Mutational Analysis of Conserved Hydrophobic Amino Acid Residues in the N-Terminal Region of DnaA Protein

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DnaA is the initiator of chromosomal DNA replication in E. coli. We previously reported that conserved hydrophobic amino acid residues in the N-terminal region of DnaA (I26 and L40) are essential for DNA replication in vivo and in vitro using mutant DnaA proteins (DnaAI26S and DnaAL40S). In this study, we introduced further random mutations to find intragenic suppressors for dnaAI26S or dnaAL40S. By direct DNA sequence, a mutation which causes substitution of the Ser (Ile, in the wild-type DnaA) with Phe (DnaAI26F or DnaAL40F) was found in all of the suppressors. Site-directed mutational analysis showed that DnaAI26L and DnaAL40L, but not DnaAI26S or DnaAL40S, were active for oriC DNA replication in cells. Furthermore, purified DnaAI26S but not DnaAL40S was active for oriC DNA replication in a crude extract. These results strongly suggest that hydrophobic amino acid residues in these positions of DnaA (I26 and L40) are important for the function of this protein as an initiator of DNA replication both in vivo and in vitro.

Key words DnaA; N-terminal region; DNA replication; hydrophobic amino acid residue

Materials and Methods

Materials [α-32P]dCTP (6000 Ci/mmol) was from Amer sham Pharmacia Biotech. Wild-type DnaA and DnaAI26F were purified as previously described.17) DnaAI26S was purified as previously described.15) A crude extract for an oriC complementation assay was prepared from the WM433 strain of E. coli, as previously described.18)

Bacterial Strains Five strains of E. coli from our laboratory stocks were used in this study. They were JM109 (recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB) F′[traD36, proAB, lacI, lacZΔM15],) KS1001 (W3110 lacZ), KS1003 (KS1001, dnaA46),19) KA450 (ΔoriC1071::Tn10, rnhA199(Am), dnaA17(Am), trpE9828(Am), tyrA (Am), thr, ilv, and thyA),20) and WM433 (dnaA204, leu19, pro19, trp25, his47, thyA59, arg28, met55, deoB23, lac11, strA56, sul1, hsdSK12).

Site-Directed Mutagenesis and Plasmid Constructions

Site-specific mutagenesis was performed as described previously.4) Briefly, uracil-containing single-stranded DNA of the M13 phage, containing the coding region of the dnaA gene, was hybridized with oligonucleotide primers representing each mutation. The complementary DNA strand was synthesized in vitro and the resultant double-stranded DNA was introduced into E. coli JM109. Direct DNA sequencing confirmed the mutation, and double-stranded DNA containing the mutation was prepared.

To construct plasmids for the over-expression of DnaA, the EcoRI-HindIII region of each dnaA gene was ligated into pMZ001,28 which contains an arabinose promoter.

Plasmids for complementation analysis were constructed by ligating the coding regions of the mutant dnaA gene (BamHI-HindIII fragment) into pMZ002,4 which contains the wild-type promoter of the dnaA gene.

Screening for Intragenic Suppressors for dnaAI26S or dnaAL40S

The mutant dnaAI26S or dnaAL40S was introduced into E. coli JM109. Direct DNA sequencing confirmed the mutation, and double-stranded DNA containing the mutation was prepared.

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Screening for Intragenic Suppressors for dnaAI26S or dnaAL40S

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**DNA Replication in a Crude Extract**

Replication of minichromosomes in a crude extract (Fraction II) was assayed, as previously described. Template DNA (M13E10) (200 ng, 600 pmol of nucleotides), 200 µg of Fraction II from WM433 (dnaA204), and DnaA were mixed with reaction cocktails and incubated for replication at 30 °C for 20 min. The reaction was terminated by chilling on ice and adding 10% TCA. Samples were then passed through Whatman GF/C glass-fiber filters. The amount of radioactivity on the filter was measured in a liquid scintillation counter and the amount of DNA synthesized (pmol nucleotides) was calculated, as previously described.

**RESULTS AND DISCUSSION**

DnaAI26S and DnaAL40S were inactive for the oriC DNA replication in vivo and in vitro. Therefore, neither dnaA126S nor dnaA40S could complement the temperature-sensitivity of a dnaA46 mutant; in other words, dnaA46 cells with plasmid containing dnaA126S or dnaA40S still showed a temperature-sensitive growth phenotype. In order to obtain intragenic suppressors for the dnaA126S or dnaA40S, we performed two-step experiments. At first, plasmids containing dnaA126S or dnaA40S were randomly mutagenised by MNNG in JM109 cells, then introduced into dnaA46 to select temperature-resistant colonies. Direct DNA sequence analysis of the dnaA gene obtained from colonies showed that all of the suppressors contained further substitution of the Ser (originally I26 or L40 in the wild-type DnaA) with Phe (Table 1).

Results in Table 1 suggest the importance of hydrophobic amino acid residues in these positions (I26 or L40 in the wild-type DnaA). This idea is supported by the fact that these amino acid residues are well conserved among various species of DnaA as hydrophobic amino acid residues (Fig. 1). For further confirmation of this idea, we constructed several mutant dnaA genes using site-directed mutation (Fig. 1). To determine whether these mutant dnaA genes could function in vivo, we conducted plasmid complementation experiments using a dnaA46 mutant. The coding region of the mutant or the wild-type dnaA genes was conjugated with a wild-type dnaA promoter on pMZ002. Each resultant plasmid was introduced into a temperature-sensitive dnaA46 strain. The ratio of transformation efficiency of colonies was assayed, as previously described. Template DNA (M13E10) (200 ng, 600 pmol of nucleotides), 200 µg of Fraction II from WM433 (dnaA204), and DnaA were mixed with reaction cocktails and incubated for replication at 30 °C for 20 min. The reaction was terminated by chilling on ice and adding 10% TCA. Samples were then passed through Whatman GF/C glass-fiber filters. The amount of radioactivity on the filter was measured in a liquid scintillation counter and the amount of DNA synthesized (pmol nucleotides) was calculated, as previously described.

**Table 1. Suppressor Mutation in the dnaA Gene from dnaA126S or dnaA40S Mutant**

<table>
<thead>
<tr>
<th>Mutant genes</th>
<th>DNA mutation</th>
<th>Amino acid mutation</th>
<th>Number of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnaA126S</td>
<td>C77T</td>
<td>S26F</td>
<td>30</td>
</tr>
<tr>
<td>dnaA40S</td>
<td>C119T</td>
<td>S26F</td>
<td>34</td>
</tr>
</tbody>
</table>

JM109 cells harboring either dnaA126S or dnaA40S were treated with MNNG. Plasmids were isolated and transformed into a temperature-sensitive dnaA46 strain. Plasmids were isolated from more than 30 temperature-resistant colonies, and DNA sequences of the dnaA gene were determined.

**Fig. 1. Amino Acid Sequence and Strategy for Site-Directed Mutations in the N-Terminal Region of DnaA**

Amino acid sequences of the N-terminal regions of DnaA (from W25 to W50) are shown. The “*” indicates an amino acid residue that is identical to that found in E. coli. These data are from Ref. 1. Eco, E. coli; sty, S. typhimurium; sma, S. marcescens; pmi, P. mirabilis; bap, B. aphidicola; ppu, P. putida; eco, S. coelicor; mlu, M. luteus; bba, B. burgdorferi. The conversion of amino acid residues in the mutant DnaA proteins is shown. We introduced indicated amino acid substitutions in the position of I26 or L40 in the wild-type DnaA.

**Table 2. Complementation Analysis of Temperature-Sensitivity of a dnaA46 Mutant with Plasmids Carrying the Mutant dnaA Genes**

<table>
<thead>
<tr>
<th>Clones</th>
<th>Colony formation efficiencies (42°C/30°C)</th>
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</thead>
<tbody>
<tr>
<td>pBR322(vector)</td>
<td>1.6×10^-8</td>
</tr>
<tr>
<td>pMZ002/dnaA</td>
<td>1.3</td>
</tr>
<tr>
<td>pMZ002/dnaA126S</td>
<td>1.1×10^-3</td>
</tr>
<tr>
<td>pMZ002/dnaA126L</td>
<td>1.3</td>
</tr>
<tr>
<td>pMZ002/dnaA40F</td>
<td>1.0</td>
</tr>
<tr>
<td>pMZ002/dnaA40S</td>
<td>8.4×10^-6</td>
</tr>
<tr>
<td>pMZ002/dnaA40L</td>
<td>1.2</td>
</tr>
<tr>
<td>pMZ002/dnaA40I</td>
<td>1.1</td>
</tr>
</tbody>
</table>

KS1003 (dnaA46) cells were transformed with pMZ002 with each dnaA gene. Cultures were diluted appropriately and spread on LB agar plates containing 100 µg/ml ampicillin. Plates were incubated at 30°C or 42°C, the numbers of colonies were counted, and the ratio of transformation efficiencies (42°C/30°C) was determined.

For biochemical analysis, we purified DnaAI26S, DnaAI26F, and wild-type DnaA from over-producing cells and examined their activities for oriC DNA replication in vivo.
vitro using an oriC complementation assay. We also tried to purify DnaAL40F, but due to its poor solubility, we could not obtain purified DnaAL40F. As shown in Fig. 2, DnaAI26F was active for oriC DNA replication as much as the wild-type protein in a crude extract. DnaAI26S was inactive for the DNA replication, as described previously.

Based on all these observations, we concluded that DnaAI26F is active for the oriC DNA replication both in vivo and in vitro.

Using random mutagenesis and site-directed mutagenesis, we here showed the importance of hydrophobic amino acid residues in the N-terminal region of DnaA (I26 and L40) for the oriC DNA replication in vivo and in vitro. These amino acid residues may form a leucine-zipper structure in which hydrophobic amino acid residues are important. Previously, we showed that these amino acid residues are essential for DnaA–DnaA interaction, which is an essential step for oriC DNA replication. Based on all these observations, we propose that the N-terminal region of DnaA is involved in DnaA–DnaA interaction using a leucine-zipper structure.

REFERENCES