studies. However, those complexes which are active replicate like those with the wild-type Polδ holoenzyme.

While pcna-79-dependent replication by Polδ on a singly primed M13mp18 template is visibly less processive than that by the wild-type Polδ holoenzyme, this lower processivity appears to be associated with discrete pause sites, which presumably are caused by extensive secondary structure in the mp18 template. Therefore, we examined processivity of pcna-79-dependent Polδ replication on an oligo(dT)-primed poly(dA) template which lacks secondary structure. Replication of $poly(dA)_{500} \cdot oligo(dT)_{22}$ by the mutant holoenzyme was severely impaired compared to wild type (Fig. 5A). However, when the replication products were size fractionated, it became evident that full-length replication products were synthesized, indicating that the mutant holoenzyme was completely processive on the poly(dA) template (Fig. 5B). Therefore, the low activity of pcna-79 Polδ holoenzyme on this template-primer is due not to a lack of processivity but to a defect in the formation of a productive holoenzyme complex.

The most likely explanation for the discrete sizes of replication intermediates in the pcna-79 reactions is that of replicative pausing at sites of stable secondary structure in the otherwise single-stranded M13mp18 DNA molecules. To test directly the susceptibility of pcna-79 holoenzyme to pausing at a known secondary structure, we compared the abilities of wild-type and mutant holoenzymes to replicate through the intrinsic hairpin formed by the M13mp7 DNA polylinker sequence (Fig. 6). The palindromic polylinker of this phage DNA forms a stem-loop structure with a 22-nt stem and a 4-nt loop (17). The wild-type holoenzyme replicated readily through this hairpin structure (Fig. 6, lanes 1 and 2), even with PCNA present at a

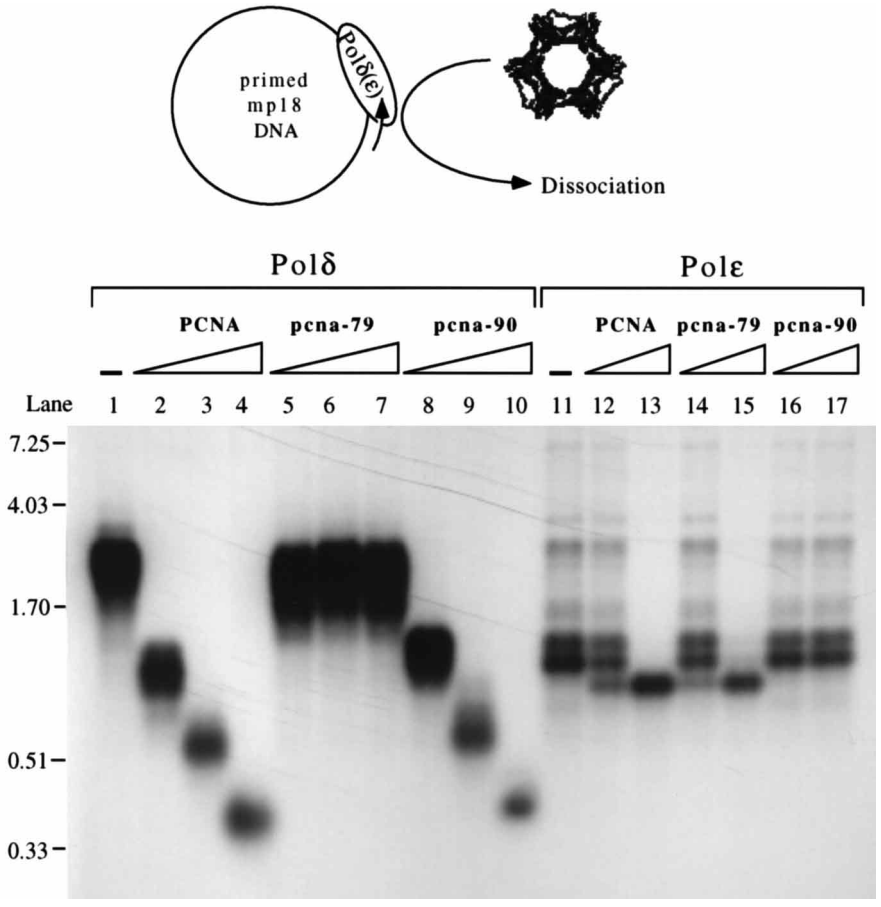

FIG. 7. PCNA-mediated inhibition of replication on circular DNA. Polδ or Polε replication on SSB-coated singly primed M13mp18 template was performed with or without added wild-type or mutant PCNA as described in Materials and Methods. Lanes 1 to 10, Polδ; lanes 11 to 17, Polε. Lanes 1 and 11, no wild-type or mutant PCNA; lanes 2, 5, and 8, 25 ng; lanes 3, 6, and 9, 100 ng; lanes 4, 7, 10, 12, 14, and 16, 400 ng; lanes 13, 15, and 17, 2,000 ng. The replication products were separated in a 1.5% alkaline agarose gel. Migration of DNA size standards (in kilobases) is indicated to right. The principle of the inhibition assay is shown at the top and described in the text.

substoichiometric level (lane 1). In contrast, most of the products of pcna-79 holoenzyme replication terminated at three major sites which are 4 to 6 nt into the stem just prior to six consecutive C and G residues (Fig. 6, lanes 3 to 5). A 50-fold molar excess of pcna-79 permitted limited replication through the stem-loop. This is in agreement with the data in Fig. 3A, which also showed that high levels of pcna-79 partially suppressed the replication defect of the mutant. Thus, DNA secondary structure likely accounts for the pause sites observed during pcna-79-mediated replication of M13mp18.

pcna-79 has reduced affinity for DNA Polδ, and pcna-90 has reduced affinity for DNA Polε. The preceding biochemical assays suggest that pcna-79 is defective in its interaction with Polδ. In contrast, pcna-90-dependent Polδ replication was only modestly defective, while this mutation severely reduced Polε replication. We used three distinct assays to either directly or indirectly assess the affinities of pcna-79 and pcna-90 for Polδ and Polε. The first assay is based on the observation that in the absence of RF-C, nonprocessive replication by Polδ on primed M13mp18 DNA is inhibited by PCNA (8). In this assay the circularity of the M13mp18 template prevents diffusional loading of PCNA onto the primer terminus. Although the mechanism of inhibition has not been established, the inhibition may be caused either by formation of a complex of Polδ and PCNA in solution with a reduced affinity for the template-primer or by direct dissociation of Polδ from the template-primer through the formation of an unproductive Polδ-PCNA complex (Fig. 7, top). Regardless of the mechanism, the inhibition assay is an indirect measure of the strength of interaction between Polδ and PCNA and, analogously, of that between Polε and PCNA. Increasing amounts of wild-type PCNA resulted in the accumulation of shorter replication products with Polδ, signifying strong inhibition (Fig. 7). In contrast, 16-fold-higher concentrations of pcna-79 caused no significant reduction in DNA synthesis by Polδ. Therefore, the affinity of pcna-79 for Polδ is dramatically reduced. In contrast, pcna-90 was indistinguishable from wild-type PCNA in sequestering Polδ. An analogous assay with Polε showed that pcna-79 was nearly as efficient as wild-type PCNA in sequestering Polε, whereas a fivefold-higher pcna-90 concentration had no inhibitory effect (Fig. 7).

The second assay directly measures binding of Polδ to wild-type PCNA coupled to agarose beads. The abilities of the mutant PCNAs to compete binding of Polδ to these PCNA-agarose beads are a measurement of their affinities for Polδ relative to that of wild-type PCNA (Fig. 8). pcna-90 was similar to wild-type PCNA in its ability to compete Polδ off the PCNA beads: a twofold excess of these PCNAs reduced binding of Polδ to the beads by about 50%. In contrast, even an 18-fold excess of pcna-79 completely failed to compete, indicating no measurable interaction between pcna-79 and Polδ (Fig. 8). As

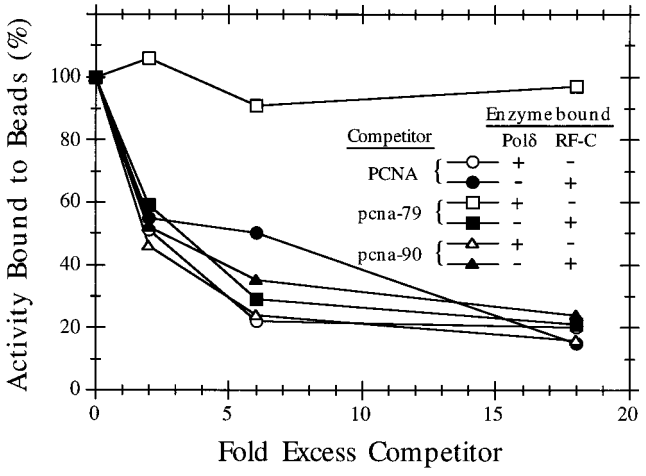

FIG. 8. Binding of Polδ and RF-C to PCNA-agarose beads. For details, see Materials and Methods.

expected from the results in Fig. 3C, the mutant PCNAs were similar to the wild type in their ability to compete RF-C from binding to the PCNA beads (Fig. 8). Binding of Polε to the PCNA beads was too weak and irreproducible to allow usage of the competition assay (data not shown).

The third assay measures the binding of the mutant PCNAs to individual subunits of Polδ. For this assay, a derivative of PCNA with an amino-terminal addition of eight amino acids which can be phosphorylated by the cyclic AMP-dependent protein kinase (see Materials and Methods) was used (40). The radiolabeled PCNA was used to probe a blot of immobilized Polδ subunits. Under these conditions, PCNA binds exclusively to the 55-kDa subunit of Polδ, the *POL32* gene product (Fig. 9) (13). Binding of the probe to the blot was competed by addition of excess unlabeled wild-type or mutant PCNAs during the probing, and the radioactivity remaining was quantitated. pcna-79 completely failed to compete binding of the probe to the blot, indicating that the interaction between the mutant and the 55-kDa subunit was reduced by at least 2 orders of magnitude (Fig. 9). Surprisingly, pcna-90, which in the previous two interaction assays was shown to have an affinity for Polδ equal to that of wild-type PCNA, was a 5- to 10-fold-less-effective competitor than wild-type PCNA in this blot assay. Apparently, the defect in the interaction between pcna-90 and the 55-kDa subunit is only a minor one, because it did not show up as a defect when binding to the three-

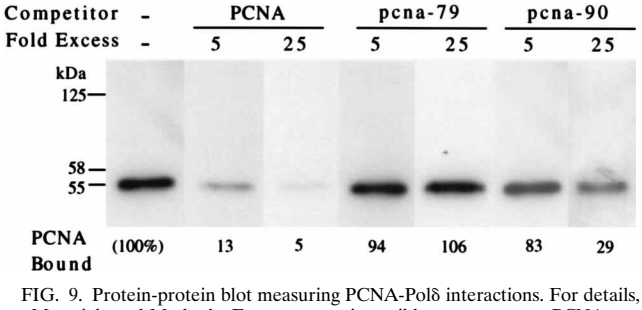

FIG. 9. Protein-protein blot measuring PCNA-Polδ interactions. For details, see Materials and Methods. Excess competitor wild-type or mutant PCNA was included during the probing with ^{32}P-labeled PCNA. Migration positions of the three subunits of Polδ are indicated.

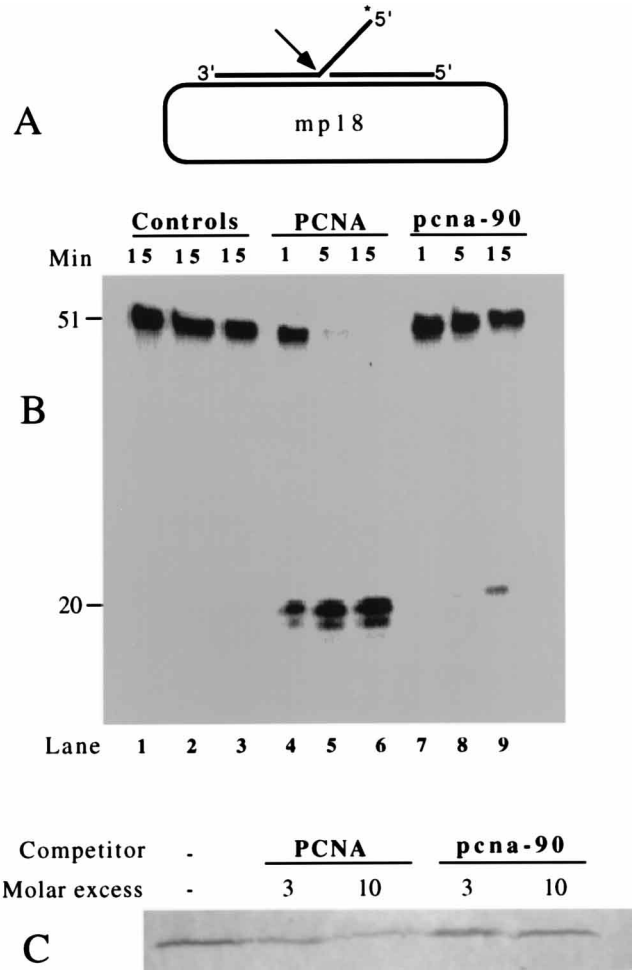

FIG. 10. Stimulation of FEN-1 activity by PCNA. (A) Diagram of the DNA FLAP substrate. The position of the label is indicated by the asterisk on the flap strand. (B) Separation of FEN-1 reaction products on a denaturing 15% polyacrylamide gel. The mobility of the cleaved product is 19 to 20 nt. Lane 1, no enzyme; lane 2, FEN-1 only; lane 3, FEN-1, PCNA, and RF-C but no ATP; lanes 4 to 9, FEN-1, RF-C, PCNA or pcna-90 as indicated, and ATP. For details, see Materials and Methods. (C) Binding of FEN-1 (50 ng) to PCNA beads. Competitor PCNA or pcna-90 was added as indicated. FEN-1 bound to the beads was released with 1 M NaCl and visualized by an immunoblot analysis. For details, see Materials and Methods and reference 32.

subunit Polδ was measured (Fig. 7 and 8), perhaps because the other subunits also contribute to binding.

pcna-90 is defective with FEN-1. FEN-1, the product of the *RTH1* gene, is a structure-specific endonuclease proposed to function in Okazaki fragment maturation and in mismatch repair (18, 21, 44, 47). Because of the known interaction between FEN-1 and PCNA and the mismatch repair defect in pcna-90, we determined whether this PCNA mutant showed a defect in the interaction with FEN-1 (32). The assay shown in Fig. 10 measures cutting of a flap structure by FEN-1. At the physiological salt concentration used in this assay (125 mM NaCl), FEN-1 activity is absolutely dependent on the presence of PCNA, which has been loaded onto the DNA substrate by RF-C and ATP (32). pcna-90 showed a severe defect in stimulating FEN-1 activity, being 30- to 50-fold less effective than wild-type PCNA (Fig. 10B). pcna-79 was as active as wild-type PCNA in this assay (data not shown). The defect with pcna-90 is at least in part due to a defect in the interaction between

FEN-1 and pcna-90. pcna-90 was approximately 5- to 10-fold less effective than wild-type PCNA in competing binding of FEN-1 to PCNA beads (Fig. 10C).

DISCUSSION

***POL30* interdomain connector loop mutations define a site critical for Polδ holoenzyme assembly.** Among the mutant *POL30* alleles characterized genetically in this study, *pol30-79* and *pol30-90* were selected for detailed biochemical characterization because these alleles conferred a moderate-to-severe sensitivity to mutagenesis and elevated spontaneous-mutation rates. The amino acid replacements introduced by the *pol30-79* allele, L126A and I128A, truncate exposed hydrophobic side chains in the midst of an extended polypeptide strand traversing the outer edge of the PCNA subunit (Fig. 1). These two amino acids are absolutely conserved in PCNAs from all organisms. In contrast, flanking charged amino acids are not conserved or are only in part conserved, and alanine substitution mutations in those amino acids (K127, E129, and E130) show no effects in vivo (Table 2). Leu126 and Ile128 form a hydrophobic cleft in which a hydrophobic side chain of an interacting protein could be inserted. Mutation of these two residues eliminates interaction with the 55-kDa subunit of Polδ and with Polδ itself in the absence of DNA but allows processive DNA synthesis on a template lacking secondary structure. Surprisingly, this PCNA mutation, which leads to a severely crippled Polδ holoenzyme in our in vitro studies, has only a minimal effect in vivo. It is likely that interactions with additional replication proteins serve to stabilize the replisome.

The replicative defect in pcna-79 suggests a hierarchy in PCNA-Polδ interactions leading to a productive holoenzyme complex. We suggest a two-step process of holoenzyme formation to account for the observed results: (i) the extended loop connecting the two domains of the PCNA subunit functions as an initial contact domain to catch the polymerase via its 55-kDa subunit during holoenzyme complex formation at the template-primer junction, and (ii) contacts with other residues subsequently stabilize the holoenzyme complex during processive elongation in the absence of a potential block to fork progression. Some of these contacts may be on the inside surface of the PCNA ring (12). These interactions are presumably mediated through the catalytic and/or 58-kDa subunit. A previous study has shown that PCNA stimulates replication by the catalytic subunit of yeast Polδ and has suggested interaction with an N-terminal domain of this subunit (6). When potential blocks to elongation are encountered, as shown in the experiments of Fig. 4 and 6, contacts with the loop are critical to stabilize the holoenzyme. Otherwise, complex dissociation occurs, as seen in the pcna-79 mutant, and traversion of the block can be accomplished only through multiple cycles of complex reloading, a step which is stimulated by high levels of pcna-79, Polδ, or polyethylene glycol (Fig. 3A and 6 and data not shown). In the T4 polymerase holoenzyme system, the same stem-loop structure caused rapid holoenzyme dissociation unless the stem was melted out by the T4 single-stranded binding protein (17).

With these considerations in mind, the low stimulatory activity of pcna-79 on poly(dA) · oligo(dT) is readily understood (Fig. 5). PCNA rapidly dissociates from linear DNA and is stabilized on the DNA only by interaction with the polymerase (34, 39). Therefore, pcna-79 trimers loaded by RF-C onto poly(dA) · oligo(dT) rapidly slide off the DNA, as they fail to be anchored efficiently by Polδ. However, those few protein molecules which do form a complex with Polδ replicate processively.

TABLE 4. Summary of biochemical activities of mutant PCNAs

Characteristic	Activity[a] of:	
	pcna-79	pcna-90
RF-C interaction	1	1
Stimulation of RF-C ATPase	1	1
Loading by RF-C	1	1
Polδ interaction	<0.01	1
55-kDa subunit interaction	<0.01	0.2
Activity with Polδ on:		
Poly(dA) · oligo(dT)	0.05	1
mp18 DNA	0.001	0.5
Processivity with Polδ on:		
Poly(dA) · oligo(dT)	1	
mp18 DNA	0.05	1
Polε interaction	1	<0.1
Activity with Polε on mp18 DNA	0.1	<0.001
FEN-1 interaction		0.2
Stimulation of FEN-1	1	0.02

[a] All values are relative to those for wild-type PCNA (defined as 1) and are estimated from the data in Fig. 3 to 5 and 7 to 10.

Whereas pcna-79 is defective predominantly in the interaction with Polδ, the mutant is also partially defective for replication with Polε, even though its physical interaction with Polε is similar to that of wild-type PCNA (Fig. 7; Table 4). Conversely, pcna-90, which is completely defective with Polε in all assays, shows a minor defect with Polδ (Table 4). Possibly, these minor defects are caused by slight structural changes in the mutant PCNAs which express themselves in the sensitive replication assays. Alternatively, in a replication complex the DNA polymerases may contact PCNA across a broad surface including the interdomain connector loop, the carboxy terminus, and perhaps also inner-ring positive amino acids (12), with the interdomain connector loop being most important for strong interactions with Polδ and the carboxy terminus being most important for strong interactions with Polε.

The demonstration of interactions between the 55-kDa subunit of Polδ and PCNA and of the loss of these interactions in the interdomain connector loop mutant pcna-79 does not preclude interactions between the loop and another subunit of Polδ. The protein-protein blot (Fig. 9) may be biased in detecting only the 55-kDa subunit, either because of the binding conditions or because the other subunits do not properly regenerate on the blot. Interestingly, the homologous interdomain connector loop in human PCNA has been implicated in interactions with p21 (16, 55). As p21 is an inhibitor of PCNA-dependent DNA replication, our data suggest that the mechanism for this inhibition may be a direct competition between p21 and Polδ for an overlapping target site in the interdomain connector loop. Paradoxically, however, the homolog of the 55-kDa subunit is missing from purified preparations of mammalian Polδ (see the introduction). This indicates that the catalytic subunit or the 48-kDa subunit of human Polδ interacts with the interdomain connector loop of PCNA and is subject to displacement by p21.

Multiple defects in carboxy-terminal *POL30* mutants. Recently, Amin and Holm (1) isolated by random mutagenesis a set of eight cold-sensitive *POL30* mutations. Six of these map to the carboxy-terminal region of the protein investigated in the present study (1). Random mutagenesis may identify amino acid changes which disturb the structure and folding of the protein, interaction with other factors, or function at the restrictive temperature. The first may be the case for the *pol30-107* mutant, as the phenylalanine-to-serine change at residue

249 resulted in cold sensitivity and MMS sensitivity, whereas replacement of the two phenylalanines at residues 248 and 249 by alanines in the *pol30-89* mutant caused no discernible phenotype (Table 2) (1). Interestingly, three of the random mutations that confer cold sensitivity are changes at residues 251 (A→V for *pol30-104* and A→T for *pol30-108*) and 253 (K→E for *pol30-100*). These overlap with the *pol30-88* and *pol30-90* mutations, which also confer a pronounced cold sensitivity. Furthermore, both the *pol30-104* and *pol30-90* mutants are mutators and are partially defective for mismatch repair (Table 3) (19). An additional PCNA mutant, the *pol30-52* mutant, which is a monomer in vitro, is also defective for mismatch repair (53). However, as this mutant also shows extensive deficiencies in DNA replication and repair, the observed mismatch repair deficiency may be very general and not due to loss of specific protein-protein interactions with the mismatch repair machinery (see below) (3).

PCNA interacts with FEN-1 but also with the MSH2-MSH3 heterodimeric complex. Both factors are required for mismatch repair in yeast (19, 20, 33). The site on PCNA of interaction with MSH2-MSH3 is not known. Furthermore, a genetic interaction between *MLH1*, another required mismatch repair gene, and PCNA has been identified (43, 53). As this interaction was determined genetically by the two-hybrid method, it is uncertain whether it is direct or indirect, e.g., through MSH2-MSH3 or FEN-1. Our data show that pcna-90 is defective for interaction with FEN-1 and suggest that this defect may be responsible for the mismatch repair defect in the *pol30-90* mutant, and perhaps also in the *pol30-104* mutant (Fig. 10). On the other hand, the observed mismatch repair phenotype observed in another PCNA mutant, the *pol30-52* mutant, whose mutation maps to the subunit interface and which is a monomer in vitro, cannot be attributed to a loss of PCNA–FEN-1 interactions. The physical interactions between pcna-52 and FEN-1 were comparable to wild type (32). However, in this case, the observed failure of pcna-52 to stimulate FEN-1 activity was caused by a failure of the monomeric mutant to encircle the DNA and thereby interact functionally with FEN-1 (32).

In addition to a defect in the interaction with FEN-1, pcna-90 is also defective in interaction with Polε (Fig. 3B and 7), indicating that the tetrapeptide motif LAPK, which is invariant in PCNA, has a dual function in the interaction with these two replication proteins. Currently, it remains unresolved whether specific or combined defects are responsible for the cold-sensitive phenotype.

Susceptibility of the *SUP4-o* locus to spontaneous mutations. The *SUP4-o* locus has been used extensively as a convenient target for determining spectra of both spontaneous and mutagen-induced mutations, mainly because almost any base change in the 89-nt tRNA gene gives a scorable phenotype (24). The spontaneous-mutation rate at *SUP4-o* in a *pol30-90 pms1*Δ strain is comparable to that in the *pms1*Δ or *pol30-90* single-mutant strain, consistent with the conclusion that *pol30-90* is defective for mismatch repair. The increase in mutation rate at the *SUP4-o* locus when mismatch repair is inactive is only 10-fold, which is low compared to that of other targets (see, e.g., references 19, 26, and 43). This discrepancy has been noted by others and may relate to the observation that, after correction for penetrance of mutations, the spontaneous-mutation rate per base pair at *SUP4-o* is higher than that at other loci, e.g., *URA3* (9). Therefore, it is possible that mismatch repair in the *SUP4-o* locus is relatively inefficient.

The mutation rates determined for the *pol30-79* mutant provide an puzzling twist to this. The *pol30-79* mutant is a marginal mutator for most targets, indicating that mismatch repair is operational in this PCNA mutant. However, the *pol30-79* mutant is a much stronger mutator than the *pms1*Δ mutant at *SUP4-o*. Moreover, the *pol30-79 pms1*Δ double mutant is not a stronger mutator than *pol30-79* alone (Table 3). The spontaneous mutations arising in the mutant are point mutations and/or single- or double-base-pair insertions or deletions which should be substrates for the mismatch repair machinery (data not shown). One explanation for these observations is that replication of pcna-79 through *SUP4-o* is accompanied by an increased rate of insertion mutations, or a decreased efficiency of proofreading, by the polymerase, perhaps in response to replication blocks at this locus. The main defect of the pcna-79 Polδ holoenzyme is its inefficient replication though secondary structures. In addition, these lesions are not processed by the mismatch repair machinery.

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ADDENDUM IN PROOF

A recent paper (D. X. Tishkoff, N. Filosi, G. M. Gaida, and R. D. Kolodner, Cell **88:**253–263, 1997) questions the involvement of FEN-1 in mismatch repair. Although this study does not affect our conclusion that the *pol30-90* mutant is defective in mismatch repair, it does make our suggestion that the defect in this mutant is due to a defective interaction with FEN-1 unlikely.

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