DNA replication efficiency is dictated by DNA polymerases (pol) and their associated proteins. The recent discovery of DNA polymerase Y family (DinB/UmuC/RAD30/REV1 superfamily) raises a question of whether the DNA polymerase activities are modified by accessory proteins such as proliferating cell nuclear antigen (PCNA). In fact, the activity of DNA pol IV (DinB) of *Escherichia coli* is enhanced upon interaction with the β subunit, the processivity factor of DNA pol III. Here, we report the activity of *Sso* DNA pol Y1 encoded by the *dbh* gene of the archaeon *Sulfolobus solfataricus* is greatly enhanced by the presence of PCNA and replication factor C (RFC). *Sso* pol Y1 per se was a distributive enzyme but a substantial increase in the processivity was observed on poly(dA)-oligo(dT) in the presence of PCNA (039p or 048p) and RFC. The length of the synthesized DNA product reached at least 200 nucleotides. *Sso* pol Y1 displayed a higher affinity for DNA compared with pol IV of *E. coli*, suggesting that the two DNA polymerases have distinct reason(s) to require the processivity factors for efficient DNA synthesis. The abilities of pol Y1 and pol IV to bypass DNA lesions and their sensitive sites to protease are also discussed.

The recently identified novel family of DNA polymerases (pol)1 (DinB/UmUC/Rev1/Rad30 superfamily) comprises proteins from different species including Bacteria, Eukarya, and Archaea (1, 2). Members of this superfamily can bypass lesions on template DNA, such as ultraviolet light photoproducts, and mismatched primer/template DNA (3–7). Thus, the new DNA polymerases seem to function by assisting the conventional DNA replicases to cope with faulty DNA templates by taking over the DNA synthesis at aberrant sites (for recent reviews, see Refs. 1 and 8–12). Common features of family Y DNA polymerases include the lack of 3′-exonuclease proofreading activity and synthesis of DNA with low fidelity in a distributive fashion. The human DNA pol γ has been shown recently to possess 5′-deoxyribose phosphate lyase activity, suggesting a role in base excision repair (13). It has been hypothesized that the very low processivity of these polymerases prevents excessive introduction of mutations because of extended error-prone DNA synthesis. This has been, however, questioned recently when DNA polymerase IV of *Escherichia coli* (pol IV) (7) was found to synthesize tracks of more than 300 nucleotides upon interaction with the β subunit of pol III (14). The β subunit and its eukaryotic counterpart, i.e. PCNA, play essential roles in processive chromosomal replication by forming a sliding platform that mediates the interaction of DNA polymerases with DNA (15). In addition, the sliding clamps also interact with a variety of proteins other than DNA polymerases involved in DNA processing and even in cell cycle control (16). It is therefore of general interest to elucidate the relationships between various members of Y family DNA polymerases and the corresponding sliding clamps.

Archaea, the third domain of life, is thought to possess a DNA replication apparatus similar to that of Eukarya, although its morphology is more prokaryotic-like (17). As evidenced by the genome sequencing project, many eukaryotic replication proteins have close homologues in Archaea (18, 19). Interestingly, the thermoacidophilic archaeabacterium *Sulfolobus solfataricus* possesses two PCNA-like sliding clamps termed *Sso* 039p (244 amino acids, 27 kDa) and *Sso* 048p (249 amino acids, 27 kDa) (20). Both proteins possess a trimeric structure, which is similar to eukaryotic PCNAs, and functions as a processivity factor that stimulates the activity of monomeric family B DNA polymerases from this organism (*Sso* DNA polymerase B1) (21–24) by enhancing processivity. In addition, a clamp-loader apparatus was recently isolated from *S. solfataricus* (25). It is a complex of a homotetramer of a small subunit (37 kDa) and a large subunit (46 kDa), and thus is named *Sso* RFC complex. It has been shown that the amount of PCNA (either 039p or 048p) required for *Sso* DNA pol B1 stimulation markedly decreased in the presence of RFC complex and ATP.

Here, we report the biochemical characteristics of the DinB homologue (Dbh) of *S. solfataricus* (26), which we term *Sso* DNA pol Y1. Although this polymerase per se is a distributive enzyme, its activity is enhanced markedly in the presence of PCNA (either 039p or 048p) and RFC complex. Because the DNA replication apparatus of Archaea is closer to that of Eu-

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**Synthetic Activity of *Sso* DNA Polymerase Y1, an Archaeal DinB-like DNA Polymerase, Is Stimulated by Processivity Factors Proliferating Cell Nuclear Antigen and Replication Factor C**

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Proliferating Cell Nuclear Antigen and Replication Factor C*
karya than Bacteria, this finding has implications for other Y-type DNA polymerases from higher organisms including humans.

**EXPERIMENTAL PROCEDURES**

*Overexpression and Purification of Native Sso pol Y1—*The *dbh* gene was amplified by polymerase chain reaction technique using the *S. solfataricus* genomic DNA as a template and cloned into the vector pET-21a (Novagen, Madison, WI) creating the expression plasmid pET-
dbh.21. Sso pol Y1 was expressed from this plasmid in *E. coli* strain BL21-CodonPlus (DE3)-RIL (Novagen) after induction with isopro-
sulfolatericus (Fig. 1B)

**Primer Extension Assay—**Standard polymerase reactions (10 μl) were performed in 30 mM potassium phosphate buffer (pH 7.4), 7.5 mM MgCl₂, 1.25 mM β-mercaptoethanol, 5% glycerol, 100 μM dNTPs, 50 mM Sso pol Y1 protein, and 50 μM annealed 5'-3'-primer/template substrate at 55 °C for 10 min unless otherwise indicated. When DNA pol IV of *E. coli*, Klonen exo−, or Klonen exo− was used instead of pol Y1, the concentration of the enzyme was 10 nM and the reaction was performed at 37 °C for 10 min. In some lesion bypass experiments, the concentration of pol IV was 20 or 100 nM as indicated in Fig. 4D. Reactions were terminated by adding one volume of stop solution (95% formamide, 20 mM EDTA, 0.025% xylene cyanol, and 0.025% bromphenol blue), and the products were resolved by electrophoresis in 12% denaturing polyacrylamide gel and visualized by phosphorim-
aging using the Molecular Imager System GS-525 (Bio-Rad). Klonen exo− and Klonen exo− were purchased from New England Biolabs (Beverly, MA). DNA pol IV of *E. coli* was purified as described previously (14). The template used for all reactions was 5'-GAAGGGATCCTTAAGACCGG-3', where N represents either A, T, G, or C and Y represents G for dNMP incorpor-
ation assays (Figs. 1B, 2, and 3). For lesion bypass assays, N represents T, and Y represents either G (no lesion) or one of the four DNA lesions, i.e., abasic site (AP site): tetrahydrofuran, 8-oxoguanine, O2-methylguanine, and uracil (Fig. 4). The primers used were 5'-CGCGCGAAGCGGC-3' for standard dNMP incorporation assays (Fig. 1B) and "running start" bypass assays (Figs. 4, A and B), 5'-CGCGCGAAGCGGTTA-3' for "standing start" bypass assays (Figs. 4, C and D), and 5'-CGCGCGAAGCGGTTAC-3' for single dNMP incorporation assays (Fig. 2A). The primer 5'-CGCGCGAAGCGGAC-
TTACX-3' was used for 3'-exonuclease assays (Fig. 2D), where X represents G, and for terminal mismatch extension assays (Fig. 3), where X represents any of the four bases. The 36-mer templates containing the DNA lesions were synthesized by Nippon Gene Ltd. (Toyama, Japan). All other oligonucleotides were synthesized by BEX Corp. (Tokyo) and double-purified by high performance liquid chromatography.

**Limited Proteolysis—**Proteins were subjected to limited digestion by subtilisin (Wako Pure Chemical, Osaka, Japan) in a buffer containing 10 mM HEPES (pH 7.5), 150 mM NaCl, 250 μM CaCl₂, and 0.005% Tween 20 at protein concentration of 1 mg/ml. The reaction was carried out for 30 min at room temperature and stopped by adding the standard Laemmli SDS-PAGE loading buffer followed by immediate denatur-
ation at 100 °C for 5 min. Protein fragments were resolved by 15% SDS-PAGE and visualized by Coo massie G-250 staining. The N-termi-
nal amino acid sequences of selected proteolytic fragments were deter-
mined at Takara Shuzo Co. (Shiga, Japan) after transfer to Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad) using the instructions provided by the manufacturer.

**Activity Enhancement Assay—**The standard reaction mixture (10 μl) contained 50 mM HEPES (pH 8.0), 250 mM NaCl, 50 mM MgCl₂, 0.2 mM dTTP, 1 mM ATP, 18.5 ng/μl poly(dA)·poly(dT)·poly(dG)·poly(dC) (70:1 template to primer molar ratio) substrate, 0.36 μM Sso RFC complex, 0.87 μM Sso PCNA 039 or 048 (as a trimer), and variable amount of purified Sso pol Y1. The Sso RFC complex, Sso PCNA 039, and Sso PCNA 048 proteins were purified as described previously (20, 25). Reactions were carried out for 10 min at 60 °C and terminated by adding stop solution in the primer extension assay. Elongation products were separated by electrophoresis in 6% denaturing polyacryla-
mine gel and visualized by autoradiography.

**Surface Plasmon Resonance Analysis—**The DNA binding assay was performed using the BLAcoreX instrument (Biacore K.K., Japan). About 150 fmol of 34-mer oligonucleotide biotinylated at its 3' end was cap-
tured on the SA chip surface and optionally annealed with complemen-
tary oligonucleotides to form either primed DNA or dsDNA. Analysis was performed by injecting the appropriate proteins at specific concentra-
tion within the range of 0.05–1 μM in the standard buffer HBS-EP (0.01 M HEPES (pH 7.4), 0.15 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20) over the chip surface. The equilibrium dissociation constants (**Kₐ**) were calculated from the kinetic traces using the BLAcoreX software version 3.0 (Biacore AB) according to the predefined model "1:1 binding with mass transfer." In the case of pol Y1, we assayed the interactions at two different temperatures (25 and 40 °C), but found no differences. All other proteins were tested at the standard temperature of 25 °C. Ssb was purchased from U. S. Biochemical Corp.

**RESULTS**

*dbh Gene Product Is a Novel Y-type DNA Polymerase—*The *dbh* gene of *S. solfataricus* was described previously as an archaeal DinB homologue based on its sequence similarity to the DinB-like proteins (26). We cloned the gene and overex-
pressed its product in *E. coli*. The protein was purified to homogeneity using four chromatographic steps as shown in Fig. 1A, and its DNA polymerase activity was demonstrated by the primer extension assay. Fig. 1B shows that DNA synthesis was dependent on the presence of template DNA and that the synthesis was very distributive (lanes 2 and 3). It possessed no RNA polymerase activity (lane 4). The activity was completely inhibited by the addition of EDTA (lane 5), but was resistant to inhibitors, i.e., dTTTP (lane 6) and aphidicolin (lane 7). The polymerase was most active over the range 55–70 °C with a longer template primer (52/34-mer) (data not shown). Hence, the *dbh* gene shares similarity with other homologues of DinB/ Umuc/Rev/Rad30 proteins at functional level.

Recently, the superfamily of DinB/Umuc/Rev/Rad30 proteins was classified as family Y of DNA polymerases next to the
family X of DNA polymerases/deoxynucleotidyltransferases (2). Based on this classification, we termed the product of dbh gene Sso DNA polymerase Y1 (Sso pol Y1) based on the previously introduced nomenclature system used for DNA polymerases from *Sulfolobus* (27). To examine whether Sso pol Y1 is a bona fide DNA polymerase obeying Watson-Crick base pairing rules, we conducted the single dNMP incorporation assay (Fig. 2A).

Indeed, the prevalently incorporated bases by pol Y1 correctly matched the template sequence. Only the incorporation of dGMP opposite C may seem unclear, but this is specific to this particular primer-template sequence where GC compression occurs and therefore the migration of primers extended by 1 or 2 G nucleotides do not shift as normally expected (compare the same sequence on Fig. 3A, lane C/G template-primer). Some level of misincorporation particularly at the +2 position seen in Fig. 2A could be because of the lack of 3′-exonuclease proofreading activity, which is demonstrated in Fig. 2B.

**Activity of Sso pol Y1 on Aberrant DNA Templates**—Certain Y-family DNA polymerases are tolerant to terminally misaligned primers in certain sequence contexts and are implicated in DNA lesion bypass (11, 28). We tested the abilities of pol Y1 to extend from all possible 12 primer-template terminal mismatches and bypass DNA lesions. As shown in Fig. 3A, the G/G (template-primer) mismatch was extended to significant degree. It should be noted that other primers containing mismatched G at the termini, i.e. G/A and G/T, were not extended. In comparison, Watson-Crick base pairs such as T/A, G/C, C/G, and A/T were extended efficiently. To determine the base incorporated next to the mismatched G/G DNA, we conducted the single nucleotide incorporation assay (Fig. 3B). Of the four dNMPs, only dTMP was efficiently incorporated (lane T) and the full extension product was shorter than those generated by Klenow exo− and exo− enzymes (lanes N, I, and 2). Thus, it is most likely that pol Y1 extends this type of mismatch in a slippage mode; the extension occurs from the correctly paired C/G (template-primer) terminus with looping out of one guanine base in the template strand.

Because the optimal growth temperature of *S. solfataricus* is 87 °C (25), and heat induces depurination and cytosine deamination in the DNA, we examined the abilities of Sso pol Y1 to bypass AP site and uracil in template DNA (Fig. 4, A and C). In addition, we tested its abilities to bypass other naturally occurring DNA lesions, i.e. 8-oxoguanine and 6-methylguanine, which may be involved in spontaneous mutagenesis (29). For comparison, we also conducted the assay using pol IV of *E. coli*, a prototype of Y-family DNA polymerase (Fig. 4, B and D). When AP site was located in the template DNA, pol Y1 as well as pol IV stopped one base before the lesion and no substantial bypass was observed (Fig. 4, A and B, lane 2). Bypass synthesis of uracil by pol Y1 appeared to be less efficient than that by pol IV (Fig. 4, A and B, lane 3). In contrast, bypass of 8-oxoguanine by pol Y1 seemed to be more efficient than that of 8-oxoguanine by pol IV (Fig. 4, A and B, lane 4). The primer extension by two polymerases was inhibited to a similar extent by the presence of 6-methylguanine (Fig. 4, A and B, lane 5). To examine the effects of sequence context, template bases 5′ next to the lesions were changed from T to A, G, or C. However, such changes did not affect the efficiencies of trans-lesion bypass synthesis by pol Y1 as well as pol IV (data not shown). Bypass efficiencies in vitro are sometimes modulated by the amounts of enzyme used and/or the incubation temperature (30–32). Thus, we varied the reaction conditions for the bypass syntheses across AP site or uracil. When the protein concentrations were increased 10 times, pol Y1 inserted dNMP opposite AP site and pol IV bypassed the lesion (Fig. 4, C and D, AP site). Bypass of AP site by pol Y1 was achieved when pol Y1 concentration was increased 20 times and the incubation was carried out at 37 °C (Fig. 4C, AP site*). Bypass synthesis of uracil by pol Y1 was still less efficient than that of pol IV even when the protein concentrations were increased twice or 10 times (Fig. 4, C and D, Uracil). The results of these side-by-side comparison suggest that Sso pol Y1 is not more competent than pol IV of *E. coli* to bypass AP site and uracil in template DNA.

**Analysis of Sensitive Sites of pol Y1 to Limited Proteolysis**—Because of the similarities both at the primary amino acid sequence level and at functional level, we next examined whether the domain organizations of pol Y1 are similar to those of pol IV of *E. coli*. For this purpose, we chose the limited proteolysis and compared the cleavage sites of pol Y1 and pol IV (Fig. 5A). The nonspecific serine protease subtilisin cleaved both Sso pol Y1 and pol IV and produced major cleavage products of ~30 kDa. In addition, minor products of 36 and 9 kDa were observed with pol Y1 and pol IV, respectively. Bovine serum albumin was used as a control. Bovine serum albumin was relatively resistant to limited proteolysis, and subtilisin did not produce any distinct band pattern, which is consistent with its globular and single-domain characteristics. To map the cleavage sites, we determined the N-terminal amino acid sequences of the cleaved products. The results showed that the major cleavage occurred for both pol Y1 and pol IV at the same site; just before the highly conserved SI(L)DE motif (Fig. 5B). The determined sequence of the minor 36-kDa cleavage product of pol Y1 was A-N-Y-E-A-R-K-L-G-V-K-A-G, suggesting that the other cleavage occurred within the motif II of the protein (Fig. 5B). Because the partial amino acid sequence of 9-kDa fragment of pol IV was M-R-K-I-I-H-V-D-M-D-(C)-F, it was probably the N-terminal 9-kDa fragment cleaved by subtilisin. These results suggest that pol Y1 and pol IV possess similar but slightly different structural organizations and the major cleaved sites, i.e. SI(L)DE motif, are exposed to the solvent.

**Stimulation of Sso DNA pol Y1 by PCNA-like Sliding Clamps**—Based on the functional and structural similarities between pol Y1 and pol IV, we next examined whether PCNA of *S. solfataricus* could stimulate the activity of pol Y1. As de-
scribed in the Introduction, this organism possesses two forms of PCNA, i.e. Sso PCNA 039 and Sso PCNA 048 (20). We carried out polymerase activity assays using an oligo(dT)-
primed poly(dA) template (Fig. 6). Strikingly, both Sso PCNA clamps stimulated the synthetic activity of pol Y1. Stimulation was clearly expressed in the presence of Sso RFC complex,
which is reported to be able to load both Sulfolobus sliding clamps onto the DNA in an ATP-dependent reaction (25). Given the length of the used poly(dA) template, we estimated that the length of the synthesized DNA product at the highest pol Y1 concentration used was at least 200 nucleotides. Thus, the new pol Y1 can also synthesize longer DNA tracks upon interaction with the processivity subunits similar to the main S. solfataricus DNA polymerase B1 and to its prokaryotic counterpart E. coli pol IV.

Interaction of Sso pol Y1 with DNA—For a better understanding of the interaction between Sso pol Y1 and DNA, we examined its interaction with DNAs (dsDNA, ssDNA, and primed DNA) using the surface plasmon resonance technique and compared the mode of DNA binding to those of reference enzymes (Fig. 7). The reference proteins included pol IV of E. coli, rat pol /H9252, and Klenow exo /H11001 as representatives of family X and family A DNA polymerases, respectively, and E. coli Ssb as a DNA-binding protein. Although pol Y1 substantially bound to DNA, it showed no clear preference of any type of DNA. In contrast, pol IV appeared to favor ssDNA but its binding was much weaker than pol Y1. pol /H9262 preferred fully dsDNA to primed and ssDNA, whereas pol I strongly favored primed DNA over dsDNA and exhibited no binding to ssDNA at all. As expected, the Ssb protein bound only to ssDNA or partially ssDNA, i.e. primed DNA. To provide quantitative evaluation of such interactions, we calculated the equilibrium dissociation constants (KD) from the kinetic traces. As shown in Table I, it is apparent that pol Y1 bound to DNA with similar affinities as pol I and pol /H9262 (KD range: 10–50 nM), whereas the interaction of pol IV with DNA was generally very weak (KD range: 0.4–7 /H9262). The interaction of SSB with ssDNA was ~2 orders of magnitude stronger than that of the tested DNA polymerases. Taken together, these results suggest that Sso pol Y1 and pol IV of E. coli have different DNA binding modes from A-type and X-type DNA polymerases and also that the two Y-type DNA polymerases are distinct with respect to their abilities to bind to DNA.

DISCUSSION

In the present study, we isolated and biochemically characterized an archaeal representative of the new Y-family of DNA polymerases. We term this new polymerase, which is encoded by the previously described dbh (DinB homologue) gene (26), Sso pol Y1. Similar to other related enzymes (33, 34), pol Y1 lacks the 3'-exonuclease proofreading activity (Fig. 2B) and is resistant to aphidicolin and ddTTP, which inhibit the activities of many of family A and family X DNA polymerases (Fig. 1B) (35). Sso pol Y1 may be in fact a good candidate for the unclas-
sified aphidicolin-resistant DNA polymerase previously detected in *S. solfataricus* (27). To our knowledge, this is the first report of detailed biochemical characterization of an archaeal DNA polymerase belonging to the Y-family, and the first evidence that PCNA and RFC stimulate its activity.

Hyperthermophiles, such as *S. solfataricus*, are strictly dependent on high temperature for optimal growth and thus face up to major problems, such as denaturation and decomposition of nucleic acids (36). Although denaturation is not a serious problem for DNA molecules as long as they are covalently closed, heat-induced depurination and cytosine deamination could have deleterious effects. The depurinated product, i.e. AP site, is a major cytotoxic lesion in DNA, whereas deamination of cytosine and 5-methylcytosine results in G/U and G/T mismatches. To examine the possible contribution of *Sso* pol Y1 to the cytotoxic effects of AP site, we conducted bypass DNA synthesis assay *in vitro* using template DNA containing AP site. However, the ability of pol Y1 to bypass AP site was not substantially different from that of pol IV of *E. coli* (Fig. 4). In fact, pol Y1 only incorporated dNMP opposite AP site and did not bypass the lesion even when the enzyme concentration was increased 10 times (Fig. 4C, AP site). In contrast, *E. coli* pol IV bypassed AP site when the protein concentration was increased (Fig. 4D, AP site). Recently, bypass of AP site by pol Y1 was reported (32). We observed the bypass synthesis by pol Y1 using the following conditions; the concentrations of pol Y1 and template/primer DNA were increased 20 times, and the reaction was carried out at 37 °C (Fig. 4C, AP site*). However, the physiological significance of the bypass remains to be elucidated because the temperature, *i.e.* 37 °C, is far below the optimal growth temperature of *S. solfataricus* as well as the optimal temperature for the activity of pol Y1.

Similarly, bypass of uracil by pol Y1 appeared to be less efficient than that of pol IV (Fig. 4, A and B). The reluctance of pol Y1 to bypass uracil was also observed when the protein concentrations were increased (Fig. 4, C and D). It is known that several DNA polymerases from Archaea including Vent and *Pfu* specifically recognize the presence of uracil in template DNA and stall DNA synthesis several bases before uracil (37). This phenomenon seems to be specific to Archaea DNA polymerases because it is not observed in *Taq* polymerase, which is derived from thermophilic eubacterium *Thermus aquaticus*. In addition, archaeabacterial DNA polymerases are strongly inhibited by the presence of small amounts of uracil-containing DNA (38). In fact, we observed that *Sso* DNA pol B1, the DNA replicase of *S. solfataricus*, also stalls before uracil in template DNA. Thus, we speculate that the reluctance of pol Y1 to synthesis across a uracil residue is due to the inherent nature of Archaea DNA polymerases.

It is proposed that the efficiency of DNA repair enzymes, such as AP endonuclease or uracil DNA glycosylase, present in *E. coli* for correcting depurination and cytosine deamination would be sufficient to withstand the roughly 3,000-fold increase in DNA decay and allow growth at 100 °C if the bacterial proteins could tolerate the elevated temperature (29). In fact, there are only a few Y-family DNA polymerases that have so far been detected in other Archaeal genomes. Thus, repair enzymes rather than bypass DNA polymerases may play important roles in the defense mechanism against cytotoxic damage induced by elevated temperature in hyperthermophiles. However, it should be noted that *Sso* pol Y1 may contribute to the genetic diversity of this organism because it can efficiently extend the primer/template DNA having G/G mismatch with the generation of potential frameshift errors (Fig. 3), and by-pass uracil, 8-oxoguanine and 6-methylguanine in DNA (Fig. 4). pol IV of *E. coli* is known to be involved in spontaneous mutagenesis of the organism (39–41). In this respect, it is important to determine whether the bypass is error-prone and, if so, what bases are incorporated opposite the lesions by this polymerase.

Archaal proteins are widely used for crystallization and structural studies because of their structural stability and ease of purification. As an first approach to analyze the structural characteristics of Y-family DNA polymerases, we used limited proteolysis of pol Y1 and the homologous pol IV. Under nonde-naturing conditions, nonspecific proteases such as subtilisin are known to preferentially cleave proteins within flexible solvent-exposed loops. Both Y-type DNA polymerases showed a similar pattern of cleavage; a major cleavage occurs just before the highly conserved SI(L)DE motif (Fig. 5B). Recently, Zhou et al. (32) determined the crystal structure of catalytic domain of pol Y1 (Dbh). According to their report, the structure is in the shape of a right hand formed from domains termed the fingers, palm, and thumb, which is similar to other polymerases. The major cleavage site, *i.e.* SIDE in motif III, is located in the hairpin loop between two β strands 5 and 6 in the palm domain. This area seems to be the active site of this enzyme because highly conserved Asp residues and Asp residues are closely located to bind to Mg ions for catalysis. Molecular modeling of DNA-bound pol Y1 structure suggests the hairpin loop is located in the bottom of cleft, in which template/primer DNA and dNTP are bound. Thus, a part of the active site of pol Y1 seems to be exposed to solvent at least in the absence of DNA. The second protease-sensitive site of pol Y1, *i.e.* ANYEAR in motif II (Fig. 5B), is located the boundary between β strand 3 and loop C in the fingers domain (32). Because this cleavage site is not clearly detected in *E. coli* pol IV, the finger domain structure might be slightly different between the two polymerases. Cleavage pattern and susceptibility sites are often changed when limited proteolysis is carried out in the presence of DNA (23). This is caused by DNA-induced conformational changes

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2 P. Grzu, F. M. Pisani, M. Shimizu, M. Yamada, and T. Nohmi, unpublished results.
Comparison of affinities of several DNA-binding proteins for ssDNA and dsDNA

The binding analysis was performed under the same conditions with five different analyte concentrations and fitted globally using the BIAeval software to obtain the most accurate $K_D$ constant values.

| Protein           | Substrate | $K_D$ |
|-------------------|-----------|-------|
| Sso DNA pol Y1    | ssDNA     | 10    |
| E. coli DNA pol IV| ssDNA     | 475   |
| Klenow fragment   | ssDNA     | No detectable binding |
| Rat DNA pol β     | ssDNA     | 54    |
| E. coli Subb      | ssDNA     | 0.2   |

and/or protection of the cleavage site by DNA. In this respect, it would be interesting to examine the limited proteolysis of these polymerases in the presence of template/primer DNA.

The use of a sliding clamp to increase the processivity of DNA polymerases is a common strategy in Archaea, prokaryotes, and eukaryotes (20, 42, 43). The clamps tether DNA polymerases to a template/primer DNA, thereby preventing the falling off of polymerases from template DNA. Typical examples of the sliding clamps include gp45 of T4 DNA polymerase, the β subunit of E. coli DNA polymerase III, and PCNA of eukaryotic DNA polymerase δ. Despite the weak similarity at the amino acid sequence level, they all assemble in a toroidal shape, which is capable of encircling dsDNA. In this study, we demonstrated that the activity of Sso pol Y1 is substantially enhanced upon interaction with PCNA (039p or 048p) and RFC complex (Fig. 6). The length of synthesized tracks was more than 200 nucleotides at the highest concentration of pol Y1. Because Sso PCNA 039p and 048p possess trimeric structures and conserve several functionally important motifs with eukaryotic counterparts (20), the results may have implications in the eukaryotic members of Y-type DNA polymerases. In fact, Harrasza et al. (44) reported that interaction with PCNA is essential for yeast DNA polymerase η function. However, Gerlach et al. (33) reported that the activity of human pol κ is not stimulated by PCNA, and thus its intrinsic moderate processivity could be the result of direct interaction with DNA via two zinc clusters located at the C-terminal part of the protein (30, 45). In this case, however, the human pol κ enzyme is fused to glutathione S-transferase on the N-terminus and to hexahistidine on the C terminus, and RFC is not included in the reaction (33). It has been suggested that fusion groups, such as maltose-binding protein, could impair the proper interaction with the processivity factors (14). In addition, as shown in Fig. 6, a small enhancement of the activity of pol Y1 was observed in the absence of RFC complex. Thus, a more comprehensive approach is desired to address the question. Recently, Kannouche et al. (46) showed that the human DNA polymerase η co-localizes with PCNA in vivo and carries a putative PCNA binding motif at its C terminus, suggesting the role of PCNA in the regulation of DNA polymerase η.

To gain an insight into the interaction between Y-type DNA polymerases and DNA, we examined the ability of Sso pol Y1 to bind to DNA by the BLAcore assay (Fig. 7, Table I). The most stable pol Y1 displayed a significantly higher affinity for DNA than did pol IV, and the affinity was compatible with those of Klenow fragment of E. coli DNA pol I and rat DNA pol β. Klenow fragment and pol β are involved in DNA repair, and the activities are not stimulated by the β subunit and PCNA, respectively. Despite the affinity for DNA, pol Y1 itself is a distributive DNA polymerase and required the Sulfolobus PCNA sliding clamp and RFC for efficient DNA synthesis (Fig. 6). This suggests that strong DNA binding does not necessarily lead to high processive DNA synthesis. In vitro studies have demonstrated that Sso RFC selectively binds to primed but not ssDNA, thereby efficiently loading PCNA on a primed template DNA (25). Thus, we suggest that Sso PCNA plus RFC are required to offer a preference to primed template DNA. In contrast, E. coli pol IV itself showed a limited affinity for DNA (Table I). This is consistent with the observation that pol IV cannot form a stable complex with DNA in the absence of the β subunit of pol III (14). Hence, one possible role of the β subunit is to complement the affinity for DNA to ensure efficient DNA synthesis. The preferential binding to ssDNA resembles the DNA binding of E. coli pol V (UmuC) and pol RI (MucB), members of another subfamily of Y-type DNA polymerases (47–49). The interaction with ssDNA may serve an important role in the loading of these lesion bypass DNA polymerases. Because pol Y1 and pol IV exhibit different DNA binding capabilities despite the conserved DNA binding motifs IV and V (Fig. 5B), we suggest that nonconserved amino acids also play important roles in the binding to DNA.

Stimulation of Sso pol Y1 and pol IV of E. coli by the sliding clamps suggests that these DNA polymerases act in concert with the main DNA replicases such as Sso DNA pol B1 and E.
coli pol III, respectively. These replicases are associated with the sliding clamps during the processive chromosomal replication. It has been suggested that more than one DNA polymerase of E. coli are held together by protein-protein interactions in sliding clamp, so that the most appropriate one can engage with DNA at any given moment (10, 12, 50, 51). Because multiple DNA polymerases are identified in eukaryotic cells (12), it might be interesting to investigate how such polymerases are replaced depending on the DNA lesions and sequence context. The archael DNA replication apparatus resembles that of eukaryotes rather than prokaryotes. Therefore, studying the more simplified archael DNA polymerase-sliding clamp system should enhance our understanding of the basic mechanism of mutasome assembly.

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Synthetic Activity of *Sso* DNA Polymerase Y1, an Archaeal DinB-like DNA Polymerase, Is Stimulated by Processivity Factors Proliferating Cell Nuclear Antigen and Replication Factor C

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