DNA Binding of PriA Protein Requires Cooperation of the N-terminal D-loop/Arrested-fork Binding and C-terminal Helicase Domains*

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PriA protein is essential for RecA-dependent DNA replication induced by stalled replication forks in *Escherichia coli*. PriA is a DEXH-type DNA helicase, ATPase activity of which depends on its binding to structured DNA including a D-loop-like structure. Here, we show that the N-terminal 181-amino acid polypeptide can form a complex with D-loop in gel shift assays and have identified a unique motif present in the N-terminal segment of PriA that plays a role in its DNA binding. We have also identified residues in the C terminus proximal helicase domain essential for D-loop binding. PriA proteins mutated in this domain do not bind to D-loop, despite the presence of the N-terminal DNA-binding motif. Those mutants that cannot bind to D-loop in *vitro* do not support a recombination-dependent mode of DNA replication induced by stalled replication forks. We propose that binding of the PriA protein to stalled replication forks requires proper configuration of the N-terminal fork-recognition and C-terminal helicase domains and that the latter may stabilize binding and increase binding specificity.

*Escherichia coli* PriA protein, a DEXH-type DNA helicase with unique C2C2-type zinc finger-like motifs interrupting the helicase domains, is an essential component of the dX174-type primosome (1, 2) and plays critical roles in RecA-dependent inducible and constitutive stable DNA replication (iSDR and cSDR, respectively) (3) as well as in recombination-dependent repair of double-stranded DNA breaks (4). PriA null cells generally exhibit low viability (5, 6), and do not form colonies on plates lacking thymine. Those mutants that cannot bind to D-loop structures resembling D-loops (15), and the *X174*-type primosome, respectively, on the chromosome (3, 13, 14).

Consistent with this proposal, PriA binds to synthetic DNA structures resembling D-loops (15), and the *X174*-type primosome was shown to be assembled on recombination intermediates of Mu phage DNA replication or on a model D-loop structure (16, 17). ATPase activity of PriA, previously demonstrated in interactions with other primosomal proteins (28), ATPase/DNA helicase-deficient mutants of PriA protein are fully active for *in vitro* replication of the *X174*-type primosome (29), whereas they are only partially active for iSDR and cSDR (27). The above two classes of mutants are proficient in binding to D-loop structures, indicating that the structural determinants for DNA binding of PriA are present in regions other than the ATP-binding regions and zinc finger structures (27).

The primary structure of PriA protein is characterized by the presence of unique zinc finger structures that interrupt the conserved motifs of DEXH-type DNA helicases (23–25). The zinc finger structures are essential for *in vitro* primosome assembly on n-pras (26) as well as for recombination-dependent DNA replication *in vivo* (27). This motif appears to be involved in interactions with other primosomal proteins (28). ATPase/DNA helicase-deficient mutants of PriA protein are fully active for *in vitro* replication of the *X174*-type primosome (29), whereas they are only partially active for iSDR and cSDR (27). The above two classes of mutants are proficient in binding to D-loop structures, indicating that the structural determinants for DNA binding of PriA are present in regions other than the ATP-binding regions and zinc finger structures (27).

In this report, we have identified a novel conserved motif in the N-terminal region of the PriA protein that is evolutionarily conserved in eubacteria and have shown that the isolated segment containing this motif is capable of structure-specific binding.

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1. The abbreviations used are: iSDR, inducible stable DNA replication; pas, primosome assembly site; cSDR, constitutive stable DNA replication.

2. T. Tanaka, K. Arai, and H. Masai, unpublished result.
pendent DNA replication and are partially deficient in other cellular functions. On the basis of these results, we discuss a possible mode of recognition of DNA by PriA protein.

**EXPERIMENTAL PROCEDURES**

**E. coli Strains, Plasmid DNA, and Medium**—The *E. coli* strains used in this study, AQP634 (thyA, priA1) and AQ8581 (thyA, priA::kan) (3), were grown in M9 medium supplemented with required amino acids (50 μg/ml), thymine (20 μg/ml), and glucose (0.4%) at 37°C, except where indicated otherwise. For selection of antibiotic-resistant cells, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml. pR454 is based on pT7-6 and carries the priA gene under control of the T7 promoter (24). All point mutants were constructed on pT7-7, a derivative of pT7-6. The oligonucleotides used in this study are listed in Table I. D-loop and bubble substrates were grown in M9 medium supplemented with required amino acids (50 μg/ml; thymine (20 μg/ml), 10% glycerol, 40 mM potassium glutamate, and 1 mM diethiothreitol), and were lysed with sonication and the supernatant was saved after centrifugation. Proteins were precipitated by adding 0.28 g of solid ammonium sulfate per ml of each soluble fraction. The pellet was resuspended in buffer A and dialyzed against the same buffer for 3 h. The dialyzed protein fraction was applied to a heparin-agarose column by fast protein liquid chromatography (Amersham Biosciences) and the peak fractions that eluted around 0.5 M KCl were used for assays.

**PriA N-terminal regions amplified with PCR using primer sets, PriA5’-1 (number 15) and PriA3’-1 (number 16) or PriA5’-1 (number 15) and PriA3’-2 (number 17), were cloned into the pET15b expression vector (Novagen), resulting in His-PriA1 (1-195) or His-PriA1 (1-181), respectively. Expression was induced by addition of 0.7 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 20 min, an aliquot of reaction mixture was directly loaded onto an open column. The resin was washed with buffers containing 20 mM Tris-Cl (pH 8.0), 300 mM KCl, and 2 mM phenylmethylsulfonyl fluoride without imidazole or with 20 mM imidazole. Histidine-tagged polypeptides were eluted with the same buffer containing 250 mM imidazole. Eluted His-tagged polypeptide, after concentration and buffer exchange using Centricon YM-10 (MWCO 10,000, Millipore), was treated with thrombin (1/100, w/v) in a buffer containing 20 mM sodium phosphate (pH 6.5), 80 mM KCl, 1 mM EDTA, 2 mM diethiothreitol, and 2 mM CaCl2 at room temperature for 1 or 2 days. Benzamidin-Sepharose resin (Amersham Biosciences) was added to the digested polypeptide and filtrated to remove thrombin. The N-terminal polypeptide without the His tag was applied onto SP-Sepharose (Amersham Biosciences) and eluted around 300–400 mM KCl. Purified fractions were saved and used in assays. Overexpression and purification of the N-terminal mutant polypeptides were conducted as described for the wild-type histidine-tagged polypeptide. Mutant His-tagged polypeptides were directly used in assays, because the presence of the His tag did not affect binding activities. For cloning of priA gene onto a mini-R1 plasmid, the coding frame of the wild-type or mutant priA genes on pRA45 or pT7-7 was amplified by PCR with the primer sets, PriA-N (number 15) and PriA3’-1 (number 16) or PriA5’-1 (number 15) and PriA3’-2 (number 17). The products were digested with EcoRI and BamHI and inserted at the EcoRI HI site of pHM6050 (3). The PriA-N (EcoRI-RBS) primer contains a ribosome-binding site in front of the initiation codon and is expected to facilitate expression of PriA on a read-through transcript from the vector. **Generation of an Antibody against PriA Protein and Western Blotting of PriA N-terminal Polypeptides**—Western blot analysis was conducted by substituting the multiple cloning sites of pT7-7 with a Ndel-ClaI fragment from that of pQE30 (Qiagen) carrying the His sequence. The PriA N-terminal region amplified from pRA45 by PCR using the primer sets, PriA-N-SaliI (number 19) and PriA N2-antisense (BamHI-EcoRI) (number 20), was digested by BamHI and inserted at the BamHI site of pT7-7-QE30. Overexpression was conducted by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 3 h to the culture and cleared lysate was fractionated on a heparin-agarose column by the fast protein liquid chromatography system. For further purification, a dialyzed peak fraction was applied to the nickel-nitritropric acid column. Eluted polypeptide was used as antigen for antibody generation. **Construction and Purification of Mutant PriA Proteins**—Mutagenesis was conducted by the Kunkel method (31). The oligonucleotides used for mutagenesis or amplification of truncated DNA segments are listed in Table I. After PCR, amplified fragments were inserted into Ndel-EcoRI or Ndel-BamHI sites in pT7-7 and the expected base substitu-
## Table I

**Oligonucleotides used in this study**

| Number of Oligonucleotide | Length | Sequence (5'-3') | Use |
|---------------------------|--------|------------------|-----|
| 1 Upper strand            | 61     | GACGCTGCCGAATTCTACCAGTGCCCTGCTAGGACATCTTTGCCCACCTGCAAGTTTCACC | D-loop or bubble |
| 2 Lower strand            | 61     | GGGTGAACCTGCAGGTCGGGCGCTGCTATCGTAGTGTGTTAGTGGTAGAATCCGGCAGCCTC | D-loop or bubble |
| 3 Invading strand         | 41     | TAAGAGCAAGATGTTCTATAAAAGATGTCATACGGAAGC | D-loop |
| 4 PriA N (EcoRI-RBS)      | 37     | GGAATTCCGAGGACGATACATAGATACGCCTCGTGCCTCC | Construction of mutant PriA on mini-R1 plasmid |
| 5 PriA C (EcoRI)          | 28     | GGAATTCCCTCTACCCATCGGATCAAC | Construction of mutant PriA on mini-R1 plasmid |
| 6 PriA C (BamHI)          | 29     | CGGATCTTAAACCCTCAATCGGATCAAC | Construction of mutant PriA on pT7-7 |
| 7 WYY-s                   | 36     | TTGCTGCTAGGGGCGCAGATGCGGCTCATCGATAG | Construction of WYY |
| 8 WYY-as                  | 36     | CGGATGATGACCGCCATCTCGCCGCCCTAGCAAGCAA | Construction of WYY |
| 9 P101L-s                 | 24     | TTTGATCTTATGCTTATTTTACTA | Construction of P101L |
| 10 P101L-as               | 24     | GCGTAGAATTAATCAGGAAAGCCATG | Construction of P101L |
| 11 HA-LD-s               | 24     | TGGCCGCTTATCTGACCCGGATCTGCGTCT | Construction of HA-LD |
| 12 HA-LD-as              | 24     | CAGATCGCGTCAAGATAGCCGCA | Construction of HA-LD |
| 13 RREH-A-s               | 74     | CTGCTATGACCCGCTACCGATACCCACAGCAGGCCCAAGGGGCGCTGGAACAGCAACTGTCGACAGTACCCGTCGATCG | Construction of RREH-A |
| 14 RRHA-A-as             | 74     | GCGGCGCGCGCGAGCTACTGCTGCAAGTGCTTCCAGCGCCCTGCTGGTATCGCGTCGATACG | Construction of RREH-A |
| 15 '5'-2                 | 24     | GGCAGCCATATCGCCGCTGGCCAC | N-terminal polypeptide (1–193 and 1–181) |
| 16 '3'-1                 | 33     | GACAGCCGATCTTAATATGCGGTGTGCCAATTTTATTTGCTCTTCGAGTGGGTTGACCAAAGGACAGC | N-terminal polypeptide (1–193) |
| 17 '3'-2                 | 33     | GACAGCCGATCTTAATATGCGGTGTGCCAATTTTATTTGCTCTTCGAGTGGGTTGACCAAAGGACAGC | N-terminal polypeptide (1–181) |
| 18 n’-pas                | 66     | AGTTATTAAACCGCGAGGCGGToluteTTAAAATTTTCTGCTGAGTGGGTTGACCAAAGGACAGC | n’-pas oligonucleotide |
| 19 PriA-N-SalI           | 35     | CGGGAATGCTGCGCATATCGCCGGTCCCAGTTGCGC | N-terminal polypeptide for antibody |
| 20 PriA-N2-antisense (BamHI-EcoRI) | 35     | CGGGAATGCTGCGCATATCGCCGGTCCCAGTTGCGC | N-terminal polypeptide for antibody |
Measurement of radioactivity on the filters was counted using a liquid scintillation counter and was corrected by the cell densities at the time of addition of \( {}^{3}H \) thymine.

Measurement of UV Sensitivity—E. coli cells were serially diluted and plated onto M9 plates. Plates were exposed to UV light at the doses indicated in the figure. After incubation at 37°C for 48 h, numbers of growing colonies on each plate were counted.

Transformation Assay of Plasmid DNA—One-hundred ng of each plasmid DNA was transformed by electroporation into AQ634 or AQ8851 electrocompetent cells, which were plated onto M9-ampicillin plates. The numbers of growing colonies were counted after incubation at 37°C for 48 h.

RESULTS

Limited Tryptic Digestion of PriA Protein—PriA protein was originally identified as a protein essential for conversion of \( \phi X 174 \) single stranded to replicative form \( \textit{in vitro} \) and was shown to bind specifically to n-pas, a stem-loop structure (33). It was later shown that PriA protein binds to structured DNAs including a D-loop-like structure (15). PriA binds efficiently to a D-loop mimicking a forked structure with an arrested nascent DNA (Fig. 1A, lanes 1–6), but binds much less efficiently to a bubble containing a fork without a hybridizing strand, as was previously shown (Fig. 1A, lanes 7–12) (22). PriA generated specific protein-DNA complexes on the D-loop in gel shift assays, whereas it generated smeared bands on bubble only at higher concentrations of PriA. The affinity of PriA to D-loop was nearly 1 order of magnitude higher than that to bubble, as estimated from the concentration of PriA protein required for half-maximum binding to each substrate (\( \sim \)10 and 90 nM, respectively; Fig. 1B).

To examine the presence of a domain(s) of PriA capable of binding to DNA, we conducted a limited tryptic digestion of the full-length PriA protein. The tryptic digests of PriA protein were separated by size fractionation on Superdex 200 and each fraction was analyzed by gel shift assays to detect polypeptides capable of binding to D-loop. D-loop binding was detected in fractions 16–19 at a molecular weight of \( \sim \)100,000 (Fig. 2). Examination of the polypeptides in each fraction by SDS-PAGE revealed comigration of two closely migrating polypeptides of about 12 kDa in size with the binding activity. Western blotting with the antibody against the N-terminal 207-amino acid polypeptide of the PriA protein showed that these fragments are strongly reactive with this antibody, indicating that they contain an N-terminal region of PriA protein. This result suggests a possibility that the N-terminal region contains a D-loop-binding domain.

N-terminal 181 Amino Acids Can Bind to D-loop on Its Own—We then constructed deletion derivatives of PriA containing N-terminal polypeptides (Fig. 3A), and examined their ability to bind to D-loop-like structures \( \textit{in vitro} \). On comparison of various PriA-related polypeptides, we have noted that the helicase domain starts from the residue 194. On the other hand, the prediction of secondary structure suggested the absence of helix or sheet structures after residue 182. Therefore, we have prepared two N-terminal polypeptides of PriA, one containing residues 1–181 and the other 1–193. Both 181- and 193-amino acid polypeptides were capable of binding to the D-loop (Fig. 3B, lanes 7–26). However, the affinity to D-loop of the N-terminal polypeptides was more than 20-fold reduced compared with the full-length PriA protein (concentration required for half-maximum binding of the 1–193 and full-length being \( \sim \)200 and 8 nM, respectively; Fig. 3C). Furthermore, both 181- and 193-amino acid polypeptides bound to a bubble structure with affinity only slightly lower than that to D-loop (Fig. 3C), in contrast to the full-length protein that exhibited high affinity binding specifically to D-loop DNA, suggesting that the C-terminal segment may contribute to the affinity and specificity of PriA binding to DNA. These results show the presence of a DNA-binding domain in the N-terminal 181 amino acids, consistent with the results of tryptic digestion.

Identification of a Novel Motif in PriA-related Proteins Conserved in Eubacteria—All the members of PriA-like proteins from various eubacteria contain N-terminal segments of \( \sim \)200 amino acids long outside of the helicase-conserved motifs, and alignment of these amino acid sequences revealed the presence of conserved amino acid residues (Fig. 4A). This motif appears to be unique to the PriA protein family. We have introduced amino acid substitutions at the conserved residues of this motif to assess whether it contributes to DNA binding activity of PriA protein.

To facilitate purification of recombinant proteins, we have added a histidine tag at the N terminus of the N-terminal 193-amino acid polypeptide. The histidine-tagged protein...
bound to D-loop with efficiency similar to that of the nontagged polypeptide (Fig. 4B, lanes 1–14). A WYY mutant, in which the three conserved residues (Trp-83, Tyr-87, and Tyr-88) were replaced with glycine, bound to D-loop with reduced affinity. The affinity of the P101L mutant to D-loop was also reduced. The concentrations required for half-maximum binding of the wild-type, WYY, and P101L His-PriA-(1–193) polypeptides were ~100, 200, and 300 nM (Fig. 4C). More notably, formation of distinct mobility shifted complexes on the gel at lower protein concentrations was significantly disrupted with the mutant proteins, and the complexes appeared as a smear, migrating faster than those generated with the wild type at the same concentrations (compare lanes 15, 16, and 22; 23 with 8, 9 in Fig. 4B), suggesting the instability of the mutant-D-loop complexes in the polyacrylamide gel. The result of the filter binding assays are also consistent with reduced affinity of the mutant polypeptides to DNA (Fig. 4D).

Motifs in DNA Helicase Domain Essential for Binding to D-loop Structures—Although the isolated N-terminal segment of PriA can bind to D-loop-like structures, its binding efficiency is lower than the full-length PriA by more than 1 order of magnitude, and the binding specificity also compromises in that it binds to both D-loop and bubble with similar affinity. Therefore, we examined mutations in the C-terminal region including DNA helicase domains for their effect on DNA binding. We have already reported that mutations in the Walker A motif, essential for ATPase and DNA helicase activities, and those in zinc finger motifs do not significantly affect the DNA binding activity of PriA (27).

We have identified lysyl-tRNA synthetase (34) as a D-loop-binding protein from extracts of fission yeast (data not shown). On comparison of its primary structure with that of PriA, we identified a small stretch of a segment on PriA that shares significant similarity with that on the lysyl-tRNA synthetase (24% identity and 40% similarity on a 38-amino acid segment; Fig. 5A). We have mutated the conserved Arg-512, Arg-517, Glu-527, and His-529 residues with alanine on a full-length PriA protein (see Fig. 7A). The resulting RREH-A mutant did not exhibit significant binding to D-loop in gel shift assays (Fig. 3).
5B, lanes 17–21), suggesting that this domain is critical for DNA binding of PriA protein.

During the course of construction of various mutant proteins, we have accidentally generated a mutant PriA protein that failed to bind to D-loop. Examination of nucleotide sequences of this mutant revealed amino acid substitutions at His-336 and Asp-337 with leucine and aspartic acid, respectively. The mutant (histidine-tagged) PriA N-terminal polypeptides (WYY, lanes 14–21; P101L, lanes 22–25), as shown in the figure, were purified (see Fig. 3A) and used in gel shift assays with D-loop as a substrate. Lanes 2–7, 8–13, 15–20, and 22–27 contain each protein at 80, 160, 320, 800, 1600, and 4000 nM, respectively. Lanes 14, 21, and 28 contains 8 μM of each protein. Lane 1, no protein added. C, the results in B were quantified as described under “Experimental Procedures” and the values were plotted against the concentrations of the added polypeptides. D, filter binding assays were conducted with the D-loop substrate in the presence of 160 nM of each polypeptide indicated. Fractions of bound proteins are shown. The values are average of three independent experiments and error bars are indicated.

The amino acid substitutions of RREH-A and HA-LD do not appear to cause drastic changes in overall structures of PriA protein, because partial tryptic digestion of the mutant proteins yielded tryptic polypeptide patterns almost identical to that of the wild-type protein (data not shown). Furthermore, the RREH-A and HA-LD mutant proteins retained ATPase activity 40–50% of the wild-type in the presence of n’-pas (Fig. 5D), indicating that the proteins still maintain the functional conformation.

Addition of excess n’-pas DNA but not D-loop DNA as an effector stimulated ATPase activity of the HA-LD mutant (Fig. 5E). Consistent with this, gel shift assays indicate that HA-LD and RREH-A can bind to n’-pas DNA and forms a shifted band, albeit with reduced affinity compared with the wild-type (Fig. 5F, lanes 15, 16, 20, and 21).

DNA Binding Activity of PriA Is Essential for Recombination-dependent Replication of the E. coli Chromosome—A unique feature of the E. coli chromosomal replication is its ability to replicate its genome in a manner dependent on recombination functions but independent of DnaA under certain conditions (13). Persistent DNA replication can be induced in the presence of a protein synthesis inhibitor by, for example, preincubating the cells in the absence of precursors for DNA replication (e.g. thymine). This iSDR depends on RecA, RecB, and RecC functions and on PriA protein (3, 9, 10). We previously reported that zinc finger motifs are essential for iSDR, and ATP hydrolysis...
activity of PriA is also required for full level activity of iSDR (27). We have examined whether DNA-binding mutants of PriA can support iSDR. We have transferred the entire coding region of the wild-type and mutant PriA proteins onto pHM6050, a mini-R1 plasmid derivative whose replication does not require PriA (30). The levels of ectopically expressed PriA proteins were examined by immunoblotting of the whole cell extracts and were shown to be constant among different mutants except for RREH-A. They were about 20 times higher than that of the endogenous protein (Fig. 6A). The level of RREH-A was markedly lower than others, almost the same as the endogenous protein level. The levels of iSDR with P101L and RREH-A mutants were almost the background level observed in priA null cells in the absence of any plasmid, whereas iSDR in the HA-LD mutant was partially compromised. WYY mutant exhibited the wild-type level of iSDR (Fig. 6B). These results are consistent with the conclusion that the DNA binding activity of PriA is essential for its ability to support recombination-dependent DNA replication.

We also examined the morphology of priA::kan null cells harboring various plasmids expressing mutant PriA proteins (Fig. 6C). It is known that 10–20% of the population of priA null cells exhibit extremely elongated morphology and SOS responses are induced in these elongated cells (8). Filamentous morphology of the priA null cells was corrected by wild-type and WYY mutant PriA, but not by P101L or RREH-A mutants. The priA null cells expressing the HA-LD mutant were still elongated, but its extent was partially rescued. Similarly, slow growth and UV-sensitive phenotypes of priA null cells were not corrected completely by P101L, but were corrected to the wild-type level by other mutants (Fig. 6D and data not shown). PriA protein is essential for replication of ColE1-type plasmids, because the lagging strand syntheses of these plasmids depend on PriA-mediated primosome assembly (35–37). Ability of the priA mutant to support replication of ColE1-type plasmids was also examined by transforming the mutant cells with ColE1-based plasmids expressing the wild-type or mutant PriA proteins (Table II). In this assay, only P101L could not rescue the maintenance of the plasmid, whereas others could support the maintenance, although HA-LD exhibited 8–10 times lower transformation efficiency compared with the wild-type. These results indicate that at least the extensive DNA replication dependent on recombination functions requires DNA binding activity of PriA, and that other in vivo functions of PriA also depends on DNA binding, but less tightly.

**DISCUSSION**

PriA protein was originally discovered as a factor essential for *in vitro* conversion of single stranded to the replicative form of dX174 DNA (1, 2). The PriA-dependent primosome is assem-
bled at n-pas within the origin of the viral DNA as well as on various plasmid genomes (21). n-pas is a small stem-loop structure and is specifically recognized by PriA. However, it has not been identified on the E. coli genome (21). Characterization of a priA null mutant demonstrated its essential function in an oriC- and DnaA-independent, RecA-dependent mode of E. coli chromosomal replication (3). This suggested that the physiological target of the PriA protein may be a structure resembling recombination intermediates (4, 7, 38). Later, it was demonstrated that PriA binds specifically to a model D-loop structure, showing that PriA is associated with a structure-specific DNA binding property (15).

In this study, we have dissected the domains on E. coli PriA protein required for structure-specific DNA binding activity. The results indicate that specific and efficient recognition of D-loop-like DNA requires proper configuration of the N-terminal DNA-binding domain and the C-terminal DNA helicase domain.

The N-terminal Domain of PriA Is Sufficient for Binding to D-loop—Partial tryptic digestion of the PriA protein revealed that an N-terminal segment of PriA is capable of binding to D-loop in gel shift assays. We then purified the N-terminal 181-amino acid polypeptide and showed that it could bind to D-loop and could generate distinct protein-DNA complexes in gel shift assays. These results demonstrate the presence of a DNA-binding domain in the N-terminal region of PriA. We further showed that amino acid substitution of residues conserved in the PriA-related proteins in the N-terminal domain (WYY and P101L) decreased the DNA binding activity of an N-terminal fragment. These results established that the N-terminal region plays a critical role in DNA binding of PriA protein.

Mutations in the C-terminal DNA Helicase Domain Abrogate DNA Binding Activity—We have identified two mutations in the DNA helicase domain that affected DNA binding activity of...
PriA protein. These mutants failed to form specific complexes with D-loop in gel shift assays despite the presence of the N-terminal DNA-binding domain. This is unexpected because the isolated N-terminal fragment can bind to D-loop on its own. The result indicates that the mutations in the helicase domain can dominantly inhibit DNA binding activity of the N-terminal segment.

RecG is another DEXH-type helicase conserved widely in eubacteria. It was proposed that PriA and RecG may collabo-rate in processing of the stalled replication forks (39). Mutational studies of RecG indicated the presence of a DNA-binding domain in its N-terminal segment (40). The x-ray crystallographic analyses of the RecG-DNA complex demonstrated recogni-tion of the arrested fork by the N-terminal segment (41). The C-terminal helicase domain was proposed to make contact with the duplex portion of the fork, poised to unwind the arrested lagging strand to generate a “chicken foot” structure. We predict that the N-terminal domain of PriA may make similar contact with the arrested replication fork-like structure. We speculate that this interaction is stabilized by the presence of the C-terminal helicase domain interacting with the duplex region adjacent to the branched point. The positions of the mutations projected onto the RecG-DNA structure are consistent with the notion that these residues are in contact with the duplex portions of the fork structure (data not shown).

The presence of the helicase domain may also make the recognition more specific, because the isolated N-terminal domain did not exhibit significant preference for D-loop over the bubble-like structure. The specific mutations we have identified in the C-terminal helicase domain may disrupt proper interaction of PriA with the duplex region, resulting in disori-entation of the helicase domain in relation to the template DNA, which ultimately inhibits the interaction of the N-termi-nal domain with the fork structure.

The importance of the relative configuration between the N- and C-terminal domains of PriA in binding to the D-loop/forked structure was also indicated by the binding properties of E. coli/Bacillus subtilis PriA chimera proteins. B. subtilis PriA also binds to D-loop/forked DNA and carries a conserved motif in the N-terminal region, suggesting that its N terminus region contains the DNA-binding domain (27, 42). The chimera pro-teins in which the N- and C-terminal domains were swapped between E. coli and B. subtilis were purified. They were signi-ficantly impaired in DNA-binding assays despite the presence of an N-terminal DNA-binding domain. This may be because of the misorientation of the helicase domain relative to the N-terminal-binding domain.

Nurse et al. (22) reported two modes of high affinity DNA binding of PriA protein: one recognizing a duplex DNA with a 3′-single-stranded extension and the other recognizing a three-strand junction with a 5′-tail. They proposed that the former binding may represent the 3′ → 5′ DNA helicase activity of PriA, whereas the latter is accounted for by specific recognition of a sharp bend generated at the junction. In light of the findings in this report, the former may involve only the C-terminal helicase domain bearing 3′ → 5′ DNA helicase activity, whereas the latter may require cooperation of both N-terminal DNA-binding and C-terminal helicase domains. It remains to be seen whether the N-terminal domain recognizes the bent DNA, as was proposed (22).

**Importance of DNA Binding Activity of PriA in Its Biological Functions**—Our results indicate that PriA contains three functionally significant domains: the N-terminal DNA-binding domain, the DNA helicase domain, and zinc finger motif embed-ded in the helicase motifs (Fig. 7A). Previous results indicate the significance of the zinc finger motif in PriA functions, because the mutants are defective in DNA replication in vitro (26) as well as in vivo recombination-dependent replication, and showed slow growth and UV-sensitive phenotypes (27). It was suggested that this motif is involved in interaction with other replication proteins essential for primosome assembly (28). On the other hand, ATPase/DNA helicase-deficient mu-tants of PriA are generally proficient in assembly of a primosome in vitro and also in supporting fast growth and UV re-sistance, although recombination-dependent DNA replication was partially impaired with ATPase-deficient mutants of PriA (27, 43). DNA binding activity was unaffected in ATPase or zinc finger mutants. In this report, we have identified a DNA-binding domain on PriA. All the DNA-binding mutants failed to support 5′SDR, indicating that the ability of PriA to recognize structured DNA is essential for persistent DNA replication dependent on recombination functions. They showed a reduced ability to complement other defects of priA null cells to varied extents, including slow growth, UV sensitivity, and filamentous morphology. Although the P101L mutant was defective in all the functions of PriA assayed and the RREH-A mutant could not rescue the abnormal morphology, the HA-LD mutant could restore both normal morphology and UV resistance.

Thus, the ability of DNA binding may be differentially required for various functions of PriA in the cells.

The HA-LD and RREH-A mutants could support replication of ColE1 plasmids, despite its inability to bind to D-loop. This could be because of their ability to bind to n’-pas, albeit with reduced efficiency. The weaker binding of HA-LD to n’-pas is
reflected in its reduced ability of supporting ColE1 plasmid replication. The differential effect of these mutations on the ability of PriA to bind to D-loop and n’-pas suggests that structural requirement for binding to these DNAs may not be identical.

Although both PriA and RecG recognize structured DNAs including those resembling arrested replication forks, no similarity can be found between the primary structures of the N-terminal DNA-binding domains of the two proteins. The phenotypes of null mutants of priA and recG are quite distinct; recG mutants exhibit a slight decrease in homologous recombination, but show growth rate similar to the wild-type (44, 45), whereas priA mutants are extremely low in viability, and show synthetic lethality with a number of genes including polA (5) and rep (46). More detailed structural analyses will resolve how the PriA protein specifically recognizes these structures and help understand its precise roles in nucleic acid metabolisms in bacteria.

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DNA Binding of PriA Protein Requires Cooperation of the N-terminal D-loop/Arrested-fork Binding and C-terminal Helicase Domains
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