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ORIGINAL PAPER

Localized melting of duplex DNA by Cdc6/Orc1 at the DNA replication origin in the hyperthermophilic archaeon *Pyrococcus furiosus*

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Abstract The initiation step is a key process to regulate the frequency of DNA replication. Although recent studies in Archaea defined the origin of DNA replication (*oriC*) and the Cdc6/Orc1 homolog as an origin recognition protein, the location and mechanism of duplex opening have remained unclear. We have found that Cdc6/Orc1 binds to *oriC* and unwinds duplex DNA in the hyperthermophilic archaeon *Pyrococcus furiosus*, by means of a P1 endonuclease assay. A primer extension analysis further revealed that this localized unwinding occurs in the *oriC* region at a specific site, which is 12-bp long and rich in adenine and thymine. This site is different from the predicted duplex unwinding element (DUE) that we reported previously. We also discovered that Cdc6/Orc1 induces topological changes in supercoiled *oriC* DNA, and that this process is dependent on the AAA+ domain. These results indicate that topological alterations of *oriC* DNA by Cdc6/Orc1 introduce a single-stranded region at the 12-mer site, that could possibly serve as an entry point for Mcm helicase.

Keywords *Pyrococcus furiosus* · DNA replication · Initiation protein · *oriC* · Unwinding

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Introduction

Chromosomal DNA must be duplicated during cell proliferation. To maintain a constant level of genetic material, DNA replication involves a series of highly regulated processes (Bell and Dutta 2002) and initiation of DNA replication is one among them. Before DNA synthesis starts, the origin of DNA replication is recognized by a specific factor(s). The origin DNA must be unwound before the replication machinery is assembled, because DNA synthesis occurs on single-stranded DNA.

In *Escherichia coli*, multiple copies of the DnaA protein bind to a repeated sequence (DnaA box) in the origin of DNA replication (*oriC*). The DnaA binding induces a topological change in the *oriC* region, and duplex unwinding occurs at a region, which is rich in adenine and thymine (Bramhill and Kornberg 1988). This process is dependent on the ATP bound form of DnaA. In the following step, DnaB, the replicative DNA helicase, is loaded onto the unwound site to extend the single-stranded region. Then, DNA primase and DNA polymerase initiate DNA synthesis on the single-stranded DNA (Baker et al. 1987).

In *Saccharomyces cerevisiae*, the origin recognition complex (ORC), composed of six subunits (Orc1–6), binds

the origin of DNA replication (Bell and Stillman 1992), and this protein complex is conserved in Eukaryota. A genomic footprinting analysis detected DNA unwinding in the autonomously replicating sequences (ARS) plasmid in *S. cerevisiae* cells (Geraghty et al. 2000). In *Schizosaccharomyces pombe*, the binding of ORC to the replication origin altered the topological structure of the DNA, although the formation of single-stranded DNA was not detected in this study (Gaczynska et al. 2004). It is unknown how ORC binds to the specific site on the chromosomal DNA in human cells, although the assembly of the human Orc1 to 6 proteins, to form ORC in vitro, is dependent on ATP binding (Siddiqui and Stillman 2007). HMGA1a, a member of the high-mobility group protein family, reportedly targets ORC to a specific site on DNA, and thus replication origins in metazoan cells might be specified by targeting ORC to DNA with chromatin proteins (Thomae et al. 2008). However, the molecular mechanism of DNA unwinding at the replication origin remains quite elusive in Eukaryota, although vigorous research has provided continuous progress in this field, as described above.

We found that *Pyrococcus furiosus,* a hyperthermophilic archaeon, has a protein with a sequence similar to those of eukaryotic Orc1 and Cdc6, and this represents the first such protein identified in Archaea, the third domain of life (Uemori et al. 1997). We designated this protein as Cdc6/Orc1, since we found that *P. furiosus* has no other gene encoding a similar sequence in the total genome database (Matsunaga et al. 2001). The origin of DNA replication (*oriC*) is located in the intergenic region upstream of the *cdc6/orc1* gene in the *Pyrococcus* genome (Myllykallio et al. 2000; Matsunaga et al. 2001), and we confirmed that Orc1/Cdc6 binds to the *oriC* region in *Pyrococcus* cells (Matsunaga et al. 2001). An analysis of archaeal genome sequences revealed conserved 13-bp repeats, which are characteristic of the archaeal *oriC*, as predicted by bioinformatics (Lopez et al. 1999). A subsequent study showed that two of the repeats are longer, and they surround a putative Duplex unwinding element (DUE) with an AT-rich sequence in *Pyrococcus* genomes (Matsunaga et al. 2003). Since all of the sequenced archaeal genomes have been found to contain genes encoding one or more Cdc6/Orc1-like protein(s) (Barry and Bell 2006), although *Methanococcus* and *Methanopyrus* have only distantly related homologs (Slesarev et al. 2002; Zhang and Zhang 2004, 2005). Furthermore, in several archaeal organisms, the homologs of eukaryotic ORC (Orc1) and Cdc6 were shown to recognize the origin of DNA replication (*Methanothermobacter thermoautotrophicus*; Capaldi and Berger 2004; *Aeropyrum pernix*; Grainge et al. 2006; *Sulfolobus solfataricus*; Robinson et al. 2004). The longer repeated sequence found in the *oriC* region, as described above, was designated as an origin recognition box (ORB), which is recognized by Cdc6/Orc1, by Bell et al. from the *S. solfataricus* study (Robinson et al. 2004). We previously reported that the Cdc6/Orc1 protein binds specifically to the ORB and miniORB (minimal version of ORB) repeats in the *oriC* region of *P. furiosus* (Matsunaga et al. 2007). In addition, our whole genome microarray analysis revealed that Cdc6/Orc1 binding is extremely specific to the *oriC* region in the cells (Matsunaga et al. 2007). Recent data showed that one of the two homologs of Cdc6/Orc1 in *Aeropyrum pernix*, called ORC1 (the authors referred to the two homologs of the initiation protein in this organism as ORC1 and ORC2), binds to each ORB as a dimer, and a transition to a higher order assembly, with alterations in both topology and superhelicity, occurs when all four ORBs in the *oriC* region are bound with the ORC1 protein (Grainge et al. 2006). Since the digestion by the single-stranded DNA-specific P1 endonuclease was limited to distinct sites, which displayed periodicity, the authors assumed that the wrapping of the DNA around ORC1 created distortions in the outer surface of the DNA duplex (Grainge et al. 2006). Two recent reports have described the crystal structures of *S. solfataricus* Cdc6-1 and Cdc6-3 forming a heterodimer bound to *ori2* DNA (one of the three origins in this organism) (Dueber et al. 2007), and that of *A. pernix* ORC1 bound to an origin sequence (Gaudier et al. 2007). These studies revealed that both the N-terminal AAA+ ATPase domain and C-terminal winged-helix (WH) domain contribute to origin DNA binding, and the structural information not only defined the polarity of initiator assembly on the origin but also indicated the induction of substantial distortion into the DNA strands. This distortion probably triggers the unwinding of the duplex DNA to start replication. In addition, these structural data provided the detailed interaction mode between the initiator protein and *oriC* DNA. The essential interaction between an arginine residue conserved in the archaeal Cdc6/Orc1 and an invariant guanine in the ORB sequence was also demonstrated, by mutation analyses of the *M. thermautotrophicus* protein (*Mth*Cdc6-1) (Majernik and Chong 2008). Despite the progress in the analyses of the initiation of DNA replication, the exact mechanism of duplex DNA unwinding at the specific site remains to be clarified. In addition, it is still unclear how the origin is unwounded with a width sufficient to load Mcm helicase, DNA primase, and DNA polymerase.

The *oriC* in *Pyrococcus* sp. consists of two ORB repeats (ORB1 and ORB2), several miniORB repeats, and adenine or thymine rich regions (A/T-rich region), predicted as a DUE, as described above (Matsunaga et al. 2003). There is another cluster of ORB (ORB3) and miniORB repeats approximately 1.5 kb away from *oriC,* and we demonstrated that Cdc6/Orc1 also specifically binds to this region, as well as to *oriC* (Matsunaga et al. 2007). However, it was still unclear how the binding of the Cdc6/Orc1 protein to

these repeated sequences results in the activation of *oriC*. In this study, we investigated the consequences of Cdc6/Orc1 protein binding to the *oriC* plasmid (the *oriC* region of *P. furiosus* genome within the pUC-derived plasmid), and demonstrated that Cdc6/Orc1 introduces localized melting of the DNA duplex in the *oriC* region, in the absence of other replication proteins, in vitro. Furthermore, we discovered the location where Cdc6/Orc1 unwinds the DNA in the *oriC* region.

Materials and methods

Plasmids

The DNA fragment containing the *oriC* region (1 kb) was amplified from the genomic DNA of *P. furiosus* by PCR, using the primers 5′-dGTGGAGACAAAATGAACATT TATGGTCATTAAAATTTG-3′ and 5′-dTAACTTTGT AGCTGCAAACCACCTGGGATG-3′. For the DNA fragment containing *oriC*, *cdc6/orc1*, and ORB3 (3 kb), the following two primers were used for PCR amplification: 5′-dTAACTTTGTAGCTGCAAACCACCTGGGATG-3′ and 5′-dGGGGTTCATGGGTATTGTCA-3′. The amplified DNA fragments were cloned in the pUC-based plasmid vector, pGEM T-easy (Promega, Madison, WI), to produce the *oriC* plasmid and the *oriC*-ORB3 plasmid, respectively. For both constructs, the nucleic acid sequences of the amplified gene fragments were confirmed by sequencing with a CEQ 2000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Plasmid DNA was purified with a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany).

Purification of the Cdc6/Orc1 protein

The Cdc6/Orc1 protein from *P. furiosus* was purified as described previously (Matsunaga et al. 2007). The detailed procedure is as follows. The Cdc6/Orc1 gene, cloned within the pPICZ vector, was expressed in yeast cells, by using the EasySelect Pichia expression system, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The harvested cells (16.4 g) were suspended in PBS buffer, mixed with glass beads, and heat-treated at 80°C for 20 min. The suspended cells were then disrupted by shaking with a vortex mixer. The lysate was mixed with buffer A, containing 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 10% glycerol, and 5 mM β-mercaptoethanol, and the insoluble fraction was separated by centrifugation (17,500×g for 10 min). The precipitate was resuspended in wash buffer, containing 2% Triton X-100, 10 mM EDTA, and centrifuged as described above. The precipitate was suspended with buffer B, containing 50 mM K-phosphate, pH 7.8, and centrifuged again under the same conditions. A denaturing buffer, containing 50 mM K-phosphate, pH 7.8, and 6 M guanidine-HCl, was added to resuspend the precipitate. The suspension was incubated at 25°C for 60 min with continuous mixing, and then the supernatant was obtained by centrifugation under the same conditions, and was applied to a Ni-NTA agarose column (QIAGEN). The column was washed with buffer B, containing 20 mM imidazole, and then the His-tagged Cdc6/Orc1 was eluted by buffer B containing 300 mM imidazole. The eluted fraction was diluted 100-fold by the addition of refolding buffer, containing 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 10% glycerol, and 5 mM β-mercaptoethanol, and was incubated at 25°C for 24 h, and then at 80°C for 20 min. The supernatant obtained by centrifugation was applied to the Ni-NTA agarose column again, and the fraction eluted by buffer B containing 300 mM imidazole was subjected to dialysis against the refolding buffer at 25°C for 24 h. Finally, the refolded His-tagged Cdc6/Orc1 was treated with thrombin (GE Healthcare, Piscataway, NJ) to cleave the histidine tag. The protein concentration was calculated by measuring the absorbance at 280 nm, using its theoretical molar extinction coefficient 36,060 M^{-1} cm^{-1}. To construct the mutant Cdc6/Orc1 lacking the AAA+ domain (Cdc6/Orc1ΔAAA+), the gene encoding the region from Glu303 to Ile420 of the Cdc6/Orc1 was amplified and cloned into pET28a. The His-tagged Cdc6/Orc1ΔAAA+ protein was produced in *E. coli* BL21 (DE3) CodonPlus-RIL (Stratagene, La Jolla, CA), with induction of the gene expression by IPTG. The total cell extracts were incubated for 20 min at 80°C, and the heat-stable fraction was subjected to Ni-NTA agarose column chromatography.

P1 endonuclease assay

The reaction mixture (50 μL) contained 60 mM HEPES KOH (pH 7.6), 8 mM Mg-acetate, 30% glycerol, and 0.32 mg/mL BSA. The nucleotide-bound form of the Cdc6/Orc1 protein was prepared by incubating the apoprotein in a buffer containing 3 μM ATP/ADP and 2.5 mM Mg-acetate for 15 min on ice. Cdc6/Orc1 was incubated in the reaction mixture containing 400 ng of *oriC* plasmid or *oriC*-ORB3 plasmid for 15 min at 45°C. When indicated, 5 mM ATP or ADP was present in the reaction. P1 nuclease (10 U, Yamasa Co., Chiba, Japan) was then added, and the reaction was incubated for 2 min at the same temperature. The reaction was terminated by adding 20 μL of stop solution (1% SDS and 50 mM EDTA). The DNA was purified by phenol/chloroform extraction and ethanol precipitation, and then was digested with a restriction enzyme (*Sca*I for the *oriC* plasmid and *Dra*III for the *oriC*-ORB3 plasmid). The resulting DNA fragments were fractionated on a 1.2% agarose gel and were visualized by staining with ethidium bromide.

Primer extension analysis

For the preparation of the template DNA, the major band produced by P1-endonuclease digestion was excised from the agarose gel and purified by using the Wizard SV Gel and PCR Clean-Up System (Promega). The primer extension reaction was accomplished by using a *TaKaRaTaq* Cycle Sequencing Kit (TAKARA BIO, Shiga, Japan). The reaction was performed according to the manufacturer's instructions, and each reaction contained 100 fmol of template DNA and 0.25 pmol of 5′-end labeled primer. The primer sequences are as follows: 5′-dTGAAAGGTAAGC AATTTTCACGAG-3′ (primer 1), 5′-dCTCCATTGGAAA TTGTGCTCCT-3′ (primer 2), 5′-dGCAGTCTACTTAAT ATGGGAGAGTGTTTG-3′ (primer 3), 5′-dGTCTTCACA GGAAATCTGAAGTTCC-3′ (primer 5), 5′-dGTGGAA GTTTTGGGCTGGGTGTGG-3′ (primer 7). Note that Fig. 2 shows the results of primer 2 (top strand) and primer 7 (bottom strand). The results with the other primers were consistent with those obtained with primer 2 and primer 7.

Topology footprint assay

The topology footprint assay was performed essentially as described by Grainge et al. (2006), with slight modifications. The reaction mixture contained purified Cdc6/Orc1 protein and supercoiled DNA. After an incubation for 15 min at 45°C, topoisomerase I (4 U) from *E. coli* (New England Biolabs, Ipswich, MA) was added to the reaction mixture, which was further incubated for 5 min at 45°C. The plasmid DNA was purified by ethanol precipitation after deproteinization by proteinase K treatment and phenol–chloroform extraction. The purified plasmid DNA was fractionated on a 0.85% agarose gel containing 0.5 μg/mL chloroquine (50 V, 220 min). DNA bands were visualized by staining with ethidium bromide.

DNA binding assay

For the preparation of the *oriC* DNA-coupled magnetic beads, the *oriC* plasmid was biotinylated by using Photoprobe Biotin (Vector Laboratories), and the biotinylated *oriC* plasmid was bound to streptavidin-coated magnetic beads by incubating 1 mg beads/17.5 pmol DNA overnight, using a Dynabeads kilobase BINDER kit (Dynal Biotech, Carlsbad, CA) according to the manufacturer's instructions. The purified Cdc6/Orc1 and its ΔAAA+ mutant proteins were incubated with the magnetic bead-bound *oriC* plasmid in the same solution (50 μL) as that used for the topology footprint assay at 45°C for 15 min. The reaction solution was diluted by 1 mL of washing buffer, containing 60 mM HEPES KOH, pH 7.6, 8 mM Mg-acetate, and 30% glycerol, and the magnetic bead-bound fraction was separated by placement in the magnet stand. This procedure was repeated three times, and after the bound fraction was subjected to SDS-12%PAGE, the proteins were electroblotted onto a polyvinylidene difluoride membrane. The western blotting images using anti-Cdc6/Orc1 antiserum were visualized by an enhanced chemiluminescence system (Millipore) and an LAS-3000 mini image analyzer (Fujifilm, Tokyo, Japan).

Results and discussion

Cdc6-dependent duplex opening in the *oriC* region

We examined whether the Cdc6/Orc1 protein from *P. furiosus* could unwind DNA containing the *oriC* sequence. We set up an assay system according to the methods used for the *E. coli* and *Thermotoga maritima oriC* plasmids (Bramhill and Kornberg 1988; Ozaki et al. 2006). This assay is based on the principle that the single strands produced by localized melting of duplex DNA can be cleaved by P1 endonuclease, a single-strand-specific nuclease. We first incubated the purified Cdc6/Orc1 protein (Fig. 1) with covalently closed plasmid DNA containing *oriC* (*oriC* plasmid, as shown in Fig. 2a). After digesting the plasmid DNA with P1 endonuclease, the proteins were removed by phenol/chloroform extraction. The purified DNA was then digested with the *Sca*I restriction endonuclease that cuts the plasmid only once, and the products were observed by agarose gel electrophoresis. As the amount of the Cdc6/Orc1 apoprotein increased, the intensity of a 2-kb band became stronger (Fig. 2b, right). Since the size of the *oriC* plasmid is 4 kb, the band should be a doublet of two 2-kb fragments, generated by the digestions

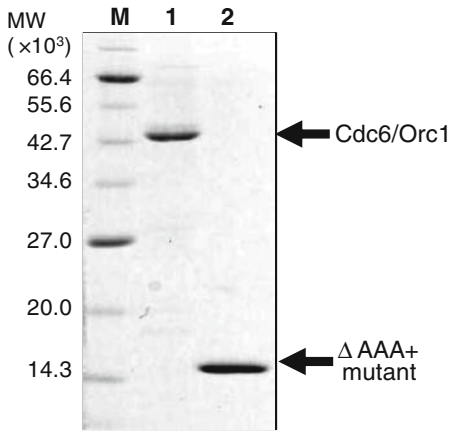

Fig. 1 Purification of wild type and ΔAAA+ mutant Cdc6/Orc1 proteins. The purified wild type and truncated Cdc6/Orc1 proteins (1 μg each in *lanes 1* and *2*, respectively) were subjected to SDS-15%PAGE, and were stained by Coomassie Brilliant Blue. *M* molecular mass standards (New England Biolabs Inc.)

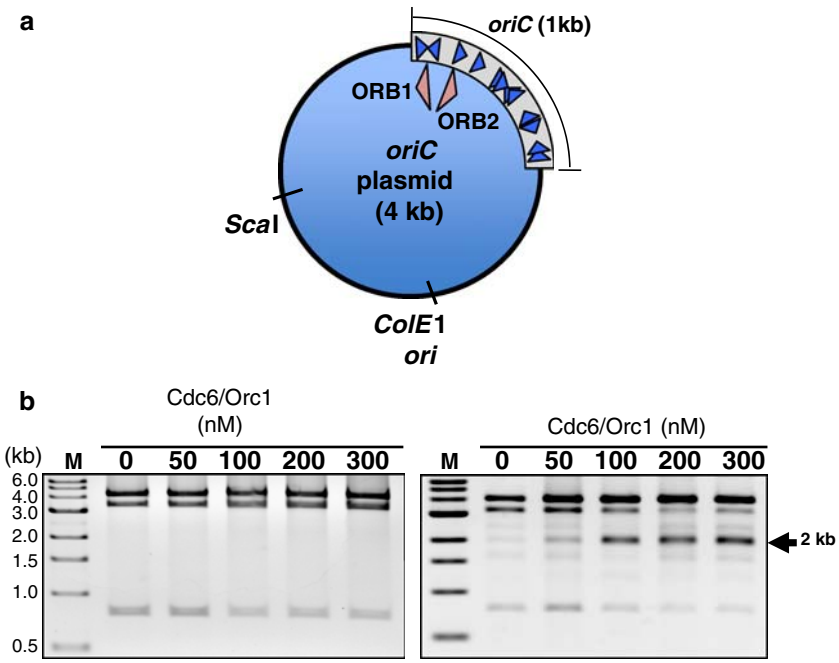

Fig. 2 Detection of strand unwinding by a P1 endonuclease assay. **a** The schematic representation of the *oriC* plasmid. The *oriC* region of the *P. furiosus* genome (1 kb) was inserted into the pGEM-T plasmid. The locations of ORB1 and ORB2 are shown in the inserted fragment, and the *arrowheads* indicate miniORBs, with the direction of the conserved sequence. **b** The indicated amount of purified Cdc6/Orc1 protein was incubated with the *oriC* plasmid DNA (*right panel*) and the control plasmid as described in the "Materials and methods" (*left panel*), respectively, and then was subjected to P1 endonuclease and *Sca*I digestions

with the P1 endonuclease and the *Sca*I. Therefore, the result indicates that the *oriC* plasmid was digested by P1 endonuclease in addition to *Sca*I, and the location of the P1-digestion site was deduced to be in the *oriC* region (Fig. 2a). We observed two additional bands (3.2 and 0.8 kb) in the P1 nuclease assay (Fig. 2b, right). The digestion site was estimated to be the *ColE1* origin by the size of the bands (Fig. 2b, right), and this result indicates that a site in the *ColE1* origin was also unwound and sensitive to P1 nuclease. However, these two bands were also observed in the absence of the Cdc6/Orc1 protein (far left lane of Fig. 2b, right). These *ColE1* origin-dependent bands were also detected from the plasmid containing the non-specific DNA fragment with the same size as the *oriC* region (1 kb) by exactly the same procedure, and the intensities of these bands did not increase with higher concentrations of Cdc6/Orc1 (Fig. 2b, left). Therefore, we concluded that these bands were generated by spontaneous denaturation of the *ColE1* origin at 45°C. In addition, two other very faint bands (2.5 and 1.5 kb) were observed in the agarose gel of the P1 assay. The sizes of these bands indicated digestion by P1 endonuclease at one of the A/T-rich regions in *oriC* that we had previously identified (Matsunaga et al. 2003). However, the presence of Cdc6/Orc1 did not enhance the intensity of these bands, and we concluded that they were also derived from the strand opening of the A/T-rich regions, by spontaneous denaturation of the plasmid at the high temperature of the assay.

The efficiency of the duplex opening in the absence of ATP reached a plateau at a 100 nM Cdc6/Orc1 concentration (30-fold excess amount on the *oriC* plasmid). Since the Cdc6/Orc1 protein is an AAA+ ATPase, we analyzed the effect of ATP in this reaction. When the ATP-bound form of Cdc6/Orc1 was incubated in the reaction buffer containing 5 mM ATP, we observed the same digestion pattern as that produced by the apoprotein (Fig. 3a). Quantification of the intensity of each band on the gels showed that the unwinding efficiency was decreased in the presence of ATP, as compared to that in the absence of the nucleotide (Fig. 3b). It is currently unknown how the ATPase of Cdc6/Orc1 functions for the initiation of the DNA replication process, and it remains unclear whether the decreased efficiency of unwinding in the presence of ATP in this experiment is a consequence of some regulation of initiation in the cells. It is well known that purified archaeal Cdc6/Orc1 proteins contain tightly bound ADP, and only denaturation in urea or guanidinium can remove the bound nucleotide (Liu et al. 2000; Grabowski and Kelman 2001; Singleton et al. 2004; Shin et al. 2008). In our study, we purified *P. furiosus* Cdc6/Orc1 from the insoluble fraction, and solubilization in a denaturing buffer, containing 50 mM K-phosphate, pH 7.8, and 6 M guanidine-HCl, was necessary in the purification procedure. Therefore, we believe that our experiments to investigate the effect of the nucleotide on the *oriC* unwinding, as described above, were appropriately controlled.

ORB3 does not affect the plasmid unwinding in vitro

We previously found another region containing the ORB (we named this ORB3) and miniORB sequences, located in the structural gene for the DP1 protein, a subunit of DNA

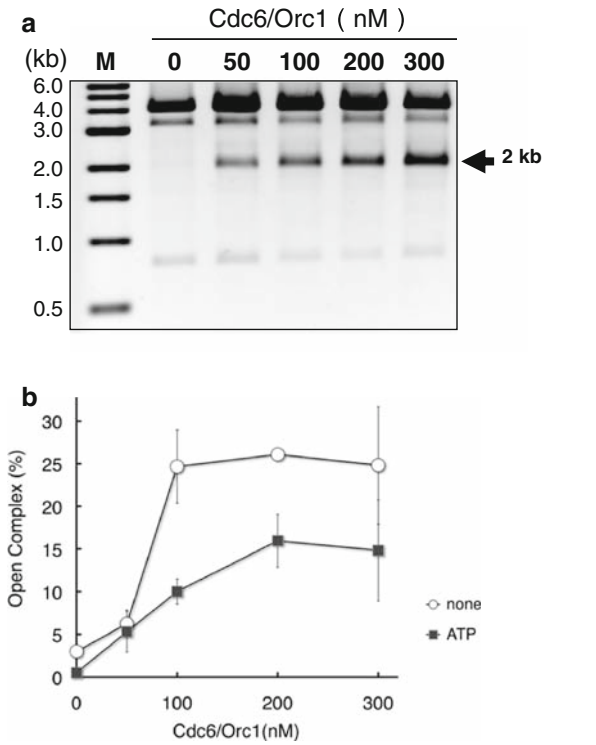

Fig. 3 Effect of ATP on strand unwinding by Cdc6/Orc1. **a** The indicated amount of purified Cdc6/Orc1 protein was incubated with the *oriC* plasmid DNA in the presence of 5 mM ATP, and then was subjected to P1 endonuclease digestion. **b** Quantification of strand unwinding. Band intensities of the gels shown in Figs. 2b and 3a were quantified by an LAS-3000 imaging system (Fujifilm). The amount of the 2.0 kb band was normalized to the total DNA, and was plotted against the concentration of Cdc6/Orc1. The average of three independent experiments is shown. The *error bar* indicates the standard deviation

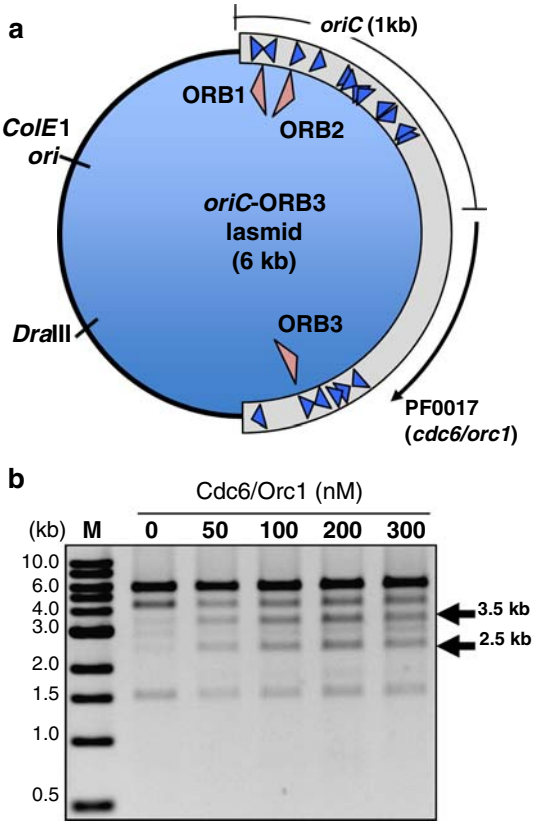

Fig. 4 Detection of strand unwinding of the oriC-ORB3 plasmid. **a** The schematic representation of the *oriC*-ORB3 plasmid. The DNA fragment containing ORB1, 2, and 3 of the *P. furiosus* genome (3 kb) was inserted into the pGEM-T plasmid. The locations of the ORBs are shown in the inserted fragment, and the *arrowheads* indicate miniORBs, with the direction of the conserved sequence. The *arrows* outside of the plasmid indicate the structural genes for Cdc6/Orc1 and DP1, respectively. **b** The indicated amount of purified Cdc6/Orc1 protein was incubated with the *oriC*-ORF3 plasmid DNA, and then was subjected to P1 endonuclease and *Dra*III digestions

polymerase D, in the *Pyrococcus* genome (Matsunaga et al. 2007). This feature is conserved in the genomes of *P. abyssi, P. furiosus*, and *Thermococcus kodakaraensis*. To investigate whether the region is also unwound by the Cdc6/Orc1 protein, we performed the same assay using the ORB3-*oriC* plasmid (Fig. 4a), which contains both the *oriC* and ORB3 regions. As shown in Fig. 4b, the amounts of two bands, with sizes of 2.5 and 3.5 kb, were increased with greater amounts of Cdc6/Orc1 by P1 and *Dra*III digestions, indicating that Cdc6/Orc1 unwound the duplex of the ORB3-*oriC* plasmid in the same region as found in the *oriC* plasmid (Fig. 4b). The unwinding efficiency reached a plateau (about 20%) at 100 nM Cdc6/Orc1, as in the case of the *oriC* plasmid. The function of ORB3 is currently unknown, although the Cdc6/Orc1 protein binds it and *oriC* in vitro with similar efficiencies, as judged from the gel shift assay in our previous study (Matsunaga et al. 2007). An in vitro reconstituted DNA replication system will be necessary to further investigate the role of ORB3 in the initiation of DNA replication.

Localized melting of duplex DNA occurs at the A/T-rich 12-mer sequence

To locate the site where Cdc6/Orc1 introduces DNA unwinding, we prepared a set of primers and analyzed the entire *oriC* region by means of a primer extension assay. The primer extension reaction stops when DNA polymerase encounters the site digested by P1 endonuclease. We found that there is one major P1 endonuclease-sensitive site, located between the miniORB repeats in the *oriC* region (Fig. 5). The deduced unwinding sequence, 5′-TA AATGTAATAA-3′, is rich in adenine and thymine. We also noted that the unwinding site is more than 70 bp away from the start position of *cdc6/orc1* mRNA synthesis (Uemori et al. 1997), indicating that this is not the site for loading RNA polymerase. There are sequences similar to the 12 mer in other regions of the *P. furiosus* genome, but the same sequence is not present in the *oriC* regions of two

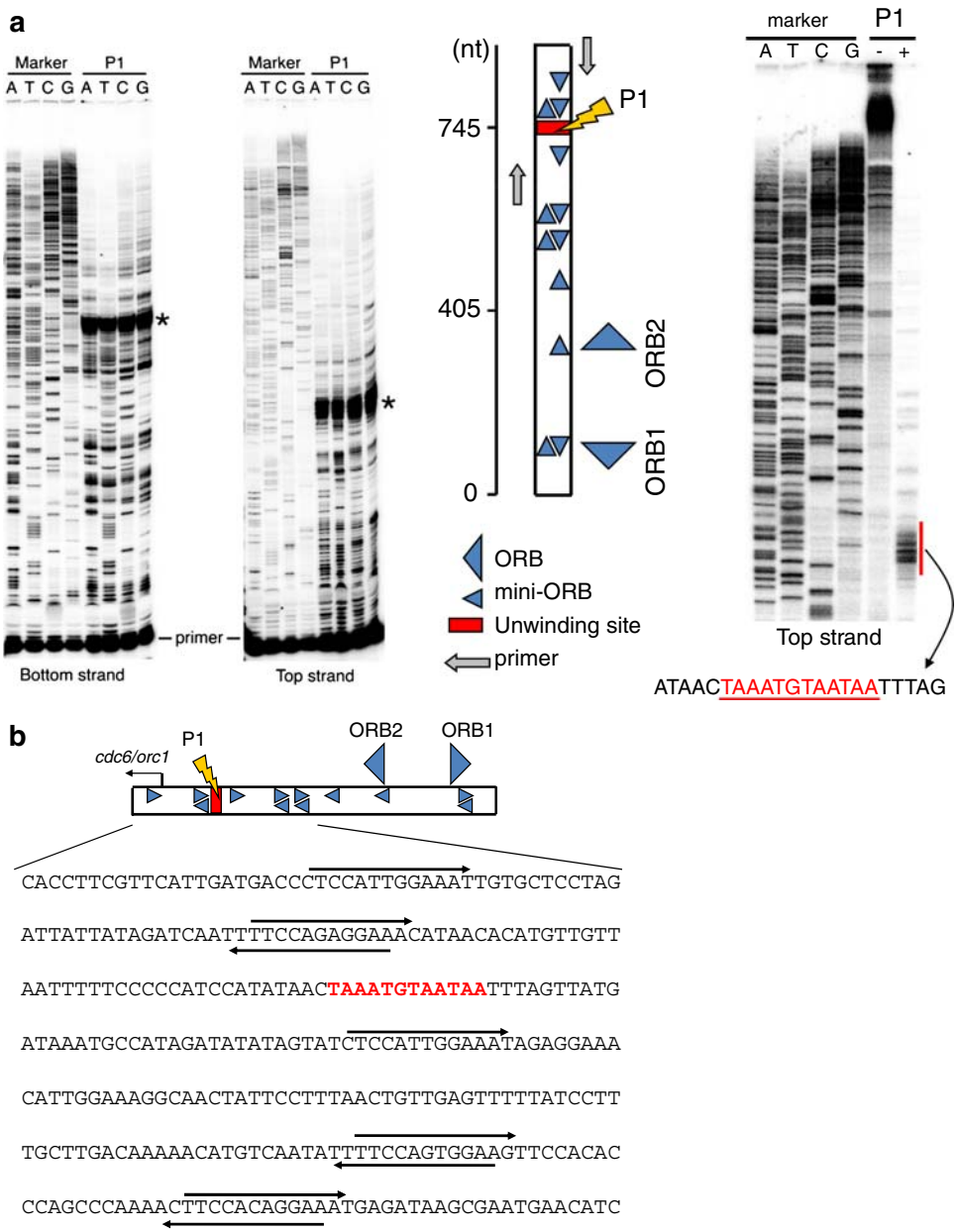

Fig. 5 Identification of the unwinding site by a primer extension assay. **a** Primer extension products (*P1*) in the presence of dideoxynucleotides, under the same conditions as those used for the sequencing reactions (indicated as *Marker*), were fractionated by denaturing polyacrylamide gel electrophoresis with 7 M urea (*left panel*). Both the *bottom* and *top* strands showed a strong P1 endonuclease-sensitive site (indicated by *asterisks*). The results of primer extension reactions without dideoxynucleotides for the *top* strand, both with and without P1 nuclease digestion, are shown in the *right panel*. The structure of the *oriC* region, including ORB, miniORB, and the P1 nuclease-sensitive site, is schematically presented in the *middle*. **b** The nucleotide sequence of the P1 endonuclease-sensitive site and its flanking region. Only the *top* strand DNA sequence is shown. The 12 bp P1-sensitive site is written by bold letter, and the *arrows* indicate miniORB

other *Pyrococcus* genomes (*P. abyssi* and *P. horikoshii*). These observations suggest that the position and/or A/T-richness of the 12 bp sequence, rather than the specific sequence, are important for determining the location of the strand-opening reaction. This speculation is in contrast to the case of *E. coli*, in which three A/T-rich sequences at the unwinding region share a 13 bp consensus sequence, 5′-GATCTnTTnTTTT-3′.

We also found that there are minor P1 endonuclease-sensitive sites in *oriC* (Fig. 5, e.g., faint bands observed on the lane of P1+ in the right panel). At these sites, the digestion by P1 endonuclease did not exceed 5 bp, and the efficiency was much lower than that of the major P1 endonuclease-sensitive site. The primer extension assay, together with the P1 endonuclease assay, clearly indicated that the 12 bp sequence described above is the major DNA-unwinding site in *oriC* of *P. furiosus*. As expected from the results of the P1 endonuclease assay, the presence of ATP or ADP did not change the strand-opening site (data not shown). We also analyzed the DNA-unwinding site in the ORB3-*oriC* plasmid. The same 12-bp sequence was located at the center of the unwinding site in this plasmid (data not shown). The same 12-mer sequence is not found in ORB3 region. Altogether, we predict that ORB3 alone will not induce localized unwinding as wide as that observed at the 12-mer sequence. However, we do not exclude the possibility that the ORB3 region bound by Cdc6/Orc1 affects topology of chromosomal DNA in physiological

condition, thereby regulating the initiation of DNA replication in the cells.

The local unwinding site described above is about 400 bp away from the predicted DUE site, which is located between ORB1 and ORB2, as described in our previous study (Matsunaga et al. 2007). The site is also 670 bp away from the transition site between leading and lagging syntheses, as determined by an in vivo RIP (replication initiation point) assay (Matsunaga et al. 2007). However, this apparent discrepancy may be explained from the fact that several events are necessary to initiate actual DNA synthesis, after starting the localized unwinding. For bidirectional replication, the replication machinery must be established at the unwound site. Although the details of the machinery are not fully understood in Archaea, it should minimally include Mcm, Gins, primase, PCNA, DNA polymerase, RPA, and topoisomerase. The assembly of these factors must be within and nearby the single-stranded region. However, it is evident that the 12-mer is not wide enough to accommodate all of these replication factors. We assume that the localized unwinding found in this study is the very first step before the recruitment of the replication factors. To assemble these replication factors, it would be important to create a single-stranded region wider than a 12-mer, by the action of the Mcm helicase complex. It is possible that the DUE sequences predicted in the previous study (Matsunaga et al. 2001) assist in this process, as the A/T-rich sequence is thermodynamically unstable, as revealed by the extra bands observed in the P1 nuclease assay. Indeed, the helicase activity of mammalian Mcm4/6/7 complex is activated by thymine stretches in vitro (You et al. 2003). At the initiation of DNA replication in *E. coli,* duplex unwinding starts from an A/T-rich 13-mer, while the subsequent RNA priming and DNA synthesis occur at different regions (Fang et al. 1999). This observation can also be explained by the same scenario as described above. In *A. pernix oriC*, the P1 nuclease-sensitive site is located between ORB2 and ORB3, and this site is proposed to function as a DUE, where the MCM helicase dodecamer, with a thickness of 240 Å, can be accommodated to expand the single-stranded region (Grainge et al. 2006). The *oriC* in *Pyrococcus* sp. has multiple repeats of ORB and miniORB, where quite a few number of Cdc6/Orc1 protein bind (Matsunaga et al. 2001). Our results may imply a somewhat different order of unwinding at the *oriC* region to start DNA replication in *Pyrococcus* organisms.

Our results indicated that negative supercoiling is important for localized unwinding in the *oriC* region. Similarly, Thomm et al. also found that negatively supercoiled plasmid is a better substrate for in vitro transcription using RNA polymerase and transcription factors from *P. furiosus* (Hethke et al. 1999). It is possible that negative supercoiling is important for such essential physiological processes in hyperthermophilic archaea. However, Forterre et al. reported that plasmid DNA is relaxed in *Pyrococcus* cells (Charbonnier et al. 1992). The inconsistency of the DNA structures can be explained by the two possibilities. Since the relaxed state of intracellular DNA in *Pyrococcus* has been determined after deproteinization of plasmid DNA, it is possible that some DNA regions, such as promoters or *oriC,* are maintained in a negatively supercoiled form in vivo by the chromatin structure of *Pyrococcus.* For example, positive wrapping of DNA in vivo by two histone homologs encoded in the *Pyrococcus* genome could induce compensatory local negative supercoiling. The other possibility is that Cdc6/Orc1 requires yet unknown factor(s) in vivo to unwind a linear or relaxed DNA molecule. To test these possibilities, it is necessary to reconstruct the replication system using relaxed plasmid and histone proteins or other accessory proteins in the next work.

Cdc6/Orc1 induces a topological change in *oriC* DNA

We found that the digestion by P1 endonuclease at *oriC* in the *oriC* plasmid is dependent on the supercoiled structure; Cdc6/Orc1 did not induce localized melting of the linearized *oriC* plasmid (data not shown). We then performed a topology footprint assay, to examine whether the binding of Cdc6/Orc1 to *oriC* causes a topological change in the plasmid structure. The Cdc6/Orc1 protein was incubated with a negatively supercoiled plasmid bearing *oriC*, which was then treated with topoisomerase I. The plasmid DNA was purified, fractionated on a 0.85% agarose gel in the presence or absence of chloroquine, and then stained with ethidium bromide to visualize the topoisomer distribution. Presence or absence of chloroquine allows us to detect different topoisomer species, as chloroquine intercalates into DNA and rewinds DNA duplex as observed previously (Mizushima et al. 1996). We found that the binding of Cdc6/Orc1 to the *oriC* plasmid changed the topoisomer distribution (Fig. 6a). The shift in the topoisomer distribution indicated that Cdc6/Orc1 binding decreases the average linking number of the topoisomer population. Similar results were obtained in the presence of ATP or ADP (Fig. 6b), in contrast to the case of *E. coli* DnaA, which is inhibited by ATP or ADP (Mizushima et al. 1996). These results indicated that the binding of Cdc6/Orc1 to *oriC* alters the topological structure to produce more negative supercoil in the plasmid. The topological change could be derived from the DNA wrapping around Cdc6/Orc1, as suggested in other archaeal (Grainge et al. 2006) and eubacterial (Bramhill and Kornberg 1988; Clarey et al. 2006; Erzberger et al. 2006; Ozaki et al. 2008) replication systems. We assume that this topological

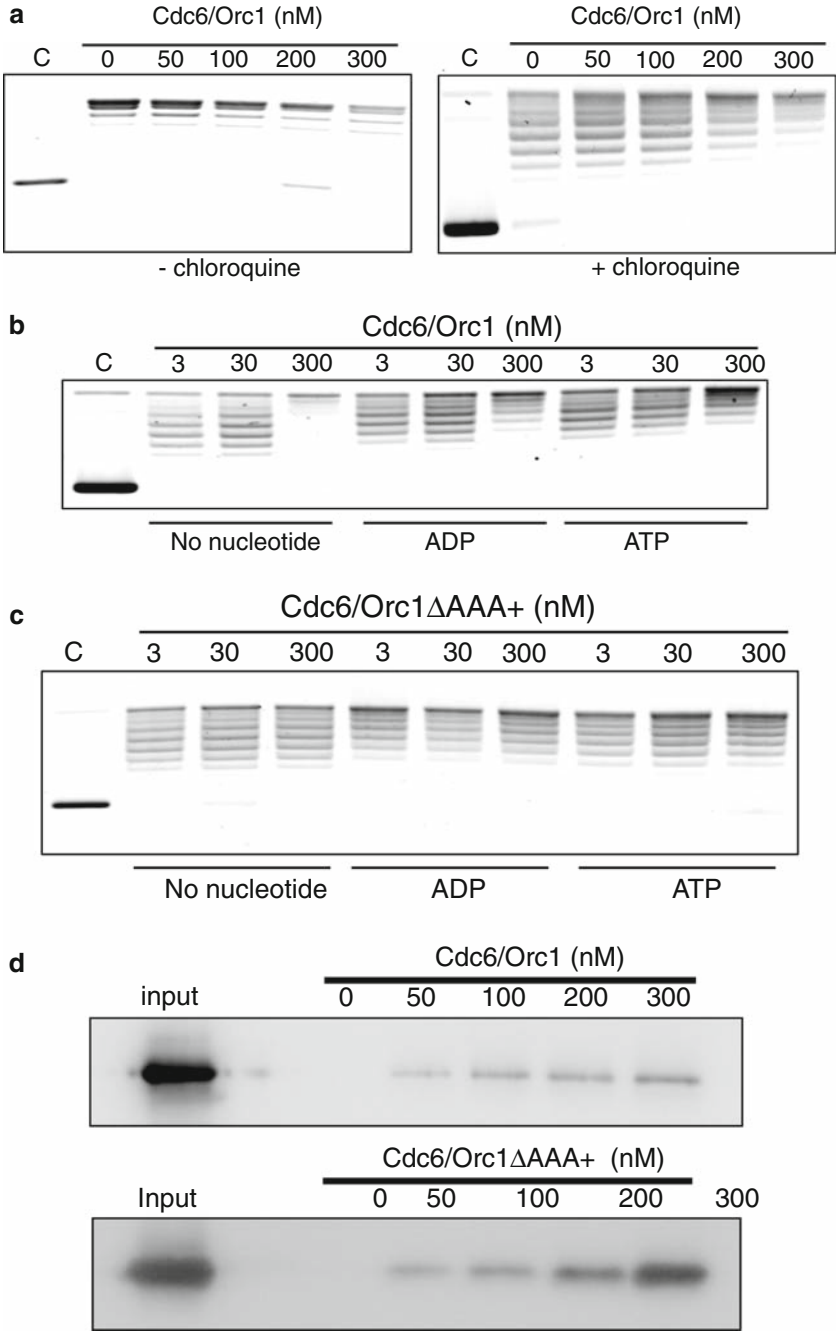

Fig. 6 Cdc6/Orc1 induces topological changes in the *oriC* plasmid. **a** The indicated amount of Cdc6/Orc1 was incubated with negatively supercoiled *oriC* plasmid, which was then treated with *E. coli* topoisomerase I. The DNA was purified and fractionated on a 0.85% agarose gel with (*right panel*) or without (*left panel*) chloroquine (0.5 μg/mL). **b** The same reaction was performed in the presence of ATP or ADP. **c** Cdc6/Orc1 lacking the AAA+ domain was used in the same reaction. *Lane C* control plasmid DNA without topoisomerase treatment. **d** DNA binding assay. The indicated amounts of the wild type (*upper panel*) and truncated (*lower panel*) Cdc6/Orc1 proteins were mixed with magnetic bead-bound *oriC* plasmid, and the magnetically separated fractions were subjected to a western blot analysis using anti-Cdc6/Orc1 antiserum

change is a key driving force to melt the DNA duplex at *oriC* in *P. furiosus*. However, the shift of the topological distribution of the *oriC* plasmid with increasing amount of Cdc6/Orc1 observed in Fig. 6 may also be explained by Cdc6/Orc1-induced unwinding. It would be interesting to perform quantitative analysis of the linking number introduced by binding of Cdc6/Orc1 to determine the extent of DNA unwinding.

We found that the AAA+ domain is essential for generating the topological alteration. The Cdc6/Orc1 protein lacking this domain (Cdc6/Orc1ΔAAA+), shown in Fig. 1, exhibited a much smaller change in the topoisomer distribution in the topology footprint assay (Fig. 6c). The DNA binding affinity of the ΔAAA+ mutant of *P. furiosus* Cdc6/Orc1 were apparently comparable or even higher than that of the wild type Cdc6/Orc1, as judged by a binding assay using a magnetic bead-conjugated DNA fragment (Fig. 6d). However, the AAA+ domain also interacts with DNA, as well as it is responsible for the interactions between Cdc6/Orc1 protomers (M. Akita et al. unpublished result). In the case of *A. pernix* ORC1, increased binding of the corresponding domain (domain III) alone causes a

topological change at very high concentration, and the domain alone can unwind DNA at the ORB sites, but not at the same sites as the wild type ORC1 does (Grainge et al. 2006). Furthermore, the AAA+ domains of *A. pernix* ORC1 and *S. solfataricus* Orc1-1/Orc1-3 are shown to interact with a part of ORB sequence from each *oriC* region in the co-crystal structures of the protein–DNA (Gaudier et al. 2007; Dueber et al. 2007). We have not examined whether Cdc6/Orc1ΔAAA+ is still able to induce unwinding. However, from these experimental data, we assume that the binding of both WH-domain and AAA+ domain of the *P. furiosus* Cdc6/Orc1 protein to DNA, as well as multimerization of Cdc6/Orc1 by protein–protein interaction via AAA+ domain, is important for altering the topological structure and correct unwinding of the *oriC* region in the DNA.

The physiological meaning of the ATP binding and hydrolysis by Cdc6/Orc1 should be elucidated, to clarify the function of this initiator protein. Since there have been several reports of a physical interaction between Cdc6/Orc1 and Mcm (De Felice et al. 2003; Kasiviswanathan et al. 2005; Haugland et al. 2006), Cdc6/Orc1 has been considered to play a major role in the assembly process of Mcm at *oriC,* as a helicase loader. A recent report suggested that one of the two Cdc6 proteins (Cdc6-2) in *M. thermautotrophicus* may function as the Mcm loader (Shin et al. 2008). We have characterized the Mcm helicase, GINS, and primase proteins from *P. furiosus* in vitro (Bocquier et al. 2001; Liu et al. 2001; Yoshimochi et al. 2008), and are currently investigating the loading mechanism of these proteins on the *oriC* region, as well as characterizing the Cdc6/Orc1 protein in more detail. As compared to the complex eukaryotic systems, with many more protein factors, *P. furiosus*, which utilizes eukaryotic-like replication factors, would be a good model organism to elucidate the principles of eukaryotic-type DNA replication.

Conclusion

P. furiosus Cdc6/Orc1 binds to *oriC*, thus altering the topological structure of the DNA and promoting localized melting over the 12 bp A/T-rich sequence in the *oriC* region. Such broad DNA unwinding had not been previously observed with the known systems utilizing the eukaryotic-type initiator proteins, ORC and Cdc6. *P. furiosus* Cdc6/Orc1 does not require other protein factors for efficient and localized unwinding, although we cannot exclude the possibility that an as yet unknown factor stimulates this process. We also found that the protein–protein interactions between Cdc6/Orc1 protomers are essential for altering the topological structure of *oriC*. As suggested from analyses of other bacterial systems and of the crenarchaeota, *A. pernix*, topological alterations of protein–DNA complexes seem to be a driving force for unwinding the origin in the euryarchaeota, *P. furiosus.* Clarifying the mechanism of Mcm loading and DNA unwinding by the MCM-GINS complex will be essential for further understanding of the initiation of DNA replication in Archaea.

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