Distinct Domain Functions Regulating de Novo DNA Synthesis of Thermostable DNA Primase from Hyperthermophile *Pyrococcus horikoshii*†

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ABSTRACT: DNA primases are essential components of the DNA replication apparatus in every organism. Reported here are the biochemical characteristics of a thermostable DNA primase from the thermophilic archaeon *Pyrococcus horikoshii*, which formed the oligomeric unit L_1S_1 and synthesized long DNA primers 10 times more effectively than RNA primers. The N-terminal (25KL) and C-terminal halves (20KL) of the large subunit (L) play distinct roles in regulating de novo DNA synthesis of the small catalytic subunit (S). The 25KL domain has a dual function. One function is to depress the large affinity of the intrasubunit domain 20KL for the template DNA until complex (L_1S_1 unit) formation. The other function is to tether the L subunit tightly to the S subunit, probably to promote effective interaction between the intrasubunit domain 20KL and the active center of the S subunit. The 20KL domain is a central factor to enhance the de novo DNA synthesis activity of the catalytic S subunit since the total affinity of the L_1S_1 unit is mainly derived from the affinity of 20KL, which is elevated more than 10 times by the heterodimer formation, presumably due to the cancellation of the inhibitory activity of 25KL through tight binding to the S subunit.

DNA primases are essential components of the DNA replication apparatus in every organism. They catalyze the synthesis of oligoribonucleotides on single-stranded DNA, which subsequently serve as primers for the replicative DNA polymerase. Proteins displaying primase activity are well-characerized in baceriophage, viral, bacterial, and eukaryotic systems. In contrast to bacterial primases, the archaeal enzymes are closely related to their eukaryotic counterparts. From eukaryotic cells, the DNA primase is copurified with DNA polymerase α. The DNA polymerase α-primase complex consists of four subunits with approximate molecular masses of 180, 70, 58, and 48 kDa, which are highly conserved in a wide range of eukaryotic organisms (*1*, *2*). The 180 kDa polypeptide is responsible for the polymerase activity (*3*, *4*). The 70 kDa protein species has no known enzymatic activity, is possibly involved in interactions with other proteins bound to the origin of replication, and is phosphorylated by cdk2/cyclin E or cdk2/cyclin A in a cellcycle-dependent manner probably to regulate the activity of the pol α-primase complex (*5*−*8*). The 70 kDa protein is also involved in the translation of the catalytic subunit of the pol α-primase and plays important roles in the translocation of the catalytic subunit to the nuclei (*9*). The DNA primase is a heterodimer of large and small subunits (58 and 48 kDa, respectively). In a number of species, the 48 kDa subunit has been shown to be the catalytic subunit for synthesizing RNA primers (*10*). The function of the 58 kDa subunit is still poorly understood, although it stabilizes primase activity (*11*, *12*) and is required for the binding of the primase heterodimer to the 180 kDa polymerase α polypeptide (*13*). The 58 kDa subunit is also involved in the transportation of the 48 kDa subunit to the nuclei (*14*).

The hyperthermophilic archaeon, *Pyrococcus horikoshii*, can grow well at around 100 °C. However, its DNA replication and repair systems working at high temperatures are not elucidated well. Using the genome information of *P. horikoshii*[1] (*15*), several proteins involved in replication/repair have been characterized (*16*−*20*). By the sequence search of the primases in the total genomes of several archaeal organisms, open reading frames homologous to the eukaryotic p48 and p58 have been found in all euryarchaeal organisms, including *Methanococcus jannaschii*, *Methanothermobacter thermoautotrophicus*, *Archaeoglobus fulgidus*, and *Halobacterium sp* NRC-1, as well as *Pyrococcals* (*21*). The gene homologues of DNA primase from *P. horikoshii* were expressed, and their biochemical characteristics were analyzed. The two subunits could form a complex and showed strong activity, whereas the catalytic S monomer had weak activity.[2] The complex could synthesize long DNA

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[1] Abbreviations: *P. horikoshii*, *Pyrococcus horikoshii*; BAP, bacterial alkaline phosphatase; dNTP, deoxynucleotide; ssDNA, single-stranded DNA; Amp, ampicillin; IPTG, isopropyl-*β*-D-thiogalactopyranoside; 2-ME, 2-mercaptoethanol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CBBR, Coomassie brilliant blue R-250; His-tag, histidine-tag; HisS, the histidine tagged small subunit of primase; L, the large subunit of primase; 25KL, the N-terminal 25 kDa fragment of L; 20KL, the C-terminal 20 kDa fragment of L; Pho-prim, primase from *P. horikoshii*; Pfu-prim, primase from *P. furiosus*; SPR, the surface plasmon resonance; K_A, an association constant; k_d, a dissociation rate constant.

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Table 1: List of Primers[a]

DPS-1	
CTTTAAGAAGGAGATATACATATGCTGCTGCGTGAGGTAACCCGTGAGGAAAGAAA	
GAACTTTTAC NdeI	
DPS-2	
TTTTGAGCTCTTTGGATCCTTAGGCCATCTTTTAAGTTCCGAGACTTTC	
BamHI	
DPL-1	
TTTTGTCGACTTACATATGGCGATCATGCTCGACCCA	
NdeI	
DPL-2	
TTTTAAGCTTTTTGGATCCTTATTCCATTCATTGGTGTAA	
BamHI	
DPL-25KL	
TTTTAAGCTTTTTGGATCCTTATTCCATTCATTGAATCTCTCC	
BamHI	
DPL-20KL	
TTTTGTCGACTTACATATGGGAAAACTAAGGCCGGAG	
NdeI	

[a] The restriction sites are underlined.

primers 10 times more effectively than RNA primers. Recently, the enzymatic characteristics of DNA primase from *Pyrococcus furiosus* were reported by Liu et al. (*21*). The primer synthesis activity of the small subunit, Pfup41, is regulated by the large subunit, Pfup46. The p41-p46 complex showed higher DNA binding activity than the catalytic p41 subunit alone. The DNA synthesized by the p41-p46 complex was much more abundant and shorter in length than that synthesized by Pfup41 alone. However, the details of the subunit interaction and the activity regulation are still unclear. To clarify the basic mechanism of the activity regulation for the DNA primase from *P. horikoshii* (Pho-prim), the N-terminal and the C-terminal halves of the large subunit (L), 25KL and 20KL, were successfully overexpressed, and the DNA binding abilities and enzymatic properties of the S and deleted L subunits were analyzed. Here, we reported domain interactions for controlling the de novo DNA synthesis.

MATERIALS AND METHODS

Chemicals. The pET11a vector, *Escherichia coli* strain BL21 (DE3) CodonPlus-RIL, and ultracompetent *E. coli* XL2-Blue MRF′ cells were purchased from Stratagene. The vector pET15b was obtained from Novagen. Vent DNA polymerase was purchased from New England Biolabs. Restriction enzymes were purchased from Promega and Toyobo (Osaka, Japan) and were used according to the manufacturer's recommendations. Bacterial alkaline phosphatase (BAP) and M13 mp18 ssDNA were from Takara Shuzo (Otsu, Shiga, Japan). An ultrapure deoxynucleotide solution (dNTPs) and poly dT were obtained from Amersham Bioscience. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Takara Shuzo. The protease inhibitor cocktail (Complete EDTA-free) was from Roche Diagnostics GmbH, Germany. Bovine DNase I was from Sigma.

Cloning of Genes and Construction of Expression Vectors. In the genome sequence of *P. horikoshii* (*15*), we found two open reading frames (PH0195 and PH0196) corresponding to the small (S) and the large (L) subunits of the DNA primase. Each subunit was cloned separately. The DNA fragment containing S was amplified by polymerase chain reaction (PCR) with two primers, DPS-1 and DPS-2, listed in Table 1, which contain NdeI and BamHI sites, respectively. The PCR products were purified with a QIAquick Spin PCR Purification Kit (QIAGEN) and digested by NdeI and BamHI. The digested fragment coding S was purified and then ligated to the insertion sites of the vector pET15b. Ultracompetent *E. coli* XL2-Blue MRF′ cells were transformed with the recombinant molecule. Transformants were screened on 2 × YT plates containing 50 μg/mL of ampicillin (Amp) incubated at 37 °C overnight. The transformant colonies were propagated in 5 mL of 2 × YT +Amp medium at 37 °C overnight, and the plasmid pET15b/S was purified after centrifugation using a Mini Plasmid Kit (QIAGEN). pET15b/S was double-digested with NdeI and BamHI, and the insert length was checked using agarose gel electrophoresis. The absence of additional mutations within the coding region of S was verified by sequencing on an Applied Biosystems 373A DNA sequencer (Taq DyeDeoxy Terminator Cycle Sequencing Kit, Perkin Elmer).

The DNA fragment containing L was amplified by PCR with primers DPL-1 and DPL-2 listed in Table 1, which contain the NdeI and BamHI sites, respectively. The PCR products were cloned into the vector pET11a in the same way as S. The resultant plasmid pET11a/L was purified from the transformants. DNA sequencing was performed to verify the absence of additional mutations.

Construction of Mutant Genes. For the L subunit, the limited digestion points by proteases were analyzed by the N-terminal sequencing of these proteolytic products. Using the information for the domain structure, the L subunit was split into the N-terminal half (25KL) and C-terminal half (20KL) as shown in Figure 1 A to investigate the function of each domain separately. The 25KL gene coding residues 1−222 was constructed with the primers DPL-1 and DPL-25KL listed in Table 1 by PCR. The 20KL gene coding residues 223−397 was also constructed with the primers DPL-20KL and DPL-2 by PCR. The DNA fragments of 25KL and 20KL were inserted into the pET11a and pET15b vectors, respectively, using the NdeI and BamHI sites. The nucleotide sequence of each mutant plasmid was examined with an Applied Biosystems 373A DNA sequencer (Taq DyeDeoxy Terminator Cycle Sequencing Kit, Perkin Elmer).

Overexpression and Purification of Native and Mutant Proteins. Histidine-tagged S (HisS) and L subunits were overexpressed and purified as follows: *E. coli* BL21(DE3) CodonPlus-RIL harboring pET15b/S or pET11a/L was propagated at 37 °C overnight as seed cultures in 2 × YT medium containing ampicillin (50 μg/mL). Forty milliliters of the seed culture was inoculated into 2 L of 2 × YT +Amp medium. The transformant was induced at $OD_{600} = 1$ with 1 mM IPTG for 4 h. The cells were collected by centrifugation and stored at −20 °C.

In the case of HisS, the frozen cells (25 g) were suspended with 50 mL of 50 mM HEPES−NaOH buffer (pH 7.8) containing 0.5 M NaCl, 10% glycerol, 0.01% Nonidet P-40, and 5 mM 2-mercaptoethanol (2-ME). The cell suspension was sonicated with a Sonifier 250 (Branson) for 10 min at an output control level of 5 and at a 50% duty cycle. After centrifugation, the supernatant was loaded onto a 1 mL HiTrap Chelating HP column (Amersham Bioscience) equilibrated with 50 mM HEPES−NaOH buffer (pH 7.8) containing 0.5 M NaCl, 10% glycerol, 0.01% Nonidet P-40, and 5 mM 2-ME. HisS was eluted with a linear gradient of 0−0.2 M imidazole and 0.5−0.1 M NaCl in the same buffer. The protein solution was applied to a 1 mL HiTrap Heparin column (Amersham Bioscience) equilibrated with 10 mM

[2] Matsui, I., Kikuchi, H., Kawarabayasi, Y., and Honda, K. (2000) *The Abstracts of Extremophiles 2000*, p 209.

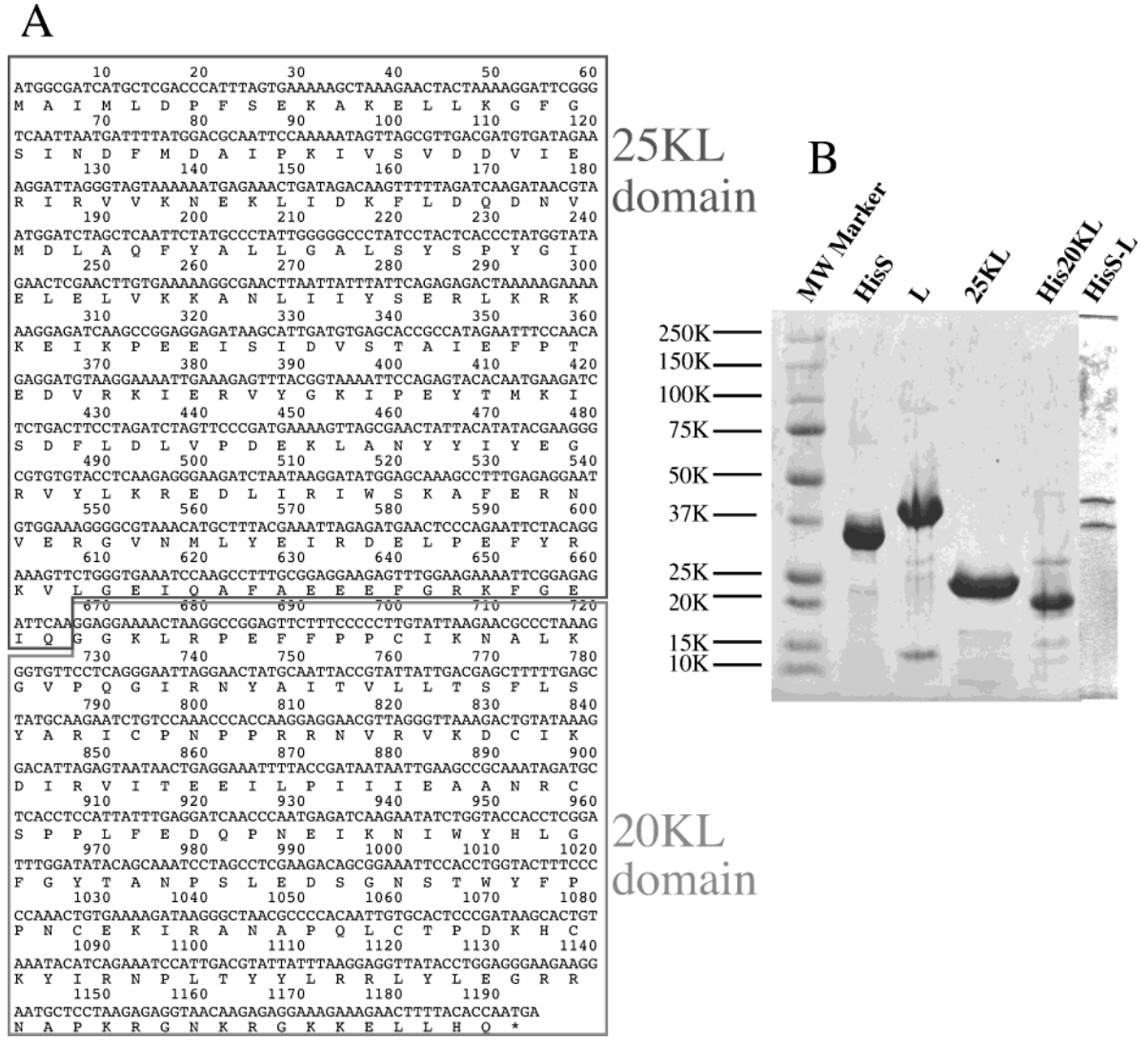


FIGURE 1: Sequence information of the domain boundary of the large subunit (A) and SDS−PAGE of the purified primase molecules (B). (A) The regions of the N-terminal 25 kDa and C-terminal 20 kDa domains are separated by rectangles. The domain boundary is present between Q222 and G223. (B) The SDS−PAGE pattern of the purified subunits with full and deleted sizes and the purified HisS-L complex.

HEPES−NaOH buffer (pH 7.8) containing 0.1 M NaCl, 10% glycerol, 0.01% Nonidet P-40, and 5 mM 2-ME. The protein was eluted with a linear gradient of 0.3−1 M NaCl in the same buffer. The protein was dialyzed with 5.5 mM HEPES−NaOH buffer (pH 7.8) containing 0.1 M NaCl, 50% glycerol, 0.0055% Nonidet P-40, and 2.8 mM 2-ME overnight and stored at −20 °C.

In the case of L, the frozen cells (25 g) were suspended with 25 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol, 5 mM 2-ME, protease inhibitor cocktail (Complete EDTA-free), and a few milligrams of bovine DNase I. The cell suspension was sonicated for 10 min and heated at 70 °C for 10 min. After centrifugation, the supernatant was loaded onto a 5 mL HiTrap SP column (Amersham Bioscience) equilibrated with 50 mM phosphate buffer (pH 6.0) containing 10% glycerol and 5 mM 2-ME. The protein was eluted with a linear gradient of 0−1 M NaCl in the same buffer. The eluted sample was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl overnight and then applied to a 5 mL HiTrap Heparin column (Amersham Bioscience) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 10% glycerol and 5 mM 2-ME. The protein was eluted with a linear gradient of 0.1−1 M NaCl in the same buffer. The protein was dialyzed with 28 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl, 50% glycerol, and 2.8 mM 2-ME overnight and stored at −20 °C.

Deleted L subunits (25KL and His20KL) were overexpressed with *E. coli* BL21(DE3) CodonPlus-RIL harboring pET11a/25KL or pET15b/20KL in the same way as L. The induced cells were collected by centrifugation and stored at −20 °C.

For 25KL, the frozen cells (25 g) were suspended with 25 mL of 50 mM Tris-HCl buffer (pH 7.0) containing protease inhibitor cocktail (Complete EDTA-free) and a few milligrams of bovine DNase I. The cell suspension was sonicated for 10 min and heated at 85 °C for 10 min. After centrifugation, the supernatant was loaded onto a HiTrap Q column (Amersham Bioscience) equilibrated with 50 mM Tris-HCl buffer (pH 7.0). The protein was eluted with a linear gradient of 0−1 M NaCl in the same buffer. The eluted sample was dialyzed against 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl overnight and stored at 4 °C.

For His20KL, the frozen cells (25 g) were suspended with 25 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol, 5 mM 2-ME, protease inhibitor cocktail (Complete

EDTA-free), and a few milligrams of bovine DNase I. The cell suspension was sonicated for 10 min. After centrifugation, the supernatant was loaded onto a 1 mL HiTrap Chelating HP column (Amersham Bioscience) equillibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, 10% glycerol, and 5 mM 2-ME. HisS was eluted with a linear gradient of 0−0.5 M imidazole in the same buffer. The eluted sample was dialyzed with 28 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl, 50% glycerol, and 2.8 mM 2-ME overnight and stored at −20 °C.

Protein Analysis. SDS−PAGE was performed with Phast-Gel Gradient 10−15 (Amersham Bioscience) on a Phast-System (Amersham Bioscience). Protein bands were made visible by Coomassie brilliant blue R-250 (CBBR) staining.

Protein concentrations were determined with Coomassie Protein Assay Reagent (Pierce, Rockford, IL). N-Terminal peptide sequencing was carried out as follows: the pure protein was separated on a 15% resolving gel by SDS−PAGE and then transferred onto PVDF membrane. N-Terminal sequencing was performed by Takara Shuzo Co. Ltd. (Otsu, Shiga, Japan) using a protein sequencer PSQ-1 (Shimadzu, Japan).

To determine the native molecular weight of the purified proteins, gel filtration was carried out with a Superose 12 column (10/300, Amersham Bioscience) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl. The proteins were eluted from the column with the same buffer.

Precipitation of Complexes with Histidine-Tag (His-Tag). The ability to form a complex between HisS and L or 25KL was compared as follows: 2 μg of HisS and 3 μg of L or 1.25 μg of 25KL were added to 180 μL of 20 mM Tris-HCl buffer (pH 7.9) containing 0.5 M NaCl, 5 mM imidazole, 1% Triton X-100, and 0.04% bovine serum albumine (BSA). After incubation of the mixture in ice for 10 min, the solution was mixed well for 10 min with 16 μL of His Bind resin (Novagen) containing Ni^{2+} and washed with binding buffer (Novagen, His Bind buffer kit) three times. The precipitate was suspended with 50 μL of SDS sample buffer and boiled for 10 min. After centrifugation, the supernatant was analyzed by SDS−PAGE.

Purification of HisS-L Complex. The stock solutions of L (281 μg) and HisS (250 μg) were mixed together and kept on ice for 10 min. The mixture was loaded onto a HiTrap SP column (Amersham Bioscience) equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 10% glycerol and 5 mM 2-ME. The protein was eluted with a linear gradient of 0−1 M NaCl in the same buffer. The eluted sample was diluted five times with 50 mM Tris-HCl buffer (pH 7.0) containing 10% glycerol and 5 mM 2-ME and then applied to a HiTrap Heparin column (Amersham Bioscience) equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 10% glycerol and 5 mM 2-ME. The protein was eluted with a linear gradient of 0.1−1 M NaCl in the same buffer. The eluted sample was dialyzed with 28 mM Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl, 50% glycerol, and 2.8 mM 2-ME overnight and stored at −20 °C.

De Novo DNA Synthesis. De novo DNA synthesis (DNA priming) was measured using single-stranded DNA (ssDNA) as a template. The reaction mixture (20 μL) contained 0.1 μM enzyme, 10 mM $MgCl_2$, 0.8 μg of poly dT, 0.1 mM dATP, and 3 μCi of [α-^{32}P]dATP in 40 mM glycine-NaOH buffer (pH 9.1). After the surface of the solution was covered with mineral oil (Sigma), the reaction was carried out at 50 °C for 20 min. When M13 mp18 ssDNA (0.5 μg) was used as the template, 0.25 mM dNTPs (dCTP, dGTP, and dTTP), 0.02 mM dATP, and 3 μCi of [α-^{32}P]dATP were used, whereas the remaining conditions were as in the reaction with poly dT as template. The reaction was carried out at 70 °C for 5 min.

Quantification Methods of Synthesized DNA. The reaction products were quantified by two methods; one is a electrophoretic method, and the other is a acid precipitation method. For the electrophoretic method, the reaction was stopped by mixing with the same volume of 90% formamide with 20 mM EDTA. After boiling for 3 min, the mixture was chilled on ice and then analyzed with a 4.75 or 15% denaturing polyacrylamide gel containing 7 M urea and 1 × TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The electrophoretic profiles were analyzed by autoradiography with a Molecular Imager FX (Bio-Rad). Oligonucleotides, 8-, 10-, 30-, 40-, 54-, and 69mer, were labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP and used as molecular markers.

For the acid precipitation method, the reaction was stopped by mixing with 100 times the volume of 10% trichloroacetic acid (TCA) containing 50 mM sodium pyrophosphate. After being kept on ice for 10 min, 150 μL of the mixture containing radioactive precipitates was spotted on a GF/C glass filter (9 × 12 cm, Whatman) with a Bio-Dot microfiltration apparatus (Bio-Rad). Each spot on the filter was washed three times with 500 μL of 6.3% trichloroacetic acid (TCA) containing 50 mM sodium pyrophosphate and twice with 500 μL of 99.5% ethanol. After drying, the autoradiograph of the filter was analyzed with a Molecular Imager FX (Bio-Rad).

Surface Plasmon Resonance (SPR) Experiments. The interaction among subunits and domains of Pho-prim and ssDNA were quantitatively analyzed on a BIAcore X apparatus (Biacore, Uppsala, Sweden) at 25 °C. For analyzing the protein interaction, the flow cell was routinely equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 1% glycerol, and 0.005% Tween 20. The proteins as ligands, HisS, His20KL, and 25KL, in 100 mM sodium acetate buffer (pH 5.0) were injected and immobilized on the surface of a Sensor Chip CM5 (Biacore) with an amine coupling kit (Biacore), resulting in resonance units (1879 for 25KL and 2952 for HisS in the interaction analysis and 374 for 20KL and 780 for HisS in the kinetic analysis). The proteins were diluted in the running buffer and injected for 120 s. The protein solutions at various concentrations from 5 to 100 were injected for the kinetic analysis, and for the interaction analysis a constant concentration, 100 nM, was used. For analyzing the interaction of ssDNA with the proteins, a biotinylated 54mer ssDNA (5′-GAGCTAGATGTCGGACTCTGCCTCAAGACGGTAG-TCAACGTGCACTCGAGGTCA-3′) was immobilized to a streptavidin−dextran layer on the surface of the Sensor Chip SA (Biacore) at 70 resonance units. The flow cell was routinely equilibrated with 50 mM Tris-HCl buffer (pH8.0) containing 50 mM NaCl, 1% glycerol, and 0.005% Tween 20. The same buffers containing 150 mM NaCl or 100 mM NaCl were used for 20KL or the HisS-L complex, respectively. To form the complex, the proteins were mixed at

equimolecular amounts. For the kinetic analysis, the proteins were injected using at least five different concentrations ranging from 1 to 1000 nM. The obtained sensograms were analyzed by the evaluation software (Biacore) on the basis of the simple 1:1 binding model.

RESULTS AND DISCUSSION

Purification of Subunits and Complexes. The amino acid sequence of the L subunit is shown in Figure 1A. The HisS and L subunits were purified as shown in Figure 1B. Although the structure/function relationship of the catalytic subunit S was characterized well (22), the molecular mechanism of the L subunit to regulate the primase activity was still unclear. To analyze the function of L, the N-terminal (25KL) and C-terminal (20KL) halves were successively expressed and purified as shown in Figure 1B since the domain boundary was predicted between G^{219} and G^{223} by the N-terminal sequencing of the proteolytic products of L, as shown in Figure 1A. The purified 25KL and 20KL were used in assays for de novo DNA synthesis and DNA binding to investigate the function of L.

Furthermore, the complex, HisS-L, was purified completely as shown in Figure 1B. The molar ratio of the L to S subunit was analyzed by a densitometer, Molecular Imager FX (Bio-Rad) using the SDS−PAGE pattern of the HisS-L complex shown in Figure 1B, and proved to be 1−1.1 according to the band intensity, indicating an oligomeric unit L_1S_1.

To analyze the native molecular forms of the purified proteins, the gel filtration of the purified HisS, L, 25KL, and the complex HisS-L were performed at room temperature with the Superose 12 column (Amersham Bioscience) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl. The protein was eluted with same buffer. The molecular weights of L and 25KL were determined to be 46 and 41 kDa using marker proteins, suggesting a monomer and dimer, respectively, due to their calculated molecular weights of monomer forms (data not shown). The native molecular weight of HisS was not determined since the molecule was not eluted from the Superose 12 column due to its unfavorable absorption to the gel matrix under the condition. The recovery of the complex HisS-L from the column was also too low to determine the native molecular weight as well as HisS alone.

De Novo DNA Synthesis is 10 Times More Effective than RNA Synthesis. DNA primers approximately 0.3 kb in length were synthesized effectively during a 5 min reaction using M13 ssDNA as template as shown in Figure 2 lane 1, although RNA primers of around a 30mer were produced with 1/10 this intensity as shown in lane 2. When the NTP mix was added to the reaction mixture for DNA primer synthesis, the length decreased slightly, but the total amount of primers increased a little as shown in lane 3. However, synthesis of the RNA primers was affected markedly by addition of the dNTP mix to the reaction mixture as shown in lane 4. The products, presumably synthesized as a DNA/RNA hybrid, were elongated up to approximately 0.4 kb, whereas the amount synthesized was not remarkably increased, as compared with the RNA primer as shown in lane 2. Further study by alkaline hydrolysis might be required to elucidate the molecular structure of the synthesized hybrid

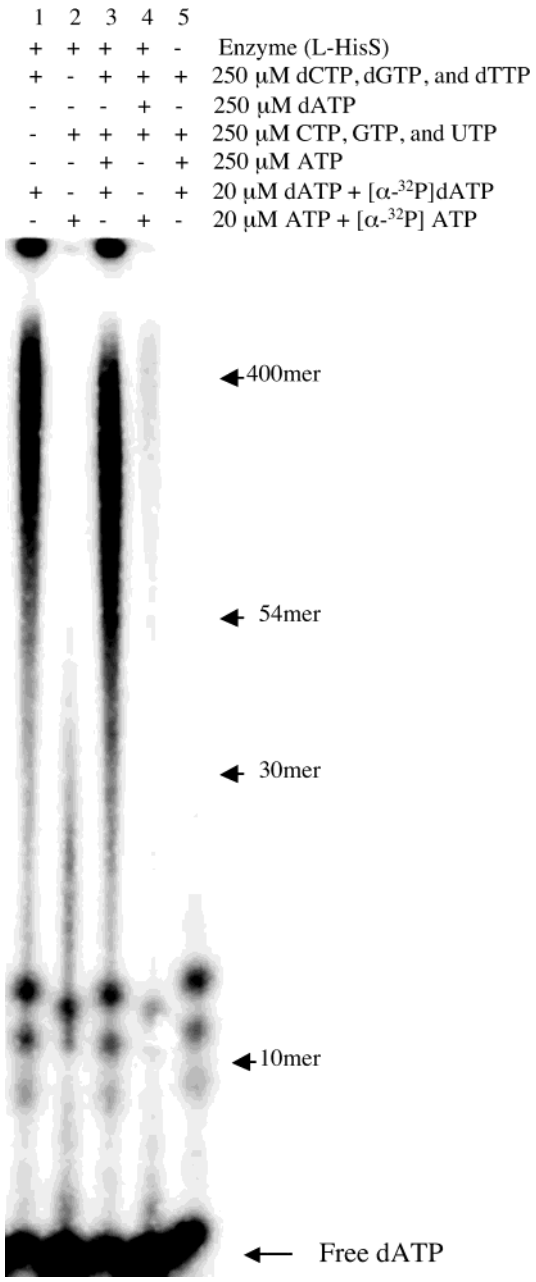


FIGURE 2: De novo DNA, RNA, and DNA/RNA hybrid synthesis by the purified complex HisS-L. For both DNA and RNA synthesis, the reaction mixture (20 μL) contained 0.1 μM the purified complex HisS-L, 0.5 μg of M13 mp18 ssDNA, and 10 mM $MgCl_2$ in 40 mM glycine-NaOH buffer (pH 9.1). To synthesize DNA, 0.25 mM dNTPs (dCTP, dGTP, and dTTP), 0.02 mM dATP, and 3 μCi of [α-^{32}P]dATP were added in the reaction mixture. To synthesize RNA, 0.25 mM NTPs (CTP, GTP, and UTP), 0.02 mM ATP, and 3 μCi of [α-^{32}P] ATP were added in the reaction mixture. The enzyme reactions were performed at 70 °C for 5 min, and the reaction products were analyzed by the denatured gel method.

primers. It would be very interesting and important to determine which primer (RNA, DNA, or DNA/RNA hybrid) is synthesized de novo in the DNA replication process in *P. horikoshii* cells. Isolation and characterization of the Okazaki fragments from the archaeal cells are necessary for further clarification. The ability of the enzyme to carry out template dependent DNA synthesis without the requirement of a primer might be useful as a tool in biotechnology.

25KL Is a Specific Domain for the Tight Binding of L to S. The formation of a heterodimer between HisS and L or

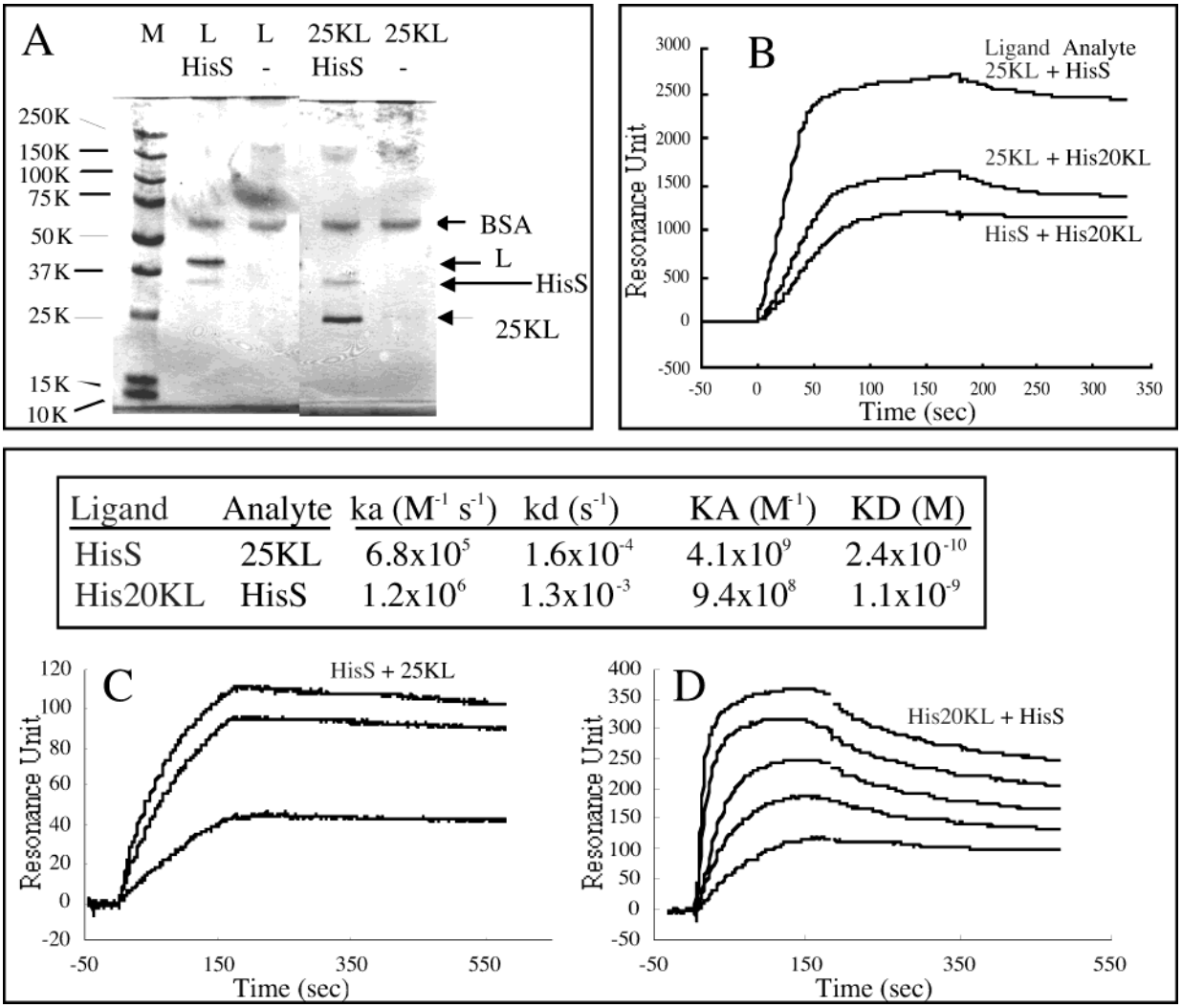
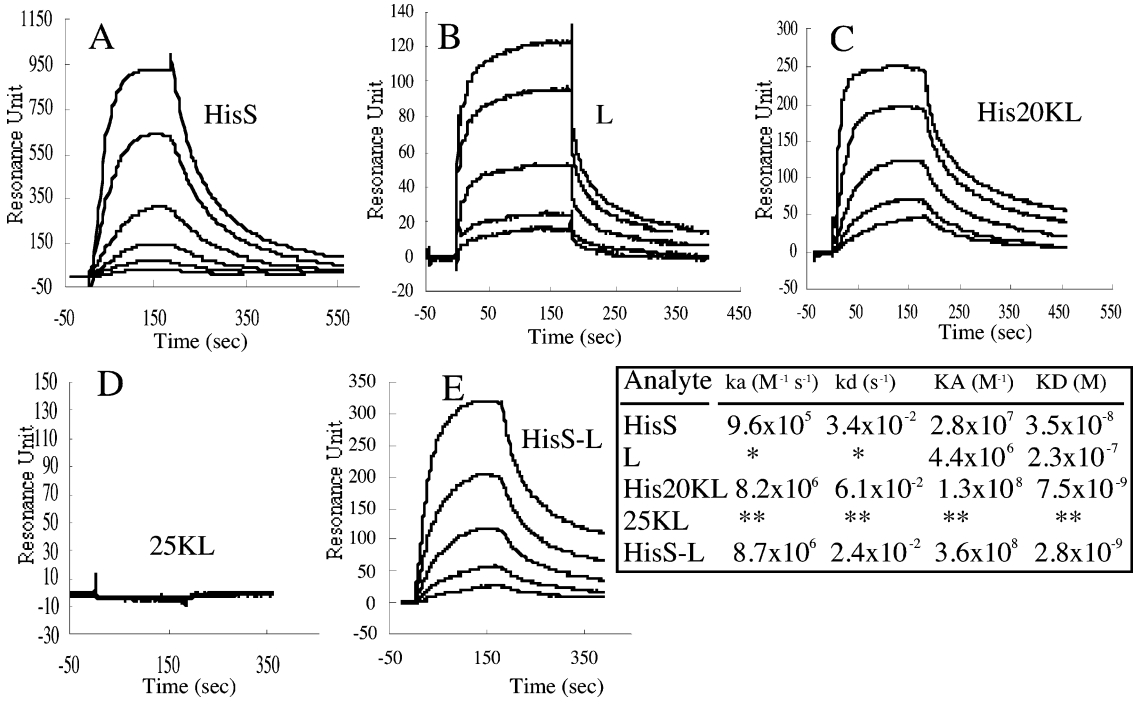


FIGURE 3: Domain interactions checked by the precipitation method with a His-tag (A) and measured in the surface plasmon resonance (SPR) experiments (B−D). With the precipitation method, it should be noted that usually less intense bands of HisSs were observed on the SDS−PAGE pattern, as compared with those of the counterparts, due to the reabsorption of His-tagged proteins by the resin even after boiling with SDS-buffer. (A) The interaction between HisS and L or 25KL; the symbol (−) and the letter (M) shown in the upper part of the panel indicate the absence of HisS in the mixture and the molecular weight marker, respectively. (B) The interaction among HisS, 25KL, and His20KL measured by SPR experiments. (C) The kinetic analysis of the interaction between HisS as ligand and 25KL as analyte. The concentration of the analyte, 25KL was varied at 5, 10, and 20 nM. (D) The kinetic analysis of the interaction between His20KL as ligand and HisS as analyte. The concentration of the analyte, HisS, was varied at 5, 10, 20, 50, and 100 nM. The kinetic parameters calculated by the experimental results of panels C and D were summarized in the inset.

25KL was checked by the precipitation method with a His-tag. The 25KL bound to HisS was retarded with nickel resin as well as the complex with L and HisS, while L or 25KL alone was not retarded as shown in Figure 3A. In this method, it should be noticeable that usually less intense bands of HisS were observed on the SDS−PAGE pattern, as compared with those of the counterparts, due to the reabsorption of His-tagged protein to the resin even after boiling with SDS-buffer. To investigate the mutual interactions among HisS, 25KL, and His20KL in more detail, the kinetic parameters of the protein interactions were determined by the surface plasmon resonance (SPR) experiments with BIAcore as shown in Figure 3B−D. The results were summarized in the inset of the figure. Both 25KL and His20KL could bind to HisS. However, the dissociation rate constant (k_d) of 25KL from HisS was 8.1 times smaller than that of His20KL from HisS. Furthermore, the association constant (K_A) value of the HisS-25KL interaction was 4.4 times larger than that of the HisS-His20KL interaction, suggesting a tighter interaction between 25KL and S than between 20KL and S. The interaction between 25KL and S was so strong that the analyte HisS was unable to be removed from the ligand 25KL even with 3 M KCl. Interestingly, the intrasubunit domains, 25KL and 20KL, could interact with each other as shown in Figure 3B. The experiments clearly indicated that 25KL rather than 20KL is contributing to the tight binding of L to S and that at least two interaction sites of L, which were located separately on the 25KL and 20KL domains, could recognize S independently.

Affinity of the 20KL Domain Is Controlled by the 25KL Domain and Is the Main Source of the Total Affinity of the L_1S_1 Unit. The SPR experiments with BIAcore indicated that 25KL alone could not bind to ssDNA, whereas His20KL and HisS could as shown in the inset of Figure 4. The affinity of His20KL was remarkably high, as compared with that of the L or HisS alone. The K_A value of His20KL was 29.5 times higher than that of the L subunit. The results indicate that the intrasubunit domain 20KL is controlled in its affinity



Analyte	ka (M^{-1} s^{-1})	kd (s^{-1})	KA (M^{-1})	KD (M)
HisS	$9.6x10^{5}$	$3.4x10^{-2}$	$2.8x10^{7}$	$3.5x10^{-8}$
L	*	*	$4.4x10^{6}$	$2.3x10^{-7}$
His20KL	$8.2x10^{6}$	$6.1x10^{-2}$	$1.3x10^{8}$	$7.5x10^{-9}$
25KL	**	**	**	**
HisS-L	$8.7x10^{6}$	$2.4x10^{-2}$	$3.6x10^{8}$	$2.8x10^{-9}$

FIGURE 4: Interaction of ssDNA with the proteins measured in the surface plasmon resonance (SPR) experiments. (A) The interaction of ssDNA with HisS as analyte in the presence of 50 mM NaCl. The ssDNA was immobilized at 240 resonance units. The concentration of the analyte, HisS was varied at 2, 5, 10, 20, 50, and 100 nM. (B) The interaction of ssDNA with L as analyte in the presence of 50 mM NaCl. The ssDNA was immobilized at 70 resonance units. The concentration of the analyte, L, was varied at 10, 20, 50, 100, and 200 nM. (C) The interaction of ssDNA with His20KL as analyte in the presence of 150 mM NaCl. The ssDNA was immobilized at 70 resonance units. The concentration of the analyte, His20KL, was varied at 5, 10, 20, 50, and 100 nM. (D) The interaction of ssDNA with 25KL as analyte in the presence of 50 mM NaCl. The ssDNA was immobilized at 230 resonance units. The concentration of the analyte, 25KL, was varied at 100, 500, and 1000 nM. (E) The interaction of ssDNA with the complex, HisS-L as analyte in the presence of 100 mM NaCl. The ssDNA was immobilized on the sensor chip at 70 resonance units. The concentration of the analyte, HisS-L, was varied at 1, 2, 5, 10, and 20 nM. The kinetic parameters calculated by the experimental results of panels A−E were summarized in the inset. *The sensogram obtained in panel B was analyzed by the evaluation software (Biocore) on the basis of the steady-state affinity model, in which the k_a and k_d values were not available. **The parameters were not obtained due to no significant signal on the sensograms as shown in panel D.

by the partner 25KL, while 25KL alone has no detectable affinity to DNA as shown in Figure 4D and the inset. However, once the complex S−L was formed, its main affinity was caused by 20KL, the small part of the complex, since the K_A value of the complex was maintained at the same level as that of His20KL alone as shown in the inset of Figure 4. The concepts matched well with the findings that the complex HisS-L or His20KL alone could bind to ssDNA strongly even at more than 100 mM NaCl as shown in Figure 4, although in both cases specific binding was not detected at 50 mM, due to high nonspecific adsorption, suggesting an identical DNA binding manner for both proteins. On the contrary, the binding of HisS or L alone to ssDNA was remarkably reduced at higher than 100 mM NaCl and only measurable at 50 mM. From these results, we concluded that the main source of the total affinity of the L_1S_1 unit was derived from 20KL and that the inhibitory activity of 25KL against the affinity of 20KL might be canceled out by the complex formation.

Since the K_A value of the protein interaction between HisS and 25KL (4.1×10^9 M^{-1} as shown in Figure 3) is 100 times higher than the K_A values of HisS and L alone against ssDNA (2.8×10^7 and 4.4×10^6 M^{-1}, respectively, as shown in Figure 4), the S−L complex easily formed by the high K_A value might trigger the enhancement of the priming activity. The affinity of the resultant complex to ssDNA might be enhanced by the cancellation of the inhibitory function of the 25KL. The activated complex with high affinity could bind ssDNA and supply it very effectively to the active center, leading to the enhancement of the priming activity.

Deletion studies of human DNA primase by Copland indicate that contacts between the p49 subunit and the p58 subunits involve amino acids in both the N-terminal and the C-terminal halves of p58 (*23*). Using photoactivable cross-linking agents, Arezi et al. reported that the N- and C-terminal halves of p58 both contain DNA binding domains (*24*). Since the N-terminal half, 25KL, has no detectable DNA binding ability as reported here, the euryarchaeal primases seem to recognize the template DNA in a slightly different manner from their eukaryotic counterparts, although the priming reaction by the cooperative actions of the two L domains seems to be conserved in these two interesting kingdoms (*25*).

25KL and 20KL Domains Located in the Same Subunit Play Opposite Roles in Regulating de Novo DNA Synthesis of the S Subunit. To clarify the function of the N- and C-terminal halves of the L domain, de novo DNA synthesis was analyzed with different combinations among HisS, L, 25KL, and His20KL at an equal molar ratio. For accuracy, the reaction products were quantified by two different assay methods, the acid precipitation method and the denatured gel method, as shown in Figure 5A,C, indicating the high reproducibility of these two methods. These molecules were mixed at an equal molar ratio and used to measure the priming activities. The HisS-L complex showed seven times

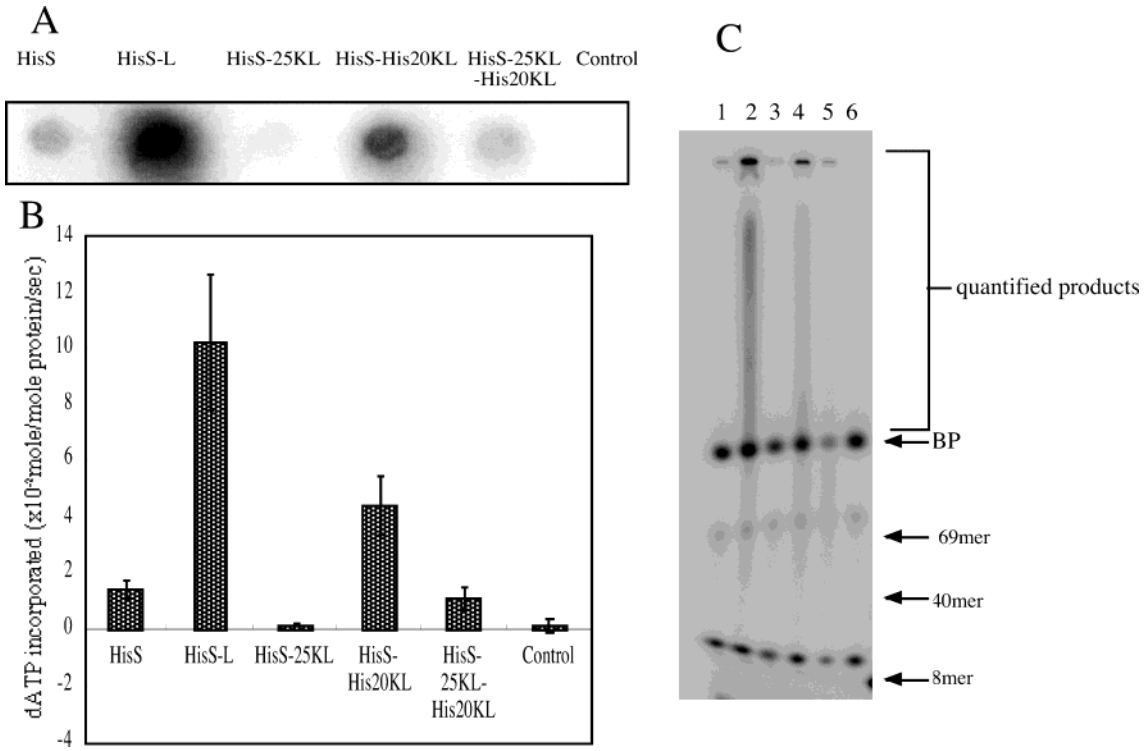


FIGURE 5: Distinct effects of 25KL and 20KL on the de novo DNA synthesis activity of HisS. (A) The autoradiogram of the synthesized DNA products trapped on the glass filter by the acid precipitation method. The reaction conditions were as follows: to form the enzyme complex, 10 pmol of the purified subunit with a full and deleted peptide length were mixed in 12 μL of 50% glycerol containing 0.1 M NaCl and kept on ice for 10 min. Immediately after the complex formation, 3 μL of the solution was added to 17 μL of the reaction mixture. The resultant reaction mixture (20 μL) contained 0.13 μM enzymes, 10 mM $MgCl_2$, 0.8 μg of poly dT, 0.1 mM dATP, and 3 μCi of [α-^{32}P]dATP in 40 mM glycine-NaOH buffer (pH 9.1). The enzyme reaction was performed at 50 °C for 20 min. As a control, the reaction was performed with the same reaction conditions mentioned above with HisS as enzyme and without the template, polydT. (B) The de novo DNA synthesis activities of the complexes quantified with the Molecular Imager FX using the trapped products shown in panel A. The mean of three independent experiments is shown with error bars. (C) The electrophoresis pattern of the reaction products prepared in panel A with the 4.75% denatured polyacrylamide gel. Lanes 1−6 correspond to the reaction products with the enzymes HisS, HisS-L, HisS-25KL, HisS-His20KL, HisS-25KL-His20KL, and control, respectively, in panel A. The abbreviation, BP, with an arrow indicates the spots of a biproduct from the HisS reaction with glycerol and [α-^{32}P]dATP. The reproducibility between the two different assay methods, the acid precipitation method and the denatured gel method, was quite high as judged by the quantified amounts of the products shown in panels A and C.

more activity than HisS alone as shown in Figure 5A,B. Surprisingly, the deleted complex, HisS-His20KL, showed three times more activity than HisS alone. On the contrary, another deleted complex, HisS-25KL, had an opposite effect on the activity. The activity was remarkably decreased, almost to the same level as a control reaction without the template. Furthermore, in the case of the triple complex, HisS-25KL-His20KL, the activity was not restored to that of the HisS-L complex but was maintained at the same level as HisS alone, probably because of the steric hindrance between the N-terminal His-tag of 20KL and the C-terminal portion of 25KL. These results clearly indicate that the 20KL domain was a key determinant in enhancing the S activity after the heterodimer formation. Since the complex HisS-25KL did not show a remarkable increase in binding ability as compared with HisS alone (data not shown), the fact that 25KL exhibited inhibitory activity against the S subunit presumably suggests the unfavorable temperate binding to S by the less cooperative action of 25KL without the interdomain linkage to 20KL.

Here, we successfully identified the opposite functions of 25KL and 20KL in regulating de novo DNA synthesis, whereas Copland reported that the N- and C-terminal halves (1−270 and 270−509, respectively) of human p58 did not support the p49 priming activity, while each half of the protein binds DNA and binds to p49 (*23*). These findings also suggest that the activities of euryarchaeal primases might be regulated by a slightly different mechanism from that of the eukaryotic counterparts. Our successful isolation of the active domains of the L subunit reported here might be valuable to analyze the structure/function of the euryarchaeal large subunit in detail.

Conclusion. The results of this study unequivocally proved that the N-terminal half of the L subunit, 25KL, has a dual function as follows: (1) depressing the large affinity of the intrasubunit domain 20KL to the template DNA until the complex (L_1S_1 unit) formation and (2) anchoring the L subunit tightly on the S subunit, probably to promote the effective interaction between the intrasubunit domain 20KL and the active center of the S subunit. On the other hand, the C-terminal half domain of the L subunit, 20KL, is a key determinant to enhance the de novo DNA synthesis activity of the catalytic S subunit since the total affinity of the L_1S_1 unit is mainly derived from the affinity of 20KL, which is elevated more than 10 times by the heterodimer formation, presumably due to the cancellation of the inhibitory activity of 25KL through tight binding to the S subunit. These distinct domain functions regulating de novo DNA synthesis represent an important piece in the puzzle of understanding the

DNA primase functions essential for the replication machinery.

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