Modulation of hyperthermophilic DNA polymerase activity by archaeal chromatin proteins

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SUMMARY

*Sulfolobus* synthesizes a large quantity of highly conserved 7-kD DNA binding proteins suspected to be involved in chromosomal organization. The effect of the 7-kD proteins on the polymerization and 3′-5′ exonuclease activities of a family B DNA polymerase (polB1) from the hyperthermophilic archaeon *Sulfolobus solfataricus* was investigated. PolB1 degraded both ssDNA and dsDNA at similar rates in vitro at temperatures of physiological relevance. The 7-kD proteins were capable of significantly inhibiting the excision and enhancing the extension of matched template primers by the polymerase. However, the proteins did not protect ssDNA from cleavage by polB1. In addition, the 7-kD proteins did not affect the proofreading ability of polB1 and were not inhibitory to the excision of mismatched primers by the polymerase. The dNTP concentrations required for the effective inhibition of the 3′-5′ exonuclease activity of polB1 were lowered from ~600 µM in the absence of the 7-kD proteins to ~50 µM in the presence of the proteins at 65 °C. Our data suggest that the 7-kD chromatin proteins serve to modulate the extension and excision activities of the hyperthermophilic DNA polymerase, reducing the cost of proofreading by the enzyme at high temperature.
INTRODUCTION

Hyperthermophiles face a challenging task of maintaining the stability of their genome while allowing efficient DNA transactions at temperatures close to or exceeding the melting point of DNA. Different hyperthermophiles appear to have evolved different strategies in accomplishing this task (1). *Sulfolobus*, a group of hyperthermophilic archaea, synthesizes copious amounts of small DNA binding proteins, classified as 7-, 8- and 10-kD proteins on the basis of their molecular weight (2 - 6). Among them, the 7-kD DNA binding proteins are the most abundant and believed to be a major chromatin component in *Sulfolobus*. Each *Sulfolobus* species encodes at least two 7-kD proteins (7- 9). Previous studies have focused on the 7-kD proteins from *S. acidocaldarius* (Sac7), *S. solfataricus* (Sso7) and *S. shibatae* (Ssh7) (10 - 12). All the 7-kD proteins are very similar, and those from *S. shibatae* (Ssh7a and Ssh7b) and closely related *S. solfataricus* (Sso7d-1, Sso7d-2 and Sso7d-3) are identical or nearly identical (7 - 9). Native 7-kD proteins are monomethylated at specific lysine residues to various extents (2, 4, 6, 7, 11, 12). The proteins bind double-stranded DNA as monomers and with little sequence specificity (3, 5, 6, 13). But, they show low affinity for single-stranded DNA (7). Fluorescence titrations and electrophoretic mobility shift assays show that the affinity of the proteins for dsDNA is in the micromolar range in low salt (9, 10, 12, 14, 15). Estimates of the binding size of the protein vary from 4 to 6.6 base pairs (9, 14, 15). Binding by the 7-kD proteins raises the Tm of DNA by as much as 40 °C (12, 14, 16). Structural analysis of the recombinant Sso7d and Sac7d proteins
by NMR and their complexes with DNA by X-ray crystallography shows that both proteins consist of a triple-stranded anti-parallel β-sheet on which a double-stranded β-sheet is packed (12, 13, 17 - 19). The proteins bind in the minor groove of DNA, causing a 61° single-step kink in the DNA helix through intercalation. In agreement with their ability to deform DNA, these proteins are capable of constraining DNA in negative supercoils (7, 20, 21).

Given their cellular abundance and ability to affect DNA structure, the 7-kD proteins may affect fundamental biological processes such as DNA replication, transcription, repair and recombination. The potential role of Sso7d in the regulation of gene expression and recombination in Sulfolobus has been implicated in recent studies (22, 23). Analysis of the effect of the 7-kD proteins on DNA transactions may help determine how these processes occur in a physiologically relevant environment and how they are adapted to high growth temperature at which Sulfolobus thrives. In this study, we have investigated the effect of the 7-kD proteins on a hyperthermophilic family B DNA polymerase (polB1) from the crenarchaeon Sulfolobus solfataricus P2 (8). The gene encoding a polB1 homologue from S. solfataricus MT4 has been cloned and expressed in E. coli (24). The recombinant polymerase has been characterized and a role for the protein in DNA replication proposed (25, 26). Like almost all archaeal family B DNA polymerases, Sulfolobus polB1 possesses intrinsic proofreading 3’-5’ exonuclease activity (27). We report here that the Sulfolobus enzyme cleaved both dsDNA and ssDNA at similar rates at high temperature, and the exonucleolytic activity of the enzyme remained
significant at dNTP levels as high as 600 µM. Binding of the 7-kD chromatin proteins substantially reduced the excision and enhanced the extension of matched template primers. However, the chromatin proteins did not seem to affect the proofreading ability of polB1. Our data suggest that the archaeal chromatin proteins play an important role in maintaining the critical balance between the polymerization and excision activities of DNA polymerases in hyperthermophiles.

**EXPERIMENTAL PROCEDURES**

**Growth of organisms**

*S. solfataricus* P2 (a generous gift from Dennis Grogan) and *S. shibatae* (purchased from the American Type Culture Collection) were grown as described previously (28).

**Proteins**

Recombinant *S. solfataricus* DNA polymerase B1 (polB1) was prepared as a fusion protein containing a His tag at its N-terminus as follows. The gene encoding DNA polymerase B1 (ORF: SSO0552) was amplified from the *S. solfataricus* genomic DNA, prepared as described previously (10), by PCR using *Pfu* DNA polymerase (Stratagene) and the following pair of primers: 5’-GCCGGATCCATGACTAAGCAACTTATTTAT/5’-CGCAGCTCTTAACCTATTTCCCTTTACTTG (BamHI and SacI sites were in bold face). The PCR product was treated with *Taq* polymerase in the presence of dATP and ligated into plasmid pGEM-T (Promega). The recombinant plasmid was cleaved with *Bam*HI and *Sac*I, and the fragment containing the polB1 gene was cloned into the same sites of the expression vector pET-30a(+) (Novagen). The resulting plasmid was
transformed into *E. coli* BL21(DE3) codon-plus RIL (Stratagene). The sequence of the cloned gene was verified by DNA sequencing.

For protein expression, the transformant was grown at 37 °C in LB medium containing 100 µg/ml kanamycin and 50 µg/ml chloramphenicol in a Bioflo 3000 fermentor (New Brunswick Scientific Co., Inc.) until the optical density at 600 nm reached ~0.6. Isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After incubation for 2.5 hrs, the induced cells were harvested and resuspended in buffer A [20 mM Tris-HCl, pH8.0, 500 mM KCl, 10% (w/v) glycerol] containing a mixture of protease inhibitors (50 µg/ml PMSF, 1 µg/ml aprotinin, 0.2 µg/ml benzamidine, 0.1 µg/ml leupeptin, 0.5 µg/ml pepstatin A). The cells were immediately disrupted by three cycles of freeze in liquid nitrogen and thawing at 4 °C. After removal of cell debris by ultracentrifugation (120,000 x g, 60 min, 10 °C), the supernatant was heat-treated for 15 min at 70 °C. The sample was clarified by ultracentrifugation (120,000 x g, 30 min, 10 °C). The supernatant was loaded onto a Hitrap chelating column (1 ml, Amersham Pharmacia Biotech) equilibrated with buffer A. The column was washed with buffer A (10 ml), and bound proteins were eluted with an imidazole gradient (0 - 500 mM) in buffer A (10ml). Fractions containing the His-tagged recombinant protein, eluted at 150 - 250 mM imidazole, were pooled, dialyzed against buffer B [20 mM Tris-HCl, pH8.0, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM KCl, 10% (w/v) glycerol]. The dialyzed sample was applied to a Resource Q column (1 ml, Amersham Pharmacia Biotech) equilibrated with buffer B. The column was washed
with buffer B (10 ml) and developed with a 15-ml linear gradient (50 - 1000 mM KCl).

Fractions containing the pure recombinant protein were combined, dialyzed against 20 mM Tris-HCl, pH8.0, 0.1 mM EDTA, 1 mM DTT, 50% (w/v) glycerol and stored at –70 °C. All purification steps were carried out at 4 °C.

Native Ssh7 proteins were prepared as described previously (7).

Protein concentrations were determined by the Lowry method using bovine serum albumin (BSA) as the standard (29).

**DNA templates and substrates**

Oligonucleotides used in this study (Table 1) were synthesized and purified by gel electrophoresis. Oligonucleotide primers were labeled at the 5’ end with T4 polynucleotide kinase (Biolabs) and \[\gamma\text{-}^{32}\text{P}\text{]ATP, and purified using a G25 microspin column (Amersham Pharmacia Biotech). Each labeled primer was annealed to template t76 at a molar ratio 1:1.5 to ensure complete hybridization of the primer to the template. Annealing reactions were carried out in 20 mM Tris-HCl, pH7.6, 100 mM NaCl.

**Primer extension/excision assays**

The standard reaction (10 µl) contained 60 nM polB1, 1 - 3 nM \(^{32}\text{P}\)-labeled primer-template substrate, 50 mM Tris-HCl, pH7.5, 2 mM β-mercaptoethanol, 100 µg/ml BSA, 3 mM MgCl\(_2\) and 5 µM dNTPs, unless otherwise specified. For primer excision assays, dNTPs were omitted from the reaction mixture. A layer of mineral oil was added to each sample to prevent evaporation. The reaction was incubated for 20 minutes at 65 °C, unless specified, and quenched by the addition of 10 µl of ice-cold 2 x
loading buffer (95% deionized formamide, 100 mM EDTA, 0.02% bromophenol blue).

When Ssh7 was included in the reaction, a solution (2 µl) containing 3% SDS, 150 mM EDTA and 15 mg/ml proteinase K was added to the sample, and the mixture was incubated for 45 min at 50 °C before the addition of the loading buffer. The mixtures were then heated for 3 min at 98 °C and immediately cooled on ice. The samples were analyzed by electrophoresis in a 15% polyacrylamide, 8 M urea gel in 1 x Tris-borate-EDTA (TBE). The gel was dried and exposed to X-ray film or analyzed by using an ImageQuant Storm phosphorimager (Molecular Dynamics, Amersham Pharmacia Biotech).

**Proofreading assays**

The proofreading ability of polB1 was analyzed by incubating 50 nM polB1 with 10 nM 5’-labeled mismatched substrate p42G/t76 in the presence or absence of Ssh7 (25 µM) for 15 min at 65 °C in 50 mM Tris-HCl, pH 6.5, 2 mM β-mercaptoethanol, 100 µg/ml BSA, 4 mM MgCl2 and 25 µM dNTPs in a total volume of 10 µl. For samples lacking Ssh7, the chromatin proteins were added back to the mixture to 25 µM after the reaction. The reaction was quenched by the addition of a solution (2 µl) containing 3% SDS, 150 mM EDTA and 15 mg/ml proteinase K. Following incubation for 1 hr at 50 °C, the samples were extracted three times with phenol/chloroform, once with chloroform and precipitated with ethanol. The DNA was digested with SaI or left untreated. The samples were mixed with ice-cold 2 x loading buffer (95% deionized formamide, 100 mM EDTA, 0.02% bromophenol blue), heated for 3 min at 98 °C and immediately cooled
on ice. The samples were analyzed by electrophoresis in a 12% polyacrylamide, 8 M urea gel in 1 x Tris-borate-EDTA (TBE). The gel was dried and exposed to X-ray film.

Proofreading by polB1 was also examined using a radiolabel incorporation assay. In this method, an unlabeled substrate (10 nM) was incubated for 15 min at 65 °C with 50 nM polB1 in 50 mM Tris-HCl, pH 6.5, 2 mM β-mercaptoethanol, 100 µg/ml BSA, 4 mM MgCl₂, 40 µM [α-³²P]dCTP and 100 µM each of dATP, dGTP and dTTP. The samples were treated as described above.

RESULTS

PolB1 excises ssDNA and dsDNA at similar rates at high temperature.

PolB1 possesses both 5’-3’ polymerization and 3’-5’ exonuclease activities. A proofreading exonuclease is capable of cleaving both ssDNA and dsDNA. But the cleavage reaction on ssDNA is minimally affected by temperature whereas that on dsDNA depends strongly on temperature (30). To examine the ability of polB1 to excise ssDNA and dsDNA at high temperature, we incubated the protein with either a template primer (p42/t76) or a primer alone (p42) in the absence of dNTPs at 65 °C. As shown in Fig. 1, the labeled 42-nt primer was degraded with similar kinetics in both cases. In a control experiment, we found that the p42/t76 substrate was not denatured at temperatures up to 72°C under our assay conditions. Moreover, both linearized and nicked plasmid DNAs were readily digested by the enzyme at 65 °C (data not shown). Therefore, it appears that the ends of duplex DNA may have become so destabilized or frayed at the test temperatures that base pairing can no longer form a significant energetic
barrier for the exonucleolytic action of polB1.

**Ssh7 inhibits the cleavage of a template primer by polB1.**

We then sought to learn how the Ssh7 proteins might affect the robust exonuclease activity of polB1. When Ssh7 was titrated into the cleavage reaction containing substrate p42/t76, the primer became increasingly less susceptible to the degradation by the enzyme (Fig. 2). The same result was obtained irrespective of the order of addition of Ssh7 and polB1 to the substrate. The cleavage of the primer was most drastically reduced when the Ssh7 level reached ~2.5 µM. It was found that Ssh7 bound short duplex DNA fragments with a Kd of 0.06 – 0.1 µM and saturated the majority of the DNA molecules in a binding reaction at concentrations greater than ~1 µM under conditions similar to those employed in the present study (9). So, it appears likely that the significant inhibition of the primer cleavage by polB1 occurs when the duplex portion of the substrate becomes saturated by Ssh7. By comparison, Ssh7 did not affect significantly the cleavage of the 42-nt primer by polB1. This is consistent with the finding that Ssh7 binds poorly to ssDNA (7). These results also suggest that Ssh7 inhibits the excision of a template primer by polB1 through its interaction with the substrate instead of the polymerase.

**Effect of Ssh7 on the polymerase and exonuclease activities of polB1.**

Binding of Ssh7 inhibited the excision by polB1 of a template primer. A question then arose as to if Ssh7 would also suppress primer extension by polB1. To answer this question, we incubated polB1 with substrate p42/t76 in the presence of dNTPs. Various
amounts of Ssh7 were included in the reaction. Both excision and extension of the primer were apparent in the presence of dNTPs. A number of prominent reaction products of different lengths were observed, suggesting that the balance between the two opposing activities of polB1 depended strongly on sequence. Addition of Ssh7 clearly enhanced the extension while inhibiting the excision of the primer, resulting in a significant increase in the amount of the full-length extension product (Fig. 3). The proteins did not appear inhibitory to primer extension even at 25 µM.

The balance between the extension and excision activities of a proofreading DNA polymerase is strongly affected by dNTP concentrations (31). Surprisingly, cleavage of the template primer by polB1 remained significant even when the dNTP levels were raised to 600 µM (Fig. 4). However, when Ssh7 was added to the reaction, the excision activity of polB1 was significantly inhibited at much lower dNTP levels (Fig. 4). The ratio of primer extension over excision achieved at 1 mM dNTPs in the absence of Ssh7 was similar to that at 50 µM dNTPs in the presence of 50 µM of the proteins.

**Effects of temperature and primer size on the Ssh7-mediated modulation of the extension/excision activities of polB1.**

Considering the ability of the 7-kD DNA binding proteins to increase substantially the melting temperature of dsDNA in vitro, we then examined how Ssh7 might affect the extension/excision of a template primer by polB1 at temperatures above the melting point of the substrate. We found that, when incubated with polB1 and dNTPs in the absence of Ssh7, much of a 30mer annealed to a 76mer (Tm ≈ 68 °C) was extended, mostly to the
full length, at 50 °C, but a large portion of the primer was excised or poorly extended at 80 °C (Fig. 5A). Addition of Ssh7 substantially reduced the excision but increased the extension of the primer at 80 °C. The extension/excision ratio of the primer in the presence of Ssh7 (4 or 8 µM) was about 30 times greater than that in the absence of the proteins. We also tested the effect of Ssh7 on the extension and excision by polB1 of template primers differing in length at a denaturing temperature. A significant amount of the full-length extension product was obtained at 80 °C even with a substrate containing a 15-nt primer (Fig. 5B). Taken together, these data suggest that binding by Ssh7 may stabilize base pairing between a primer and its template at an otherwise denaturing temperature, providing a primer terminus suitable for extension by the DNA polymerase.

**Effect of Ssh7 on the proofreading ability of polB1.**

In view of the effect of Ssh7 on the excision and polymerization activities of polB1, it would be of interest to examine how the chromatin proteins may influence the proofreading ability of the polymerase. So we conducted a primer extension/excision assay on a substrate containing a mismatched base at the 3’ end of the primer (p42G/t76). The assay conditions (15 nM polB1 and a reaction time of 30 sec) were chosen such that less than 50% of the substrate would be excised or extended at the end of the reaction. As shown in Fig. 6A, cleavage of the mismatched primer resulted in the appearance of fragments that were slightly shorter than the primer whether or not Ssh7 was present. In comparison, these cleavage products were hardly detectable when the assay was
performed on the matched substrate under the same conditions. It appears that the mismatch altered the primer structure or destabilized the template primer, enhancing the excision activity of polB1. Interestingly, excision of the mismatched primer was not suppressed by Ssh7. To determine if the extension product of the mismatched substrate still contained the mismatch, we increased the amounts of both polB1 and dNTPs in the assay (to 50 nM and 25 µM, respectively), and allowed the reaction to proceed for 15 min. Under these conditions, the majority of the input primers were extended (Fig. 6B). The extension products were then treated with SalI. Since the mismatch occurred at the SalI site of the substrate, an extension product containing the mismatch would not be sensitive to cleavage by SalI whereas an extension product in which the mismatch had been corrected would be digested by the restriction enzyme. When the extension products were cleaved by SalI, a labeled 38-nt fragment would result. As shown in Fig. 6B, extension products from both matched and mismatched substrates were sensitive to the cleavage by SalI, suggesting that the mismatch was efficiently corrected by B1 in the presence or absence of Ssh7. As a control, products of extension from the mismatched substrate by an exonuclease-deficient polB1 were not cleaved by SalI (data not shown). We also tested the ability of polB1 to correct the other two types of mismatches at the 3’ terminus of the primer (p42T/t76 and p42C/t76) in the presence of Ssh7 using a label incorporation assay. Both types of mismatches were also efficiently corrected (Fig. 6C). Based on these results, we conclude that the strong proofreading ability of B1 is not affected by Ssh7.
DISCUSSION

A proofreading DNA polymerase is known to coordinate its polymerase and exonuclease activities to achieve high extension fidelity without incurring a high cost resulting from excessive cleavage of correctly incorporated nucleotides. In this regard, mesophilic DNA polymerases normally cleave double-stranded DNA much less efficiently than single-stranded DNA due to the requirement for melting of the duplex terminus and translocation of the DNA from the polymerase site to the exonuclease site (32). How might a thermophilic DNA polymerase balance its polymerization and excision activities at high temperature which favors melting or destabilization of duplex DNA? In the present work, we show that DNA polymerase B1 from the hyperthermophilic archaeon S. solfataricus is capable of cleaving dsDNA as efficiently as ssDNA at temperatures of physiological relevance. Similar observations have been reported for family B DNA polymerases from Pyrobaculum islandicum and Thermococcus litoralis, and a split polB from Mathanobacterium thermoautotrophicum ΔH (33 - 35). It is worth noting that, since both ssDNA and dsDNA were similarly cleaved by polB1 at temperatures significantly below the melting point of duplex DNA, the enzyme does not appear to require the substrate to be extensively denatured for efficient exonucleolytic cleavage.

Furthermore, deoxynucleoside triphosphates are known to affect the pol/exo balance of a DNA polymerase in favor of polymerization. Although primer extension by polB1 occurred efficiently at a low level (2 µM) of dNTPs, significant excision by the enzyme
remained even at 600 µM dNTPs, a concentration far exceeding that found in the cell (≤ 100 µM) (36). In comparison, DNA polymerase from the thermophile *Thermococcus litoralis* also displays a strong 3’-5’ exonuclease activity on both ssDNA and dsDNA, but the activity on dsDNA was suppressed in the presence of moderate amounts of dNTPs (50% inhibition at 1 µM dNTPs) (34). The strong exonuclease activity of polB1 on the paired primer terminus suggests that hyperthermophiles must have evolved mechanisms to reduce the cost of proofreading, which would otherwise be extraordinarily high at the growth temperatures of the organisms.

In *Sulfolobus*, DNA is densely bound, if not entirely covered, by the 7-kD DNA binding proteins (1, 2). Given the effect of these proteins on the structure and duplex stability of DNA, they are likely involved in the modulation of the pol/exo balance of DNA polymerase. Indeed, as demonstrated in this study, the pol/exo balance of polB1 on a primed template was significantly changed in favor of primer extension in the presence of Ssh7 as compared to that in the absence of the proteins. Titration experiments suggest that the level of Ssh7 at which primer cleavage by the polymerase became substantially inhibited was similar to that required for the maximum binding of the proteins to the duplex region of the substrate. In addition, cleavage of single-stranded DNA by polB1 was not inhibited by Ssh7. These data indicate that Ssh7 influences the extension and excision activities of polB1 by affecting the structure of the substrate, and not through protein-protein interactions. As demonstrated by the ability of the 7-kD proteins to raise the Tm of dsDNA (12, 16), binding by these proteins would
presumably stabilize the annealing of a primer to its template at high temperature, thereby
reducing the availability of unpaired primer ends for the excision by the DNA polymerase.
We also found that, in the presence of small amounts (e.g. 4 µM) of Ssh7, the dNTP
levels required for the effective suppression of the exonuclease activity of polB1 were
lowered to within the physiologically relevant range. In addition, the effect of Ssh7 on
primer extension and excision by polB1 was pronounced on substrates containing short
primers (e.g., 15-nt primer), suggesting that the 7-kD proteins may serve to protect
nascent DNA chains from degradation by the exonuclease activity of polB1 at high
temperature. Importantly, despite its strong inhibitory effect on the cleavage of dsDNA
by polB1, the chromatin proteins did not affect the proofreading ability of the DNA
polymerase.

As the most abundant DNA binding proteins in Sulfolobus chromatin, the 7-kD DNA
binding proteins conceivably play an important role in various DNA transactions that take
place under the stressful conditions of high temperature. It is of interest to study these
reactions in vitro both in the presence and in the absence of the 7-kD proteins. Results
from such comparative studies will provide a physiologically relevant view of these
processes and clues to the adaptation of these processes to high temperature.

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FIGURE LEGENDS

Figure 1. Kinetics of cleavage of a template primer and a primer alone by polB1. polB1 (15 nM) was incubated with labeled p42 or p42/t76 (2 nM) under the standard assay conditions for various lengths of time. Reactions were terminated by the addition of a loading buffer, and samples were resolved by electrophoresis in a sequence gel in 1 x TBE. The gel was dried and exposed to X-ray film or analyzed using a phosphorimager. The percentage of the primer excised during the reaction is shown at the bottom of each lane. Each number represents an average of two independent measurements. Lane C, no enzyme.

Figure 2. Effect of Ssh7 on the excision of DNA by polB1. PolB1 (60 nM) was incubated with labeled p42 or p42/t76 (2 nM) for 15 min under the standard assay conditions in the presence of indicated levels of Ssh7. SDS, EDTA and proteinase K were then added to final concentrations of 0.5%, 25 mM and 2.5 mg/ml, respectively. After incubation for 45 min at 50 °C, samples were mixed with a loading buffer and processed as described in the legend to Fig. 1.

Figure 3. Effect of Ssh7 on the polymerase and exonuclease activities of polB1. PolB1 (60 nM) was incubated with labeled p30/t76 or p42/t76 (2 nM) for 20 min in the presence of indicated levels of Ssh7 in the standard extension/excision reaction mixture containing 5 µM dNTPs. The samples were treated as described in the legend to Fig. 2. Lanes C1 and C2, no enzyme.

Figure 4. Effect of dNTP concentrations on the polymerase and exonuclease activities of
polB1 in the presence and absence of Ssh7. PolB1 (60 nM) was incubated with labeled p42/t76 (2 nM) for 20 min in the presence of various amounts of dNTPs in the standard extension/excision reaction mixture either containing 25 µM Ssh7 or lacking it. The samples were treated as described in the legend to Fig. 2. Lane C, no enzyme.

**Figure 5.** Effects of temperature and primer length on the ability of Ssh7 to modulate the activity of polB1. (A) Temperature effect. PolB1 (60 nM) was incubated for 20 min at indicated temperatures with labeled p30/t76 (2 nM) in the presence of dNTPs (25 µM) and various amounts of Ssh7 under the standard assay conditions. Reactions were treated as described in the legend to Fig. 2. The ratio of the amount of the primer extended to that excised (extension/excision) during the reaction is indicated at the bottom of each lane. (B) Primer length effect. PolB1 (60 nM) was incubated with labeled p15/t76 (2 nM) for 20 min at indicated temperatures in the presence or absence of 25 µM Ssh7 in the standard assay mixture containing 25 µM dNTPs. Reactions were treated as described above. The extension/excision ratios obtained in the presence and absence of Ssh7 are indicated by black and grey bars, respectively. Each number represents an average of three independent measurements.

**Figure 6.** Effect of Ssh7 on the proofreading ability of polB1. (A) A rapid quench experiment showing the effect of Ssh7 on the excision and extension of a 3′-mispaired template primer. PolB1 (15 nM) was incubated with 5′-labeled p42G/t76 or p42/t76 (2 nM) for 30 sec at 65 °C in the standard assay mixture containing an increasing amount of dNTPs in the presence or absence of Ssh7 (25 µM). Reactions were terminated and
processed as described in the legend to Fig. 2. (B) Effect of Ssh7 on the ability of polB1 to correct a 3’-mismatched primer terminus. PolB1 (50 nM) was incubated with 5’-labeled p42/t76 or p42G/t76 (10 nM) for 15 min at 65 °C in the standard reaction mixture containing dNTPs (25 µM) in the presence or absence of Ssh7 (25 µM). After reactions were stopped, Ssh7 was added to samples lacking the proteins to 25 µM. All samples were treated with proteinase K, extracted with phenol and chloroform. DNA was precipitated with ethanol. Half of each DNA sample was digested with SalI. Both digested and undigested samples were analyzed by electrophoresis in a sequencing gel in 1 x TBE. The gel was dried and exposed to X-ray film. (C) Correction of various mismatches by polB1 in the presence of Ssh7. PolB1 (50 nM) was incubated with p42T/t76 or p42C/t76 (10 nM) for 15 min at 65 °C in the standard assay mixture containing [α-32P]dCTP (40 µM) in addition to dATP, dGTP and dTTP (100 µM each) in the presence or absence of Ssh7 (25 µM). Samples were treated as described above.
| Primers | Template |
|---------|----------|
| p15     | 5’ CAGTGAATTCGAGCT 3’ |
| p30     | 5’ CAGTGAATTCGAGCTCAGCTGTACCCGGGGATC 3’ |
| p42     | 5’ CAGTGAATTCGAGCTCAGCTGTACCCGGGGATCCTAGAGTCGA 3’ |
| p42G    | 5’ CAGTGAATTCGAGCTCAGCTGTACCCGGGGATCCTAGAGTCGG 3’ |
| p42T    | 5’ CAGTGAATTCGAGCTCAGCTGTACCCGGGGATCCTAGAGTCGT 3’ |
| p42C    | 5’ CAGTGAATTCGAGCTCAGCTGTACCCGGGGATCCTAGAGTCG 3’ |
| t76     | 3’ TTTTTGTCACCTTAAGCTCGAGCAGGAAGGCCCTAGGAGATCGAGCTGAGCGTGACGCGACCGCAGTTTT5’ |
FIG. 1.
FIG. 3.
FIG. 4.
FIG. 5.
FIG. 6.
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