Chapter 4
Archaeal Orc1/Cdc6 Proteins

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Abstract The initiation of DNA replication in most archaeal genomes is mediated by proteins related to eukaryotic Orc1 and Cdc6. Archaeal replication origins have been mapped and their interactions with Orc1/Cdc6 proteins have been characterized at the biochemical level. Structural and biophysical studies have revealed the basic rules of sequence recognition by archaeal initiators.

Keywords DNA replication • Helicase loader • Archaea • Evolution • Initiators

4.1 Introduction

First put forward in 1963, the replicon hypothesis posits that defined sequences within genomes serve as *cis*-acting "replicator" or origin sequences and *trans*-acting "initiator" factors act upon these sites to mediate replication initiation (Jacob et al. 1963). In bacteria, the broadly-conserved DnaA protein fulfills the role of initiator. The eukaryotic counterpart of DnaA is the six-subunit origin recognition complex (ORC) composed of Orc1–Orc6. As detailed in Chap. 3 of this book, ORC interacts with origins and leads to the recruitment of the MCM replicative helicase, in a reaction that is dependent upon two additional factors, Cdc6 and Cdt1 (Bell and Dutta 2002). Interestingly, Orc1 and Cdc6 show a degree of sequence conservation, suggesting that they may have evolved from a common ancestor. When the first archaeal genome sequences became available, it was instantly apparent that archaea have a DNA replication machinery that is closely-related to that of eukarya and

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clearly distinct from the analogous apparatus in bacteria. More specifically, genes encoding homolog(s) of the MCM helicase subunits, the two-subunit core DNA primase, the sliding clamp PCNA, the clamp loader RFC, the flap endonuclease Fen1 and the ATP-dependent DNA ligase I were found to be highly conserved between archaeal species (Edgell and Doolittle 1997). Intriguingly, however, the first archaeal genome to be sequenced, that of *Methanocaldococcus jannaschii*, did not reveal any clear candidates for initiator proteins. However, subsequent genomes of other archaeal species revealed one or more genes encoding proteins that were homologous to both Orc1 and Cdc6, possibly representative of the ancestral gene from which the distinct eukaryotic proteins evolved. In the following, I shall refer to the archaeal proteins generically as Orc1/Cdc6. Unfortunately, there has been no consensus policy adopted for the naming of these genes in archaeal genomes. This has resulted in a confusing and non-unified nomenclature with some projects calling orthologous proteins either Orc1 or Cdc6. To add to the confusion, some workers have named multiple Orc1/Cdc6 paralogs Orc1, Orc2, Orc3, etc., implying a non-existent relationship with the eukaryotic-specific ORC components Orc2, Orc3, etc.

Thus, with the exception of *M. jannaschii* and its relatives in the Methanococcales, archaea possess one or more Orc1/Cdc6 paralogs. To date the protein or proteins responsible for defining replication origins within the Methanococcales remain unknown.

4.2 Origins of DNA Replication in the Archaea

Four principal phyla of archaea have been identified thus far, the Crenarchaeota, Euryarchaeota, Thaumarchaeota and Korarchaeota (Brochier-Armanet et al. 2008; Elkins et al. 2008). While genes for Orc1/Cdc6 proteins are found in all four phyla, biochemical studies of these proteins have been restricted to the Crenarchaeota and Euryarchaeota and structural studies have been confined to crenarchaeal proteins. Nevertheless, as detailed below, the degree of sequence conservation both of the initiators and their DNA binding sites suggests that some general conclusions may be drawn despite the limited phylogenetic range of proteins sampled to date.

All archaea studied so far possess simple circular chromosomes that contain polycistronic transcription units and are thus reminiscent of the chromosome organization of most bacteria. This apparent parallel was strengthened with the first characterization of the replicon architecture of the chromosome of the euryarchaea from the genus *Pyrococcus*. Bioinformatic studies, in conjunction with *in vivo* DNA labeling studies, revealed that this organism, like bacteria, had a single origin of replication in its chromosome (Myllykallio et al. 2000). Furthermore, this origin was tightly linked to the gene for the single Orc1/Cdc6 homolog in *Pyrococcus*, again reminiscent of the linkage of *dnaA* genes with origins in many bacteria. However, as alluded to above, many archaea possess multiple Orc1/Cdc paralogs. For example, members of the genus *Sulfolobus* encode three such genes, now called *orc1–1, orc1–2* and *orc1–3*, in their single chromosome (She et al. 2001).

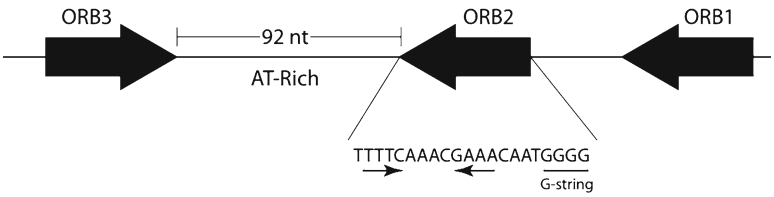

Fig. 4.1 Cartoon of the architecture of *S. solfataricus oriC1*. The three ORB element binding sites for Orc1–1 are indicated and the sequence of ORB2 is shown. The conserved dyad symmetric element (TTTC….GAAA) is indicated by *arrows* as is the polarity defining G-string element. Many archaeal origins share this arrangement where a central AT-rich region is flanked one or more ORB elements on each side

The *Sulfolobus orc1–1* gene is the clear ortholog of the single *Pyrococcus* Orc1/Cdc6. Neutral-neutral 2D agarose gel analyses of *S. solfataricus* revealed two replication origins: one, *oriC1*, adjacent to *orc1–1* as in *Pyrococcus*, and the second, *oriC2*, adjacent to *orc1–3*. No evidence for an origin within 15 kb of the *orc1–2* gene could be obtained (Robinson et al. 2004). A subsequent whole genome marker frequency analysis confirmed the existence of *oriC1* and *oriC2* and revealed a third origin in *Sulfolobus*, about 80 kb from the *orc1–2* gene (Lundgren et al. 2004). The position of *oriC3* was further mapped at high resolution by 2D gel analyses (Robinson et al. 2007). Remarkably, *oriC3* lies beside a gene encoding a divergent homolog of the eukaryal DNA replication initiation factor Cdt1. Studies using synchronized *Sulfolobus* cells have revealed that all three origins fire in every cell in every cell cycle during exponential growth. Furthermore, *oriC1* and *oriC3* fire highly synchronously while *oriC2* fires over a slightly broader temporal window (Duggin et al. 2008). How the coordinate control of origin firing is achieved is currently unknown. The existence and use of multiple replication origins per chromosome is not restricted to *Sulfolobus* species; two replication origins have been identified in another crenarchaeon, *Aeropyrum pernix*, and the main chromosome of the euryarchaeon *Haloferax volcanii* is replicated from at least two replication origins (Grainge et al. 2006; Norais et al. 2007; Robinson and Bell 2007). However, the stoichiometry of firing and timing of use of the origins in these species has yet to be evaluated.

Analysis of the sequence composition of the various replication origins reveals conservation of certain motifs between archaeal species (Fig. 4.1). The single origin of *Pyrococcus*, two origins of *Haloferax* and *oriC1* from *Sulfolobus* and *Aeropyrum* all contain conserved Origin Recognition Box (ORB) elements. These possess a dyad symmetric sequence flanked on one side by a run of three or more G bases (G-string). The G-string element therefore ascribes a polarity to the ORB element. Several origins have a common architecture where a central A-T rich region is flanked by ORB elements of inverted polarity in the two arms (Fig. 4.1) (Robinson et al. 2004).

Interestingly, *Sulfolobus oriC2* has a related mini-ORB element that lacks the G-string. Mini-ORB elements are also found at the single origin of replication in

the euryarchaeon *Methanothermobacter thermautotrophicum* (Capaldi and Berger 2004; Majernik and Chong 2008; Robinson et al. 2004). The ORB and mini-ORB elements are specific recognition sequences for *Sulfolobus* Orc1–1 and its orthologs from other species. The conservation of the binding site is sufficient to allow *Sulfolobus* Orc1–1 to bind specifically to the *Pyrococcus* origin *in vitro*, despite the phylum level divide between these organisms (Robinson et al. 2004). In addition to the Orc1–1-binding mini-ORB sites in *Sulfolobus oriC2*, this origin also possesses "C3" binding sites for Orc1–3. Orc1–3 has 35% sequence identity to Orc1–1 and its binding site contains a TTTC element that corresponds to one arm of the mini-ORB dyad. As described below, *oriC2* contains adjacent mini-ORB and C3 sites that bind Orc1–1 and Orc1–3 with a degree of positive cooperativity.

4.3 Orc1/Cdc6 Structure

Sequence analysis of the Orc1/Cdc6 proteins reveals that they possess a N-terminal AAA+ ATPase domain and a C-terminal winged-helix (wH) domain. This organisation is reminiscent of the bacterial DnaA protein that also contains a AAA+ fold followed by a DNA-binding domain, although in the case of DnaA this latter domain is a helix-turn-helix. AAA+ domains can be classified into seven distinct clades specified by characteristic embellishments on the core AAA+ fold (Erzberger and Berger 2006; Iyer et al. 2004). Importantly, DnaA, Orc1/Cdc6 and the eukaryotic Orc1 and Cdc6 all fall into the "Initiator" clade of AAA+ proteins – defined by the presence of an additional α-helix, termed the Initiator Specific Motif (ISM), that precedes the second α-helix of the core AAA+ fold. In DnaA it has been proposed that this additional α-helix serves as a steric wedge that helps drive the DnaA protein into a filamentous structure upon oligomerization (Erzberger et al. 2006).

The structures of Orc1/Cdc6 proteins from a number of archaeal species have been solved by X-ray crystallography. The first structure to be determined was that of Orc1/Cdc6 from the crenarchaeon *Pyrobaculum aerophilum* (Liu et al. 2000). This structure revealed a monomeric protein that had ADP bound in its active site. The tight ADP binding of this protein is found in many other archaeal Orc1/Cdc6s; indeed, a number of studies with recombinant Orc1/Cdc6s have found that it is necessary to employ a guanidinium hydrochloride-mediated denaturation/renaturation protocol to effect efficient exchange of ADP for ATP (Singleton et al. 2004).

This may be reflective of a switch-like regulation of the activity of the protein. Presumably, the protein when synthesized will bind to ATP, which it will then hydrolyse to ADP. If the cell regulates the timing of the synthesis of Orc1/Cdc6 during the cell cycle, a situation could be envisaged where a short window in time would be generated in which the ATP-bound form would be present. If one assumes that the ATP-bound form of the protein is the active form for initiation of replication, then a permissive period of the cell cycle would be dictated by the timing of synthesis and kinetics of ATP hydrolysis by the protein. It is also conceivable that

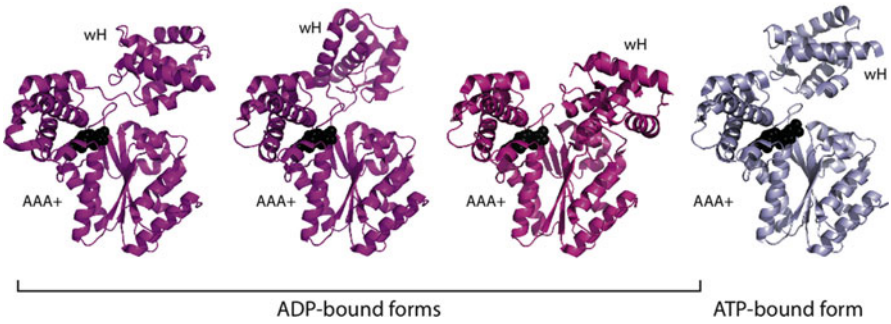

Fig. 4.2 Conformational variation of *A. pernix* Orc1–2 protein in different nucleotide bound states. ADP-bound forms of the protein are shown in *magenta* with the ADP in *black*. The ADPNP-bound form of the protein is shown in *blue*. The proteins were aligned on their AAA+ domains to highlight the distinct relative placement of the wH domains. Figure prepared using PDB files 1WSS and 1WST

specific nucleotide exchange factors may impinge upon this process in the cellular context, however, there is currently no evidence for the existence of such factors.

What are the consequences of ATP binding for the protein? Wigley and colleagues were able to determine the structures of an *A. pernix* Orc1–2 bound in the apo form, bound to ADP and bound to a non-hydrolysable analog of ATP, ADPNP (5′-adenylyl-β,γ-imidodiphosphate), by subjecting the protein to a denaturation/renaturation regimen before crystallization (Singleton et al. 2004). Interestingly, and in contrast to the situation with the *P. aerophilum* protein, the ADP-bound form of the *A. pernix* protein showed a range of distinct conformations (Fig. 4.2). While little change was observed within the AAA+ domain of the protein, the relative positioning of the wH domain varied, suggesting a degree of conformational flexibility in the ADP-bound form of the protein. In contrast, the ADPNP-bound form of the protein appeared to be much more conformationally constrained, with a locked position not seen in any of the ADP-bound forms of the protein being adopted.

4.4 Structures of Orc1/Cdc6s Bound to DNA

A major step forward in our understanding of the function of these proteins came in 2007 with the publication of two papers describing the structures of ADP-bound forms of Orc1/Cdc6s in complex with DNA (Dueber et al. 2007; Gaudier et al. 2007). One, from Wigley and colleagues, described the structure of *A. pernix* Orc1–1 bound to an ORB element derived from *A. pernix oriC1* (Fig. 4.3a). The second paper, from Berger and colleagues, described the complex of a heterodimer of *S. solfataricus* Orc1–1 and Orc1–3 bound to adjacent mini-ORB and C3 elements from that organism's *oriC2* (Fig. 4.3b). A key finding of both papers was the observation that the

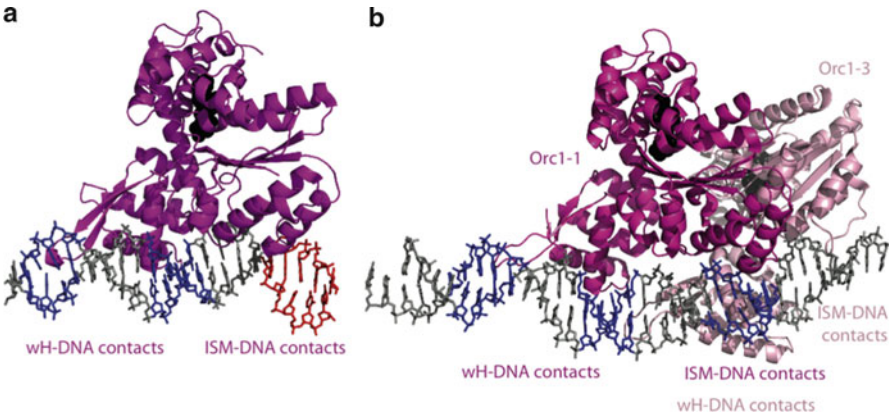

Fig. 4.3 Structures of Orc1/Cdc6 proteins bound to origin DNA. (**a**) *A. pernix* Orc1–1 bound to ORB4 from that organism's *oriC1* (PDB File 2V1U). The dyad symmetric residues in ORB4 (see Fig. 4.1) are shown in *blue* and the G-string in *red*. (**b**) The heterodimer of Orc1–1 (*magenta*) and Orc1–3 (*pink*) from *S. solfataricus* bound to adjacent mini-ORM and C3 sites from oriC2. The conserved dyad element of the mini-ORB and a related TTTC of the C3 site are shown in *blue*. Positions of contact between the proteins in DNA are shown below the diagram and colour coded as above

wH domain is not the sole DNA-binding interface in the proteins. All three proteins made additional contacts with DNA mediated by the ISM, the initiator clade signature alpha helix in the AAA+domain. In the case of the *A. pernix* Orc1–1, this contact was made with the G-string element that is found on one side of the ORB element. Thus the *A. pernix* protein makes two sets of contacts with the DNA. This first is mediated by the wH domain. The recognition helix inserts deeply into the major groove, widening it by over 2 Å and the wing of the wH makes contact with the minor groove, also resulting in significant widening of over 5 Å. Intriguingly, only four base pairs within the recognition site are directly contacted by the protein, although there are a number of additional contacts made with the phosphodiester backbone. These unanticipated additional contacts between ISM and DNA are mediated by a short loop immediately following the ISM alpha helix that inserts into the minor groove of the G-string. This makes a single sequence-specific contact with one of the G-string's guanine residues and has the consequence of widening the minor groove. Thus, the net effect of Orc1–1 binding to the ORB element leads to considerable under-winding of the DNA in the complex and also to the introduction of a bend in the DNA of about 35°. Footprinting studies revealed that the wH domain in isolation was still able to bind to DNA, albeit with a lowered affinity and a loss of protection of the G-string when compared with the protection pattern generated by the full-length protein (Gaudier et al. 2007).

The second paper revealed the structure of the heterodimer of the ADP-bound forms of *Sulfolobus* Orc1–1 and Orc1–3 in complex with adjacent mini-ORB and C3 elements from *oriC2*. As in the *A. pernix* structure, both of the *Sulfolobus* proteins

have bipartite DNA-interaction surfaces, composed of wH domain and ISM. The two proteins abut one another on the DNA, burying about 360 Å^2 of surface in a protein-protein interface and generating an extensive positively-charged surface of about 2,500 Å that interacts with 28 base pairs of DNA. Despite this extensive interface, a total of only five bases are contacted specifically by the proteins (Dueber et al. 2007). Thus, the paucity of sequence specific contacts appears a general feature of Orc1/Cdc6-DNA interactions. As in *Aeropyrum*, the *Sulfolobus* complex reveals considerable protein-induced under-winding of the DNA. It seems possible therefore that in addition to the modest sequence-specific contacts, the binding of Orc1/Cdc6 proteins is also modulated by the innate deformability of its recognition sequence. If this is the case, the archaeal proteins may represent an evolutionary stepping stone between the tight, highly-sequence-dependent interactions of the bacterial initiator DnaA and the apparently much less sequence-dependent binding of ORC in most eukaryotes. There also appears to be a degree of malleability in the structures of the proteins themselves upon interaction with DNA. Examination of the disposition of *Aeropyrum* Orc1–1 and *Sulfolobus* Orc1–3 on DNA reveal that the ISM makes equivalent contacts with the minor groove of DNA. In contrast, in the *Sulfolobus* Orc1–1/Orc1–3–DNA structure, while the wH domains of both proteins make essentially equivalent interactions with DNA, the respective ISMs do not. More specifically, the interaction between Orc1–1 and Orc1–3 results in the ISM of Orc1–1 being repositioned into the adjacent major groove, altering the angle between AAA+ and wH domains in comparison with the disposition of these domains in Orc1–3. All the DNA bound structures of Orc1/Cdc6s are of the ADP-bound form of the proteins. AAA+ proteins typically function as higher order multimers with the ATP-binding site being found at the interface between protomers. Indeed, residues from both neighbours contribute to binding. The nucleotide is principally bound in a "*cis*-acting" cleft in one protomer but is additionally coordinated by "*trans*-acting" residues in the neighbour, the classic such residue is the arginine finger. The arginine finger coordinates the γ-phosphate of ATP and thus provides a means for receiving information from, and effecting conformational changes between, protomers during the nucleotide binding, hydrolysis and release cycle of the active site.

In the Orc1–1/Orc1–3–DNA structure, the *cis*-face of Orc1–1 points towards the *trans*-face of Orc1–3, however, the arginine finger of Orc1–3 points away from the bound ADP. Some degree of repositioning would therefore be required in order to allow the arginine finger to appropriately coordinate an ATP moiety bound by Orc1–1. Given the extensive nature of the protein-DNA contacts, it seems highly likely that any significant conformational alteration within the Orc1/Cdc6 will either remodel the protein-DNA interaction and/or possibly impact upon the protein-induced DNA deformation. Interestingly, if one superimposes the wH domain of the ADPNP bound form of Orc1–2 of *Aeropyrum* onto that of the DNA-bound Orc1–1, then the resultant predicted structure has the ISM some distance removed from the path of the DNA in the structure (Fig. 4.4). This raises the tantalizing possibility that the ATP-bound form of the protein may have its AAA+ domain disengaged from the DNA and thus potentially available for ATP-mediated contacts with the AAA+ domains of adjacent protomers.

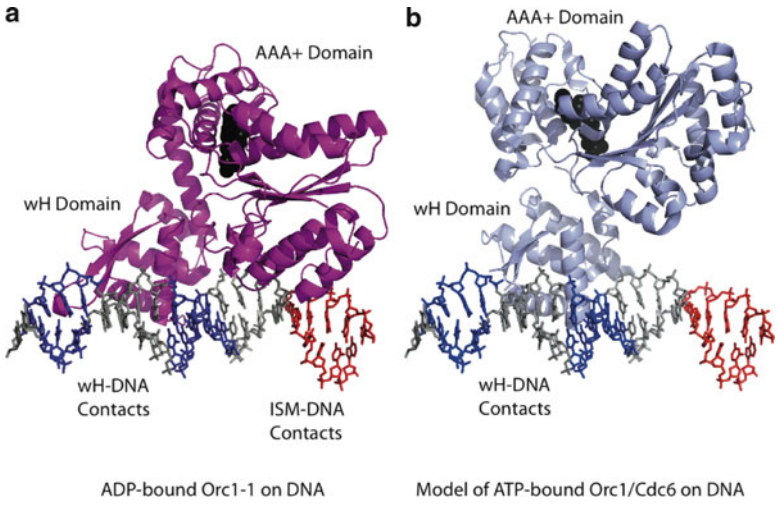

Fig. 4.4 Comparison of the disposition of the AAA+ domain in the known structure (**a**) of ADP-bound Orc1–1 (from *A. pernix* Orc1–1 on DNA; PDB 2V1U) with a model (**b**) of how the ATP-bound form may interact with DNA. The model was generated by superimposing the structure of the ADPNP form of *A. pernix* Orc1–2 (PDB 1WST) onto the DNA-bound structure of the ADP-form of Orc1–1. Colour coding of the DNA is as in Fig. 4.3; ADP and ADPNP are shown in *black*. The superimposition was generated using the wH domains as the initial point of alignment. The resultant model suggests that the AAA+ domain of the ATP-bound form may be some distance removed from the DNA and thus could potentially be available for additional protein-protein contacts. To date, no actual structure of the ATP-bound form of an Orc1/Cdc6 protein has been determined

A recent study investigated the rules of attraction between *Sulfolobus* Orc1/Cdc6s and origin DNA using a combination of biophysical and molecular-biological methodologies (Dueber et al. 2011). The analysis focused on the mini-ORB and C3 binding sites for Orc1–1 and Orc1–3 at *Sulfolobus oriC2*. The affinities of the isolated proteins for their cognate sites were 390 and 27 nM respectively. Orc1–1 showed a 12-fold lower affinity for a non-specific DNA oligonucleotide; Orc1–3 showed greater powers of discrimination, with a 280-fold difference in affinity. Mutation of conserved residues in either wing or helix of the initiators had dual impacts; the affinity of the mutant protein for DNA was significantly reduced and the ability of the protein to discriminate between specific and non-specific DNA sites was also impaired. A pair of conserved residues in the ISMs of Orc1–1 and Orc1–3 were also targeted for mutagenesis, these residues (G120 and L121 in Orc1–1 and G126 and I127 in Orc1–3) make non-sequence specific van der Waals contacts with DNA. Mutation of these residues had the anticipated effect of reducing the affinity of the initiators for their cognate sites. Surprisingly, the residues also proved important for determining the specificity of binding, despite the absence of direct contacts with the bases. These data suggest that the ISM plays a key role in reading an as yet unidentified aspect of the inherent geometry or deformability of the origin DNA. The affinity data for individual sites were complemented by footprinting

studies using the DNA conformation-sensitive reagent, copper phenanthroline. Although the resultant data were complex, some general principles could be gleaned. First, ISM mutations impacted on the DNA geometry, as revealed by altered hypersensitivity to the footprinting reagent. Second, some ISM mutations, most notably Orc1–1 (G120L, L121D) resulted in an extended region of protection, suggesting that impairing the ability of the ISM to interact with DNA actually facilitated the recruitment of a second protomer of the protein to an adjacent, presumably non-specific, site on the origin. This situation arising from mutation of the ISM is, of course, reminiscent of the model proposed above for an ATP-induced disengagement of the AAA+ domain from DNA (Fig. 4.4).

4.5 Beyond Binding Origins – What Do Orc1/Cdc6s do?

The Orc1/Cdc6s clearly bind to archaeal replication origins but how do they mediate replication initiation? Are they simply passive recruitment platforms for the replication machinery or do they actively mediate origin unwinding prior to helicase recruitment? The latter possibility would be analogous to the situation in bacteria where DnaA mediates localised DNA unwinding before the DnaB•DnaC (helicase•helicase loader) complex is recruited to the newly-exposed single-stranded DNA. However, there is little unambiguous data to support a role for archaeal Orc1/Cdc6s in mediating appropriate origin melting. Wigley and colleagues revealed that high concentrations of *A. pernix* Orc1–1 led to periodic sensitivity to nuclease P1 across the entire origin region *in vitro* (Grainge et al. 2006). However, it was not clear whether this was due to helical distortion or true melting of DNA. More recently, Ishino and colleagues have reported *P. furiosus* Orc1–1 mediated melting of DNA at the single *Pyrococcus* origin of replication *in vitro* as detected by nuclease P1 sensitivity assays (Matsunaga et al. 2010). Puzzlingly, however, this apparent melting was inhibited by ATP. Furthermore, the site of melting was 670 nt removed from the *in vivo* start site of replication, mapped previously by the same authors, raising questions regarding the physiological relevance of this observation.

It may not be too surprising that as yet there is no clinching proof for relevant origin melting by the archaeal initiators. Orc1/Cdc6 is not orthologous to bacterial DnaA and the organization of archaeal origins, with a distinct number of discrete DNA binding sites, clearly differs from the densely packed DnaA boxes in bacterial origins. Furthermore, recent studies in the orthologous eukaryotic system have provided strong support for ORC•Cdc6 mediating loading of MCM onto double stranded DNA rather than onto a pre-melted origin (Evrin et al. 2009; Remus et al. 2009). Perhaps the most parsimonious model for the archaeal system would be that Orc1/Cdc6 proteins do not lead directly to DNA melting in archaea either and that the MCM helicase is, as in eukarya, recruited to double stranded DNA. Melting could take place at a later stage during the activation of the MCM helicase (Bell 2011). Although number of laboratories have reported direct interactions between archaeal Orc1/Cdc6s and MCM, the mechanism of the putative loading reaction remains elusive to date.

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