Architecture of the *Saccharomyces cerevisiae* Origin Recognition Complex Bound to Origins of DNA Replication

by

Daniel Gyejun Lee

B. Sc. Genetics
University of Alberta, 1993

Submitted to the Department of Biology
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ABSTRACT

The Origin Recognition Complex (ORC) is thought to be required for the initiation of DNA replication in all eukaryotes. *In Saccharomyces cerevisiae*, ORC is bound to origins of DNA replication throughout the cell cycle and directs the assembly of higher-order protein-DNA complexes during G1. I have investigated the architecture of yeast ORC bound to origin DNA. Determination of DNA residues important for ORC-origin association indicated that ORC interacts preferentially with one strand of the *ARS1* origin. DNA binding assays using ORC complexes lacking one of the six subunits demonstrated that the DNA binding domain of ORC requires the coordinate action of five of the six ORC subunits. Protein-DNA crosslinking studies suggested that recognition of origin sequences is mediated by two groups of ORC subunits making sequence-specific contacts with two distinct regions of the DNA. Thus, the DNA-binding surface of ORC is formed by the coordinate action of multiple subunits.

Electron microscopy (EM) of ORC showed that the complex has a shape consistent with the structure determined by protein-DNA crosslinking. These EM studies found that ORC is an elongated molecule with three lobes. Estimates of the molecular mass of ORC using EM were consistent with this elongated complex containing one copy of each of the six ORC subunits. ORC bound origin DNA along its long axis and interacted with approximately 50 base pairs as predicted from DNase I protection assays of ORC bound to origin DNA.

To examine the fate of ORC when origin DNA is unwound during replication initiation, I determined the effect of single-stranded DNA (ssDNA) on ORC. I showed that ORC binds ssDNA and that the ssDNA-bound form of ORC is distinct from that bound to double-stranded origin DNA. EM studies demonstrated that ssDNA stabilizes a bent conformation of ORC whereas origin DNA stabilizes an extended form of ORC. In addition, ssDNA stimulates the ORC-ATPase activity, whereas origin-containing DNA inhibits it. I propose that the unwinding of origin DNA activates an ssDNA-controlled ORC conformational switch that contributes to the remodeling of the origin-associated protein complexes assembled during G1.

Thesis Supervisor: Stephen P. Bell
Title: Associate Professor of Biology

Dedicated,
with love

to my parents,
Jai-Hyung and Boon-Ok Lee

and

to my wife,
Tallessyn

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Chapter I

Introduction

The replication of a genome is a logistically complex task. The process itself is an impressive feat, involving the synthesis of tens or thousands of megabases of DNA in eukaryotes, with high fidelity, in a short period of time (~20 minutes for yeast and as little as 3-4 minutes for fruit fly embryos). Moreover, the process must be precisely regulated each cell cycle to ensure that the entire genome is duplicated but that no portion is over-replicated. Both too little and too much replication can have deleterious consequences for an organism.

In 1964, Jacob, Brenner and Cuzin proposed a simple, yet powerful model for the initiation of DNA replication that continues to be influential today (Jacob et al., 1964). The replicon model postulated that the initiation of DNA replication would require a positive trans-acting factor called the initiator protein, that would activate replication via a cis-acting sequence called the replicator. The replicator is therefore a genetically defined element that is required for replication, whereas the more frequently used term, replication origin, refers to the actual initiation site of DNA synthesis as determined by physical mapping techniques. Although the replicator and the origin need not necessarily overlap (reviewed in Stillman, 1993), the two elements are generally coincident and I will hereafter refer only to origins.

The replicon model has since been supported by the study of genomic replication in bacteria, phage and eukaryotic viruses. These experiments have indicated that the initiator generally performs three functions (first proposed by Bramhill and Kornberg, 1988; reviewed in Baker and Bell, 1998). First, initiator proteins bind their cognate origins of replication in a sequence specific manner and thereby select the site at which DNA replication will begin. Second, binding of the initiator often induces distortions in the origin DNA, facilitating the generation of the single-stranded DNA template for

polymerase action. Finally, initiator proteins recruit other replication proteins required for the assembly of replication forks at the origin.

The initiator protein and the early steps of replication are likely targets for the regulatory mechanism that ensure the fidelity of genomic replication. From a practical standpoint, it makes sense to regulate steps that occur before the stable complex of replication proteins bound to origins is converted into a moving replication machine. If replication elongation was a major target of cell cycle regulation and was routinely interrupted, the resulting replication intermediates would generate undesirable regions of genomic instability. Indeed, studies of related processes such as transcription and translation have shown that the initiation phase is a key target for regulation (Sachs and Buratowski, 1997). Furthermore, studies of the replication of eukaryotic viruses such as the Simian Virus 40 (SV40) have demonstrated that the viral genome replicates more than once per cell cycle. Because SV40 replication requires only one viral-encoded protein (the SV40 initiator protein, T-Antigen) and all other activities are provided by the mammalian host cell (reviewed in Stillman, 1994), the simplest explanation is that proteins involved in initiation and not the DNA synthesis machinery are the major target for cell-cycle regulation.

The Replicon Model in Eukaryotes

Studies of DNA replication in the budding yeast *Saccharomyces cerevisiae* strongly support the hypothesis that eukaryotic cells adhere to the replicon model. *S. cerevisiae* is the only eukaryote that has both well-defined origins of replication and a putative initiator protein. Yeast origins were first identified as genomic DNA sequences capable of supporting the autonomous replication of episomal DNA (Autonomous Replicating Sequences or ARSs; Hsiao and Carbon, 1979; Stinchcomb et al., 1979).

Many of these elements were subsequently shown to act as origins of replication in their normal chromosomal context (reviewed in Newlon and Theis, 1993). Yeast origins are modular in nature and contain the conserved 11 base-pair ARS Consensus Sequence (ACS) that is essential for ORC-DNA binding and origin function *in vivo*, as well as additional elements that enhance origin function (referred to as B elements; Bell, 1995). The structure of yeast origins will be discussed in greater detail in Chapter II.

A candidate eukaryotic initiator protein was first identified in *S. cerevisiae* as a six protein complex called the Origin Recognition Complex (ORC). ORC was purified as an activity that bound specifically to the *ARS1* origin *in vitro* in the presence of ATP (Bell and Stillman, 1992). ORC was subsequently shown to bind yeast origins of replication *in vivo* (Aparicio et al., 1997; Diffley and Cocker, 1992; Santocanale and Diffley, 1996; Tanaka et al., 1997). The six ORC subunits are referred to as Orc1p through Orc6p in order of decreasing mass and all six proteins are essential for the viability of yeast cells (Bell et al., 1993; Bell et al., 1995; Li and Herskowitz, 1993; Loo et al., 1995). Conditional mutations in ORC subunits result in cell-cycle abnormalities and high plasmid loss rates consistent with defects in DNA replication (Foss et al., 1993; Loo et al., 1995; Micklem et al., 1993). These mutations also lead to decreased origin usage *in vivo* (Fox et al., 1995; Liang et al., 1995) and decreased origin binding *in vivo* (Aparicio et al., 1997).

Although origins of replication have been difficult to define in multi-cellular eukaryotes (reviewed in Diffley, 1996), analogs of ORC subunits have been discovered in numerous species (reviewed in (Dutta and Bell, 1997). In *Xenopus laevis* and *Drosophila melanogaster*, these proteins form a six-protein complex similar to that seen in yeast. Futhermore, studies of replication either *in vivo* or *in vitro* strongly suggest that ORC is required for DNA replication in all eukaryotic species (Chesnokov et al., 1999; Landis et

al., 1997; Pasero et al., 1997; reviewed in Dutta and Bell, 1997). In addition to the putative initiator protein, other replication proteins involved in the initiation process (see below) are conserved throughout evolution as are the components of the DNA synthesis machinery (reviewed in Baker and Bell, 1998). This high degree of conservation among replication proteins argues that the mechanisms for both the initiation and the elongation phases of DNA replication are similar in all eukaryotes.

A Two-Step Process for Regulating Initiation in Yeast

Multiple aspects of replication must be controlled to ensure faithful genomic duplication. First, all eukaryotic DNA is restricted to the appropriate time during the cell cycle. In normal cycling cells, DNA synthesis begins only after cell division has been completed, and replication does not occur again until there has been an intervening mitotic phase. Second, the large size of the eukaryotic genome combined with its organization into multiple chromosomes requires that bidirectional replication be initiated from multiple origins (reviewed in Diffley, 1996). Furthermore, origins of replication are temporally regulated: rather than being activated simultaneously at the beginning of each S-phase, origins initiate replication at characteristic times during S-phase (reviewed in Diller and Raghuraman, 1994; Simon and Cedar, 1996). The problem of multiple origins firing throughout S-phase requires mechanisms to ensure that each origin initiates only once per cell cycle and that an origin that is passively replicated does not initiate. These regulatory mechanisms are enforced by controlling the assembly of replication complexes (see below).

ORC is known to possess at least two of the three general properties of initiator proteins: it specifically binds origin DNA and it recruits other replication proteins to origins. In yeast, ORC binds to origins of replication throughout most or all of the cell

cycle and directs the assembly of higher order complexes prior to the initiation of DNA replication (Figure 1). *In vivo* DNase I protection assays and chromatin immunoprecipitation (CHIP) experiments suggest that ORC alone is present at the origin during the G2- and M-phases to form what has been termed the postreplicative complex (post-RC; Aparicio et al., 1997; Diffley et al., 1994; Tanaka et al., 1997). In G1, ORC recruits additional replication proteins to origins, including Cdc6p and the MCM proteins (in that order), to form the prereplicative complex (pre-RC). Initially, the pre-RC was defined as a structure that extended the ORC-specific DNase I footprint at origins during G1 (Diffley et al., 1994). This larger region of protection was dependent on the activity of the Cdc6p protein (Cocker et al., 1996). Subsequent *in vivo* chromatin immunoprecipitation assays demonstrated that Cdc6p as well as the MCMs are recruited to origins by ORC (Aparicio et al., 1997; Tanaka et al., 1997), and the term pre-RC has since come to include MCMs.

After the formation of pre-RCs, Cdc45p and the replicative polymerases are recruited to origins in a manner correlated with the time of replication initiation (Aparicio et al., 1997; Zou and Stillman, 1998). This higher-order complex containing Cdc45p and the polymerases has been named the replicative complex (RC). During S-phase, Cdc6p is degraded (Piatti et al., 1995), and MCM proteins and Cdc45p are released from origins and appear to move with the DNA polymerases as part of the replication fork (Aparicio et al., 1997). ORC remains at the origin to repeat this process in the following cell cycle. The requirement for an ORC-dependent assembly of replication proteins on DNA is likely conserved throughout evolution, as replication in *Xenopus* extracts requires chromatin association of ORC, Cdc6p and MCM proteins, with the same dependence as seen in yeast (reviewed in Diffley, 1996).

Cell Cycle Regulation of Replication Complexes

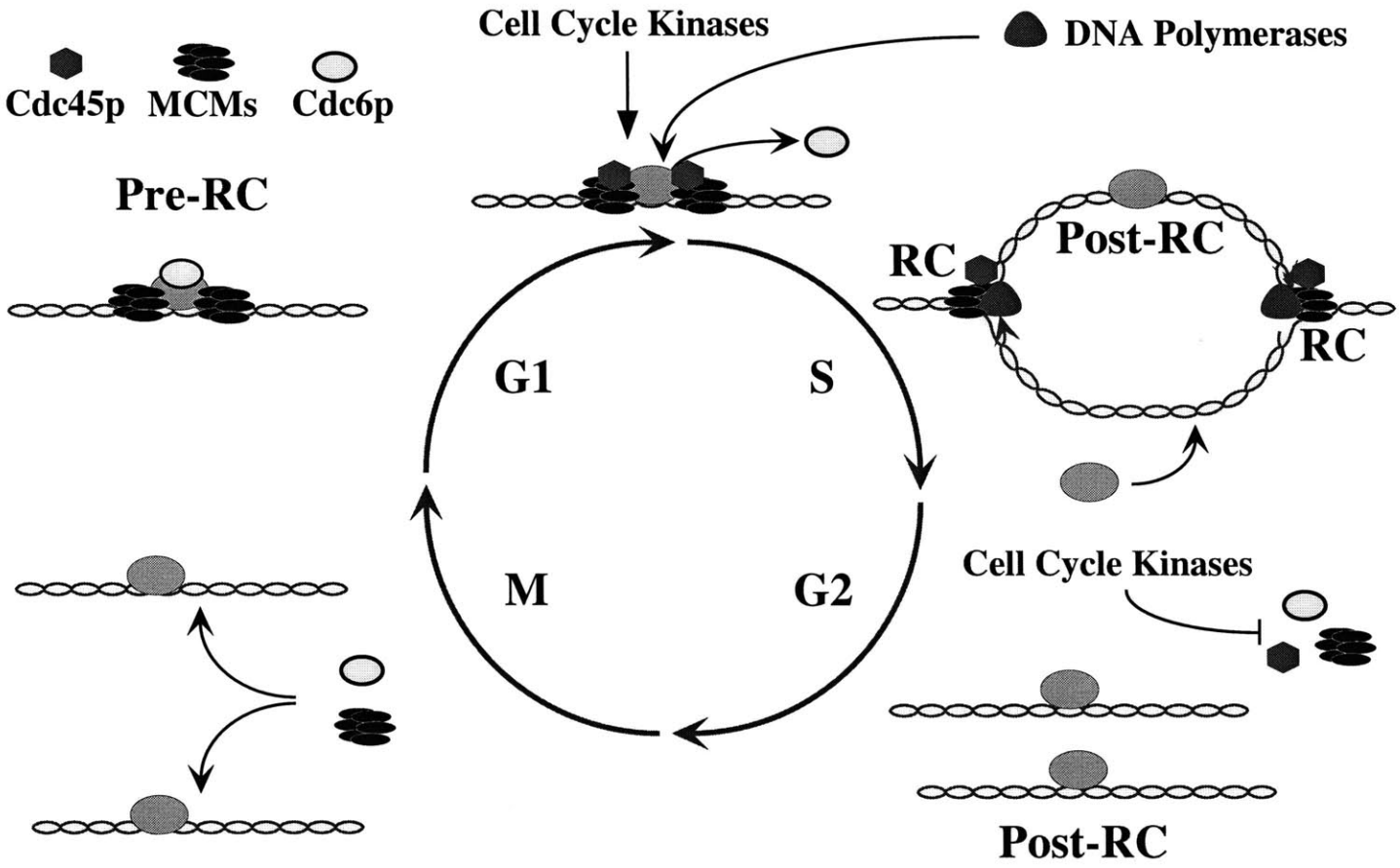

Figure 1

Figure 1. Cell-Cycle Regulation of Replication Complexes. In G2, ORC (green oval) is bound to origin DNA to form the postreplicative complex (post-RC). During G1, ORC recruits Cdc6p and MCM proteins to the origin to form a prereplicative complex (pre-RC). Close to the G1-S transition when B-type cell-cycle kinases are activated, Cdc45p and the DNA polymerases are recruited to origins to form a replicative complex (RC). The activity of kinases activate the RC, remodeling its components to release the polymerases, as well as the MCMs and Cdc45p which move as part of the replication fork. High levels of kinase activity during S-, G2- and M-phases prevent the formation of further pre-RCs. See text for more details.

During each cell cycle, the assembly and disassembly of origin-associated complexes is controlled by the action of cell-cycle regulated kinases. In yeast, the assembly of pre-RCs occurs during G1 when Cdc6p is abundant and when levels of B-type cyclin-dependent kinase (CDK) activity are low (B-type CDKs are active during S-, G2- and M-phases; see below). Entry into S-phase requires the activation of the S-phase CDKs (Clb5p- and Clb6p-associated CDKs) and the activity of the Cdc7p/Dbf4p kinase. In a CDK-dependent event, each origin is activated, resulting in the dismantling of the RC. ORC re-binds origin DNA to form a post-RC and must be prevented from re-forming new pre-RCs and re-initiating replication until the next G1-phase. Interestingly, the activities of the B-type CDKs prevent pre-RC formation during S-, G2-, and M-phases (Dahmann et al., 1995; Piatti et al., 1996; Tanaka et al., 1997). Thus, the cell cycle can be split into two phases with respect to kinase and pre-RC activity. During G1, when B-type kinase activity is low, pre-RCs can form but cannot be activated. When Clb5p- and Clb6p-associated kinase activities peak during S-phase, pre-RCs can be converted into RCs and activated, but no further pre-RC formation is allowed until the next G1-phase when B-type CDKs return to low levels.

Properties of the Known pre-RC and RC Components

As described above, ORC remains bound to origin DNA throughout the cell cycle, and the first component of the pre-RC recruited to DNA by ORC is Cdc6p (Aparicio et al., 1997; Cocker et al., 1996; Tanaka et al., 1997). Cdc6p is required for an early event in DNA replication (Hartwell, 1976) and interacts both genetically and physically with ORC (Liang et al., 1995; Wang et al., 1999; reviewed in Dutta and Bell). Both the mRNA and protein levels for Cdc6p fluctuate during the cell cycle, resulting in a peak of Cdc6p at the M-G1 transition and a second at the G1-S transition (Piatti et al.,

1995). The instability of Cdc6p is likely due to a combination of kinases and the cell-cycle regulated degradation machinery. Cdc6p is a target for phosphorylation by the Clb5-associated CDK *in vivo* and *in vitro* (Elsasser et al., 1996; Piatti et al., 1996). Furthermore, Cdc6p is degraded by the Cdc4/34/53 ubiquitin-mediated proteolysis pathway involved the regulated destruction of proteins at the G1-S transition (Drury et al., 1997; Piatti et al., 1996). No causal relationship has yet been determined for phosphorylation and degradation of Cdc6p; however, the related *Schizosaccharomyces pombe* protein, Cdc18p, has been shown to be targeted for degradation by CDK phosphorylation (Jallepalli et al., 1997).

Although Cdc6p may normally be destroyed prior to the initiation of replication, the requirement for its removal remains unclear. A stable variant of Cdc6p that is no longer a substrate for Cdc4/34/53 mediated degradation has no consequence for cell-cycle progression is budding yeast (Drury et al., 1997). These data are in contrast to work in *S. pombe* in which stabilized forms of Cdc18p are potent activators of re-replication (Jallepalli et al., 1997). This difference suggests that redundant mechanisms operate in *S. cerevisiae* to ensure that replication occurs once and only once. It should be noted that *Xenopus* and mammalian Cdc6p are stable proteins that appear to be controlled by regulated nuclear localization. These proteins are nuclear in G1 but are relocalized outside of the nucleus around the time of DNA replication (Coleman et al., 1996; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998).

Cdc6p and ORC are both required for the origin association of the next component of the pre-RC, the mini-chromosome maintenance (MCM) proteins (Aparicio et al., 1997; Donovan et al., 1997; Tanaka et al., 1997). The MCMs are a family of six related proteins (named Mcm2p through Mcm7p) first identified in yeast by screening for mutations defective in the maintenance of ARS-containing plasmids or cell-cycle

progression (Maine et al., 1984; Moir et al., 1982). These six proteins are similar in sequence, particularly in a region that contains a putative DNA-dependent ATPase domain (Koonin, 1993). Despite these sequence similarities, the MCM proteins cannot functionally substitute for one another as they are each essential for cell viability (reviewed in Dutta and Bell, 1997). MCM family members have been identified in organisms from *S. pombe* to humans, and in each case, data suggests that the MCM proteins function together in a complex. Various sub-complexes of MCMs have been observed in yeast, *Drosophila*, *Xenopus* and human cells (reviewed in Dutta and Bell, 1997), although recent work has detected a hetero-hexameric complex containing all six MCM proteins *from S. pombe* and human cells (Adachi et al., 1997; Fujita et al., 1998).

Recent data has suggested that MCM proteins are the replicative helicase. A weak helicase activity has been detected for a complex containing the human Mcm4p, Mcm6p and Mcm7p in the presence of hydrolyzable ATP or dATP (Ishimi, 1997). Furthermore, *in vivo* chromatin immunoprecipitation assays in yeast have shown that, after being loaded at origins, MCMs appear to move away from origins during S-phase with similar kinetics as the DNA polymerases, arguing that MCMs are a component of the replication fork (Aparicio et al., 1997). Less compelling arguments derive from the observation that many other replicative helicases (including those from *Escherichia coli*, SV40, and the bacteriophages T4 and T7) form hexamers that can encircle DNA (reviewed in Baker and Bell, 1998). Electron microscopy of purified MCM proteins from *S. pombe* has revealed a globular structure with a central cavity, suggesting that this complex may also encircle DNA (Adachi et al., 1997). Furthermore, Cdc6p, which is required for loading MCMs onto DNA, has recently been shown to be related to prokaryotic and eukaryotic clamp loaders, protein complexes that assemble a ring-shaped processivity factor around the DNA to improve the processivity of the DNA polymerase

(Perkins and Diffley, 1998). In isolated chromatin preparations following MCM loading, Cdc6p and ORC can be removed from the DNA by salt treatments that only partially remove the MCMs (Donovan et al., 1997). These data are consistent with the hypothesis that MCMs are loaded onto DNA by Cdc6p to form a hexameric ring that encircles the DNA and is resistant to removal by salt due to its topological linkage.

In addition to Cdc6p, MCM proteins are also putative targets for regulation by cell-cycle dependant kinases. Cdc7/Dbf4p is a kinase required for entry into S-phase (see below). A point mutation in *mcm5* was isolated as a bypass suppressor of *cdc7* null mutants (and *dbf4* mutants; Hardy et al., 1997). These data argue either that Cdc7p/Dbf4p activates Mcm5p and that the *mcm5* mutation causes it to be constitutively activated, or that Mcm5p has both positive and negative roles in DNA replication and that kinase activity overcomes the repressive function. Mcm2p also interacts genetically with Cdc7p/Dbf4p (a screen for suppressors of an *mcm2-1* mutation identified a mutation in *dbf4*; Lei et al., 1997), and a negative role for Mcm2p has been suggested by the finding that murine Mcm2p inhibits the helicase activity of the human Mcm4p, 6p, and 7p complex (Ishimi et al., 1998). If yeast Mcm2p also acts to repress DNA replication, then phosphorylation by Cdc7p/Dbf4p may relieve this repression. Finally, various MCM proteins have been shown to interact physically with or serve as *in vitro* substrates for Cdc7p kinase activity in yeast, *Xenopus* and human cells (Lei et al., 1997; Roberts et al., 1999; Sato et al., 1997).

The activity of CKDs may also negatively regulate MCM function at the end of S-phase. In mammalian cells, Mcm2p and 4p are typically hyperphosphorylated and released from chromatin in G2 (Fujita et al., 1998). When a cell line carrying a temperature-sensitive mutation in the Cdc2 kinase (the mammalian CDK required for B-type kinase activity) was tested at the non-permissive temperature, both phosphorylation

of Mcm2p and 4p and the release of MCMs from chromatin were impaired. Other work has shown that the use of the serine-threonine protein kinase inhibitor 6-dimethylaminopurine (DMAP) allows nuclei isolated from G2 HeLa cells to replicate in *Xenopus* egg extracts, whereas untreated G2 nuclei do not normally replicate (Coverley et al., 1996). DMAP treatment allows the MCMs that are normally released from chromatin in G2 nuclei to reassociate with chromatin DNA and allows for replication even in extracts that are immuno-depleted of *Xenopus* MCM proteins (Coverley et al., 1998).

Following MCM loading, Cdc45p is recruited to origins. *CDC45* was first isolated as a mutation defective for cell cycle progression and showed genetic interactions with two MCM genes (Moir et al., 1982). Further characterization of this gene product demonstrated that it has a role in the initiation of DNA replication (Zou et al., 1997) and that this protein likely acts at the same time as Cdc7p/Dbf4p during replication initiation (Owens et al., 1997). Cdc45p is recruited to origins at a time that correlates with the loading of the replicative DNA polymerases and the firing of origins (Aparicio et al., 1997; Zou and Stillman, 1998). Like the MCMs, Cdc45p also appears to move from the origins in a manner that suggests it is part of the replication fork (Aparicio et al., 1997). In *Xenopus* cells, Cdc45p is required for the loading of DNA Polymerase α and it co-localizes with the polymerase, consistent with a role for Cdc45p in the moving replication fork (Mimura and Takisawa, 1998). Unlike Cdc6p and MCMs, which are found to load onto both early and late-firing origins at the same time, the association of Cdc45p with chromatin is temporally regulated such that it binds to late origins after it has been recruited to early origins (Aparicio et al., 1999). Furthermore, cell-cycle checkpoints that block DNA replication in response to DNA damage or stalled replication forks prevent the association of Cdc45p with late origins. Since Cdc45p is required for loading DNA polymerases onto DNA in yeast (Aparicio et al., 1999) as well as *Xenopus*,

it is a likely target for the regulatory mechanisms that prevent late origins from firing until the appropriate time during S-phase or in the presence of activated cell-cycle checkpoints.

Properties of the Cell-Cycle Regulated Kinases

Cell cycle progression is controlled by the action of cyclin dependent kinases (CDKs) whose activities rise and fall in a characteristic manner during the cell-cycle (reviewed in Murray and Hunt, 1993). These enzymes are activated by associating with a regulatory subunit called a cyclin, and the levels of cyclins are regulated in a cell-cycle dependent manner. Thus, the oscillating levels of cyclins (in part) lead to the oscillating levels of CDK activity. Cyclins fall into three general categories depending on the cell-cycle stage in which they are most active: G1 cyclins, S-phase cyclins, and mitotic cyclins . In *S. cerevisiae* the S-phase and mitotic kinase activities depend on six B-type (or CLB) cyclins, CLB1-6 (reviewed in Mendenhall and Hodge, 1998; Roberts, 1999). Normally, Clb5p and Clb6p stimulate S-phase kinase activity and Clb1p – Clb4p are associated with mitotic kinase activity. As described above, B-type cyclins both activate pre-RCs at the G1-S transition (either directly or indirectly) and prevent pre-RC formation during S-, G2- and M-phases. B-type cyclins may also perform a function required during the elongation phase of DNA replication since ongoing DNA synthesis in a soluble yeast system requires the activity of the Clb5-Cdc28 kinase (Duncker et al., 1999).

Analyses of clb5 and clb6 mutant cells have supported their role in promoting S-phase. In a *clb5* mutant, DNA replication initiates at the appropriate time but lasts almost twice as long as a conventional S-phase (Epstein and Cross, 1992; Kuhne and Linder, 1993). A *clb6* mutant has a normal onset and duration of S-phase, but the start of S-

phase in a *clb5 clb6* double mutant is substantially delayed (Schwob and Nasmyth, 1993). Interestingly, the length of S-phase in these double mutant cells (driven by the mitotic cyclins, Clb1p-Clb4p) is similar to that in wild-type cells, implying that the prolonged S-phase phenotype of the *clb5* mutant is suppressed by the additional mutation in *clb6*. Clb5p is also involved in temporal regulation of DNA replication (Donaldson et al., 1998). Clb6p normally activates early but not late origins; therefore, in a *clb5* cell (in which only Clb6p is active during S-phase), late origins are not activated, resulting in a longer S-phase. Clb5p can activate both early and late origins, resulting in an apparent normal S-phase in *clb6* cells (in which only Clb5p is active during S-phase). Finally, in *clb5 clb6* double mutants, the onset of DNA replication is delayed because S-phase cannot be initiated until the mitotic kinases are active. However, since the mitotic kinases activate both early and late origins in the correct sequence, the duration of S-phase is normal. The mechanism by which early and late replication origins are selectively activated remains unclear. It is not yet known whether Cdc45p loading onto late origins is prevented in *clb5* cells in which these origins do not initiate.

In addition to the CDKs, a new class of cell-cycle regulated kinases required for cell-cycle progression have been identified (reviewed in Johnston et al., 1999). Cdc7p is a kinase required in *S. cerevisiae* for DNA replication (Hartwell, 1973). This enzyme interacts with Dbf4p, a protein whose levels oscillate during the cell-cycle in a manner reminiscent of cyclins (Cheng et al., 1999). The levels of Dbf4p increase at the onset of S-phase and decline rapidly as cells exit from mitosis and begin the next cell cycle. Cdc7p and Dbf4p-related proteins have been identified in fission yeast and metazoans, and this class of proteins has therefore been named the Dbf4p dependent kinases (DDKs).

Rather than being required simply to trigger DNA replication, Cdc7p/Dbf4p appears to act directly on individual origins throughout S-phase (Bousset and Diffley,

1998; Donaldson et al., 1998). The requirement for Cdc7p activity at each origin is consistent with a previous finding that Dbf4p could be localized to the *ARS1* origin (Dowell et al., 1994). These authors used a "one-hybrid" assay in which a library was screened to identify genomic fragments fused to a transcriptional activator that could induce transcription of reporter genes downstream of the *ARS1* sequence – Dbf4p was identified multiple times in this screen. The origin-interacting domain of Dbf4p can be separated from its Cdc7p-interacting domain; however, both are essential for yeast cell viability.

Various mutations in the *ARS1* sequence interfered with the ability of Dbf4p to be recruited to the origin (Dowell, et al., 1994). A mutation in the essential ACS element or the important B1 element (see Chapter II, Figure 1) that abolishes or reduces ORC binding, respectively, also abolished or reduced Dbf4p-origin association. Interestingly, a mutation in the B2 element also reduced the strength of the Dbf4p-origin association. Unpublished chromatin immunoprecipitation data from our lab has demonstrated that the same mutation in B2 reduces MCM loading at the *ARS1* origin (O. Aparicio and S. P. B., unpublished observations). Given the numerous direct interactions observed between Cdc7p and MCM proteins (Lei et al., 1997; Roberts et al., 1999; Sato et al., 1997), it is likely that Cdc7p/Dbf4p is brought to origins via an interaction that requires MCM proteins.

Overview

The overall goals of my thesis research were to understand the mechanisms underlying the initiation of DNA replication and the regulation of this process in eukaryotes. I chose to focus on elucidating the biochemical properties of the *S. cerevisiae* Origin Recognition Complex, the central player in the initiation process and a

possible target of cell-cycle regulation. When I began this work, little was known about the functions of ORC, except than that it bound origin DNA in the presence of ATP and that it displayed genetic interactions with multiple other genes involved in DNA replication. I therefore began by characterizing the known property of ORC, DNA binding. In Chapter II, I describe the elucidation of the architecture of ORC bound to origin DNA, with descriptions of the DNA residues important for ORC-origin association, the subunits of ORC directly contacting DNA and the arrangement of these subunits with respect to each other and the origin sequences. In Chapter III, I have taken advantage of electron microscopy (EM) to examine individual molecules of ORC and to address questions regarding the shape of ORC and stoichiometry of its subunits, and to directly examine the structure of the associated origin DNA. Finally, in Chapter IV, I have determined that ORC likely exists in two distinct states during the cell-cycle. In addition to the form of ORC bound to double-stranded origin DNA, ORC adopts a different conformation (as assayed by EM) when bound to single-stranded DNA. This change in conformation is associated with a switch in (at least) one biochemical property of ORC and may be a key mechanism in the regulation of DNA replication. Thus, the experiments described in this thesis are relevant for understanding both the mechanism of replication initiation and the manner in which this process is controlled.

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Chapter II

Architecture of the Origin Recognition Complex Bound to Yeast Origins of Replication

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SUMMARY

The replication of DNA in many organisms requires the binding of a protein called the initiator to DNA sites referred to as origins of replication. Analyses of multiple initiator proteins bound to their cognate origins have provided important insights into the mechanism by which DNA replication is initiated. To extend this level of analysis to the study of eukaryotic chromosomal replication, I have investigated the architecture of the *Saccharomyces cerevisiae* Origin Recognition Complex (ORC) when bound to yeast origins of replication. Determination of DNA residues important for ORC-origin association indicated that ORC interacted preferentially with one strand of the *ARS1* origin of replication. DNA binding assays using ORC complexes lacking one of the six subunits demonstrated that the DNA binding domain of ORC required the coordinate action of five of the six ORC subunits. Protein-DNA crosslinking studies suggested that recognition of origin sequences is mediated primarily by two different groups of ORC subunits that make sequence-specific contacts with two distinct regions of the DNA. Implications of these findings for ORC function and the mechanism of initiation of eukaryotic DNA replication are discussed.

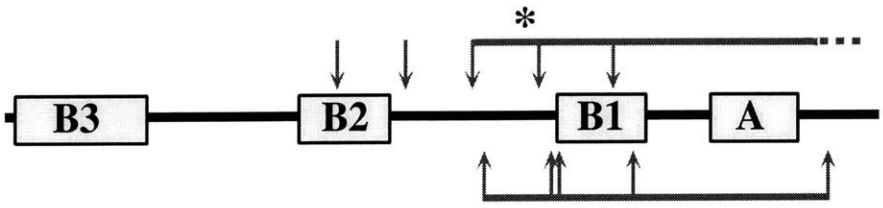
INTRODUCTION

The initiation of DNA replication is a complex process involving multiple regulated steps, including the selection of the initiation site on the DNA, unwinding of the DNA helix, and the assembly of a multi-protein replication machine. Studies of the replication of bacteria, phage, and eukaryotic viral genomes have established that a protein called the initiator binds its cognate origin of DNA replication in a sequence-specific manner. Once bound, initiator proteins often participate in other aspects of replication initiation including facilitating origin unwinding and the recruiting other replication proteins to the origin (reviewed in Baker and Bell, 1998; Kornberg and Baker, 1992). Detailed analyses of initiator proteins bound to their cognate origins have been important in determining how these proteins function during replication initiation. Our aim is to extend this type of analysis to a putative eukaryotic chromosomal initiator protein bound to an origin of DNA replication.

The *Saccharomyces cerevisiae* Origin Recognition Complex (ORC) clearly performs at least two of the three general functions of initiator proteins. As described in Chapter I, ORC binds to origins of DNA replication *in vitro* and *in vivo* and ORC recruits other replication proteins to origins to assemble the pre-RC. In this chapter, I describe work aimed at characterizing the first activity, sequence-specific DNA binding. The ability of ORC to bind origins is essential for yeast cell viability. Mutations in origin sequences that reduce or eliminate origin function also reduce or eliminate ORC binding *in vitro* (Bell and Stillman, 1992; Rao and Stillman, 1995; Rowley et al., 1995) and *in vivo* (Aparicio et al., 1997; Tanaka et al., 1997). In addition, most conditional mutations in ORC genes lead to decreased origin usage (Fox et al., 1995; Liang et al., 1995) and decreased origin binding *in vivo* (Aparicio et al., 1997; O. Aparicio and S. P. B., unpublished observations).

S. cerevisiae contains the best defined eukaryotic origins of DNA replication. These elements were first identified as genomic DNA sequences capable of supporting the autonomous replication of episomal DNA (Autonomous Replicating Sequences or ARSs) (Hsiao and Carbon, 1979; Stinchcomb et al., 1979). Many of these elements were subsequently shown to act as origins of replication in their normal chromosomal context (reviewed in Newlon and Theis, 1993). Yeast origins are modular in nature and contain a conserved 11 base-pair ARS Consensus Sequence (ACS) that is essential for ORC-DNA binding and origin function *in vivo*, as well as additional elements that enhance origin function (generally referred to as B elements; Bell, 1995). *ARS1*, the first well-characterized origin, has three such elements (B1, B2 and B3; see Figure 1). *In vitro* and *in vivo*, DNase I protection assays of *ARS1* demonstrated that ORC protects approximately fifty base pairs of DNA that include the ACS and B1 sequences (Bell and Stillman, 1992; Diffley and Cocker, 1992; Santocanale and Diffley, 1996). The DNase I cleavage pattern also contains several sites that become hypersensitive to digestion in the presence of ORC. These sites are spaced roughly 10 base-pairs (bp) apart, suggesting that ORC wraps DNA around itself or that the DNA lies along a flat surface created by ORC (Travers and Klug, 1987). The A and B1 elements direct ORC-DNA binding at *ARS1* and at *ARS307* (Rao et al., 1994; Rowley et al., 1995), and together represent the smallest functional region of either origin. I will refer to this minimal region required for ORC-DNA binding and origin function as the Core origin.

The role of the other B elements in origin function is not yet clear. The B2 element may contribute origin melting during the initiation process (reviewed in Bell, 1995). The origin of bidirectional replication (OBR, the site at which discontinuous replication switches to continuous replication) has been mapped to a site in between the B1 and B2 elements of *ARS1* (Bielinsky and Gerbi, 1998; Bielinsky and Gerbi, 1999),



length (nt):	18	29	11	26	13	9	11
function:	ABF1 binding		MCM loading? DNA unwinding?		ORC binding		ORC binding
conserved at other origins?	NO		NO		"yes"		YES

Figure 1. Properties of Functional Elements at *ARS1*. The *ARS1* elements are as previously described (Marahrens and Stillman, 1992). The A element contains the conserved ARS Consensus Sequence (ACS) and is essential for replication, while the B elements enhance replication function. The ACS and B1 elements together comprise the ORC binding site and form the Core origin. B1-like elements are found at other yeast origins and can functionally substitute for the *ARS1* B1 element. The B2 element is required for loading MCM proteins onto the *ARS1* origin (O. Aparicio and S. P. Bell, unpublished results). B3 is the binding site for the transcriptional activator, ABF1; it can be functionally substituted by a binding site for other transcriptional activators. The region of DNA protected from DNase I cleavage by ORC is indicated by red horizontal lines above and below the ARS1 schematic. ORC-induced sites of increased DNase I cleavage are indicated by arrows. The position of the origin of bidirectional replication (OBR) is indicated by the blue asterisk (Belinsky and Gerbi, 1999).

consistent with initial origin unwinding occurring near B2. Furthermore, a linker substitution mutation in B2 that decreases origin function also inhibits MCM loading at *ARS1* (O. Aparicio and S. P. B., unpublished observations). If MCMs function as the replicative helicase (see Chapter I), they would likely be loaded onto origins at sites of initial origin unwinding. The B3 element is a binding site for the ABF1 transcriptional activator and it can be functionally substituted by a binding site for other transcriptional activators (Marahrens and Stillman, 1992). One possible role for these transcription factors in origin function is to regulate chromatin structure. The *ARS1* origin was shown to have a unique chromatin structure important for origin function, with a nucleosome free region encompassing the A and B elements and precisely positioned nucleosomes to either side (Simpson, 1990; Thoma et al., 1984). Recent work from our lab has suggested that ORC and ABF1 are required to establish this specific chromatin structure (discussed in Chapter V).

We have a general understanding of the DNA sequence requirements for the association of ORC with origins (Bell and Stillman, 1992; Rao and Stillman, 1995; Rowley et al., 1995) but previous studies have not examined the requirements of different ORC subunits for DNA binding. A thorough understanding of ORC bound to yeast origins of replication can address three important questions: (1) How does ORC interact with DNA? In particular, I wanted to determine which of the approximately 50 base pairs protected from DNase I cleavage are important for ORC-DNA binding and how the structure of origin DNA is affected by interaction with ORC. Additionally, since none of the six ORC subunits contain a canonical DNA binding motif, I wanted to identify the ORC proteins are required for DNA binding. (2) How do ORC subunits interact with each other? ORC is a pre-assembled complex in the absence of DNA and I wanted to understand the organization of ORC subunits both in solution and in DNA-complexes.

An understanding of the spatial arrangement of ORC subunits is also relevant to the third question. (3) How does ORC interact with other proteins? In addition to genetic and physical interactions with the CDC6 gene product (Liang et al., 1995), ORC subunits show multiple genetic interactions with other essential genes required for DNA replication (reviewed in Dutta and Bell, 1997). Since ORC plays a central role in assembling higher order structures at origins, the arrangement of ORC subunits will undoubtedly influence the formation of these larger protein-DNA complexes.

In the experiments described in this chapter, I used DNA modification to identify specific residues in *ARS1* involved in ORC-DNA binding. DNA-bending studies were used to investigate ORC-induced structural changes in origin DNA. In addition, I used analysis of ORC complexes lacking one of the six subunits and protein-DNA crosslinking to determine which ORC subunits are required for DNA binding and how these subunits are arranged along the origin DNA. Together these studies provide a detailed view of ORC bound to origin DNA.

RESULTS

Residues of *ARS1* Required for ORC-DNA Binding

To identify residues of *ARS1* important for ORC-DNA binding *in vitro*, DNA modification-interference and missing-contact assays were performed. In both assays, the geometry of the DNA is altered at particular sites to identify important protein-DNA interactions. Modification-interference analysis involves changing the shape of DNA by adding an adduct to a DNA structure. In contrast, missing-contact assays involve the removal of a base (leaving the phosphate backbone intact). The two assays are complementary: if a particular site inhibits protein-DNA binding in both assays (either by being modified or by being removed), then it is likely to represent an important region of the DNA for protein association. Since, in the case of ORC-DNA binding I found that modification-interference and missing-contact experiments identified similar residues (Figure 2), I will describe both the addition of an adduct and the removal of a base simply as modification of a residue. In these assays, DNA fragments end-labeled on either the top or bottom-strand of *ARS1* were modified with one of five reagents prior to ORC-DNA binding (see Figure 2A legend for a description of reagents and their resulting DNA modifications). The modified DNA was incubated with purified ORC and electrophoresed on a gel to separate bound and unbound DNA molecules. DNA modifications that inhibit ORC-DNA binding are reduced or absent in the bound DNA populations.

The individual residues whose modification most strongly inhibited ORC-DNA binding were located in a region spanning the ACS and B1 elements of *ARS1* (Figure 2A, and highlighted residues in Figure 2B). ORC-DNA binding was extremely sensitive to modification of any residue within the ACS, consistent with the essential role of this element in both ORC-DNA binding and origin function. In contrast, only some of the

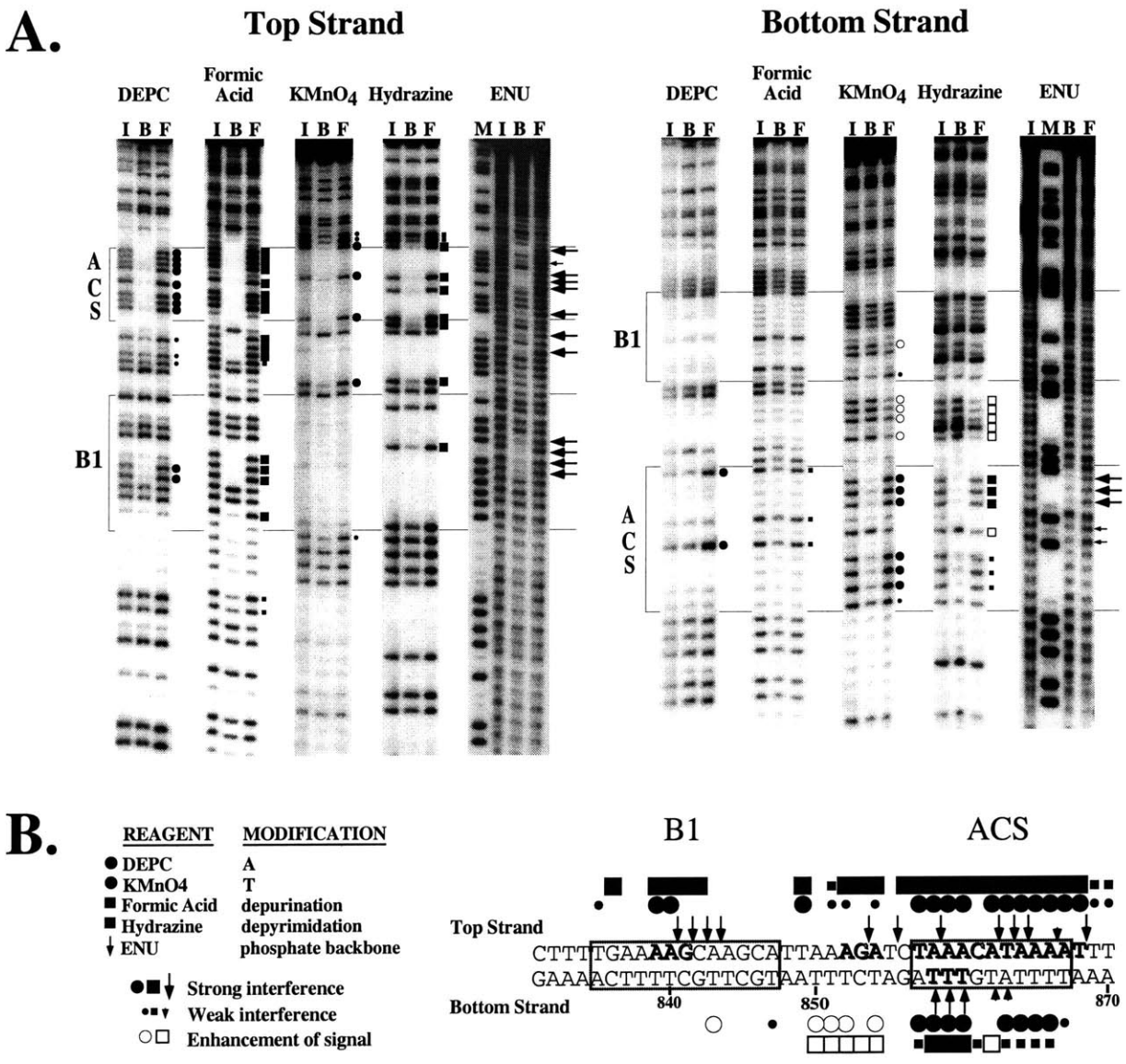

A.
Top Strand
Bottom Strand
DEPC
Formic Acid
KMnO4
Hydrazine
ENU
I B F
M I B F
I M B F
A C S
B1
B.
REAGENT
MODIFICATION
DEPC
A
KMnO4
T
Formic Acid
depurination
Hydrazine
depyrimidation
ENU
phosphate backbone
Strong interference
Weak interference
Enhancement of signal
B1
ACS
Top Strand
Bottom Strand
CTTTTTGAAAAAGCAAGCATTAAAAGATCTAAACATAAAATTT
GAAAACTTTTCGTTCGTAATTTCTAGATTTGTATTTTAAA
840
850
870

Figure 2

Figure 2. Residues of *ARS1* required for ORC-DNA binding. (A) Modification-interference and missing-contact assays were performed on a minimal *ARS1* fragment, end-labeled on either the top-strand (left half of figure) or the bottom-strand (right half). DNAs were subjected to modification by diethyl pyrocarbonate (DEPC, major groove carbethoxylation of adenines), formic acid (depurination), KMnO4 (major groove modification of thymines), hydrazine (depyrimidation), and ethyl nitroso-urea (ENU, for phosphate backbone ethylation). Treated DNAs were incubated with 600 ng of purified ORC, and bound and unbound molecules were separated. In each panel, input DNA (I), bound DNA (B), and free DNA (F) were cleaved at sites of modification and analyzed on a sequencing gel. Modified residues that inhibited or enhanced binding are labeled with filled objects or open objects respectively to the right of each panel. Results from modification-interference assays are indicated by circles for base modification and arrows for phosphate ethylation, and missing-contact data are represented by squares. Strong and weak interference is distinguished by large and small objects respectively. The position of the ACS and B1 elements are indicated by brackets. (B) Summary of modification-interference and missing-contact data. Clusters of residues that most strongly inhibit ORC-DNA binding in both assays are outlined. Numbers below the sequence correspond to *ARS1* coordinates used by Marahrens and Stillman (Marahrens and Stillman, 1992).

bases in the genetically defined B1 element inhibited ORC-DNA binding when modified, suggesting that the remainder of this element contributes to an origin function not involved in ORC-DNA binding (Rao and Stillman, 1995). The modification data also exhibited strand-specific differences. Modification of the top-strand consistently affected ORC-DNA binding to a greater extent than modification of the bottom-strand. Within the ACS, top-strand modification interfered with ORC-DNA binding more than bottom-strand modification. Within the B1 element, the residues whose modification interfered with ORC-DNA association were exclusively on the top-strand. Strand specific differences were most striking in a region between the ACS and B1, where modification of the two strands had opposite effects. Top-strand modification interfered with ORC-DNA binding whereas bottom-strand modifications were over-represented in the bound DNA population.

To determine if the region of *ARS1* between the ACS and B1 is important for origin function, I mutated residues 852 to 854, changing AGA to either GAG or CTC. Both mutations were tested for plasmid stability and ORC-DNA binding *in vitro* (Table 1). The AGA to GAG mutation had no detectable defect *in vivo* or *in vitro*, whereas the AGA to CTC substitution resulted in a 2-fold decrease in both plasmid stability and binding *in vitro*. This region had not been identified in a previous genetic analysis of *ARS1* presumably because a linker substitution of the sequences between the ACS and B1 did not make transversion mutations in the important AGA (Marahrens and Stillman, 1992). Thus, this region contributed to *ARS1* function and appeared to show a preference for purines on the top-strand and pyrimidines on the bottom-strand.

TABLE 1. Effect of Mutations in Region 852-854 of ARS1

Plasmid Name	Sequence (850-855)[a]	Plasmid Stability			Defect in Mobility Shift Activity
		Loss Rate per Generation (%)	SD	no. of Samples	
ARS1/WtA	AAAGAT	4.6	0.5	6	none
ARS1/GAG	AA**gag**T	4.3	0.9	11	none
ARS1/CTC	AA**ctc**T	10.8	1.1	6	2.4-fold
ARS1/844-850[b]	AAAGAT	13.3	2.3	3	*n.d.*
ARS1/835-842[b]	AAAGAT	27.9	1.7	3	13-fold

n.d. = not determined

[a] Sequences differing from wild type are indicated with bold-face, lower-case letters

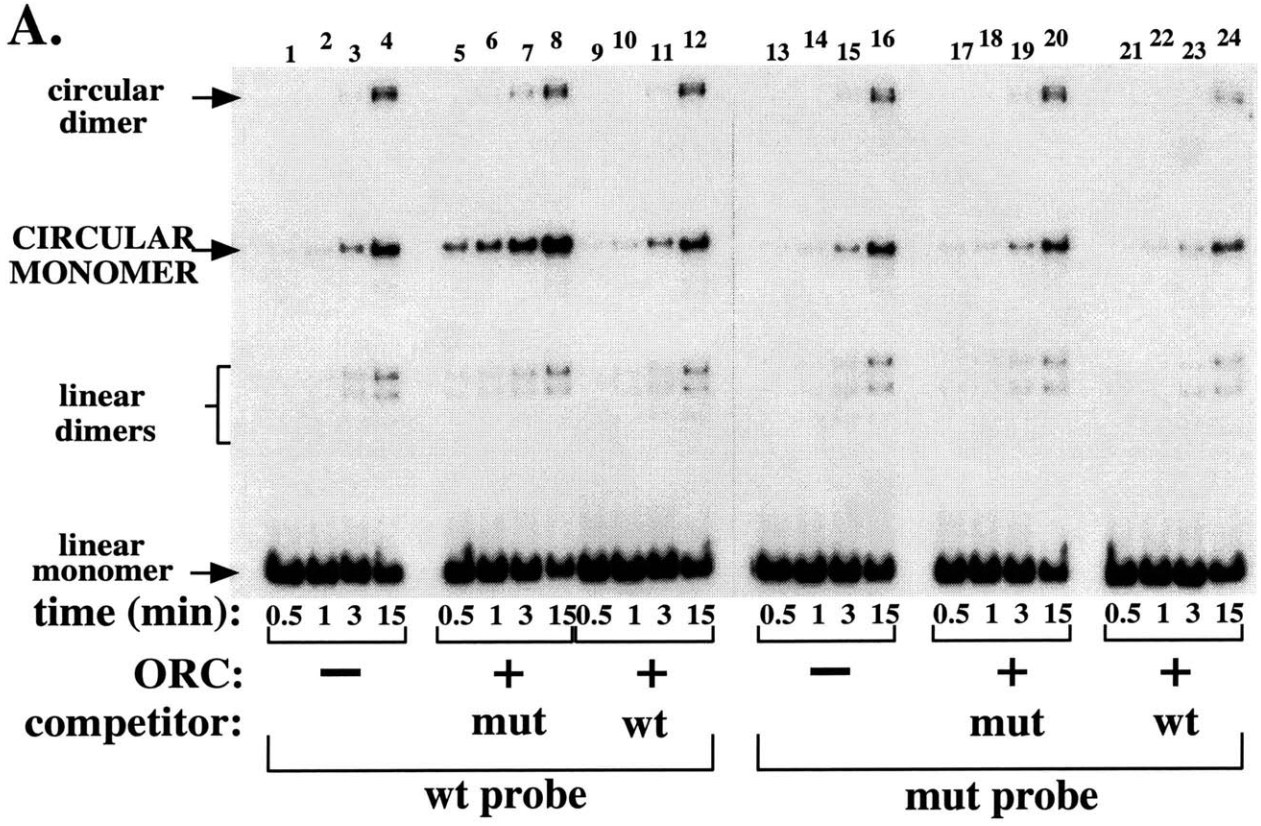
[b] Linker substitutions of the B1 element, previously described (Marahrens and Stillman, 1992)

ORC Bends DNA at Some but not All Origins

Because some initiator proteins induce DNA bending at origins as a precursor to DNA unwinding (Borowiec and Hurwitz, 1988; Bramhill and Kornberg, 1988), I investigated whether ORC bends yeast origin DNA. A radio-labeled *ARS1* fragment with cohesive ends was incubated with DNA ligase, and the rate of formation of circular monomers was monitored, either with or without ORC (Figure 3A). In the presence of ORC, the rate of monomer circle formation was stimulated 3-fold, consistent with the interpretation that ORC bends *ARS1* DNA, thereby facilitating the ligation of the two DNA ends. The observed stimulation of ligation required specific ORC-DNA binding as demonstrated by competition experiments with wild-type or mutant competitor DNAs (Figure 3A, compare lanes 9-12 with lanes 5-8) and by experiments using a DNA-bending template containing a mutated ORC binding site (Figure 3A, lanes 13-24). To determine if bending of origin DNA was a general property of ORC, two other ARS elements were examined. *ARS121* and *ARS305* are both active chromosomal origins of replication (Huang and Kowalski, 1996; Walker et al., 1991). Although ORC bound strongly to these origins (data not shown), neither was bent by ORC (Figure 3B). Thus, bending of DNA by ORC appears to occur at *ARS1* but is not a general property of ORC interaction with all origins of replication.

Orc6p is Not Required for DNA Binding

To determine the role of individual ORC subunits in ORC-DNA binding, I produced six different mutant ORC complexes, each missing a different ORC subunit (referred to as partial ORC complexes). Insect cells were co-infected with baculoviruses expressing five of the six ORC polypeptides, and these mutant ORC complexes were partially purified. The integrity of all six partial complexes was examined by


A.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
circular dimer
CIRCULAR MONOMER
linear dimers
linear monomer
time (min): 0.5 1 3 15 0.5 1 3 15 0.5 1 3 15 0.5 1 3 15 0.5 1 3 15 0.5 1 3 15
ORC: − + + − + +
competitor: mut wt mut wt
wt probe
mut probe

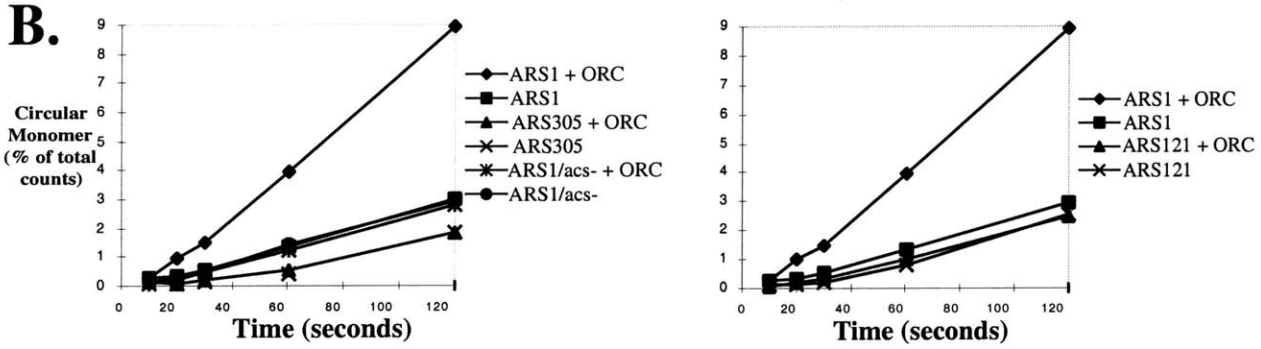

B.
Circular Monomer (% of total counts)
Time (seconds)
ARS1 + ORC
ARS1
ARS305 + ORC
ARS305
ARS1/acs- + ORC
ARS1/acs-
Time (seconds)
ARS1 + ORC
ARS1
ARS121 + ORC
ARS121

Figure 3

Figure 3. ORC induced DNA bending at *ARS1*. (A) Circular monomer formation of radiolabeled *ARS1* fragments in the presence or absence of ORC. After addition of DNA ligase, monomeric linear *ARS1* fragments are converted to circular monomers, circular dimers, and linear dimers. The three electrophoretic species of linear dimers are the result of a weak intrinsic bend in the B3 element (removal of this DNA bend using a B3 linker substitution yielded identical results; data not shown). The rate of circular monomer formation was tested in the presence (lanes 5-8 and 9-12) or absence of ORC (lanes 1-4). ORC-*ARS1* binding and ligation reactions were carried out in the presence of a fifty-fold excess of unlabeled competitor DNA containing a wild-type *ARS1* binding site (lanes 9-12) or a mutant *ARS1* binding site (lanes 5-8). A radiolabeled DNA probe containing a mutated *ARS1* binding site was also tested (the ARS1/acs-b2- mutation; lanes 13-24). (B) Circular monomer formation was measured for *ARS305* (left graph) and *ARS121*(right graph) in the presence or absence of ORC (triangles or X's respectively). Both graphs also show quantitation of cyclization rates for wild-type *ARS1* DNA in the presence or absence of ORC (filled diamonds and filled squares respectively) and the left graph includes data for an *ARS1* DNA fragment with a linker substitution in the ACS for comparison (pARS1/858-865 with and without ORC, asterisks and filled circles respectively). The amount of circular monomer produced at each time point was expressed as the percentage of total counts in each lane.

determining the ORC subunits present in the partially purified fractions. In two cases, the omission of one subunit clearly compromised complex integrity; the omission of Orc3p reduced the amount of Orc2p present, and the lack of Orc5p led to a loss of Orc4p from the complex (Figure 4B). Protein fractions containing the six partial complexes were next assayed for ORC-DNA binding activity. Of the six partial complexes tested for *ARS1* DNA binding, five were inactive. A complex lacking Orc6p, however, was capable of binding origin DNA in a sequence-specific (data not shown) and ATP-dependent manner (Figure 4A). This protein-DNA complex is likely to contain all five of the ORC proteins present in the partial complex fraction as all five subunits co-immunoprecipitate and antibodies to three of the subunits (Orc1p, Orc2p and Orc4p) can supershift the partial ORC-DNA complex (data not shown, and Appendix I). Protein-DNA complexes formed with the protein fractions containing the other partial ORC complexes are likely due to other DNA binding proteins. None of these protein-DNA complexes was ATP-dependent (Figure 4A), DNA sequence specific, or supershifted with monoclonal antibodies directed against ORC subunits (data not shown). Thus, only Orc6p is dispensable for the formation of a stable DNA binding complex and ORC-DNA binding activity is not readily attributable to any single polypeptide or small sub-assembly of polypeptides.

Organization of ORC Subunits at *ARS1*

The partial complex experiments described above implicate Orc1p, Orc2p, Orc3p, Orc4p and Orc5p as being important for DNA binding. To define the subunits that are in close proximity to origin DNA and to determine how these subunits are arranged along the length of *ARS1*, I performed UV protein-DNA crosslinking studies. In these experiments, I generated a series of ten *ARS1* crosslinking probes that were modified with

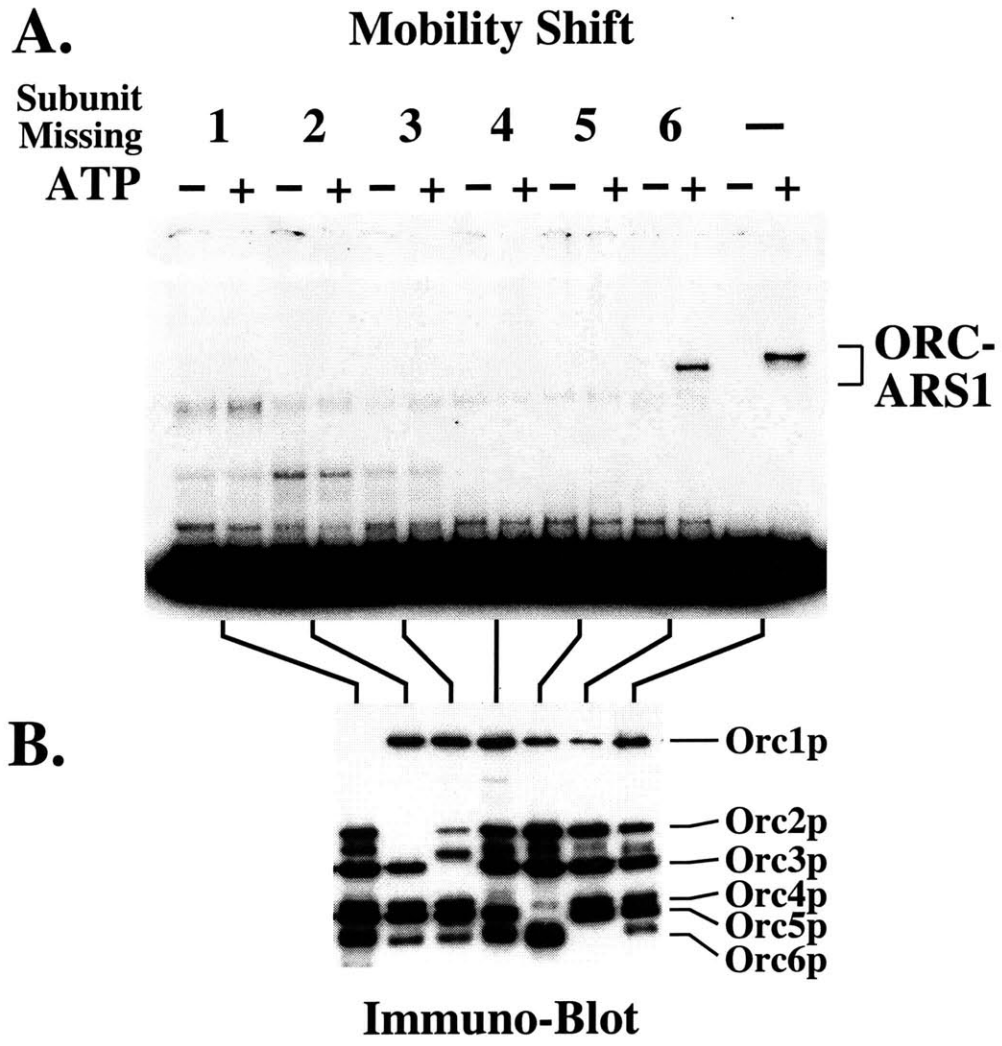


Figure 4. DNA binding properties of partial ORC complexes. (A) Electrophoretic mobility shift analysis of mutant ORC complexes lacking one subunit incubated with a radiolabeled *ARS1* fragment. Partially purified ORC complexes missing Orc1p through Orc6p are tested in the first 12 lanes (left to right), and purified wild-type ORC is shown in the last two lanes as a control. Each pair of lanes shows DNA binding reactions for each ORC complex in the presence or absence of ATP. The positions of ORC-DNA complexes are indicated by the bracket to the right. (B) Integrity of mutant ORC complexes. Peak fractions from an S-Sepharose ion-exchange column for each mutant complex were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose. Wild-type ORC is shown in the last lane. ORC proteins were identified by incubation of blots with monoclonal ORC antibodies.

a photo-reactive azido group on one or two specific phosphate residues. The azido group is coupled to thio-phosphate groups in the DNA backbone through the use of the reagent 4'-azido phenacyl bromide (4'-AZPB) which constrains the photo-reactive group to a distance of approximately 5 to 10 Å from the phosphate backbone. Proteins crosslinked to a particular modified residues are therefore within this distance, and I will describe such proteins as being in close proximity to the DNA at this site. The portion of *ARS1* examined using this set of ten probes is 92 base pairs long and includes the entire region protected from DNase I digestion by ORC (see Figure 5B).

The crosslinking studies demonstrate that different ORC subunits are distributed over different regions of *ARS1*, with a predominant clustering of subunits over the ACS (Figure 5A, and summarized in Figure 8). Orc1p is crosslinked to the right half of the ACS and to regions flanking the ACS on the right (probes I and J). Orc2p and Orc3p are co-localized to the entire ACS and to DNA that extends leftwards toward the B1 element (probes E, F, G, and I); they are the only ORC subunits crosslinked to residues located between the ACS and B1 that contribute to *ARS1* function (probes F and G). Because Orc4p and Orc5p have similar electrophoretic mobilities and are difficult to resolve, I modified Orc4p by fusing an additional 168 amino-acids derived from the Sp1 transcription factor onto its N-terminus (Figure 6A). This modified ORC4 protein migrated above the Orc2p subunit. Crosslinking was performed with a wild-type ORC complex and a complex containing the modified Orc4p and only one crosslinked protein was altered in its mobility, allowing unambiguous identification of Orc4p and Orc5p (Figure 6B). In the wild-type ORC complex, Orc4p is crosslinked strongly to the ACS (Figure 5A; probe H) and more weakly to the right of the ACS (probe J). Orc5p is the only subunit crosslinked to the region of B1 important for ORC-DNA binding (probe D),

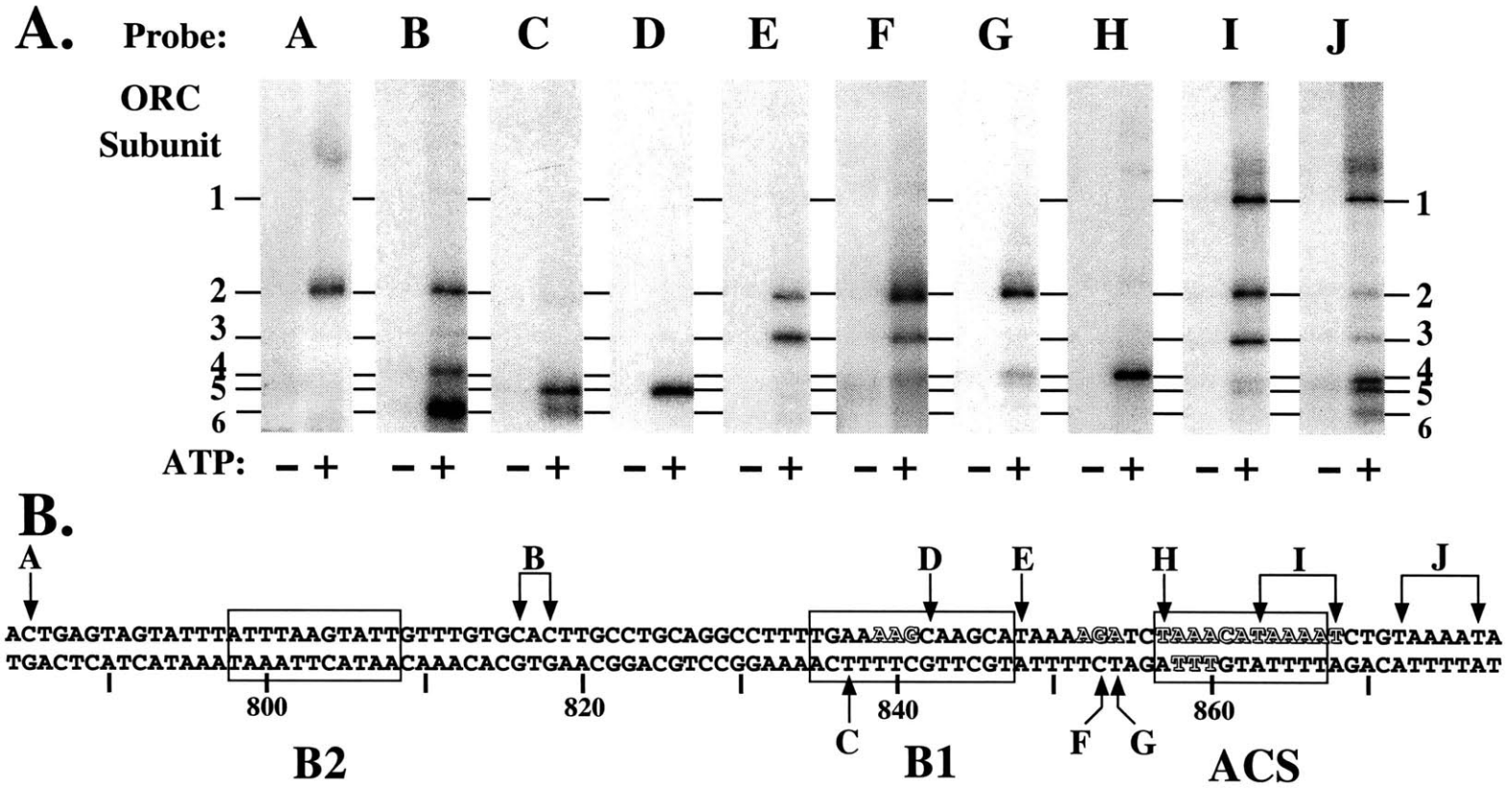


Figure 5. 4'-AZPB crosslinking of ORC subunits to *ARS1*. (A) UV crosslinking of ORC subunits to ten *ARS1* fragments (probes A through J), each incorporating a photo-reactive crosslinker at a distinct site. Purified ORC was incubated with each probe in the presence or absence of ATP. Gels were silver-stained to determine the position of ORC subunits, dried, and exposed to film. In all cases, the observed protein crosslinking was ATP-dependent. Crosslinking to ORC subunits was also UV-dependent, and sensitive to competition by a wild-type *ARS1* fragment (data not shown). (B) Positions of crosslinking nucleotides in *ARS1* probes A through J. Arrowheads identify the modified position; the phosphate coupled to the photo-reactive crosslinker is 5' to the residue indicated. Each probe has either one or two modified bases as indicated.

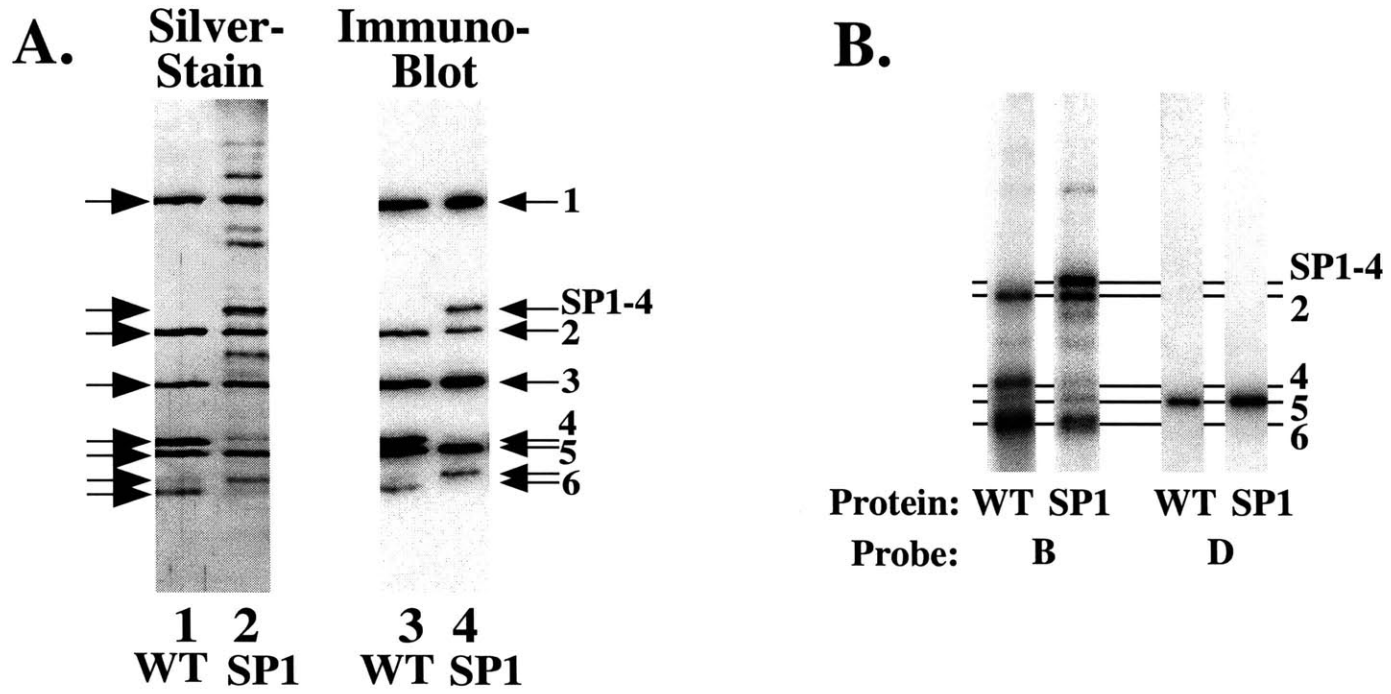


Figure 6. 4'-AZPB crosslinking of a tagged Orc4p-containing ORC. (A) Protein composition of the Sp1/Orc4p fusion complex. I altered the electrophoretic mobility of Orc4p by fusing it to the C-terminal 168 amino acids of the Sp1 transcription factor. This fusion complemented a deletion of ORC4 in yeast cells and an ORC complex with this Sp1/Orc4p hybrid as the sole copy of Orc4p showed normal DNA binding properties (data not shown). The Sp1/Orc4p containing complex was partially purified and electrophoresed beside purified wild-type ORC. Silver staining and immuno-blot analysis indicates that the Sp1/Orc4p fusion migrates above the Orc2p band as expected for a protein of 82 kDa. Orc6p normally runs as doublet due to phosphorylation, and the hybrid complex contains mostly the phosphorylated form. We have detected no differences in *in vitro* properties of wild-type complexes before and after treatment with phosphatase (R. Austin and S.P.B., unpublished data). (B) UV crosslinking of wild-type ORC and an ORC complex containing the Sp1/Orc4p fusion to *ARS1* crosslinking probes B and D. Crosslinking was performed as described in the legend to Figure 5B.

and Orc6p is crosslinked to a residue at the end of the B1 element as well as to residues between B1 and B2 (probes B and C).

Not all ORC subunits were localized to a discrete region of *ARS1*. Although Orc1p, Orc3p and Orc6p are crosslinked to only one region of the DNA, Orc2p, Orc4p and Orc5p are each crosslinked to two distinct regions of *ARS1* with no crosslinking to the intervening sequence (Figure 5A, and summarized in Figure 8). Orc2p, Orc4p and Orc5p are crosslinked to sites on the DNA separated by 78, 54, and 35 base pairs respectively. Crosslinking of subunits to two distinct regions of DNA could be explained by ORC subunits with an elongated shape, by multiple complexes or individual subunits bound at each origin, or by DNA bending at the origin. Based on comparison with protein-DNA crosslinking at *ARS305* (see below), the latter possibility is the most likely (see Discussion).

ORC Subunit Organization at *ARS305*

To determine if the subunit organization of ORC is similar at other origins of DNA replication, *ARS305* was examined with selected probes that placed crosslinkers at positions analogous to those tested for *ARS1* (determined relative to the ACS). Four crosslinking probes that examined the Core binding region of *ARS305* generated similar results to those seen at *ARS1* (compare Figure 5 and Figure 7). Just as in *ARS1*, the ACS of *ARS305* is crosslinked to Orc1p, Orc2p, Orc3p and Orc4p (Probe I^{305}). Orc1p and Orc4p also crosslink to the right of the ACS (Probe J^{305}) and Orc3p crosslinks near the B1 element of *ARS305* (probe E^{305}). Importantly, in the central portion of the *ARS305* B1 element, Orc5p is the sole ORC subunit crosslinked (Probe D^{305}). At *ARS305*, the interaction of Orc2p with the DNA does not extend as far as observed at *ARS1* since this subunit is not crosslinked next to B1 by probe E^{305}. Aside from this difference, the

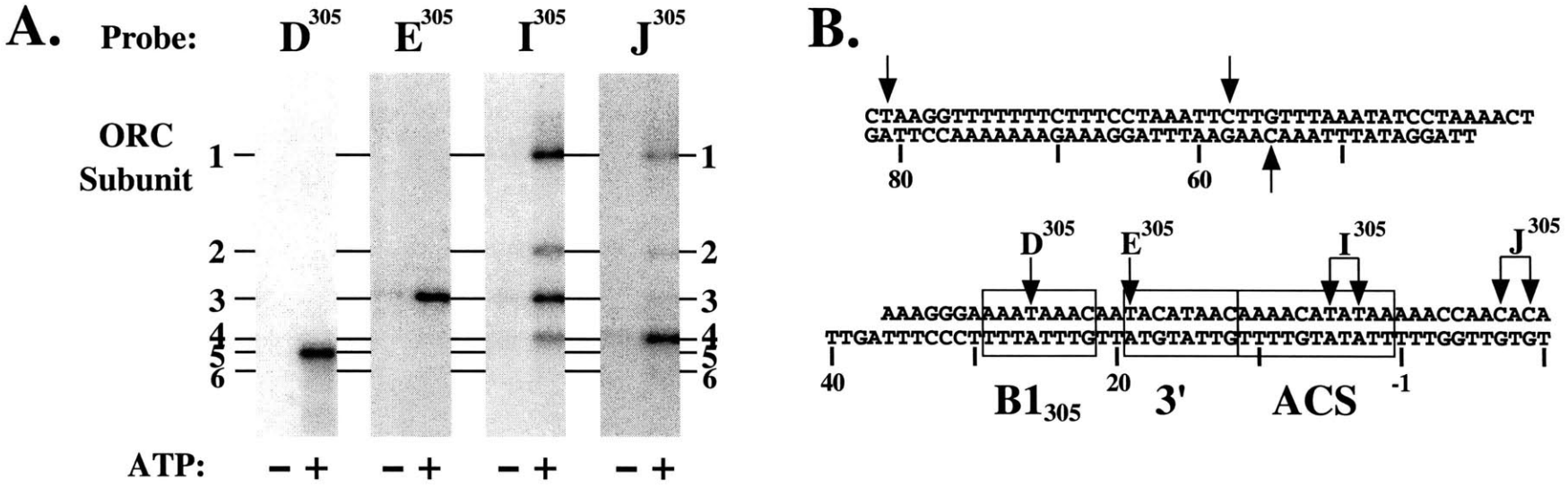


Figure 7. 4'-AZPB crosslinking of ORC subunits to *ARS305*. (A) UV crosslinking of ORC subunits to four *ARS305* fragments analogous to *ARS1* probes D, E, I, and J. *ARS305* crosslinking probes were designed to incorporate crosslinkers at identical or similar sites with respect to the ACS as the *ARS1* probes. (B) Positions of crosslinking nucleotides in *ARS305* crosslinking probes. Symbols are as described in the legend to Figure 4b. Also shown are the positions of crosslinkers in three *ARS305* probes tested for ORC subunit proximity to regions outside of the Core binding site (unlabeled arrowheads, see text). *ARS305* coordinates and elements affecting plasmid stability when mutated (boxed sequences) are as described by Huang and Kowalsky (Huang and Kowalski, 1996).

organization of ORC subunits over the Core DNA binding site is similar at both origins of replications (see Figure 8 for summary).

An interesting difference between *ARS1* and *ARS305* is that the Orc5p subunit is only crosslinked to one discrete site at *ARS305* (it is present only at B1) and not at two sites as in the case of *ARS1*. To determine if the other subunits that crosslinked to two distinct regions of *ARS1* (Orc2p and Orc4p) were also localized to only one region of *ARS305*, I examined sequences beyond the Core *ARS305* binding site. *ARS305* crosslinking probes analogous to *ARS1* probes A and B that crosslinked Orc2p and/or Orc4p, as well as a third probe that modified a nearby residue on the opposite strand, were synthesized (see Figure 7B). These three *ARS305* probes showed no evidence of specific crosslinking (data not shown). Thus, these crosslinking data argue that the association of ORC subunits with the Core origin is likely to be similar at all origins, however ORC-DNA interactions outside of the Core binding region differ.

ORC Subunits in the Major Groove of *ARS1*

Because the crosslinking experiments described above examine a region within a 5 to 10 Å radius from the phosphate backbone, they cannot distinguish between proteins that are directly interacting with DNA and those that are merely in close proximity to the DNA. To identify ORC subunits within van der Waals distance of thymines in the major groove of the DNA, BrdU crosslinking was performed at *ARS1*. DNA fragments that incorporated BrdU and radiolabeled nucleotides into either the top or the bottom-strand of *ARS1* were generated and crosslinked to ORC. DNA fragments modified on either strand both efficiently crosslinked to Orc2p and Orc4p (Figure 9). Orc1p crosslinked to thebottom-strand of *ARS1*, although weak Orc1p crosslinking was occasionally observed

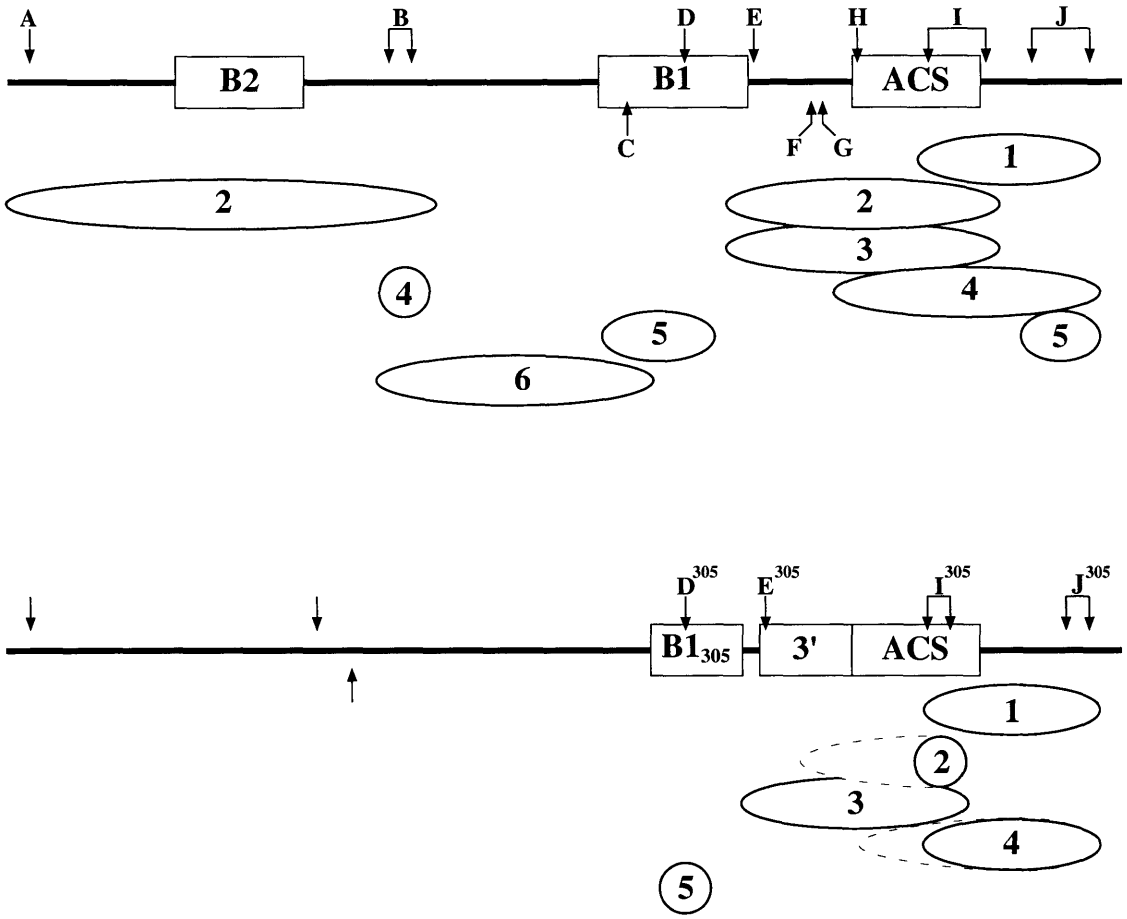


Figure 8. Summary Of ORC subunit arrangement at *ARS1* and *ARS305*. ORC subunits crosslinked to each position are schematized for *ARS1* (top) and *ARS305* (bottom). Dark outlines indicate the minimal extent of each subunit. Thinner outlines indicate hypothesized extents of subunits at *ARS305* in regions not tested. Unlabeled arrows along *ARS305* represent positions of crosslinkers on probes tested for which no sequence-specific or ATP-dependent ORC crosslinking was detected.

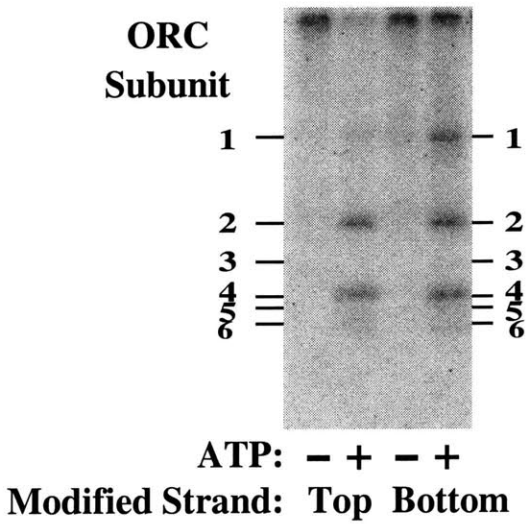


Figure 9. BrdU crosslinking at *ARS1*. *ARS1* fragments incorporating BrdU into either the top-strand or the bottom-strand were incubated with purified ORC. Crosslinking was performed as described in the Figure 5A legend, in the presence or absence of ATP as indicated.

with the top-strand (data not shown). BrdU crosslinking with both strands of *ARS305* also detected these three ORC subunits (data not shown).

DISCUSSION

The interaction of ORC with origin DNA involves six proteins with a combined molecular mass of 414 kDa specifically recognizing a region of DNA approximately 30 base pairs long. I found that the coordinate action of five of the six ORC subunits is required for this interaction (Figure 10). Recognition of critical DNA residues at two genetically defined regions of origins (the ACS and B1-like elements) is likely to be mediated by non-overlapping ORC subunits or groups of subunits. Although I have no evidence of direct DNA contact by Orc5p, the failure to crosslink any other ORC subunit to the B1 region of both *ARS1* and *ARS305* implicates this subunit in the interaction with B1 residues. At the ACS, Orc1p, Orc2p and Orc4p interact with the major groove as all three subunits are crosslinked by BrdU to both *ARS1* and *ARS305* and are within 10 Å of the ACS of both origins. The ORC subunits bound at B1 and the ACS interact physically as partial complex analysis indicates that Orc4p requires Orc5p to associate stably with the remainder of the complex. Together, our studies provide a picture of the ORC-origin association that forms a foundation for other structures assembled at origins.

Conservation of ORC-DNA Interactions at the Origin Core

Our studies argue that the manner in which ORC interacts with its binding site is similar at different yeast origins of replication. A comparison of nine yeast origins shows that the four most highly conserved residues outside of the ACS fall within regions of *ARS1* that strongly inhibited ORC-DNA binding when chemically modified (residues A839, A840, A852 and A854; Figure 10). These residues also fall within regions required for efficient origin function in origins whose structure has been well characterized. The residues at positions 839 and 840 of *ARS1* are conserved in the

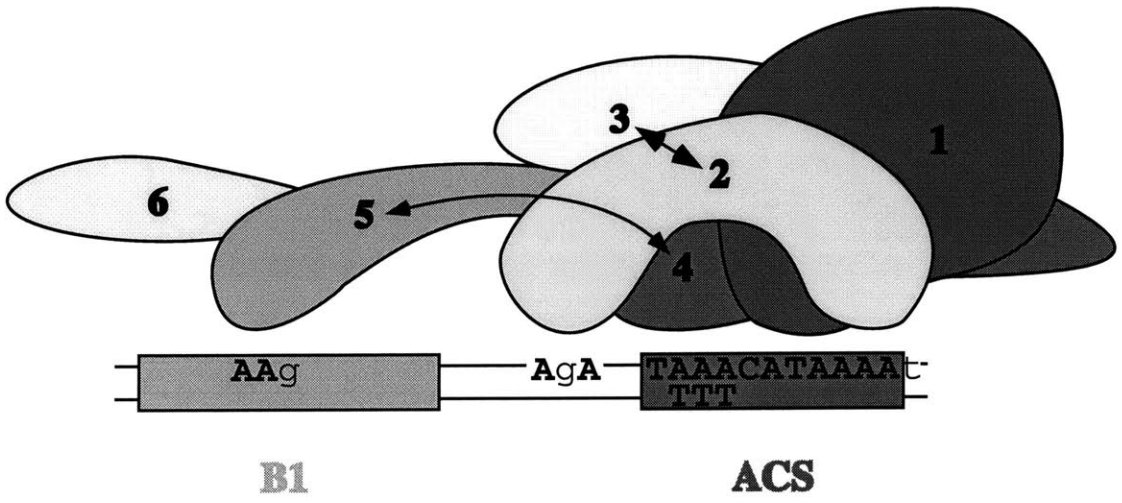


Figure 10. Model of ORC-origin Core interactions. Residues of *ARS1* whose chemical modification strongly interferes with ORC-DNA binding are indicated, and matches to the conserved ARS consensus sequence are shown in bold upper-case letters. Outside of the ACS, bold upper-case letters represent residues that are conserved in seven of nine yeast chromosomal origins of replication examined (A839, A852 and A854 of *ARS1*). A residue conserved in six of nine origins is also shown as an upper-case letter (residues A840). Protein-DNA contacts are made primarily with the top-strand of origins at three clusters of conserved DNA residues. Specific binding of the ACS is mediated by the combined major groove interactions of Orc1p, Orc2p and Orc4p. Orc5p is likely to contact important residues in B1, and Orc6p is dispensable for specific ORC-DNA binding. Physical interactions between Orc2p and Orc3p and between Orc4p and Orc5p (indicated by double arrowheads) are deduced from two lines of evidence: (1) omission of one subunit results in the loss of the second subunit from the complex (Figure 4B, partial complexes lacking Orc3p and Orc5p respectively). (2) Individual 4'-AZPB probes crosslink both subunits. The nine origins used for sequence comparison are *ARS1, ARS121, ARS305, ARS306, ARS307, ARS1413*, the *2 μm ARS*, the *Histone H4 ARS*, and the *HMRE ARS.*

B1-like elements of *ARS305* (Huang and Kowalski, 1996) and *ARS307* (Rao et al., 1994; Theis and Newlon, 1994), and residues at positions 852 and 854 of *ARS1* fall within the Box 3' element of *ARS305* (Huang and Kowalski, 1996), the extended A element of *ARS307* (Rao et al., 1994; Theis and Newlon, 1994) and the extended Core region of *ARS121* (Walker et al., 1991). The similar organization of ORC subunits over the Core binding site at *ARS1* and *ARS305* also argues that features of ORC-origin binding are conserved (Figure 8) and that this view of the ORC-Core origin interaction is likely to be generally applicable to all yeast origins.

The conservation of ORC subunit arrangement, however, does not extend beyond the Core binding region. At *ARS1*, in addition to being crosslinked to a region within the Core origin, Orc2p, Orc4p and Orc5p are crosslinked to a second site in the flanking DNA. This interaction with two distinct regions of DNA is consistent with one of the following interpretations: (1) Orc2p, Orc4p and Orc5p are elongated proteins, (2) multiple copies of Orc2p, Orc4p and Orc5p are present in each ORC complex, (3) multiple ORC complexes bind to *ARS1*, or (4) the DNA at *ARS1* is bent such that distant regions of the DNA are in close proximity to the same polypeptide. Although the different protein-DNA crosslinking observed at *ARS1* and *ARS305* could be explained by different stoichiometries of ORC or ORC subunits bound at these two origins, this interpretation is unlikely as ORC-DNA complexes migrate similarly when *ARS1* and *ARS305* DNA fragments are used in electrophoretic mobility shift assays (data not shown). DNA-bending is the most likely explanation for the discrepancy in crosslinking results, an interpretation that is consistent with the ability of ORC to induce DNA-bends at *ARS1* but not at *ARS305* (Figure 3B). Thus, the association of ORC subunits with the Core origin is likely to be similar at all origins, but higher level interactions such as DNA bending may strongly influence crosslinking outside of the Core ORC-DNA binding site.

Implications of ORC-Origin Architecture for ORC Function

ORC interacts with yeast origins by making multiple protein-DNA contacts, a strategy commonly used by Initiator proteins to induce DNA distortion. For example, the Epstein-Barr virus Initiator protein, EBNA1, forms a dimer that must bind cooperatively to two adjacent binding sites for origin function *in vivo* (Harrison et al., 1994). The crystal structure of the EBNA1 dimer bound to DNA has been solved and modeling of two dimers bound to two adjacent binding sites has been performed (Bochkarev et al., 1996). Because the DNA binding sites are only separated by three base-pairs, two dimers can not co-occupy these adjacent sites unless the DNA in between is distorted to prevent collision of the proteins. Typically, Initiator proteins are homo-multimers that make multiple protein-DNA contacts by interacting with repeated DNA elements. ORC, however, is a hetero-multimer which likely binds as a single complex to yeast origins. Thus, ORC makes multiple contacts by utilizing different sets of subunits to contact distinct regions of a large DNA binding site. It remains to be determined if these multimeric interactions function only in the specificity of the ORC-DNA interaction or are also required for downstream steps of DNA replication initiation (e.g. unwinding).

The observation that ORC is much more sensitive to modifications of the top-strand of *ARS1* than the bottom-strand is intriguing since preferred interactions with only one strand of DNA is a mechanism used by Initiator proteins to stabilize an unwound region of the origin. After the *Escherichia coli* DnaA protein binds to its origin, it induces melting of adjacent DNA (the repeated 13-mer site) in a process called open complex formation (Bramhill and Kornberg, 1988). In the open complex, DnaA preferentially interacts with one of the single strands of the unwound 13-mer region (Bramhill and Kornberg, 1988; Hwang and Kornberg, 1992). The SV40 Initiator, T-

antigen, forms a double hexamer structure encircling two DNA elements that are subsequently unwound or untwisted (reviewed in Borowiec et al., 1990) . Although the T-antigen hexamers encircle both strands of the DNA in these distorted regions, each hexamer contacts only one strand (SenGupta and Borowiec, 1994). Since ORC is bound to origins throughout most of the cell cycle, ORC-DNA binding is unlikely to be sufficient for origin unwinding. Therefore, I imagine three possible models whereby preferred interaction with one strand of DNA may be utilized by ORC. (1) ORC is responsible for initial DNA unwinding at origins but must interact with or be modified by another protein at the appropriate time in the cell cycle for this activity to occur. (2) Another protein or proteins recruited to origins performs the unwinding function but ORC stabilizes the melted region of the duplex by binding to one of the single strands. (3) ORC is passive in the unwinding process but uses single-stranded DNA binding to remain associated with origins of replication after they are unwound. It also remains possible, however, that ORC simply prefers to interact with one strand of a duplex and does not bind single-stranded DNA.

ORC Subunit Interactions

Our studies provide information regarding ORC subunit arrangement within the complex (Figure 10). The 4'-AZPB crosslinking studies identify ORC subunits in close proximity to the same modified DNA residue, and these results are consistent with the subunit organization that I have derived from partial complex experiments. The composition of the partial complex lacking Orc5p suggests that Orc4p and Orc5p physically contact each other (Figure 4B), and 4'-AZPB crosslinking demonstrated that these two subunits can be crosslinked to the same DNA site (Figure 5A, probe J). Similarly, a complex lacking Orc3p is also deficient in Orc2p (Figure 4B), and both

subunits are crosslinked by a number of *ARS1* and *ARS305* probes (Figure 5A, probes E, F, I, and Figure 7A, probe I^{305}).

UV crosslinking studies can only describe the arrangement of ORC subunits in the presence of DNA. ORC, however, is a pre-assembled complex in the absence of DNA, and the conformation and relative positions of subunits may be different when ORC is free in solution or associated with origins. ORC binds to DNA in a sequence specific manner only in the presence of ATP or ATP-γ-S (Bell and Stillman, 1992; Klemm et al., 1997). Furthermore, binding of ORC to ATP and of ORC to DNA are coordinated processes since specific DNA binding affects both association of Orc1p with ATP and the subsequent rate of ATP hydrolysis by this subunit (Klemm et al., 1997). Such coordinate action is likely to be mediated by allosteric changes within the complex. Thus, I am interested in determining how the relative positions of ORC subunits with respect to each other are affected by nucleotide and/or origin DNA binding.

Our view of ORC-origin association is necessarily limited by the static nature of the DNA-binding assay *in vitro*. Our understanding must ultimately be expanded to incorporate various cell-cycle contexts and the effects of proteins whose association with ORC is cell-cycle regulated. These initial studies will provide a foundation to understand changes in ORC properties that are induced during the cell cycle. Functional elements within yeast origins are arranged asymmetrically, and accordingly, ORC binds to origins by distributing its subunits asymmetrically along the DNA. Higher order complexes assembled at origins during G1 also reflect this asymmetry as comparisons with the post-RC demonstrate that the pre-RC has an added region of DNase I protection on only one side of the region protected throughout the cell cycle (Diffley et al., 1994). How the asymmetry inherent in the ORC-origin complex is ultimately translated into the assembly

of two symmetric replication forks at origins of bi-directional DNA replication remains to be understood.

EXPERIMENTAL PROCEDURES

Plasmids and Competitor DNA

pDL01, used in modification-interference and missing-contact assays, was prepared by inserting the following sequence into the *Eco RV* site of pBS/SK+ : 5' AAGGATCCAA AGTGCACTTA ACTGCAGAAC TTTTGAAAAG CAAGCATTAA AGATCTAAAC ATAAAATTTG TTAACTATCT AGATG 3'. This sequence encodes a minimal ORC binding site that contains 74 base pairs of *ARS1* sequence, including the ACS and B1 elements. This DNA is bound by ORC in a similar manner as the wild-type *ARS1* sequence as judged by DNase I protection and mobility shift assays (data not shown). Plasmids pARS1/GAG and pARS1/CTC were constructed by PCR mediated mutagenesis of pARS1/WTA. pARS1/a-b2- was generated by replacing the *Bgl II* to *Hind III* fragment of pARS1/858-865 (a linker substitution of the ACS) with the same fragment from pARS1/798-805 (a linker substitution of the B2 element; these plasmids were previously described in Marahrens and Stillman, 1992). Plasmid stability assays were performed as described (Bell et al., 1995). Plasmids pARS1/WTA and pARS1/acs-b2- were used as templates for production of wild-type and acs-b2- competitor DNAs respectively. Competitor DNAs were synthesized by 25 cycles of PCR using universal forward and reverse sequencing primers and PCR products were purified on a 2% agarose gel (1X TBE). DNA was recovered by electroelution followed by ethanol precipitation. All other DNA fragments and DNA probes were purified by electrophoresis on a native 4.8% polyacrylamide gel (24:1 acrylamide:bisacrylamide, 1X TBE) and recovered by electroelution.

Expression of ORC in Insect Cells

Expression and purification of wild-type ORC from insect cells was performed as described (Klemm et al., 1997) except that 10 mM MgOAc was included at all steps. The expression of partial ORC complexes lacking one subunit required the use of baculoviruses co-expressing two ORC subunits (previously described in Bell et al., 1995) and viruses expressing only one subunit. Baculoviruses expressing single ORC subunits were named bvORC1 through bvORC6. Each of the six partial ORC complexes was expressed in Sf9 insect cells by co-infection with two viruses each expressing two ORC subunits and one virus expressing a single subunit. These partial ORC complexes were purified through the S-Sepharose step in the ORC purification scheme (Klemm et al., 1997). The Sp1/Orc4p fusion protein was constructed by PCR amplifying DNA encoding the C-terminal 168 amino acids of the Sp1 transcription factor and fusing this PCR product to the coding sequence of the N-terminus of Orc4p . Coding sequences for Orc3p and the Sp1/Orc4p fusion were cloned into pFastBac Dual (Gibco BRL) to generate plasmid pFBD/ORC3/N-SP1-ORC4 and baculovirus was produced from this plasmid using the Bac-to-Bac Expression System (Gibco BRL). ORC complexes containing the Sp1/Orc4p fusion protein as the sole copy of Orc4p were expressed in insect cells and purified up to the Mono-Q chromatography step. Immuno-blot analysis was performed as described (Bell et al., 1993) except that monoclonal antibodies directed against individual ORC subunits were used to detect ORC proteins (SB16, SB46, SB3, SB6, SB5 and SB49 for detection of Orc1p through Orc6p respectively).

Electrophoretic Mobility Shift Assays

Unless noted, ORC-DNA binding conditions for all experiments were as follows. Reactions (15 μl) contained 12.5 mM Hepes-KOH (pH 7.5), 2.5 mM magnesium acetate,

2.5 mM DTT, 5 mM EGTA, 0.66 mg/ml poly d(G-C), 2 mg/ml BSA, 1 mM ATP (where indicated), 20 ng of ARS1/a-b2- competitor DNA, 0.22 ng of radiolabeled probe (300 cps), and 12 ng of ORC. Binding reactions were incubated for 10 minutes at room temperature. The *ARS1* probe used in the mobility shift assay was generated by digesting pARS1/WTA with *Eco RI*, 3' end-labeling using the Klenow fragment of DNA Polymerase I, and digesting with *Hind III*. The labeled 244 bp *Eco RI - Hind III* fragment was gel purified as described above. All ORC mobility shift assays were performed as described (Rao and Stillman, 1995) except that gels and running buffers included 80 μg/ml BSA, and gels were run at 4°C for 4 hours at 200 V.

Modification-interference and Missing-contact Assays

Chemical modifications of labeled pDL01 DNA was carried out as follows. Diethyl pyrocarbonate (DEPC) carbethoxylation was performed essentially as described (Herr, 1985), except end-labeled DNA was heated for 5 min at 90°C prior to DEPC incubation which was carried out for only 5 min at 90°C. Following two ethanol precipitations, DEPC-modified DNA was resuspended in hybridization buffer (10 mM Tris-HCl, 1 mM EDTA, 30 mM NaCl, pH 8.0), heated to 95°C for 3 min, incubated at 65°C for 10 min, and allowed to re-anneal by slow cooling to room temperature. Formic acid depurination was performed as described (Brunelle and Schleif, 1987). $KMnO_4$ modification was carried out as described (Truss et al., 1990), except that treatment with $KMnO_4$ was performed for 15 min at room temperature. Re-annealing of melted DNA was performed as described above. Hydrazine depyrimidation was carried out as described (Lee et al., 1991), except that hydrazine incubation was carried out for 30 min at room temperature. Phosphate backbone ethylation using ethyl nitroso-urea was performed as described (Hendrickson and Schleif, 1985).

Prior to separation of bound and unbound DNA molecules, an aliquot of each modified DNA sample was reserved for chemical cleavage (input samples, "I" in Figure 2A). The remainder was incubated with purified ORC in the standard binding buffer, using between 6 and 10 ng of modified DNA and 600 ng of protein in a 30 μl reaction. Bound and unbound DNA molecules were separated by electrophoretic mobility shift as described above, gels were exposed to film for 30 min, and bound and free DNA was excised from the gel. DNA was recovered by electroelution and ethanol precipitation. Recovered DNA molecules (Bound and Free molecules, as well as Input DNA aliquots) were cleaved at sites of modification using one of two methods. For ENU modification, ethylated phosphates were cleaved by heating in the presence of NaOH as described (Hendrickson and Schleif, 1985). For all other modifications, precipitated DNA was cleaved with piperidine (Lee et al., 1991). Cleaved DNA was resuspended in a formamide-dye mixture and separated on a 10% DNA sequencing gel. Gels were dried and exposed to film.

ORC-induced DNA Bending Assay

DNA fragments used for ORC-induced bending studies were generated by PCR from yeast genomic DNA (*ARS305*) or from plasmids (p19AB121 for *ARS121*, and pARS1/WT, pARS1/a-b2- and pARS1/858-865 for wild-type and mutant *ARS1* fragments). Oligonucleotides were designed to add *Xba I* sites to both ends of the following regions amplified from each ARS: nucleotides -69 to +145 of *ARS305* (Huang and Kowalski, 1996), 285 to 498 of *ARS121* (Walker et al., 1990), and 726 to 939 of *ARS1* (Marahrens and Stillman, 1992). PCR reactions were carried out for 25 cycles in the presence of 0.2 μM α-^{32}P-dATP. The PCR products were digested with *Xba I* overnight and the resulting 220 bp fragment was gel purified as described above.

Bending probes were incubated with purified ORC according to the mobility shift assay ORC-DNA binding conditions with the following modifications. Reactions were performed in 40 μl, including 13 ng of radiolabeled origin probe and a 50-fold molar excess of unlabeled competitor DNA (containing either the wild-type *ARS1* sequence or the ARS1/a-b2- sequence). 150 ng of ORC was added where indicated, and all reactions contained 96 μg of BSA. Circularization of origin probes was performed as described (Kahn and Crothers, 1992), using 56 Units of T4 DNA ligase per reaction. Samples were removed at the indicated times, and reactions were stopped and electrophoresed on a 20 cm 5% native acrylamide gel (1:40 acrylamide:bisacrylamide, 1X TBE) for 830 V-hrs. Circular species were distinguished from linear molecules by their resistance to *Exonuclease III* digestion. Gels were dried and exposed to film, or exposed to Molecular Dynamics PhosphorImager screens for quantitation using ImageQuaNT software.

Protein-DNA Crosslinking

The production of 4'-AZPB UV crosslinking probes combined methods described by Bell and Stillman (Bell and Stillman, 1992) and Yang and Nash (Yang and Nash, 1994). The *Eco RI* to *Hind III* fragment of pARS1/WTA was subcloned into M13mp18 and M13mp19 replicative forms, cut with the same enzymes, and single-stranded DNAs were produced for use as templates for *ARS1* crosslinking probes. An M13mp18 derivative of *ARS305* was generated by cloning the *ARS305* PCR product used for ORC-induced bending studies into this vector. The -40 universal sequencing primer and a second oligonucleotide were annealed to single-stranded templates. Extension using T4 DNA Polymerase in the presence of α-S-dCTP or α-S-TTP and labeled α-^{32}P-dATP or α-^{32}P-TTP resulted in the incorporation of one or two thio-phosphate nucleotides and several radio-labeled nucleotides immediately following the second primer. The

extension was chased with an excess of unlabeled, unmodified nucleotides to complete DNA synthesis. The resulting double-stranded circles were precipitated and 4'-azidophenacyl bromide (4'-AZPB) was coupled to the incorporated thio-phosphates as described (Yang and Nash, 1994). Free 4'-AZPB was removed using a 1 ml G-50 spin column. The purified DNA was digested with *Eco RI* and *Sal I* to remove any modified or labeled dNTPs incorporated following the universal primer, and the liberated restriction fragment was gel purified. DNA fragments for BrdU crosslinking were synthesized as described (Ausubel et al., 1994) using the same single-stranded DNA templates as the 4'-AZPB crosslinking probes, digested with *Eco RI* and *Sal I*, and gel purified.

ORC-DNA binding conditions for crosslinking experiments were as described above, with the following changes. The amount of purified protein added per binding reaction was increased two-fold for 4'-AZPB crosslinking, and the amount of ARS1/a-b2-competitor was increased three to five-fold for BrdU and 4'APZB crosslinking respectively. Reaction mixtures were transferred to a microtiter plate and irradiated with a 254 nm light source (UVP, Model UVG-54) at a distance of 1 cm for either 2 min (4'-AZPB crosslinking) or 30 min (BrdU crosslinking). Crosslinked proteins were treated with DNase I (Worthington) and micrococcal nuclease (Worthington) as described (Bell and Stillman, 1992), precipitated with trichloroacetic acid, and resolved on 10% SDS-polyacrylamide gels. Gels were silver stained, dried, and exposed to film. We had previously reported that Orc1p did not crosslink to *ARS1* (Klemm et al., 1997); however, I subsequently discovered that the micrococcal nuclease used to digest DNA after crosslinking to ORC was contaminated with a protease. Orc1p and Orc2p were the most sensitive of the ORC subunits to this protease (data not shown). Boiling of the

micrococcal nuclease eliminated the contaminating protease activity and Orc1p and Orc2p crosslinking became observable.

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Chapter III

Electron Microscopy of ORC

The work described in this chapter was performed in the laboratory of Jack D. Griffith at the Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC. The initial observations and EM images were collected by D. G. Lee and Alexander M. Makhov, and all of the quantitation was performed by A. M. M.

I thank Jeff Gelles for providing the plasmid containing the C-terminal fragment of the *E. coli* BCCP.

SUMMARY

Electron microscopy (EM) analysis of ORC was performed using three different preparatory methods to minimize EM artifacts. We found that ORC is an elongated molecule with three lobes, and this structure is similar when ORC is bound to origin DNA. Estimates of the molecular mass of ORC using EM were consistent with the elongated complex containing one copy of each of the six ORC subunits. ORC binds origin DNA along its long axis, interacting with approximately 50 base pairs. The feasibility of using EM to map the positions of ORC subunits within the complex is discussed.

INTRODUCTION

Biologists, and biochemists in particular, often make inferences about the properties of individual molecules based on the behavior of a population; however, sometimes a key feature of a biological system is obscured by this approach. For example, if a population consists of two (or more) distinct subpopulations with different properties, the average behavior of the entire population can be misleading. A population of cells that together produce 20% of the wild-type levels of a diffusible product may in fact consist of 20% of the cells generating wild-type quantities of product and 80% of the cells generating no product, rather than all of the cells showing an 80% reduction in product formation. These two different scenarios would lead to two distinct models of the regulation of this product. The ability to observe single molecules directly or monitor the consequences of single events can circumvent this problem and lead to important new insights.

Electron microscopy (EM) is a powerful method for determining the properties of individual proteins or protein complexes. In particular, EM analysis of replication proteins (such as the *E. coli* DnaA protein or the SV40 T Antigen) interacting with their cognate origin has contributed to the understanding of how these proteins function during replication (Dean et al., 1987; Fuller et al., 1984; Funnell et al., 1986; Mastrangelo et al., 1989; Wessel et al., 1992). The opportunity to collaborate with an EM lab with prior experience in DNA replication allowed me to ask some simple yet important questions about the properties of ORC. First, what is the shape of ORC, and how well does it relate to the structure that I had determined based on protein-DNA crosslinking (Chapter II)? Second, what is the molecular mass of ORC as determined by EM? All of our biochemical data was consistent with a monomer of ORC containing a single copy of each subunit for a combined mass of 414 kDa; however, none of our data could

definitively distinguish between a monomer or a dimer of ORC binding DNA. Thus, I was interested in determining the oligomeric state of ORC, both in solution and bound to origin DNA. Finally, does ORC binding have consequences for the shape of the DNA? DNase I protection assays of origin DNA bound by ORC had suggested that the DNA may be wrapped around the protein (see Chapter I and below), which should result in an observable reduction in the end-to-end length of DNA.

RESULTS

ORC samples were prepared for electron microscopy using three different preparatory methods to minimize the effects of experimental artifacts. "Direct mounting" EM (Griffith and Christiansen, 1978) involves adsorbing gluteraldehyde crosslinked proteins (and protein-DNA complexes) onto thin carbon supports followed by sequential washes in increasing concentrations of ethanol (up to 100% ethanol) to remove water from the samples. Although this method is rapid, the ethanol dehydration steps can sometimes lead to perturbations of native protein structure (A. Makhov, personal communication). In contrast, cryo-fixation and freeze-drying (Bortner and Griffith, 1990) involves rapid freezing of the sample on carbon supports in liquid ethane chilled in liquid nitrogen and freeze-drying. Cryo-EM is thought to better preserve the conformation of proteins but is a time-consuming process. Both direct mounting and cryo-EM involve visualization of the protein and protein-DNA complexes by rotary shadowcasting with tungsten, producing images of high contrast. However, uneven or heavy deposition of metal can lead to loss of detailed structural information. The third method, negative staining with uranyl acetate, can be performed with unfixed samples and is the least likely to alter the native structure of proteins. Since the electron-dense stain is applied in a thin layer, this method provides the highest resolution and greatest amount of detail; however, it cannot produce the high degree of contrast achieved with rotary shadowcasting. Our EM analysis of ORC relied primarily on direct mounting and negative staining due to the more rapid turnover time; however, cryo-fixation and freeze-drying was also attempted on two separate occasions. All three methods yielded results that were in excellent agreement with each other, arguing that the observations are not the results of EM artifacts.

ORC is an Elongated Complex with Three Lobes

Electron microscopy of ORC revealed that the complex has an elongated structure with three lobes. High resolution negative staining revealed two larger outer lobes (one slightly larger than the other) and a small middle lobe (Figure 1A). The dimensions of ORC stained with uranyl acetate were 17 (+/- 1) nm by 7 (+/- 1) nm (N=30). Lower resolution tungsten shadowcasting also clearly showed the two outer lobes, but depending on the orientation of the complex and possibly the thickness of the metal, the middle lobe was often not visible (Figure 1B). The overall structure of ORC was found to be similar when bound to origin DNA (Figure 2). To exclude the possibility that the observed multi-lobed structure was a multimer of two or more ORC complexes, we estimated its molecular mass from negatively stained samples, using the Herpesvirus ICP8 protein (132 kDa) as a size standard. By comparing the projected area of ORC with ICP8 (see Experimental Procedures), ORC was calculated to have a molecular weight of 456 kDa. This value is more consistent with a monomer of ORC (414 kDa) than with a dimer (828 kDa). Thus, ORC is likely to exist as a monomer with multiple lobes, both on and off DNA.

Characterization of dsDNA Binding

EM analysis of ORC bound to long (Figure 2A, B) or short (Figure 2C) origin-containing DNAs also allowed us to determine whether ORC binding resulted in DNA wrapping. As described in Chapter I, the ORC-induced DNase I protection pattern at all origins tested thus far includes a number of hypersensitive sites that are periodically spaced at roughly every turn of the DNA helix. Such a pattern is consistent with the DNA either being wrapped around the protein or the DNA laying flat against a surface (Travers and Klug, 1987). The end-to-end length of the DNA was not significantly

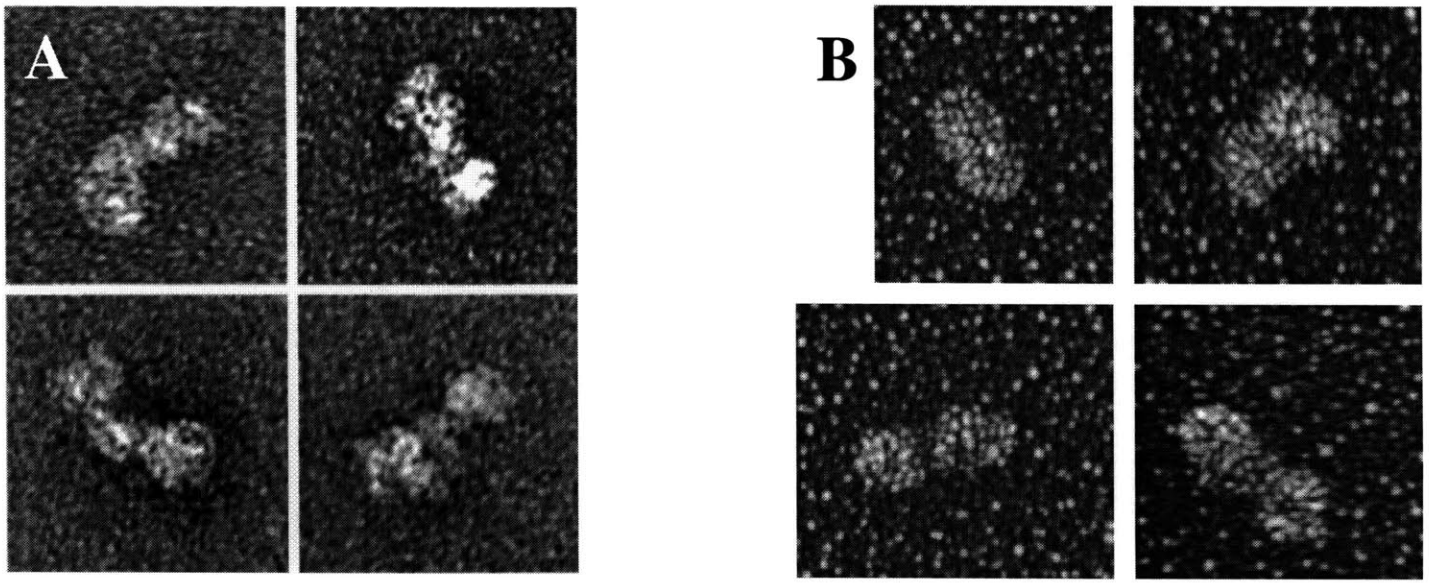

Figure 1. ORC is an Elongated Molecule with Three Lobes. (A) Uranyl acetate negative staining of ORC reveals a three-lobed structure. (B) Rotary shadowcasting of ORC with tungsten shows a similar structure, although the details of the smallest (middle) lobe are less clear using this lower-resolution technique. Samples were prepared for EM as described.

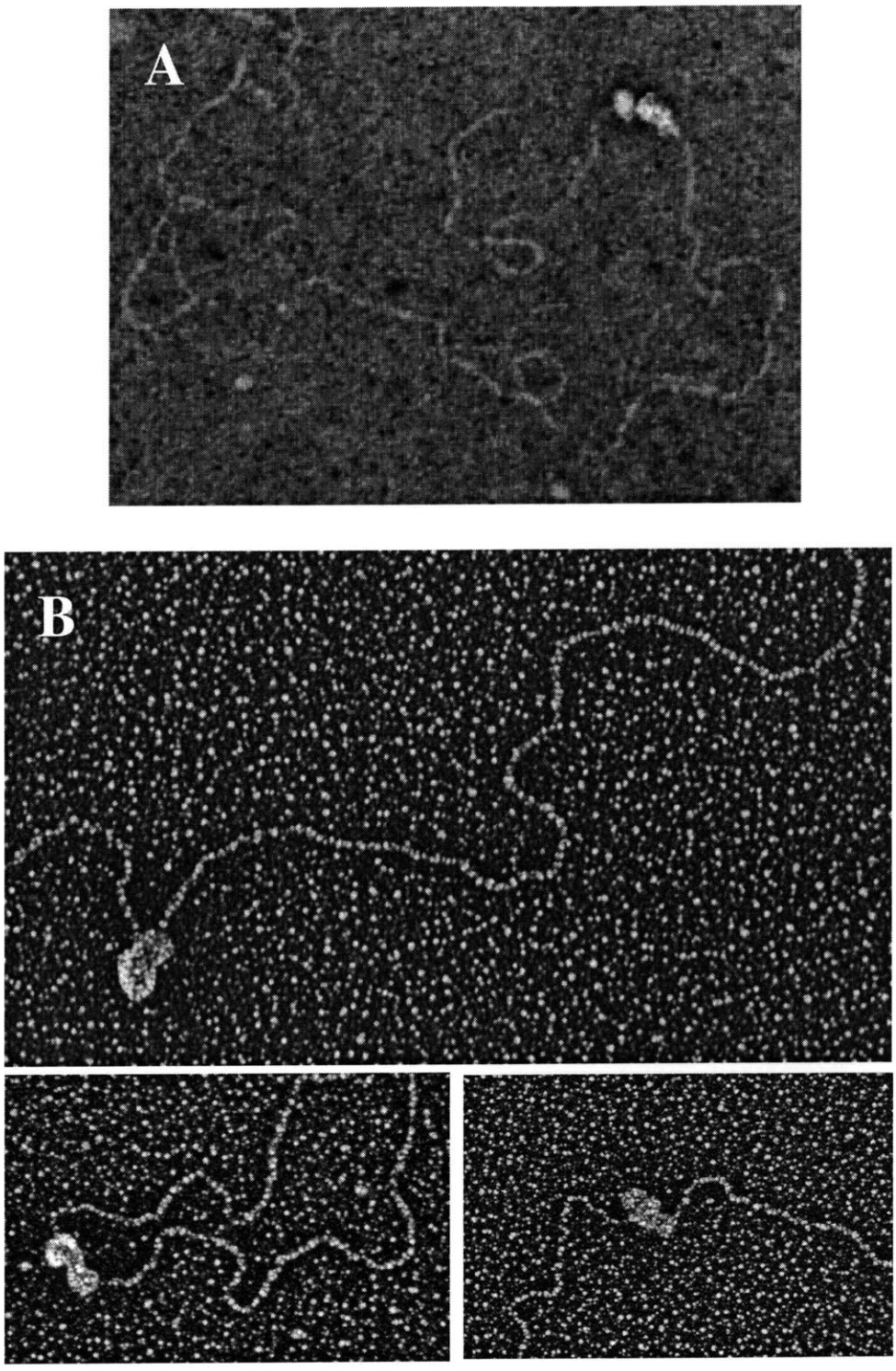

Figure 2

C

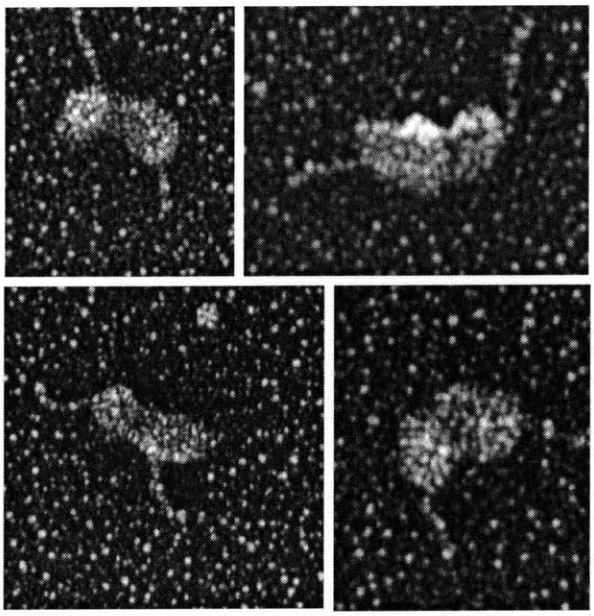

Figure 2. The Shape of ORC is Similar When Bound to Origin DNA. ORC was incubated with *ARS1*-containing DNA and ATP, crosslinked with gluteraldehyde, and prepared for EM as described. (A) Uranyl acetate negative staining of ORC bound to a 5.7 kb *ARS1*-containing fragment. (B) Rotary shadowcasting of ORC bound to a 5.7 kb *ARS1*-containing fragment. (C) Rotary shadowcasting of ORC bound to a 344 bp *ARS1*-containing fragment.

changed by ORC binding (data not shown), indicating that the latter scenario is correct. The DNA is closely juxtaposed to ORC along its length, and given that ORC is approximately 17 nm long (see above), the maximum amount of DNA covered by ORC is ~50 base pairs (assuming approximately 0.34 nm per bp of DNA). This number is in agreement with the size of the DNase I protection pattern, which is also approximately 50 base pairs in length.

EM was also used to examine the specificity of dsDNA binding by ORC. A 2.2 kb DNA fragment containing the *ARS1* origin was biotinylated at one end to allow for orientation of the DNA. ORC was then added to the DNA, and the reaction was prepared for EM by gluteraldehyde fixation, direct mounting and rotary shadowcasting (Figure 3). Eighty-four ORC-DNA complexes were examined, and the position of ORC relative to the modified DNA end was measured (Figure 4). The distribution of ORC molecules along the length of the DNA peaks at a position consistent with *ARS1*, indicative of sequence-specific binding. As many as 50% (42/84) of ORC molecules were found to be bound at other sites, including yeast sequences flanking *ARS1*, sequences corresponding to the bacterial plasmid, and yeast sequences near the *CEN4* centromere sequence. However, all of these non-specific binding events were clustered near the *ARS1* sequence – no binding was observed at distal sites on the DNA fragment.

The biased distribution of non-specific binding events close to the ORC binding site can reflect one of the following possibilities: (1) Sequence-specific binding of ORC sometimes results in dissociation and rebinding of ORC close to the origin. An apparent bias in the sites to which ORC-rebinds could result if ORC tracks along the DNA rather than dissociating prior to reassociating. (2) The particular sequences to which ORC is bound in this experiment contain near-matches to the ORC binding site and they are all fortuitously positioned close to the *ARS1* sequence in this plasmid. (3) Errors in

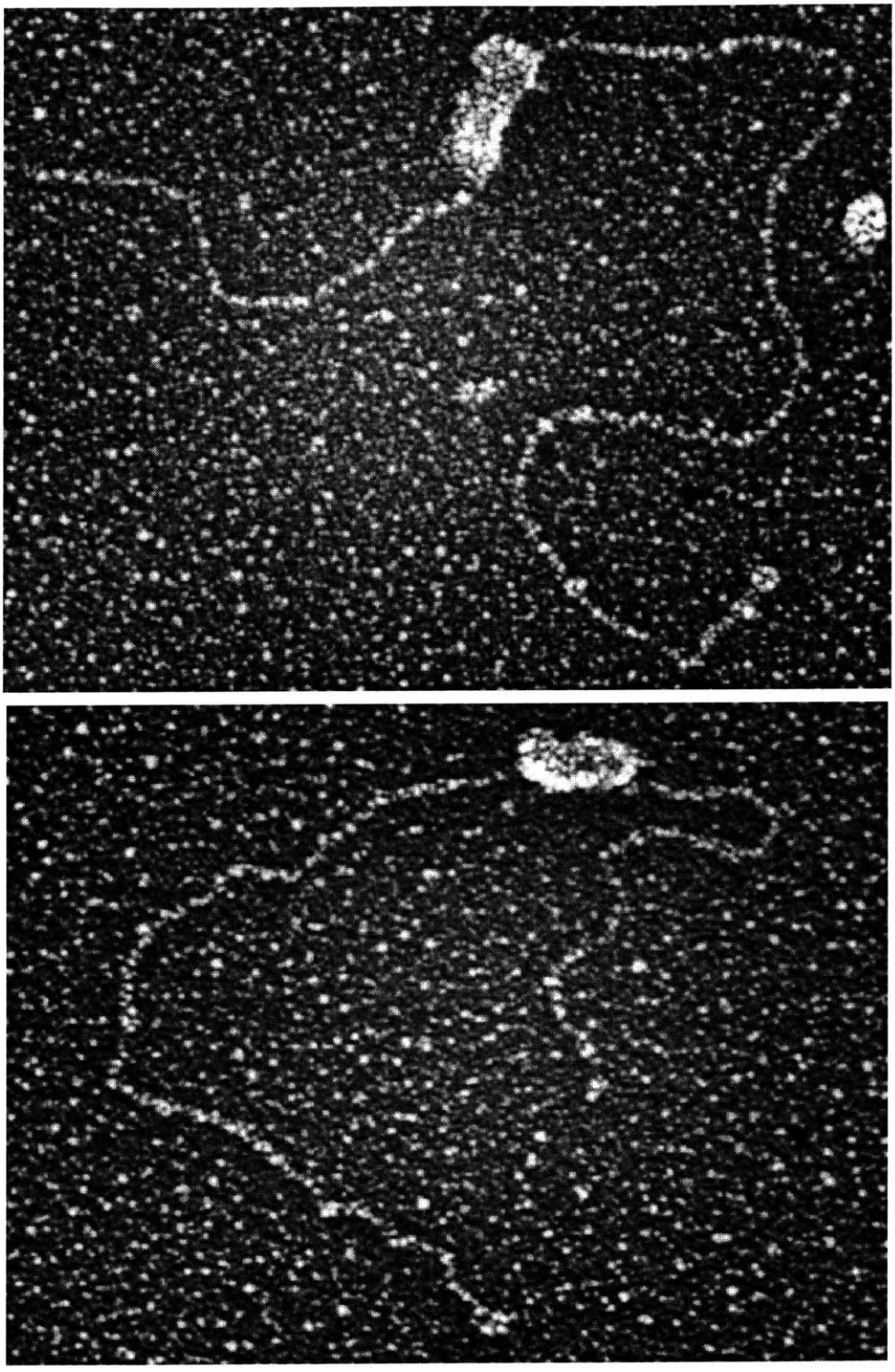

Figure 3

Figure 3. ORC Bound to ARS1-Containing Fragments Marked at One End. A 2.2 kb *ARS1*-containing fragment was labeled with biotinylated dNTPs at the end of the molecule farthest from the *ARS1* site. The DNA was incubated with ORC and with streptavidin to label the biotinylated site (visible as a thickening of the DNA at one end). The reaction mixture was crosslinked with gluteraldehyde and prepared for EM by direct mounting and rotary shadowcasting.

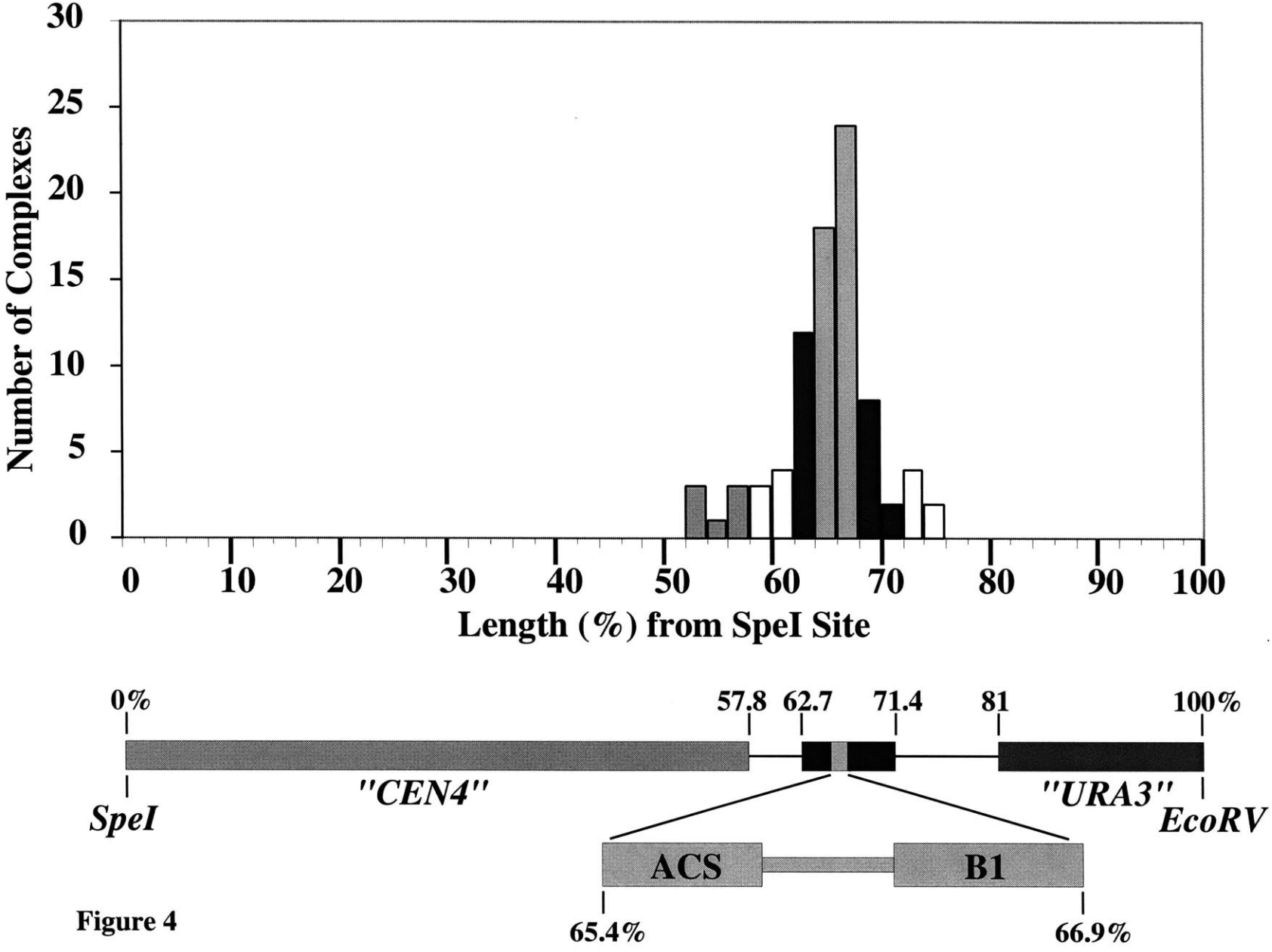
Number of Complexes
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
100
Length (%) from SpeI Site
0%
57.8
62.7
71.4
81
100%
SpeI
"CEN4"
"URA3"
EcoRV
ACS
B1
65.4%
66.9%

Figure 4

Figure 4. Sequence-Specific Binding of ORC to ARS1 DNA. ORC was bound to a biotinylated 2.2 kb *ARS1*-containing *SpeI-EcoRV* fragment labeled with streptavidin (shown and described in Figure 3). The length of each arm of the DNA was measured, and the position of ORC relative to the biotinylated *SpeI* site was determined (shown as % of the total distance from the *SpeI* site). The histogram shows the combined data from 84 ORC-DNA complexes. A schematic of the DNA fragment is shown below the histogram. Colored rectangles denote yeast DNA sequences, whereas the thin black line represents DNA derived from the bacterial plasmid. Sequences derived from the chromosomal *ARS1* site are represented by the dark blue boxes, and the positions of the ACS and B1 sites are indicated by light blue rectangles. A yeast genomic fragment containing the *CEN4* centromere (green) and a fragment containing the *URA3* gene (red) are also shown.

measurements resulted in a number of specific binding events scored as non-specific. To address these various possibilities, it will be important to repeat the experiment using a fragment that contains a mutation in the *ARS1* sequence to determine if the binding to flanking sequences is dependant on the presence of *ARS1*. Furthermore, the use of fragments containing different origins will address the generalizability of this clustered binding phenomenon. Finally, the experiment will be repeated using shorter DNA fragments and increasing the sample size to reduce the relative amount of experimental error.

DISCUSSION AND FUTURE PERSPECTIVES

Electron microscopy has provided us with the first direct observations of ORC. In addition to describing basic characteristics of ORC (its dimensions, its monomeric state on DNA, and the absence of DNA wrapping around ORC), EM analysis can potentially be used to map the positions of ORC subunits. In contrast to the protein-DNA crosslinking experiments used to map ORC subunits (Chapter II), the proposed EM assays can be performed in the presence and absence of DNA.

The proposed experimental approach involves the generation of variants of ORC in which an individual subunit is tagged at one end with an epitope that can be mapped by EM. These modified ORC complexes will be assayed in solution or bound to DNA containing an asymmetrically positioned origin to orient the protein. A candidate mapping epitope is the 87-amino acid C-terminus of the *E. coli* biotin carboxyl carrier protein (BCCP). This protein fragment is efficiently biotinylated *in vivo* and, when fused to a protein of interest, can lead to the efficient biotinylation of the chimeric protein when expressed in *E. coli* or in insect cells (Berliner et al., 1994; Young et al., 1995). The high affinity of streptavidin for biotin is then utilized to manipulate the biotinylated protein or, in my case, to determine its position. I produced and expressed twelve different ORC complexes, each containing a single ORC subunit fused to the BCCP biotinylation sequence at either its N- or C-terminus (Figure 5A). The individual modification did not destabilize the complex and resulted in the co-purification of all six subunits. More importantly, in 11 out of 12 instances, the modified complexes retained near wild-type levels of DNA binding activity – only the complex modified on the N-terminus of Orcp3 showed reduced activity (Figure 5C).

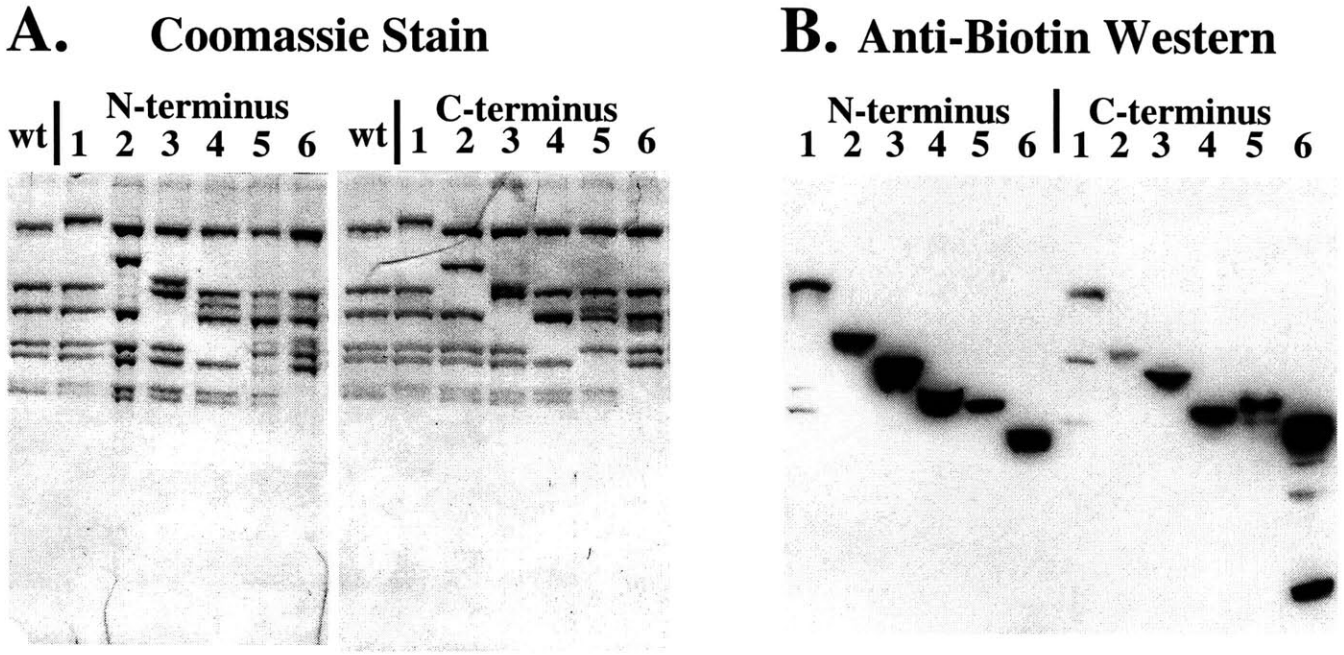

Figure 5A and 5B. Purification of Biotinylated ORC Complexes. ORC subunits were fused to a biotin acceptor peptide at their N- or C-termini. Co-expression in insect cells of each modified subunit with the five remaining wild-type subunits resulted in the production of ORC complexes with a unique, modified site and the *in vivo* biotinylation of this site. (A) Coomassie stained protein gel of the 12 purified biotinylated complexes. The position of the biotin tag is at the N- or C-terminus of each subunit as indicated. Wt = wild-type, unmodified ORC. (B) Anti-biotin western blot of the biotinylated complexes. The 12 modified complexes were electrophoresed on a 10% SDS-PAGE and transferred to nitrocellulose. The membrane was probed with horseradish peroxidase-conjugated streptavidin and treated with chemiluminescence reagents (ECL) to visualize the biotinylated ORC subunit.

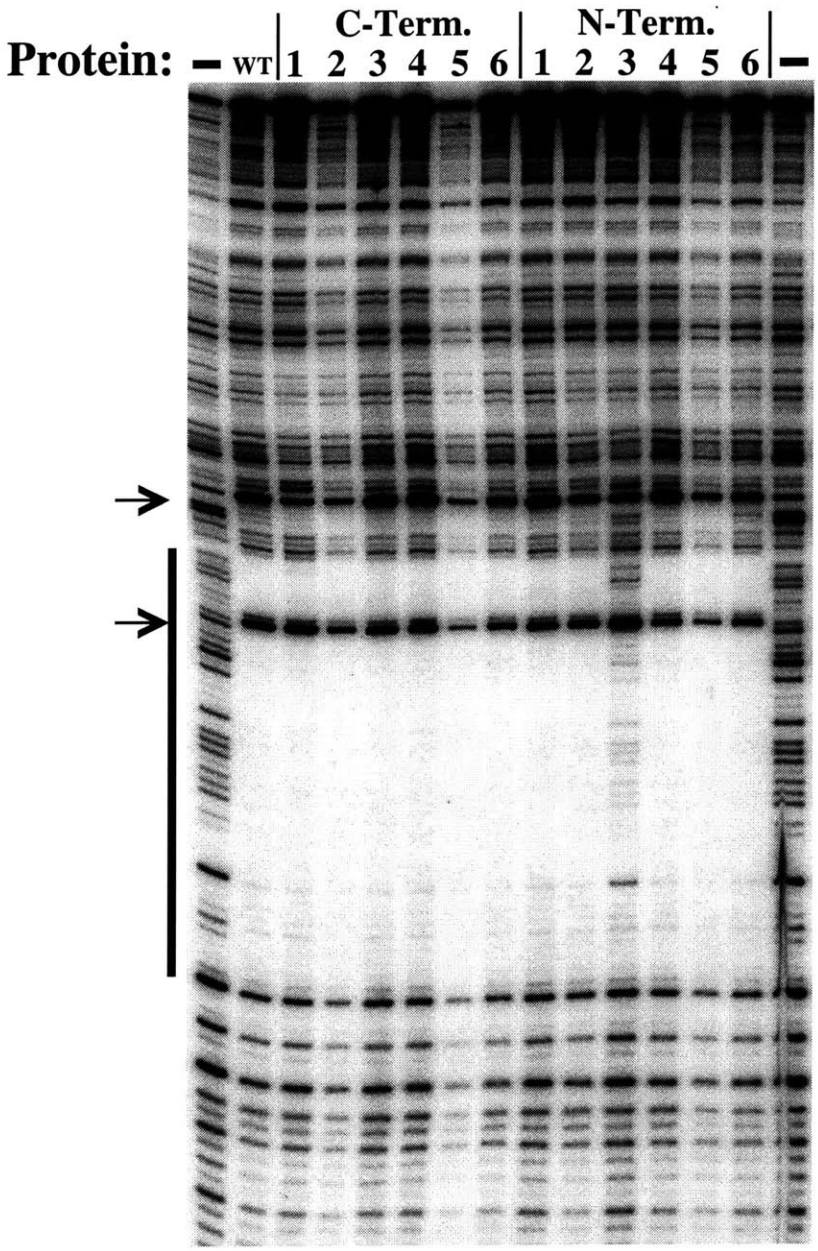

Figure 5C. DNA Binding Activity of Biotinylated ORC Complexes. Modified ORC complexes were tested for *ARS1* binding activity in a DNase I protection assay. An equivalent amount of ORC was used in each lane except for the outer two lanes which represent the DNase I cleavage pattern of naked DNA. The heavy lines to the left of the gel indicate the region of *ARS1* protected by Wild-type ORC and arrows indicate the positions of ORC-induced hypersensitive sites. All *ARS1* binding reactions were performed in the presence of ATP. The ATP-dependence of origin binding of each of the modified complexes has not yet been examined.

The biotinylation state of the modified complexes was determined by performing Western blot analysis using horseradish peroxidase-conjugated streptavidin (Figure 5B). Two modified subunits (Orc1p and Orc2p modified at the C-terminus) showed weak levels of biotinylation relative to the others. This was true only when they were co-expressed in insect cells with the other five subunits to assemble the ORC complex. In contrast, when the modified subunits were expressed individually, all were biotinylated to similar extents (data not shown). These data argue that the low level of biotinylation in the Orc1p-C-term and Orc2p-C-term subunits is due to decreased accessibility of the BCCP tag in the context of an ORC complex. The remaining 10 modified complexes may have sufficient levels of biotinylation to allow the position of the tag to be mapped by EM; however, the absolute efficiency of biotinylation for each complex has yet to be determined.

The key component of the proposed experiment that remains to be developed is a reliable, high resolution method of determining the position of the biotin tag. I had attempted to use a conjugate of streptavidin and gold as a molecular marker; however, the combined molecular mass of the gold particle coupled to multiple streptavidin molecules resulted in a complex that was roughly half the size of ORC (data not shown). Due to the large size of the streptavidin-gold conjugate and the presence of multiple streptavidin molecules, a precise determination of the contact point between the biotin and the streptavidin was not possible. An ideal molecular marker would involve a small protein with a rigid rod-like structure fused to a single copy of streptavidin to generate a protein "pointer". As a pilot experiment to examine the feasibility of this approach, it should be possible to end-label a short fragment of DNA with a biotinylated nucleotide and add the DNA to the biotinylated ORC complexes in the presence of streptavidin. Since streptavidin is multivalent (each streptavidin molecule can bind up to four biotin

molecules), the protein will be able to bind biotinylated ORC and biotinylated DNA simultaneously, allowing the DNA to serve as a rough pointer to the site of modification on ORC.

Electron microscopy promises to be a powerful tool for elucidating the structure of this multi-protein complex. In addition to the subunit mapping experiments, the collection of a greater number of high-quality EM images will lead to a computer simulation of an averaged 3-dimensional structure. Such information will greatly facilitate the long-term goal of determining a high-resolution crystallographic structure of ORC.

EXPERIMENTAL PROCEDURES

ARS1-Containing DNA

Three different *ARS1*-containing DNAs were used for EM studies. The short 344 bp fragment was a PCR product using the plasmid pARS1/WTA (Marahrens and Stillman, 1992) as the template and universal forward (cgccagggttttcccagtcacgac) and reverse (agcggataacaatttcacacagg) sequencing oligos as PCR primers. The 5.7 kb DNA and the 2.2 kb DNA were *ApaI-NcoI* and an *SpeI-EcoRV* fragments of pARS1/WTA, respectively. All DNAs were purified as previously described (Lee and Bell, 1997). The 2.2 kb DNA was biotinylated at the *SpeI* site by incubating 10 μg of DNA with 10 units of the Klenow fragment, biotinylated dATP and dCTP (8 μM final), and unmodified dGTP and dTTP (100 μM final) in a 100 μl reaction volume (containing 1X Klenow buffer). The DNA synthesis reaction was performed for one hour at room temperature, followed by two extractions using phenol/chloroform (1:1) and ethanol precipitation.

EM Methods

ORC-DNA complexes were formed in a 50 μl reaction containing 20 mM Hepes-KOH (pH 7.6), 2 mM EDTA, 2 mM EGTA, 5 mM magnesium acetate, 0.15 M KCl, and 100 μM ATP. For the short 344 bp DNA, 1.7 pmol ORC was incubated with 90 fmol DNA; for longer DNAs, 120 fmol ORC was incubated with 55-80 fmol DNA. Binding reactions were performed at room temperature for 17 minutes. When the biotinylated 2.2 kb fragment was used, streptavidin was added (5 μg/ml final) 6 minutes after the addition of ORC and the binding reaction was allowed to continue for another 11 minutes (i.e. – 17 minutes total). Gluteraldehyde was then added (0.6% final) and crosslinking was performed at room temperature for 10 minutes.

For uranyl acetate negative staining, the crosslinked samples were diluted five-fold or ten-fold in the same binding buffer, mounted on glow charged carbon supports and stained with 2% aqueous solution of uranyl acetate as described (Makhov et al., 1996). In other experiments, samples were stained without gluteraldehyde crosslinking with no obvious differences in the protein-DNA complexes observed (data not shown). For direct mounting and cryo-fixation/freeze-drying, the crosslinked samples were purified on a 2 ml BioGel A5m column (BioRad Inc.) equilibrated in TE prior to mounting on carbon supports. The samples were prepared for rotary shadowcasting with tungsten as described (Griffith and Christiansen, 1978; Griffith et al., 1995). All EM analyses were performed using a Philips CM12 transmission electron microscope. The dimensions of ORC and measurements of the position of ORC along the length of a biotinylated 2.2 kb DNA fragment were determined using a digitizer board and software developed by J. D. Griffith. Molecular mass estimates of ORC and ICP8 were performed as described (Griffith et al., 1995), using using 50-70 molecules of each protein.

Fusion of Biotinylation Tags to the N-Terminus of ORC Subunits

The C-terminal 87 amino acids of the *E. coli* biotinyl carboxy carrier protein (BCCP) were PCR amplified from the plasmid pEY4 (obtained from Jeff Gelles' laboratory) using PCR primers 5' BCCP-Bam HI (tgcgggatccatggaagcgccagcagcag) and 3' BCCP-SalI (tgcggtcgactcgataacaacaagcggttc). The PCR product was cut with *BamHI* and *SalI* and cloned in to the plasmid pFBD-Bcl cut with the same enzymes (pFBD is an expression vector for use in insect cells and it contains two multiple cloning sites for the simultaneous expression of two proteins). The resulting plasmid was cut with *SalI* and a 2.4 kb *SalI* fragment from pSPB65 was cloned into this site (this 2.4 kb *SalI* cassette was removed in the next cloning step and was included to facilitate a *SacI-SalI* digest of two

sites that would otherwise be too close together for efficient cleavage). This plasmid was then digested with *SacI* and *SalI* and the coding sequence for each of the ORC genes was cloned into these sites, generating an in-frame fusion of the BCCP fragment to the ORC gene. The inserts encoding ORC1 through ORC6 were *XhoI-SalI* fragments from plasmids pSF320/ORC1/SP1 – pSF320/ORC6/SP1, respectively. To insert a second (unmodified) ORC gene into the second polylinker of the dual expression vector, the resulting plasmids were digested with *XhoI,* and *XhoI-SalI* inserts derived from plasmids pSF320/ORC1/SP1 through pSF320/ORC6/SP1 were introduced. The ORC genes were paired such that Orc1p was co-expressed with Orc6p, Orc2p with Orc5p, and Orc3p with Orc4p.

Fusion of Biotinylation Tags to the C-Terminus of ORC Subunits

To generate a vector for the expression of chimeric proteins with the BCCP fragment fused to the C-terminus of each ORC gene, pEY4 was used to PCR amplify the BCCP C-terminus using primers 5' BCCP-SacI (tgcggagctccatggaagcgccagcagcag) and 3' BCCP-NotI (tgtgcggcggccgctcattcgataacaacaagcgg). This PCR fragment was digested with *SacI* and *NotI* and cloned into pFBD-Bcl cut with the same enzymes. The resulting plasmid cut with *SalI* and and a 2.4 kb *SalI* cassette from pSPB65 was cloned into this site. This plasmid was then digested with *SacI* and *SalI* and the coding sequence for each of the ORC genes was cloned into these sites, generating an in-frame fusion of the BCCP fragment to the C-terminus of each ORC gene. Inserts containing the six ORC gene sequences (lacking a stop codon) were XhoI-SacI fragments from plasmids pSF322/ORC1/SP1 through pSF322/ORC6/SP1, respectively. To insert the second (unmodified) ORC gene into the dual expression vector, the resulting plasmids were digested with *XhoI,* and *XhoI-SalI* inserts derived from plasmids pSF320/ORC1/SP1

through pSF320/ORC6/SP1 were introduced. The ORC genes were paired such that Orc1p was co-expressed with Orc6p, Orc2p with Orc5p, and Orc3p with Orc4p.

Expression and Purification of Biotinylated ORC Complexes

Each modified ORC subunit was co-expressed in insect cells with the remaining five (unmodified) ORC subunits as described (Klemm et al., 1997). In contrast to published results, efficient *in vivo* biotinylation did not require the addition of d-biotin and hydroxyecdysone to insect cell media (Berliner et al., 1994; Young et al., 1995). In fact, addition of d-biotin and hydroxyecdysone was found to inhibit growth of the insect cells (data not shown). Biotinylated complexes were purified as described (Lee and Bell, 1997), using S-sepharose and Mono-Q ion exchange columns. Peak mono-Q fractions were analyzed by resolving 1 μg of protein for each sample on a 10% SDS-PAGE gel and staining with coomassie brilliant blue. Protein fractions were also analyzed by staining with silver nitrate (data not shown).

Anti-Biotin "Western" Blot Analysis

The 30-50% ammonium sulfate pellet from nuclear fractions contained the bulk of the modified ORC and was used as the starting material for chromatography. One one thousandth of each 30-50% ammonium sulfate fraction was resolved on a 10% SDS-PAGE, the proteins were transferred to nitrocellulose, and the membrane was probed with horseradish peroxidase-conjugated streptavidin (PIERCE; diluted 1:30,000). An ECL chemiluminescence kit was used and the membrane was exposed to film.

DNase I Protection Assays

DNase I protection assays were performed as described previously (Bell et al., 1995; Bell and Stillman, 1992; Klemm et al., 1997) using ~120 fmol of protein where indicated.

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Chapter IV

Regulation of ORC Conformation and ATPase Activity: Evidence for a Single-Stranded DNA-Controlled Conformational Switch

An earlier version of this chapter was submitted for publication. The authors were Daniel G. Lee, Alexander M. Makhov, Richard D. Klemm, Jack D. Griffith and Stephen P. Bell. The initial EM observations were made by D. G. L. and A. M. M. in the lab of J. D. G. at the University of North Carolina, Chapel Hill. All quantitation of EM data was performed by A. M. M. The initial observation that ORC is an origin DNA-regulated ATPase was made by R. D. K. who also provided the mutant the ORC complexes defective for ATP binding that were used as controls and the wild-type ORC used in the EM experiments.

I thank Mark Biggin for advice on native gel separation of ssDNA, Richard Austin for the anti-ORC polyclonal sera, Anthony Schwacha for purified yeast RPA, and Tania Baker and Carl Pabo for critical reading of the submitted manuscript.

SUMMARY

The *Saccharomyces cerevisiae* Origin Recognition Complex (ORC) is bound to origins of DNA replication throughout the cell cycle and directs the assembly of higher-order protein-DNA complexes during G1. To examine the fate of ORC when origin DNA is unwound during replication initiation, I determined the effect of single-stranded DNA (ssDNA) on ORC. I show that ORC can bind ssDNA and that the ssDNA-bound form of ORC is distinct from that bound to double-stranded origin DNA. Electron microscopy studies demonstrated that ssDNA stabilizes a bent conformation of ORC whereas origin DNA stabilizes an extended form of ORC. In addition, ssDNA stimulates the ORC-ATPase activity, whereas origin-containing DNA inhibits it. I propose that the unwinding of origin DNA activates an ssDNA-controlled ORC conformational switch that contributes to the remodeling of the origin-associated protein complexes assembled during G1.

INTRODUCTION

Origins of DNA replication are sites at which multiple polypeptides must be assembled in a highly regulated manner and subsequently disassembled to allow formation of a mobile DNA replication machine. Studies of chromosomal replication in a number of organisms, including bacteria, phage, and eukaryotic viruses, have demonstrated that a protein called the initiator participates in multiple early steps in this process (reviewed in Baker and Bell, 1998). Initiator proteins bind origins of replication and thereby select the site at which replication is to begin. These proteins also frequently facilitate the unwinding of origins required to generate the single-stranded DNA (ssDNA) template for polymerase action. Finally, initiator proteins recruit other replication proteins required for the assembly of replication forks at the origin.

In eukaryotic cells, the strongest candidate for an initiator protein is the Origin Recognition Complex (ORC; Bell and Stillman, 1992; reviewed in Dutta and Bell, 1997). ORC was first identified in the yeast *Saccharomyces cerevisiae* and consists of six polypeptides (Orc1p – Orc6p) that are each essential for yeast viability (Bell et al., 1993; Bell et al., 1995; Li and Herskowitz, 1993; Loo et al., 1995) . Analogs of ORC subunits have been identified in a number of organisms, and in *Xenopus laevis* and *Drosophila melanogaster*, they have been shown to form a six-protein complex similar to that seen in yeast (reviewed in Dutta and Bell, 1997). Studies of replication either *in vivo* or *in vitro* strongly suggest that ORC is required for DNA replication in all eukaryotic species (Chesnokov et al., 1999; Landis et al., 1997; Pasero et al., 1997; and reviewed in Dutta and Bell, 1997).

In yeast, ORC binds to origins of replication throughout most or all of the cell cycle and directs the assembly of higher order complexes prior to the initiation of DNA replication. *In vivo* DNase I protection assays and chromatin immunoprecipitation

(CHIP) experiments suggest that ORC alone is present at the origin during the G2- and M-phases to form what has been termed the postreplicative complex (post-RC; Aparicio et al., 1997; Diffley et al., 1994; Tanaka et al., 1997). In G1, ORC is required to recruit additional replication proteins to origins, including Cdc6p and the MCM proteins (in that order), to form a prereplicative complex (pre-RC). Finally, Cdc45p and the replicative polymerases are recruited to origins in a manner correlated with the time of replication initiation (Aparicio et al., 1997; Aparicio et al., 1999; Zou and Stillman, 1998). During S-phase, Cdc6p is degraded (Piatti et al., 1995), and MCM proteins and Cdc45p appear to be released from origins and move with the DNA polymerases as part of the replication fork (Aparicio et al., 1997). ORC remains at the origin to repeat the process in the following cell cycle. The requirement for an ORC-dependent assembly of replication proteins on DNA is likely to be conserved throughout evolution, as replication in *Xenopus* extracts requires chromatin association of ORC, Cdc6p and MCM proteins, with the same dependence as seen in yeast (reviewed in Diffley, 1996).

In addition to recognizing origin DNA, yeast ORC binds and hydrolyzes ATP, and these three activities are tightly coupled (Klemm et al., 1997). ATP binding to the Orc1p subunit is required for the complex to bind origin DNA. Interaction with origin DNA inhibits the Orc1p-dependent ATP hydrolysis activity, reducing it to a rate that is consistent with roughly one turnover event per cell cycle. ATPase repression requires origin-containing double-stranded DNA (dsDNA) as non-origin dsDNA has no effect on the rate of ATP hydrolysis. Thus, ATP binding and sequence-specific DNA binding are coordinate processes that together regulate ATP hydrolysis by ORC.

The coordinate action of five of the six ORC subunits is required for origin binding (only the Orc6p subunit is dispensable for DNA binding; Chapter II; Lee and Bell, 1997). ORC subunits are arranged in two groups that each contact distinct regions

of the DNA, and ORC contacts more residues on one strand of the dsDNA than the other. This mode of DNA binding occurs throughout most of the cell cycle. However, during the initiation of replication, the origin must become unwound to allow access of the enzymatic machinery to the DNA substrate. The consequence of this DNA unwinding event for ORC-DNA interactions as well as other proteins assembled at the origin is not understood.

In each cell cycle, the assembly and disassembly of origin-associated complexes must be carefully regulated to ensure that the entire genome is replicated once and only once (reviewed in Diffley, 1996). In yeast, the assembly of pre-RCs occurs during G1 when Cdc6p is abundant and when levels of B-type cyclin-dependent kinase (CDK) activity are low. Entry into S-phase requires the activation of the S-phase CDKs (Clb5- and Clb6-associated CDK) and the activity of the Cdc7p/Dbf4p kinase. As each origin is activated, the pre-RC must be remodeled to release components of mobile replication forks and to inactivate this origin. ORC re-binds origin DNA to form a post-RC and must be prevented from re-forming new pre-RCs and re-initiating replication until the next G1-phase. Interestingly, the activities of the B-type CDKs are required to prevent pre-RC formation during S-, G2-, and M-phases (Dahmann et al., 1995; Detweiler and Li, 1998; Piatti et al., 1996; Tanaka et al., 1997). Thus, the same kinases involved in activating pre-RCs are also responsible for preventing their assembly until the appropriate time in the next cell cycle.

In contrast to our understanding of the regulation of pre-RC formation, little is known about the mechanism by which this complex is remodeled to release replication fork components and is inactivated. This event is likely to be critical for both the initiation process and the prevention of re-replication. In this chapter, I present evidence that ORC is involved in this process. In the course of experiments designed to determine

how ORC interacts with a DNA target whose structure changes during the cell cycle, I found that ORC can bind ssDNA. Furthermore, ssDNA alters ORC conformation (as determined by electron microscopy) and stimulates the ORC ATPase. I propose a model in which unwinding of origin DNA stimulates changes in ORC that are coupled to the remodeling and disassembly of origin-associated complexes and to the inactivation of pre-RCs assembled at passively replicated loci.

RESULTS

ORC Binds ssDNA in an ATP-Independent Manner

To determine if ORC was capable of continued association with origin DNA after unwinding, I first asked if ORC could bind ssDNA. I performed electrophoretic mobility shift assays using radiolabeled ssDNA and dsDNA, each containing the wild-type *ARS1* origin, as well as labeled dsDNA containing a mutated origin (Figure 1). As had been previously described, the binding of purified ORC to dsDNA was both sequence-specific (compare lanes 5 and 14) and dependent on the presence of ATP (compare lanes 1 and 5). I found that when ORC was incubated with labeled ssDNA, the mobility of the ssDNA was altered. The shifted species included ORC because it could be supershifted by ORC-specific monoclonal antibodies (Appendix A). Thus, ORC is able to bind ssDNA. As with ORC-dsDNA binding activity, the binding of ORC to ssDNA did not require the presence of the Orc6p subunit (Appendix A).

Unlike binding of ORC to dsDNA, this new DNA binding activity of ORC did not require specific yeast origin sequences or the presence of ATP. Although initial experiments used ssDNA containing the *ARS1* sequence, I have seen that ORC can bind every naturally occurring ssDNA that I have tested, including that from yeast, bacteriophage M13, bacteriophage lambda, and humans (data not shown). Furthermore, ORC-ssDNA binding occurred in the absence of ATP (Figure 1, lanes 6-9). To further demonstrate that ATP was not required for ORC-ssDNA binding, two mutated ORC complexes that have lost the ability to bind ATP were also tested (see Experimental Procedures). Neither of these mutant complexes showed any defect in ssDNA binding (data not shown). In addition, the affinity of ORC for ssDNA (see below) was unaffected by the presence of 42 μM ATP or ATPγS (a non-hydrolyzable analog of ATP; data not

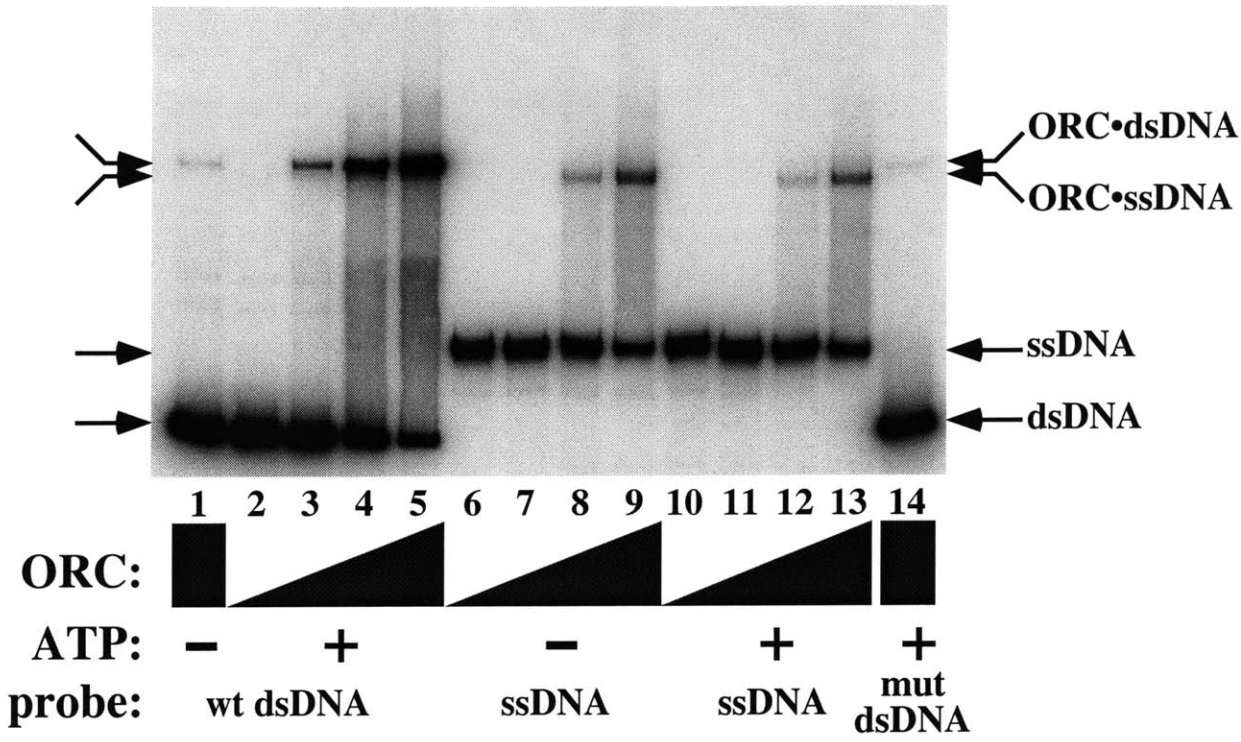

Figure 1. ORC Binds ssDNA in an ATP-Independent Manner. ORC mobility shift assays were performed using a radiolabeled 244 bp dsDNA containing a wild-type *ARS1* origin (wt dsDNA, lanes 1 to 5), a labeled 295 nucleotide ssDNA containing wild-type *ARS1* (ssDNA, lanes 6 to 13), and a labeled 244 bp dsDNA containing *ARS1* with a mutation in the ORC binding site (mut dsDNA, lane 14). ATP (50 μM final) was added to reactions in lanes 2-5 and 10-14. Two-fold titrations of ORC resulted in 2.5 ng (lanes 2, 6 and 10), 5 ng (lanes 3, 7 and 11), 10 ng (lanes 4, 8 and 12), or 20 ng of ORC per reaction (lanes 1, 5, 9, 13 and 14). The binding reactions were electrophoresed on a native polyacrylamide gel to separate bound and unbound DNA.

shown), indicating that ATP binding does not inhibit ssDNA binding. Thus ssDNA binding occurs equally well whether ORC is bound to ATP or free of nucleotide.

The relative affinities of ORC for ssDNA or dsDNA depended on the presence or absence of ATP. The electrophoretic mobility shift data suggested that, in the presence of ATP, the affinity of ORC was highest for specific dsDNA, weaker for ssDNA, and weaker still for non-specific dsDNA (Figure 1, compare lanes 5, 13 and 14 respectively). However, in the absence of ATP, ORC bound ssDNA more tightly than specific or non-specific dsDNA (compare lane 9 with lane 1). I used the mobility shift assay to determine the concentration of unbound ORC at half maximal binding to specific dsDNA (in the presence of ATP) and to ssDNA – the inverse of this value is the apparent affinity constant. The association of ORC for dsDNA in the presence of ATP (apparent K_A = 1.4 x 10^9 M^{-1}) was indeed slightly higher than that of ORC for ssDNA (apparent K_A = 0.9 x 10^9 M^{-1}). The apparent association constant for ORC and ssDNA was also compared to that of the *S. cerevisiae* ssDNA binding protein, ScRPA, and was found to be within an order of magnitude (apparent K_A for ScRPA and ssDNA = 3.0 x 10^9 M^{-1}, consistent with published results for ScRPA; reviewed in Wold, 1997). Therefore, ORC has an affinity for ssDNA that is somewhat weaker but comparable to an established yeast ssDNA binding protein.

ORC-ssDNA Binding and ORC-dsDNA Binding are Mutually Exclusive

To examine whether ORC uses a similar binding site to interact with ssDNA and dsDNA, competition experiments were carried out (Figure 2). Binding of ORC to radiolabeled substrates (wild-type dsDNA and ssDNA) was assayed in the presence or absence of ATP, and in the presence or absence of one of three unlabeled competitor DNAs (present at a ten-fold molar excess): an M13 ssDNA circle, wild-type dsDNA, and

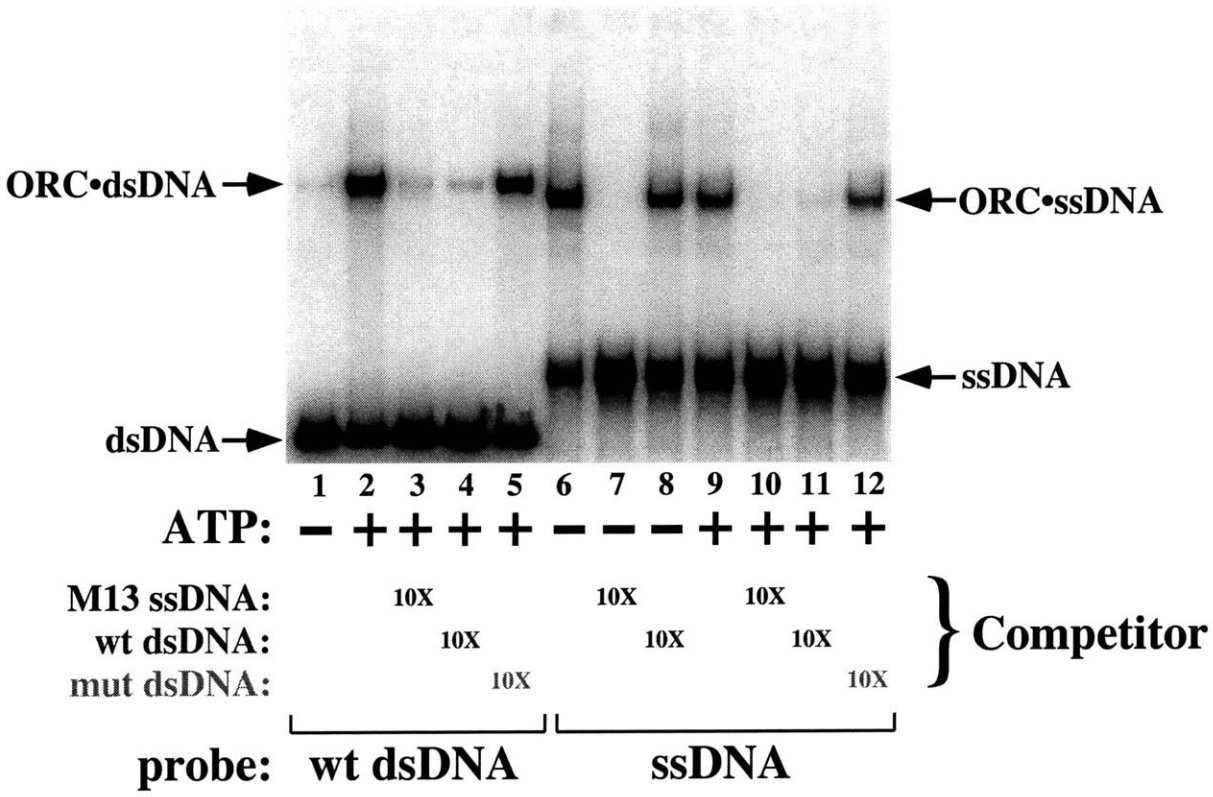

Figure 2. ORC-ssDNA Binding and ORC-dsDNA Binding are Mutually Exclusive. The ssDNA and dsDNA mobility shift assay was repeated in the presence or absence of one of three unlabeled competitor DNAs: an ssDNA M13 circle (laness 2, 7 and 10), wild-type dsDNA (lanes 4, 8 and 11) and mutant dsDNA (lanes 5 and 12). Competitor DNAs were present at a ten-fold molar excess over the labeled DNA, and ATP (50 μM) was included as indicated.

dsDNA containing a mutation in the ORC binding site (mut dsDNA). Again, ORC-dsDNA binding was dependent on origin sequences and on ATP. The binding of ORC to labeled wild-type dsDNA (in the presence of ATP, lane 2) was efficiently competed by unlabeled wild-type dsDNA (lane 4) but not the mutant dsDNA (lane 5). M13 ssDNA was also an effective competitor of ORC-dsDNA binding, resulting in a loss of the ORC-dsDNA signal without the appearance of a slower mobility species indicative of ORC simultaneously binding dsDNA and ssDNA (lane 3). Binding of ORC to radiolabeled ssDNA (lanes 6 and 9) was efficiently competed by dsDNA, but only when the wild-type sequence was used as the competitor (compare lanes 11 and 12) and ATP was present (compare lanes 11 and 8). Thus, binding of ORC to ssDNA and to dsDNA are mutually exclusive, arguing that the two binding sites are at least partially overlapping or that ORC undergoes a conformational change such that one DNA molecule acts as an allosteric inhibitor of binding to the other.

ORC Prefers to Bind ssDNA Molecules 90 Nucleotides or Longer

To determine the optimal length of ssDNA bound by ORC, I incubated ORC with a mixed population of ssDNA molecules of different lengths. Subsequent immunoprecipation of ORC allowed us to compare the ssDNA bound by ORC (B) to the unbound or free ssDNA (F, Figure 3). When ORC was incubated with end-labeled ssDNA molecules that were either 113 or 295 nucleotides long, each probe co-immunoprecipitated with ORC with similar efficiencies (Figure 3A, compare bound and free for each probe). These ssDNA fragments were then treated with the single-strand specific S1 nuclease to generate a population of different sized molecules. Incubation of ORC with the cleaved ssDNA followed by immunoprecipation of ORC showed that the bound population (B) consisted primarily of molecules ~90 nucletides or longer, and that

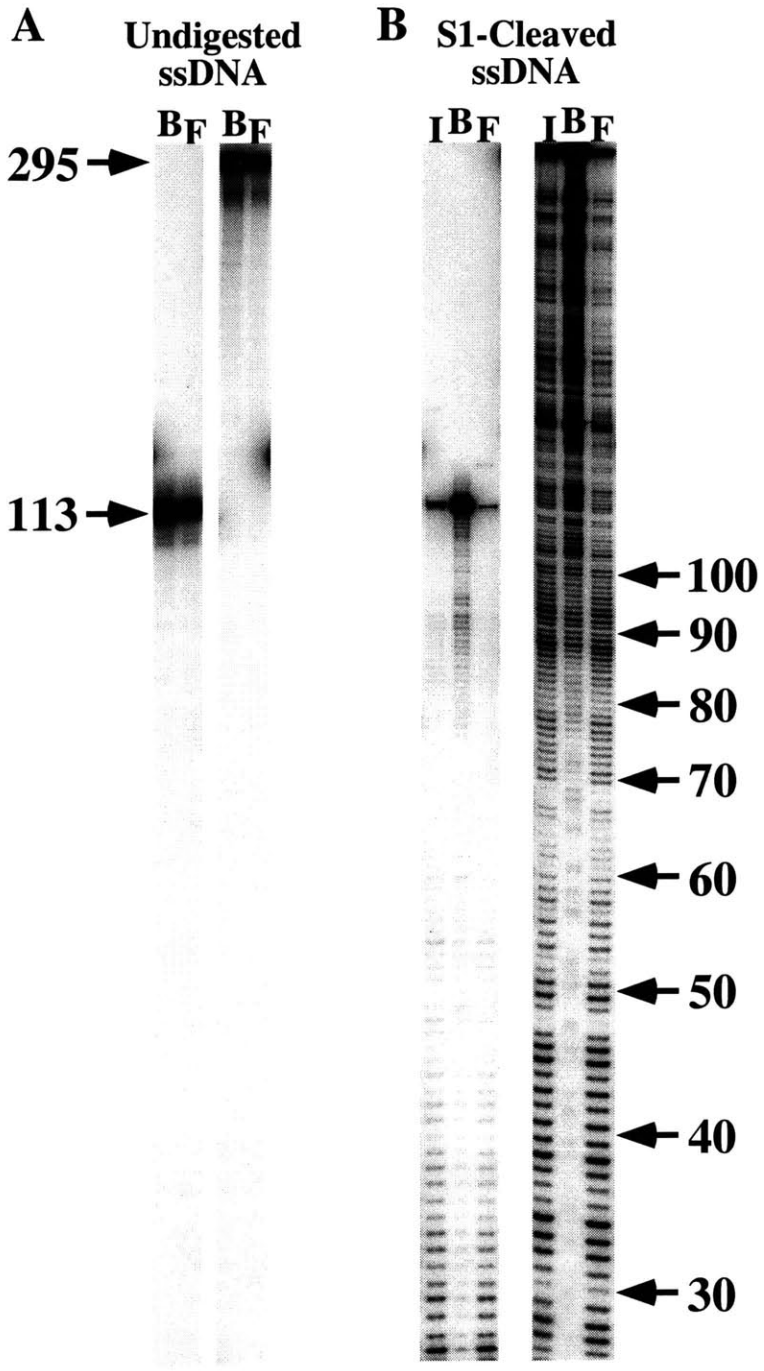
A
Undigested
ssDNA
B
S1-Cleaved
ssDNA
B F B F
I B F I B F
295
113
100
90
80
70
60
50
40
30

Figure 3

Figure 3. ORC Preferentially Binds ssDNA ~90 Nucleotides or Longer

(A) ORC was incubated with end-labeled with ssDNA that was 113 nt or 295 nt long. Anti-ORC polyclonal sera and beads coupled to Protein G were used to immunoprecipitate ORC and the associated ssDNA. The bound DNA (B) and the unbound or free DNA (F) were electrophoresed on a denaturing polyacrylamide gel. (B) 113 nt and 295 nt ssDNAs were cleaved with S1 nuclease to generate random populations of ssDNA molecules of different length (input DNA, I). The resulting ssDNA was then incubated with ORC and immunoprecipitated as before. Input, bound and free DNAs were electrophoresed on a denaturing polyacrylamide gel. ssDNA lengths (in nucleotides) are shown on the right.

the relative amounts of molecules in this size range were reduced in the free population (F) as compared to the starting input material (I). Because similar results were obtained with two DNAs of unrelated sequence, the observed threshold of ~90 nucleotides is unlikely to be due to the presence of a specific sequence. Thus, ORC has a preference for binding ssDNAs that are greater than 90 nucleotides in length.

To test if ORC is capable of binding shorter ssDNA molecules, oligonucleotides of various lengths were examined using the electrophoretic mobility shift assay. Binding of ORC was not detected with a molecule 20 nucleotides long (data not shown), weak binding was detected with a 30-mer and a 39-mer (DL11), and in general, the affinity of ORC for ssDNA increased as the length of the ssDNA increased. Oligonucleotides for which apparent association constants were calculated are shown in Figure 4A in order of increasing affinity, ranging from 1.6×10^7 M^{-1} (for the 39-nt DL11 oligo) to 7.7×10^7 M^{-1} (for the 96-nt DL15 oligo). The correlation between ssDNA length and affinity is not perfect, however. For example, the 50-nt DL12 oligo was bound more tightly by ORC than the 60-nt DL13 oligo, and I suspect that the different nucleotide sequences of these unrelated oligonucleotides are responsible for this discrepancy.

ssDNA and dsDNA Have Opposite Effects on ORC-ATPase Activity

Previous studies of the ORC ATPase indicated that origin dsDNA (but not non-origin dsDNA) strongly inhibits this activity. To determine if ssDNA also affected the ORC ATPase, I measured the rate of ORC-ATP hydrolysis in the absence of DNA, in the presence of origin-containing dsDNA, and in the presence of ssDNA (Figure 4B). I found that ssDNA and origin DNA had opposite effects: in contrast to the ~6-fold inhibition by the origin, ssDNA stimulated ATPase activity up to 2-fold. Consistent with previous findings, the ATP hydrolysis activity that occurred in the presence of ssDNA

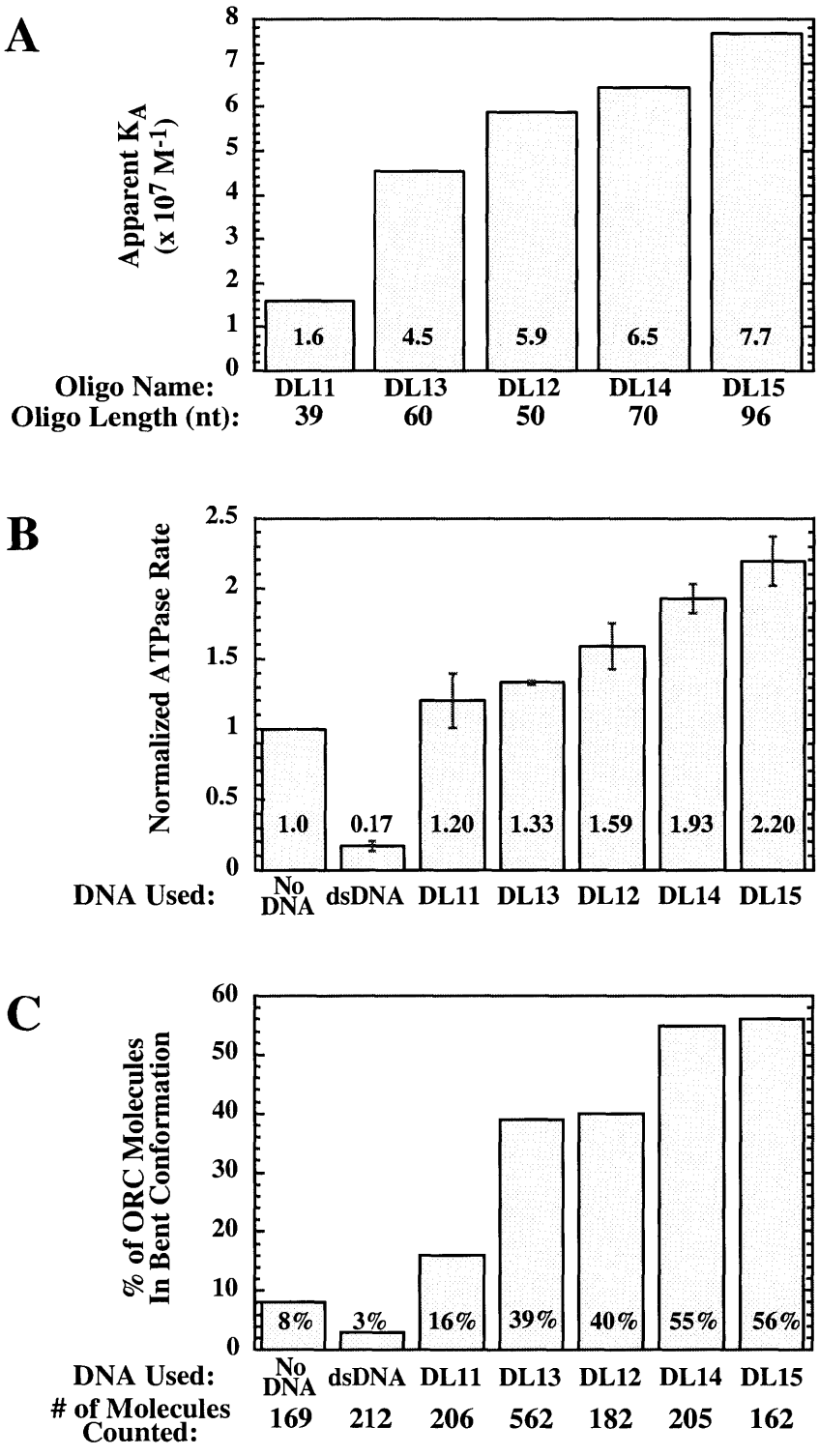
A
Apparent K_A ($\times 10^7 M^{-1}$)
8
7
6
5
4
3
2
1
0
1.6
4.5
5.9
6.5
7.7
Oligo Name:
DL11
DL13
DL12
DL14
DL15
Oligo Length (nt):
39
60
50
70
96
B
Normalized ATPase Rate
2.5
2
1.5
1
0.5
0
1.0
0.17
1.20
1.33
1.59
1.93
2.20
DNA Used:
No DNA
dsDNA
DL11
DL13
DL12
DL14
DL15
C
% of ORC Molecules In Bent Conformation
60
50
40
30
20
10
0
8%
3%
16%
39%
40%
55%
56%
DNA Used:
No DNA
dsDNA
DL11
DL13
DL12
DL14
DL15
# of Molecules Counted:
169
212
206
562
182
205
162

Figure 4

Figure 4. ssDNA Stimulates ORC ATPase Activity and Induces a Conformational Change in ORC.

(A) Apparent association constants of ORC for ssDNA oligonucleotides of indicated lengths were determined using the electrophoretic mobility shift assay. The K_A was calculated by taking the inverse of the concentration of free ORC at half maximal binding. Oligonucleotides are shown in order of increasing affinity (lengths in nucleotides are indicated below each oligonucleotide).

(B) The rate of ATP hydrolysis by ORC was measured in the absence of DNA, in the presence of origin-containing dsDNA (the 244 bp dsDNA, see Experimental Procedures), or in the presence of oligonucleotides of various lengths. The ssDNA oligonucleotides are again shown in order of increasing affinity for ORC. ATPase rates were normalized to the rate of hydrolysis seen in the absence of DNA. The averages and standard deviations for three experiments are shown.

(C) Quantitation of the percent of ORC molecules in the bent conformation as determined by electron microscopy (Figure 5). ORC was examined in the absence of DNA, in the presence of dsDNA, or in the presence of ssDNA of various lengths. The total number of ORC molecules counted in at least two experiments is shown below each bar.

was dependent on the activity of the Orc1p subunit, (see Experimental Procedures). To determine if the strength with which ORC binds a particular ssDNA was related to the ability of the DNA to stimulate ORC-ATPase activity, I tested oligonucleotides of various lengths in this assay (Figure 4B). ssDNA molecules with a higher affinity for ORC resulted in a greater stimulation of ATP hydrolysis. Thus, just as the strength of dsDNA binding correlates with the extent of inhibition of ATP hydrolysis (Klemm et al, 1999), I found that the strength of ssDNA binding correlated with the degree of ATPase stimulation.

ssDNA Alters the Conformation of ORC

The switch from a state of ORC in which it stably binds ATP to a state in which it hydrolyzes ATP may involve allosteric changes among ORC subunits. To test this hypothesis directly, I examined the effect of dsDNA and ssDNA on the conformation of ORC using transmission electron microscopy in collaboration with Jack Griffith's laboratory at the University of North Carolina, Chapel Hill. In the absence of DNA, ORC stained with uranyl acetate had a tri-lobed structure, with the smallest lobe in the middle and one of the outer lobes slightly larger than the other. A representative field of ORC molecules showed that the majority of complexes were straight or extended (Figure 5A and C). However, a minority of ORC complexes (8%, N = 169) adopted a more bent or compact structure (Figure 4C). When ORC was bound to dsDNA (in the presence of ATP), the structure of ORC was similar to that of ORC in solution, with an even smaller fraction of the complexes (3%, N = 212) adopting the bent conformation (Figure 4C). In contrast, incubation of ORC with ssDNA resulted in a dramatic increase in the number of bent complexes: in the presence of the 96-nt DL15 oligonucleotide, 56% of ORC complexes were bent (N = 162; Figures 4C, 5B and 5C).

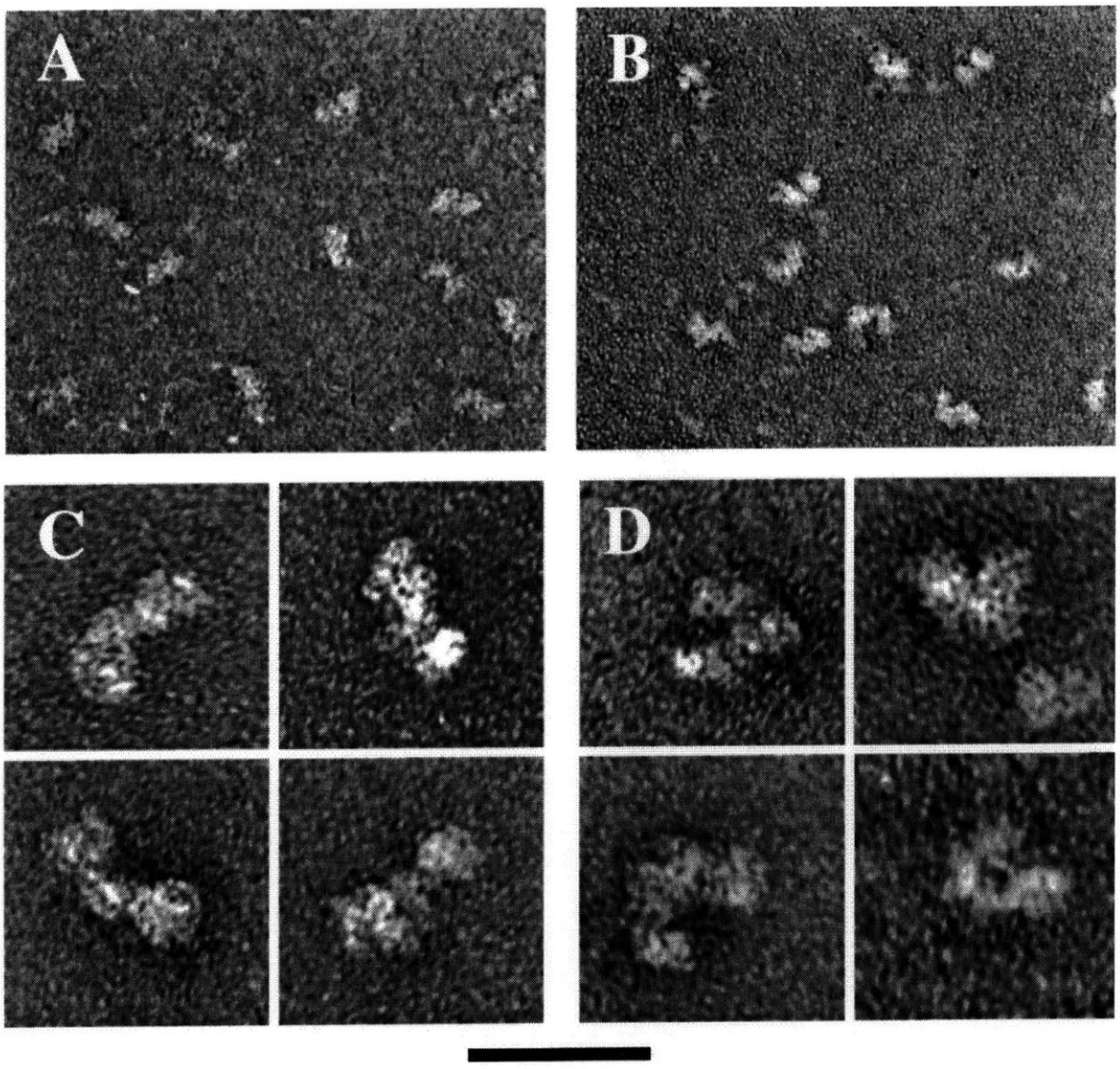

Figure 5

Figure 5. Electron Microscopy of ORC.

Transmission electron microscopy of ORC was performed on samples negatively stained with uranyl acetate. Low magnification images show that, in the absence of DNA, ORC is primarily in a straight or extended conformation (A), whereas in the presence of ssDNA, many complexes adopt a bent or curved conformation (B).

(C) High magnification images of straight ORC molecules in the absence of DNA.

(D) High magnification images of bent ORC molecules in the presence of ssDNA.

The scale bar represents 40 nm for panels A and B and 17 nm for panels C and D.

To further characterize the ssDNA-stimulated change in ORC conformation, we measured the angle formed between the two arms of ORC. When ORC complexes in the straight conformation were measured, the angle was 166 +/- 11 degrees (N = 100), whereas the angle for complexes in a bent conformation was 106 +/- 13 degrees (N = 100). We then chose 100 molecules at random (regardless of conformation) from the samples containing ORC alone: the average angle for this population of molecules was 151 +/- 26, consistent with a majority of complexes in a straight conformation. In contrast, for ORC in the presence of the 96-nt DL15 ssDNA, the average angle for a population of molecules was 127 +/- 31 (N = 100), consistent with roughly half of the complexes present in the bent conformation. Finally, different oligonucleotides were tested to see if the fraction of ORC complexes bent in the presence of ssDNA was dependent on ORC's affinity for the DNA. We again observed a correlation between the strength of ssDNA binding and the percent of bent ORC molecules (Figure 4C), similar to the correlation seen between the strength of binding and the rate of ATP hydrolysis (Figure 4B). Thus, the abilities of a ssDNA molecule to stimulate ATPase activity and stabilize the altered ORC conformation are likely related (see Discussion).

DISCUSSION

In addition to the well-characterized ORC-dsDNA binding activity, I have found that ORC also binds ssDNA *in vitro*. Unlike the binding of ORC to dsDNA, ORC-ssDNA interaction does not require specific sequences or ATP. Binding of ORC to dsDNA and ssDNA are mutually exclusive, consistent either with binding sites that are (at least partially) overlapping or with different conformational states of ORC that are specific for ss- or dsDNA. Finally, ssDNA binding has a significant effect on two potentially related properties of ORC: ATP hydrolysis and ORC conformation.

The rate of ATP hydrolysis by the Orc1p subunit is stimulated by ORC-ssDNA binding resulting in an ATPase activity that is roughly 13-fold higher than that of ORC bound to the origin. Currently, I do not know if the ATPase activity seen with ORC in the presence of (sequence-specific) dsDNA is due to a slow rate of hydrolysis by ORC bound to dsDNA, or if hydrolysis can only occur when ORC is unbound from DNA (either due to a small population of ORC that is not bound to origin DNA or to dissociation of ORC from the origin DNA). If hydrolysis requires that ORC is free from dsDNA, then our measurement of the ATPase activity of origin-bound ORC bound is an overestimate, and the true difference between the rate of hydrolysis for ORC on dsDNA versus ssDNA would be larger.

ssDNA also stimulates ORC to adopt a more bent or curved conformation, resulting in a shift from 3% of the molecules bent in the presence of origin DNA to 56% bent in the presence of ssDNA. Although this change is significant, the difference between the percentage bent in the presence of dsDNA and ssDNA may also have been underestimated. First, since short ssDNA was not visualized by uranyl acetate staining, some of the straight complexes counted may not have been bound to ssDNA. In contrast, ORC in the presence of dsDNA was only scored as bent or straight if associated DNA

was observed. Second, bent complexes that were rotated 90 degrees with respect to the visual plane may have been incorrectly scored as straight. Although this latter scenario would result in underestimates for all conditions (ORC alone, ORC with dsDNA and ORC with ssDNA), the samples with the greatest absolute number of bent complexes would have been affected the most. For example, if only 80% of all bent complexes were in the appropriate orientation, then the actual percent of bent complexes would be 10% (instead of 8%) for ORC alone, 4% (instead of 3%) for ORC with dsDNA, and 70% (instead of 56%) for ORC with the 96-nt ssDNA.

The conformational state of ORC (straight or bent) correlates with the ability or inability of ORC to hydrolyze ATP. When ATPase activity is lowest, the percent of bent complexes is at a minimum, and when ATPase activity is highest, the greatest amount of bending is observed (compare Figures 4B and 4C). This correlation suggests that the two phenomena affected by ORC-ssDNA interaction are functionally related. Since the percent of ORC complexes bent in the presence of ssDNA is unaffected by the addition of ATPγS or by the use of an ATPase-defective mutant ORC complex (data not shown), ATP hydrolysis is unlikely to be required for the observed conformational change. Instead, I hypothesize that the ssDNA-induced conformational change is required for ATPase activity, although our current data cannot prove that a causal relationship exists.

Does ORC Interact with ssDNA *in vivo*?

If ORC does bind ssDNA *in vivo*, when and where would this interaction occur? The initiation of DNA replication results in dramatic changes in the structure of origin DNA and represents the most likely time for ORC to bind ssDNA. Recent data suggest that initial DNA melting at the *ARS1* origin occurs immediately adjacent to the ORC binding site (Bielinsky and Gerbi, 1999), possibly leading to its partial or complete

disruption. In the absence of its preferred dsDNA binding site, our data suggests that ORC would bind the newly formed ssDNA as the apparent affinity for ssDNA is higher than that for non-specific dsDNA. Since origin DNA binding involves more numerous interactions with the A-rich strand (Chapter II; Lee and Bell, 1997), the transition from dsDNA binding to ssDNA binding may not require dissociation of ORC from the DNA. Furthermore, the continued association of ORC with origin DNA in the unwound state may facilitate rapid re-binding of ORC to the origin after it is replicated and before other proteins such as histones have the opportunity to bind.

The amount and distribution of ORC within the nucleus suggest that ORC binds ssDNA only at origins. Estimates of the amount of ORC present in yeast cells indicate that there are roughly equal numbers of ORC molecules and origins of replication (Rowley et al., 1995). Furthermore, *in vivo* DNase I protection assays and *in vivo* chromatin-immunoprecipitation of both active and inactive origins have shown them to be bound by ORC throughout most or all of the cell-cycle (Aparicio et al., 1997; Diffley et al., 1994; Tanaka et al., 1997). Thus, the majority of ORC present in the cell is likely to be bound to origin DNA, and the amount of ORC available to bind ssDNA at non-origin sequences is likely to be low.

The Transition Between the Two States of ORC may be Coupled to Origin Unwinding.

Our data suggests that ORC exists in two states, one that has a straight conformation and binds ATP, and one that has a bent conformation and hydrolyzes ATP. Since these two states are stabilized by two different forms of DNA, I hypothesize that the transition from one state of ORC to the other is coupled to the transition from dsDNA to ssDNA that occurs during origin unwinding. Based on these data and previous work

from our lab and other groups, I propose the following model for ORC function at the origin (Figure 6): (1) ORC, ATP, and origin DNA form a tripartite complex soon after the origin has been replicated to form the post-RC. The binding of ORC to dsDNA inhibits the Orc1p-associated ATPase activity resulting in a stable complex. (2) During G1, ORC mediates the assembly of the pre-RC. (3) Melting and unwinding of the origin disrupts the ORC binding site and simultaneously generates ssDNA to which ORC binds. (4) The loss of dsDNA binding and the concomitant binding to ssDNA results in a stimulation of ORC-ATPase activity. (5) The hydrolysis of ATP renders the ssDNA-associated ORC inactive for re-binding dsDNA until it has re-bound ATP. Furthermore, I hypothesize that the observed conformational change in ORC may alter its association with other proteins in the pre-RC.

Origins of replication must accomplish two things. They must direct the assembly of a stable multi-protein machine with high sequence specificity but subsequently allow this complex to be remodeled to release proteins that are components of replication forks (potentially including MCM proteins and Cdc45p; Aparicio et al., 1997). It is attractive to speculate that the remodeling of the pre-RC occurs, in part, via a conformational change in ORC that is coupled to the origin unwinding step. If ORC monitors and responds to changes in the structure of the origin DNA, then the cell could ensure that pre-RC remodeling would not occur at any origin until unwinding has occurred. Indeed, the length dependence of the ORC-ssDNA interaction may serve as a measure of DNA unwinding to postpone remodeling until the appropriate length of ssDNA is present at the origin. ORC could then irreversibly commit that origin to completing the initiation process by temporarily losing its ability to bind dsDNA (due to the hydrolysis of ATP) and by releasing the components of the replication fork from the origin. This particular ORC molecule could only re-bind dsDNA after re-binding ATP

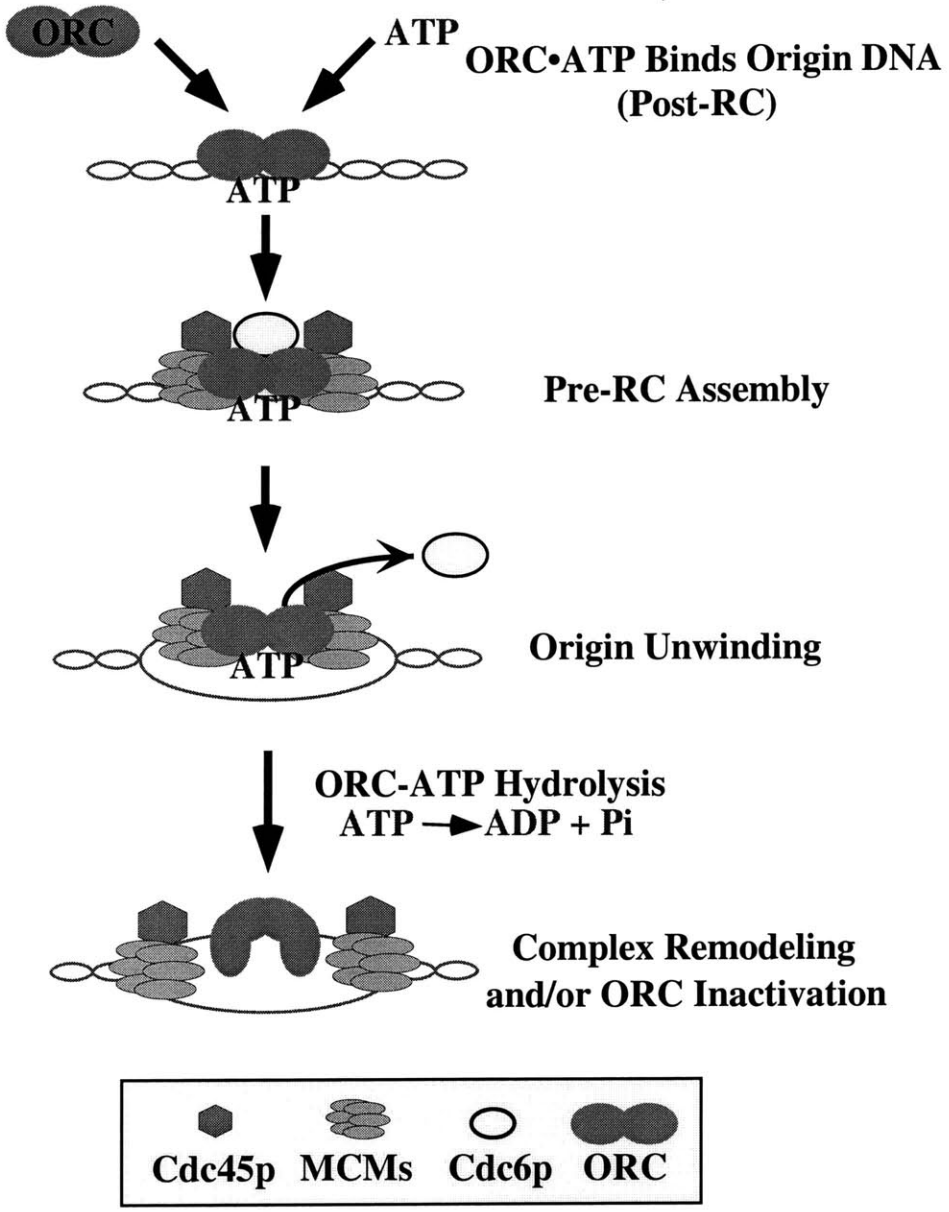

Figure 6. Model for an ssDNA-Regulated Conformational Switch in ORC

ORC binds to origin DNA in an ATP-bound state with an extended conformation. This form of ORC is competent to recruit replication proteins to the origin to assemble the prereplicative complex (pre-RC). Origin unwinding allows ORC to interact with the resulting ssDNA which in turn induces a conformational change in ORC and stimulates ATP hydrolysis. This conformational switch may be required to inactivate ORC (to prevent re-replication from activated origins) and/or to release components of replication forks from the origin. See text for more details.

and would then exist only in the post-RC state. The inhibitory effects of S-phase CDKs would prevent pre-RC formation until the subsequent cell cycle (Dahmann et al., 1995; Detweiler and Li, 1998; Piatti et al., 1996; Tanaka et al., 1997).

In addition to preventing activated origins from re-firing during the same S phase, an ssDNA-regulated conformational switch in ORC may also inactivate pre-RCs that are formed at passively replicated origins. Many origins fire during only a percentage of cell cycles (Yamashita et al., 1997) yet it is imperative that the pre-RCs assembled at these origins not fire if they are passively replicated. Indeed, it is known that an inactive origin such as *ARS301* can assemble a pre-RC even though it does not initiate DNA replication (Santocanale and Diffley, 1996). Although the movement of a replication fork through an origin that has not fired may be sufficient to dismantle the pre-RC, the ssDNA generated by a moving replication fork (Park et al., 1998) could also be sensed by ORC to ensure complete inactivation of the pre-RC. How can the same conformational switch in ORC be used to trigger DNA replication at pre-RCs formed at active origins but to inactivate pre-RCs formed at passively replicated loci? One likely explanation is that the recruitment of Cdc45p to origins is temporally regulated (Aparicio et al., 1999). Cdc45p, a protein essential for the initiation of replication (see Chapter I), is not loaded onto late-replicating origins until later in S-phase, close to the time at which they are activated. Therefore, when ssDNA formed at a passively replicated locus triggers ORC to remodel the associated pre-RC components, the lack of Cdc45p (and likely the lack of DNA polymerases, see Chapter I) results in an inactivation rather than activation of these replication proteins.

In addition to its role in replication initiation, ORC may also be an important component of the regulatory mechanisms that prevent re-initiation and ensure the fidelity of genomic replication. A rigorous test of this model will require a detailed

understanding of how the properties of ORC (including ssDNA binding, ATP hydrolysis, and conformational change) are affected by its interaction with other proteins that are recruited to origins. Ultimately, these studies can lead to an *in vitro* reconstitution of a functional pre-RC that will allow for the dissection of the various steps in the initiation pathway and the role of changes in the conformation of ORC during this process.

EXPERIMENTAL PROCEDURES

Wild-type and Mutant ORC Complexes

Wild-type ORC was expressed in insect cells and purified as described (Lee and Bell, 1997). Mutant ORC complexes containing point mutations in the ATP-binding domain of the Orc1p subunit (ORC-1A complex) or the Orc5p subunit (ORC-5A complex; Klemm et al., 1997), were also compared to wild-type ORC in ssDNA binding assays and in ATPase assays. The Orc5p subunit binds ATP, but does not hydrolyze ATP and is not regulated by DNA. I found that neither the ORC-1A complex nor the ORC-5A complex showed defects in ssDNA binding and that the stimulation of ORC-ATPase activity seen in the presence of ssDNA required wild-type Orc1p function but not Orc5p function (data not shown). The ORC-1A complex was also examined by electron microscopy and found to have wild-type levels of an ssDNA-stimulated change in conformation (data not shown).

dsDNA and ssDNA

All DNA prepared during the course of these experiments was purified as described (Lee and Bell, 1997) unless otherwise noted:

1. 244 bp dsDNA (wild-type and mutant): an *EcoRI-HindIII* fragment of pARS1/WT (Marahrens and Stillman, 1992) or pARS1/a-b2- (Lee and Bell, 1997) respectively. When used as radiolabeled probes, these DNAs were end-labeled with T4 Polynucleotide Kinase (New England Biolabs) and γ^{32}P-ATP at the *HindIII* site.
2. 575 bp dsDNA: an *ARS1* containing PCR product of pARS1/WT using oligos FSP24 and BUBLR1 as primers.
3. 244 nt ssDNA: the end-labeled 244 bp dsDNA (wild-type) was resuspended in 51 µl of TE containing 9% DMSO, heated to 95°C for 10 minutes to denature the DNA, and quick

chilled in ice water. An equal volume of ice cold 0.1 M NaOH was added, and the entire mixture was separated on a 5% acrylamide gel (50:1 acrylamide:bisacrylamide, 0.56 x TBE) cooled to 4°C. Electrophoresis was performed for 6,000 Volt-hours at 4°C, and the gel-purified labeled ssDNA was detected by exposing the wet gel to film. The *HindIII* labeled ssDNA was separated from the other single strand and from duplex DNA (data not shown). Since this ssDNA was derived from the end-labeled dsDNA, the two DNAs had identical specific activities.

4. 113 nt ssDNA and 295 nt ssDNA: these ssDNAs were generated by combining 50 pmol of an end-labeled oligonucleotide with 8 μg of a plasmid cut with a restriction enzyme in a standard PCR mixture (Ausubel et al., 1994) and subjecting the reaction to 30 cycles of linear amplification with Taq polymerase. The reaction was precipitated with ethanol, resuspended in a formamide-dye mixture, and electrophoresed on a 6% sequencing gel to purify the end-labeled ssDNA. For the 113 nt ssDNA, oligo DLX833-814 was used with XbaI digested pARS1/WT, and for the 295 nt ssDNA, oligo RSP23 was used with EcoRI digested pARS1/WT. 120 cpm each of 113 nt and 295 nt ssDNA was digested with S1 nuclease (0.6 U, Boehringer Manheim) for 1 minute at room temperature. The reaction was stopped by the addition of 200 ml of 1% SDS/20 mM EDTA, and the DNA was purified by phenol extraction and precipitation with ethanol.

The sequences for the oligonucleotides used in this study were:

FSP24: cgccagggttttcccagtcacgac

BUBLR1: cgttcttccttctgttcggag

DLX833-814: aaggcctgcaggcaagtgca

RSP23: agcggataacaatttcacacagg

DL11: gttaccatggcatcgagttckttcaacaagactacaatgg

DL12: acttgctcgagatgtccttacttgcttacaagcaaacagaagacttatac

DL13: attatttttttttggaagtgtttttcgacagaagttgcatcatcgatgaattcgagctcg

DL14: aggttctcgagatgtccccaaagaagaagaggaaggtcttagcggagtccaaagccatcctggcactat

DL15:acctgtcgtgccagctgcattaatgaatcgcgacccccccattcaagaacagcaagcagcattgagaactttggaatcca gtccctcttccacctgc

Electrophoretic Mobility Shift Assays (EMSA)

ORC-DNA binding reactions were carried out in 10 µl of binding buffer [100 mM Hepes-KOH (pH 7.6), 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, and 0.15 M KCl, 10 % (vol./vol.) glycerol, 0.01% (wt./vol.) NP-40, 1 mM DTT, 0.1 mg/ml pd(G-C)]. ATP was included as indicated at 50 µM (unless otherwise noted). For gel shifts comparing dsDNA and ssDNA binding at various concentrations of ORC (Figure 1), 15 fmol of DNA were incubated with 2.5, 5, 10, or 20 ng of ORC (6, 12, 24 and 48 fmol of ORC respectively). The radiolabeled DNAs corresponded to the 244 bp dsDNA (wild-type and mutant) and the 295 nt ssDNA. Competition experiments (Figure 2) were performed using 24 fmol of ORC with 15 fmol of labeled (wt) 244 bp dsDNA or 36 fmol of ORC with 15 fmol of labeled 295 nt ssDNA. Where indicated, 150 fmol of competitor DNA were used (i.e.- a 10-fold molar excess of competitor to probe DNA). The unlabeled competitor DNAs were the 244 bp dsDNA (wild-type and mutant) and an M13 ssDNA circle. Binding reactions were incubated at room temperature for 7 minutes, at 4°C for 7 minutes, and EMSAs were performed as described (Lee and Bell, 1997). Apparent affinity constants were determined from EMSAs by plotting binding curves, determining the free concentration of ORC at half-maximal binding and taking the inverse of this value.

DNA Co-Immunoprecipitation Assays

1 μg of ORC was incubated with ~40 cpm of ssDNA (undigested or digested with S1 nuclease as described above) in 50 μl of binding buffer lacking pd(G-C). Reactions were incubated at room temperature for 10 minutes and at 4°C for 10 minutes. The volume was increased to 200 μl with binding buffer, 1 μl of polyclonal anti-ORC antisera was added, and each reaction was incubated at 4°C for 1 hour with constant mixing. 20 μl of protein-G coupled sepharose beads (Pharmacia) were added, followed by an additional incubation (with mixing) for 30 minutes. Beads were precipitated by centrifugation and the supernatant was removed. Unbound ssDNA in the supernatant was isolated by precipitating with ethanol. The beads were washed four times with 1 ml of binding buffer, and the bound ssDNA was eluted by adding SDS to 1%, removing the beads and proteins by phenol extraction, and precipitating the ssDNA with ethanol. ssDNA samples were separated on an 8% DNA sequencing gel. For S1 cleaved samples (Figure 3B), equal amounts of radioactivity were loaded for the input, bound and free samples. The co-immunoprecipitation of ssDNA was dependant on the presence of ORC and on the presence of anti-ORC antisera (data not shown).

ATPase assays

ATP hydrolysis assays were performed as described (Klemm et al., 1997) in a 25 μl reaction containing 1 pmol of ORC, and 4 pmol of DNA where indicated. In each experiment, the rate of ATP hydrolyzed was normalized to the activity seen in the absence of ORC. The average and standard deviations for three independent experiments are shown.

ORC Electron Microscopy (EM)

ORC-ssDNA complexes were formed in a 10 µl reaction containing 20 mM Hepes-KOH (pH 7.6), 2 mM EDTA, 2 mM EGTA, 5 mM magnesium acetate, 0.15 M KCl, 725 fmol of ORC, and 8 pmol of oligonucleotide ssDNA. ORC-dsDNA binding reactions were performed in 50 µl of the same buffer, with 530 fmol of ORC, 53 fmol of *ARS1*-containing dsDNA (575 bp PCR DNA) and 100 µM ATP. Binding reactions were incubated at room temperature for 10 to 17 minutes. Samples were either not diluted, diluted five-fold or diluted ten-fold in the same buffer, mounted on glow charged carbon supports and stained with 2% aqueous solution of uranyl acetate as described (Makhov et al., 1996). A Philips CM12 transmission electron microscope was used to analyze the samples. Quantitation of the percent of bent complexes was performed by counting complexes directly on the microscope screen or from negatives of images. These experiments were performed at least twice for each DNA. ORC-dsDNA complexes were counted only if dsDNA was detected in association with ORC. In other experiments, ORC-dsDNA samples were treated with 0.6% gluteraldehyde for 10 minutes at room temperature, purified on a 2 ml BioGel A5m column (BioRad Inc.) prior to mounting on carbon supports, and analyzed by EM using uranyl acetate staining or rotary shadowcasting with tungsten as described (Griffith and Christiansen, 1978). Neither fixation and column purification nor rotary shadowing altered the percent of bent ORC complexes when bound to dsDNA (data not shown).

Measurements of the angle between the two arms of straight or bent ORC complexes were performed by analyzing ORC alone or ORC plus DL15 oligo samples respectively using NIH Image software. To determine the average angle for a population of molecules in each of two experimental conditions (ORC alone or ORC plus ssDNA), 100 molecules were randomly selected for measurements regardless of their

conformation. Figures were prepared for publication by scanning the negatives using a Nikon Film Scanner LS-4500AF (Nikon Corporation, Japan) and Adobe Photoshop software was used to adjust the brightness and contrast.

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ChapterV

Conclusions

During the course of my graduate studies, tremendous advances have been made towards understanding the regulation eukaryotic chromosomal replication. The initiation of replication is preceded by the step-wise assembly of a prereplicative complex (pre-RC) at the origin whose known components are being extensively characterized. The formation of the pre-RC is controlled by cell-cycle kinases to ensure that genomic duplication occurs after mitosis and only once per cell cycle. Pre-RC formation requires the absence of B-type kinase activity and therefore occurs after these kinases have been inactivated at the completion of mitosis. This same class of kinases then activates origins at the G1-S transition and their activity prevents further pre-RC formation as the cells proceed into mitosis. The targets of Clb-kinase activity have not yet been identified; however, these kinases clearly affect pre-RC formation either directly or indirectly. At the heart of the pre-RC, is the Origin Recognition Complex, the putative initiator protein in all eukaryotes examined thus far.

In this chapter, I will discuss the implications of my work for the understanding of ORC regulation and describe future directions for the study of ORC. I will summarize the experiments that demonstrated that ORC requires coordinate action of its subunits to bind origin DNA. Furthermore, I had shown that ORC-DNA binding, ORC ATPase activity and ORC conformation are related properties that together serve to regulate ORC function during the cell-cycle. The control of ORC activities through the use of an ATPase switch has implications for the functions of related proteins such as Cdc6p. Therefore, I will describe recent findings regarding the putative roles of ATP binding and ATP hydrolysis for Cdc6p function, highlighting the similarities between ORC, Cdc6p, and finally, other members of a larger family of ATPases involved in DNA replication. Finally, I will discuss future directions for ORC biochemistry, leading towards a high-

resolution structure and an understanding of ORC activities in the context of other proteins and specific nuclear environments.

The Subunits of the Origin Recognition Complex Act in a Coordinate Fashion

Perhaps the most unexpected outcome of my studies on the architecture of the *Saccharomyces cerevisiae* Origin Recognition Complex was that it requires coordinate action of its subunits for function. Not only is ORC a pre-assembled complex in the absence of DNA, but this assembly appears to be required for the stability and possibly the correct folding of the component polypeptides. When individual ORC subunits are expressed in insect cells, the yields of full-length protein are lower than when all six subunits are simultaneous co-expressed and individual subunits are often more insoluble (D. G. L. and S. P. B., unpublished observations). The assembly of ORC has not been extensively studied, but preliminary *in vitro* studies have shown that this process is likely complex. The addition of purified Orc1p to a partial ORC complex lacking Orc1p does not reconstitute DNA binding activity (D. G. L. and S. P. B., unpublished observations), arguing that the assembly process may be coupled to protein synthesis and involves the activity of proteins with chaperone-like activities .

I have shown that ORC requires five of its six subunits to form an active DNA-binding surface (Chapter II). The absence of any canonical DNA binding motif in all ORC subunits is consistent with this observation. Four of the six ORC subunits, Orc1p, Orc2p, Orc4p and Orc5p, make specific DNA contacts. Orc5p mediates recognition of nucleotides within the B1 element, whereas Orc1p, Orc2p and Orc4p each contact the essential 11-base pair ACS. A fifth subunit, Orc3p, is required for Orc2p to remain stably associated with the other proteins and is likely involved in correctly positioning the three subunits contacting the ACS. Only Orc6p is dispensable for DNA binding.

Therefore, ORC represents an unusual example of an efficient DNA-binding surface formed by the coordinate action of multiple proteins. Ultimately, the determination of the crystal structure of the ORC-DNA interface will be required to understand this interesting protein-DNA interaction (see below).

I have also demonstrated that affecting interactions between ORC subunits is likely important for the regulation of ORC function within the context of the cell cycle (Chapter IV). A conformational switch and an accompanying stimulation of ATP hydrolysis triggered by ssDNA potentially links changes in ORC function to the progression of the initiation pathway. Although ORC is required for the stable assembly of pre-RCs during G1, the ssDNA generated by origin unwinding could remodel the origin-associated complex and release the components of the moving replication fork. In addition to activating and disassembling origin complexes at triggered origins, this conformational switch can also be employed to inactivate pre-RCs formed at loci that become passively replicated and thereby prevent over-replication. Thus, ORC may be required, not only for the triggering of origins but also for the regulatory mechanisms that ensure the faithful replication of the genome.

Regulation of Cdc6p via an ATPase Switch

The regulation of ORC function through the use of an ATPase switch may have implications for the manner in which other related proteins are controlled. Orc1p, the subunit of ORC required for ATP binding and hydrolysis, is a member of the AAA+ family of ATPases (ATPases Associated with a variety of cellular Activities; Neuwald et al., 1999). This family includes a variety of chaperones and proteins involved in DNA replication, including Cdc6p, Orc4p, Orc5p, Mcm2p, the *E. coli* DnaA protein, and subunits of the processivity factor (sliding-clamp) loaders of *E. coli*, bacteriophage T4,

and eukaryotes (Guenther et al., 1997; Neuwald et al., 1999; Perkins and Diffley, 1998). Due to this sequence similarity, is has been suggested that ORC and Cdc6p are both regulated by ATPase switches. Furthermore, because of their similarities to replication clamp loaders that remodel ring-shaped processivity factors and assemble them around the DNA (see below), it has been proposed that ORC and Cdc6p also serve to assist in the remodeling of other proteins (Neuwald et al., 1999; Perkins and Diffley, 1998). In this model, ORC acts as a loading factor for Cdc6p that then loads MCM proteins onto origins.

Cdc6p, like ORC, contains a consensus bipartite ATP binding motif (Walker A and B motifs; Koonin, 1993; Walker et al., 1982). A Cdc6p mutation in the conserved Walker A motif (thought to be involved in ATP binding) results in a non-functional protein that is reduced in its ability to associate with chromatin, does not generate the extended pre-RC DNaseI footprint *in vivo* and fails to load MCM proteins (Perkins and Diffley, 1998; Weinreich et al., 1999). This particular point mutant (K114E) is also defective in a recently characterized interaction between Cdc6p and Orc1p (Saha et al., 1998; Wang et al., 1999). Thus, the motif that is presumably required for ATP binding by Cdc6p is essential for its normal function.

The conserved Walker B motif is thought to be involved in ATP hydrolysis. Although alanine substitutions in the Walker B domain have no phenotype *in vivo* (Weinreich et al., 1999), a glutamate to glycine mutation in this motif (E224G) shows an interesting dominant-negative effect (Perkins and Diffley, 1998). The dominant-negative *CDC6-d1* allele supported the formation of pre-RCs as assayed by *in vivo* DNase I protection assays, yet it blocked the loading of Mcm5p onto chromatin even in the presence of wild-type Cdc6p. The ability of *CDC6-d1* to exert its dominant negative effects depended on an intact Walker A motif. Together, these data have led to a model

in which Cdc6p, acting as a loading factor for MCMs, requires ATP binding to form a productive and stable interaction with ORC at the origin. Hydrolysis of ATP is then required for a subsequent aspect of MCM loading.

This model of ATP-regulated Cdc6p function is further suggested by a mutant isolated by Liang and Stillman (Liang and Stillman, 1997). The *Cdc6-3* allele was identified in a screen for temperature-sensitive mutations in Cdc6p and it caused the accumulation of a greater than 2C DNA content in cells. This over-replication phenotype was due to persistent loading of MCMs onto chromatin and origin activation in G2- and M-phases when Clb-kinase levels were high. *Cdc6-3* contains two substitutions, an H144T mutation that maps in a non-conserved region of the protein and an L258S substitution in a conserved position in a motif called Sensor 1 (this amino acid is a hydrophobic residue in >70% of all AAA+ family members; Neuwald et al., 1999). Crystallographic analysis of related proteins suggests that residues in this motif may form hydrogen bonding interactions with the terminal phosphate of ATP and respond to nucleotide binding and/or hydrolysis.

If ATP hydrolysis is required for MCM loading, then the *Cdc6-3* mutation could be explained by an inability of the protein to respond appropriately to the hydrolysis of ATP (due to the Sensor 1 mutation) and the continued activity of Cdc6p after the successful deposition of MCMs onto origins. It is also possible, however, that the mutation in the non-conserved residue (L258) is responsible for the over-replication phenotype of *Cdc6-3*. Another mutation in the same Sensor 1 motif (a G260E substitution in the *Cdc6-1* allele; Wang et al, 1999) does not lead to over-replication, and furthermore, a stronger over-replication allele identified by Stillman and Liang (*cdc6-2*) does not contain mutations in any of the conserved AAA+ motifs. The amino acid

substitutions in *Cdc6-3* must therefore be separated to determine if either of the two mutations can lead to the over-replication phenotype on its own.

The model that ATP binding and ATP hydrolysis regulate separable function of Cdc6p is based on the predicted effects of mutations in the conserved Walker A or B motifs. That ATP binding is required for efficient Cdc6p-origin association (i.e.- pre-RC formation) is inferred from the properties of the K114E mutation in a critical lysine required for ATP binding in other proteins. However, this mutant protein has not been purified and tested for ATP binding activity or for proper folding. Indeed, a recombinant Cdc6p containing the K114E substitution is more insoluble than the wild-type protein (R. J. Austin and S. P. B., unpublished observations), and increased unfolding of the protein could explain both its reduced ability to form pre-RCs and to bind Orc1p.

This model of Cdc6p function also relies on the properties of the dominant negative *CDC6-1d* mutation, hypothesized to bind but not hydrolyze ATP. This mutation is thought to result in defective pre-RCs that are locked in a non-productive state and are incapable of loading MCMs, thereby blocking the function of wild-type Cdc6p. The properties of this mutant, however, are more complex than this simple model would predict. For example, a yeast strain containing *CDC6-1d* as the only form of Cdc6p is fully viable when the gene is expressed from the endogenous *CDC6* promotor (R. J. Austin and S. P. B., unpublished observations). Purification of this mutant protein will be required to directly test whether or not the protein is able to bind ATP (and presumably to bind Orc1p) but is specifically defective for ATP hydrolysis. Also, if ATP hydrolysis is required for MCM loading, then the over-replication alleles of *CDC6* (*cdc6-2 and cdc6-3*) may have elevated levels of ATPase activity as compared to wild-type Cdc6p. Again, purification of these mutant proteins will allow this hypothesis to be tested.

ATPase Switches in Other Proteins Involved in DNA Metabolism

The control of a protein's function via nucleotide binding and hydrolysis can be thought of in two general ways. Hydrolysis of nucleotides can provide the energy to power motion as in the case of helicases and other motor proteins (reviewed in Baker and Bell, 1998; Block, 1998; Goldman, 1998; Lohman et al., 1998). Conversely, nucleotides can regulate a molecular switch, allowing a protein to alternate between two functional states. The molecular switch model is exemplified by the GTPase superfamily of proteins that are active in their GTP-bound form and inactivated by GTP hydrolysis (reviewed in Bourne et al., 1990 and Bourne et al., 1991). For example, the bacterial elongation factor (EF) Tu binds aminoacyl tRNAs in its GTP-bound form and transports them to ribosomes (reviewed in Stellwagen and Craig, 1998). When the mRNA codon and the tRNA anti-codon are correctly paired, the ribosome stimulates the GTPase activity of EF-Tu by four orders of magnitude as compared to cases in which the codon and anti-codon are mismatched (Rodnina et al., 1996). The hydrolysis of GTP results in the tRNA and dissociation of EF-Tu. Therefore, the molecular switch is activated only when the components of the protein synthesis machinery are poised to continue along the translation pathway. The result of this GTPase switch is that the pathway proceeds by one step and EF-Tu is recycled to repeat this process.

Although proteins that hydrolyze nucleotides for energy also alternate between different conformational states, the putative molecular switch proteins that I will compare below may be considered distinct in that the transition from one state to the other is more tightly regulated. In these molecular switches, the activation of nucleotide hydrolysis typically requires a precise trigger that, in effect, signals to the protein that a critical step of a pathway has successfully been completed. The ensuing switch in activity results in a commitment to continue along the pathway. In the case of ORC, origin unwinding (and

more precisely, unwinding of the appropriate amount; Chapter IV) may act as a signal that alters its properties and allows the associated replication proteins to complete the initiation process and begin the elongation phase of DNA replication. Recent mechanistic studies of AAA+ family members such as the *E. coli* DnaA initiator protein and the γ-complex clamp loader, as well as the bacteriophage Mu regulator of transposition (MuB), suggest that this general theme seen in GTPases is also conserved among certain ATPases.

The *E. coli* DnaA protein requires ATP for replication activity and hydrolysis of ATP is necessary to inactivate it at the appropriate time. A mutation of DnaA that binds but fails to hydrolyze ATP is lethal and leads to over-replication in *E. coli* (Mizushima et al., 1997). DnaA binds the OriC origin and requires ATP to melt DNA and form the Open Complex (Bramhill and Kornberg, 1988; Mizushima et al., 1998; Sekimizu et al., 1987). The site of unpaired DNA serves as the entry site for loading of the DnaB helicase that unwinds the DNA and allows for the assembly of the DNA Polymerase III holoenzyme (reviewed in Kornberg and Baker, 1992). Recent work has shown that the DnaA ATPase activity is maximally stimulated by a combination of the loading of the β–clamp (that increases the processivity of the DNA polymerase), the presence of a protein called IdaB, and DNA Pol III activity (Katayama et al., 1998). Thus, the successful assembly of the replicative polymerase, and more precisely, the act of initiating replication may provide the signal that triggers ATP hydrolysis and inactivates DnaA, serving as one mechanism to prevent re-replication until the next cell cycle.

ATP binding and hydrolysis also regulates the activity of the *E. coli* γ-complex required for loading the β-clamp processivity factor onto DNA. The γ-complex is comprised of five different subunits (γ, δ, δ', χ and ψ) and is itself a component of the DNA Pol III holoenzyme (Maki and Kornberg, 1988). The β-clamp is a homo-dimeric

ring-shaped molecule that encircles DNA and increases the processivity of the DNA polymerase. To load the ring onto DNA, interactions between the two subunits of β must first be broken. β-clamp binding and ring opening activity has been observed for the δ subunit alone; however, in the context of the γ-complex, no ring-opening is observed, arguing that the δ subunit is normally inaccessible (Naktinis et al., 1995; Turner et al., 1999). The binding of ATP to the γ subunit alters the conformation of the γ-complex and frees δ to bind and open the β-clamp. Binding of the clamp to the γ-complex then inhibits ATP hydrolysis by the γ subunit. Through a series of protein-protein and protein-DNA interactions, the γ-complex is brought to the site of primed template DNA (Yuzhakov et al., 1999). Interaction with primed template DNA (but not unprimed ssDNA) stimulates the ATPase activity of γ, resulting in a return to the conformation in which δ-β binding is inhibited (Turner et al., 1999). The γ-complex is ejected from the DNA and the β-clamp is left to close around the primed DNA. Therefore, the interaction with primed template DNA triggers ATP hydrolysis and results in a conformational switch that recycles the γ-complex and loads β-clamps at the proper DNA site.

MuB, a protein involved in the transposition of the bacteriophage Mu genome, is an ATP-dependent DNA binding protein. Efficient Mu transposition requires the activities of both the MuA transposase and MuB, which stimulates MuA activity (reviewed Mizuuchi and Craigie, 1986). MuB (when bound to ATP) binds DNA and marks potential target sites for Mu transposition. The DNA in turn inhibits MuB ATPase activity (Adzuma and Mizuuchi, 1991). When MuA and MuB interact, the target DNA is delivered to MuA and the transposase activity of MuA is stimulated (Baker et al., 1991). Furthermore, the combination of DNA and MuA stimulates MuB to hydrolyze ATP (Adzuma and Mizuuchi, 1991; Maxwell et al., 1987). This switch in MuB is thought to result in the release of the target DNA and its transfer to the MuA transposase for the

next step in catalysis (Yamauchi and Baker, 1998). Finally, MuB is released from the transpostion complex and recycled to bind other potential target sites. Thus, the MuB ATPase is triggered by its simultaneous association with target DNA and MuA, allowing for progression along the transposition pathway and the concomitant inactivation of MuB.

This molecular switch in MuB activity has also been implicated in the phenomenon of target immunity (Yamauchi and Baker, 1998). Mu transposition into integrated Mu genomes and the surrounding sequence is inhibited, resulting in the spread of Mu to other regions of the host genome and preventing the disruption of Mu genomes (reviewed in Craig, 1997). Since the stimulation of MuB hydrolysis (by MuA and DNA) releases MuB from DNA, a biased distribution of MuB at sites away from the Mu genome could result due to the higher local concentration of MuA at the ends of Mu DNA sequences.

A comparison of the regulation of ORC, DnaA, the γ-complex, and MuB highlights the similarities among the use of ATP binding and ATP hydrolysis by these proteins (Figure 1). In each case, ATP binding allows the protein to carry out an early step in the process (i.e. – Open Complex formation in the case of DnaA) or to bind to a target molecule (DNA or another protein). Often, interaction with this target inhibits the intrinsic ATPase activity of the protein. The ATP-bound form of the protein is required for subsequent steps in the process, leading to a stage of the pathway that stimulates ATPase activity and activates a switch in the activity of the protein. In the case of ORC and DnaA, the net result of this switch is an inactivation of the protein and may play a role in the prevention of re-replication. For proteins that may function multiple times in the same cell-cycle such as the γ-complex and MuB, the net result is release and recycling of the protein. RFC, the eukaryotic clamp loader and member of the AAA+

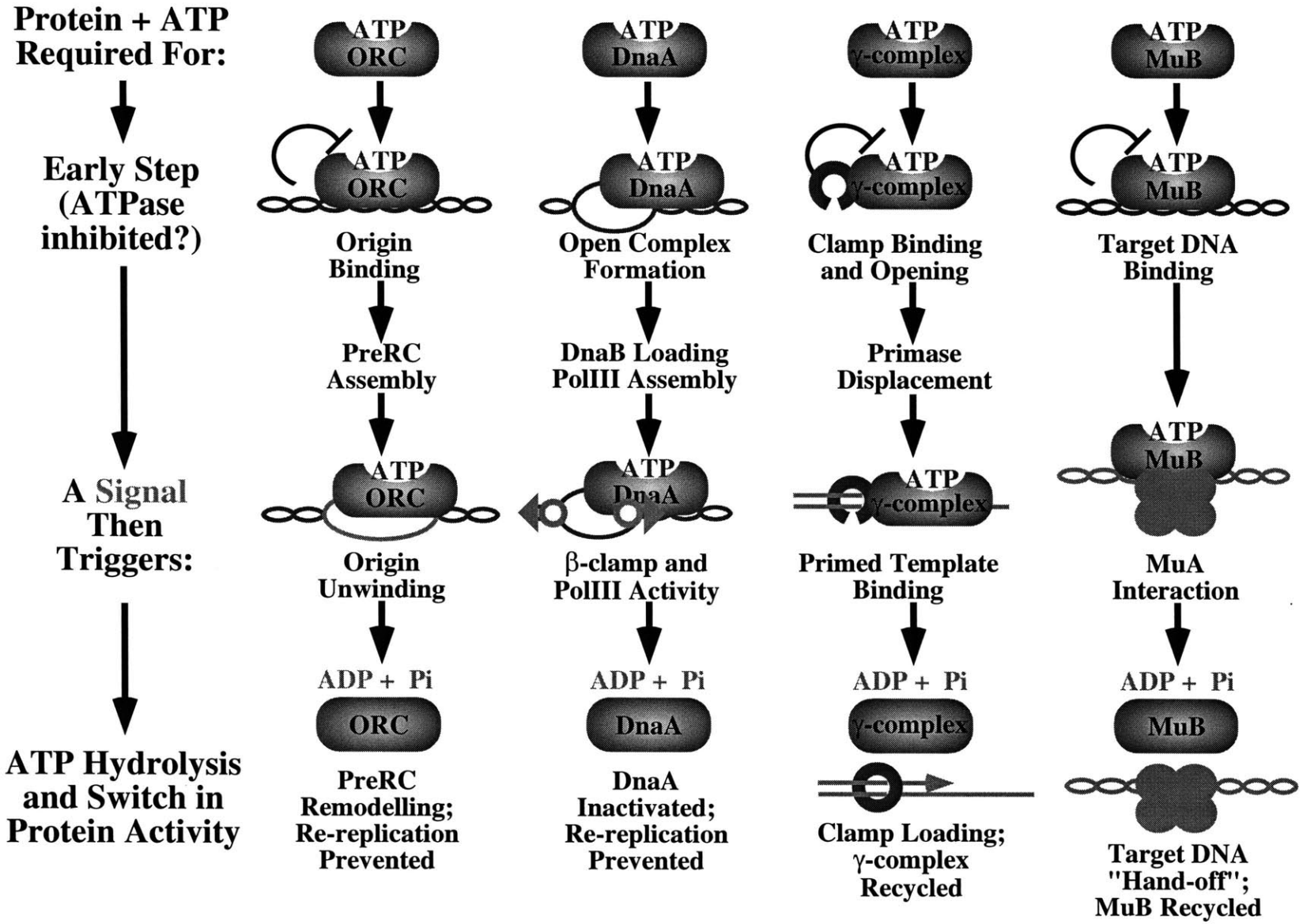
Protein + ATP Required For:
Early Step (ATPase inhibited?)
A Signal Then Triggers:
ATP Hydrolysis and Switch in Protein Activity
ATP ORC
ATP ORC
Origin Binding
PreRC Assembly
ATP ORC
Origin Unwinding
ADP + Pi
ORC
PreRC Remodelling; Re-replication Prevented
ATP DnaA
ATP DnaA
Open Complex Formation
DnaB Loading PolIII Assembly
ATP DnaA
β-clamp and PolIII Activity
ADP + Pi
DnaA
DnaA Inactivated; Re-replication Prevented
ATP γ-complex
ATP γ-complex
Clamp Binding and Opening
Primase Displacement
ATP γ-complex
Primed Template Binding
ADP + Pi
γ-complex
Clamp Loading; γ-complex Recycled
ATP MuB
ATP MuB
Target DNA Binding
ATP MuB
MuA Interaction
ADP + Pi
MuB
Target DNA "Hand-off"; MuB Recycled

Figure 1

Figure 1. Common ATPase Switches in Proteins Involved in DNA Replication Processes. The regulation of four proteins involved in DNA replication is compared. These include the *S. cerevisiae* initiator protein, ORC, the *E. coli* initiator protein, DnaA, the *E. coli* β-clamp loading γ-complex, and the phage Mu regulator of transposition, MuB. In each case, the protein requires binding of ATP for an early step in the process, such as open complex formation (DnaA), or ligand (DNA or protein) binding. In the case of ORC, the γ-complex and MuB, binding to the correct ligand inhibits ATPase activity. The successful attainment of a later step in the pathway (shown in green) acts as a signal that triggers ATP hydrolysis and other changes in the activity of the protein. In this manner, the ATPase switch is activated only at an appropriate time and commits the protein to continue along the pathway. See text for more details.

superfamily, also exists in an ATP bound form that is active for an early step in the loading process and requires ATP hydrolysis for some aspect of the final step (reviewed in Perkins and Diffley, 1998). Whether ATPase activity is specifically stimulated by a signal consistent with successful attainment of a critical stage in the pathway remains to be seen.

A High-Resolution Structure of ORC

The experiments that describe the architecture of ORC subunits with respect to DNA (Chapter II) and the proposed EM mapping experiments (Chapter III) will provide important information to facilitate the determination of a high-resolution crystal structure. The structure of ORC will potentially be interesting to structural biologists and biologists in general: I have already discussed how the protein-DNA interface is likely unusual (Chapter II). Furthermore, a detailed understanding of the change in conformation of ORC when bound to ssDNA may provide additional clues as to the role of the putative conformational switch during initiation. The ability to initiate X-ray crystallography studies is complicated by the large size of ORC and its heterogeneity when produced in insect cell expression systems. The six subunits of ORC combine for a total molecular mass of 414 kDa. These six polypeptides are co-expressed in insect cells to form the complex, however, subassemblies of ORC complexes that lack entire subunits or regions of subunits (due to proteolysis) also form. Although the purification of ORC removes the bulk of these partial complexes, the small fraction of remaining contaminants or subsequent proteolysis of the intact complex may inhibit the formation of high-quality crystals. In addition, three of the six ORC subunits are phosphorylated *in vivo*. Orc1p contains a single putative phosphorylation site in its N-terminus, Orc2p contains six

putative sites and Orc6p contains four such sites. Heterogeneity in the pattern of phosphorylation among different ORC molecules may also be problematic.

One approach to facilitate the formation of ORC-DNA co-crystals is to generate a truncated form of ORC that still retains sequence-specific binding activity. I have demonstrated that Orc6p is not required for origin DNA binding (or for ssDNA binding; see Chapter II and Appendix A). During the course of my studies, deletion analyses of Orc1p showed that the N-terminus was also dispensable for replication function (Bell et al., 1995). Subsequent experiments have shown that an ORC complex that combines a truncation of Orc1p with the absence of Orc6p still retains both dsDNA- and ssDNA-binding activity (R. D. Klemm and S. P. B., unpublished observations, and Appendix A). In addition to reducing the overall mass of the complex, this smaller form of ORC (dubbed "ORC Lite") also removes five of the putative eleven phosphorylation sites. The remaining phosphorylation sites reside in Orc2p and a future goal is to purify a form of ORC Lite containing a mutated Orc2p in which all of the putative phosphorylation sites have been mutated. Unpublished work from Joachim Li's lab indicated that such an Orc2 mutant shows no phenotype *in vivo* (personal communication), arguing that DNA binding is likely unaffected.

A true minimal binding domain of ORC can be derived from ORC Lite by continuing deletion analyses of Orc2p, Orc3p, Orc4p and Orc5p or by a complementary limited proteolysis treatment of the intact ORC Lite complex. Limited digestion using proteases that cleave frequently has been used to separate different functional domains of proteins (Konigsberg, 1995). Preliminary experiments using V8 protease treatment of wild-type ORC have demonstrated that smaller subcomplexes are generated that retain both dsDNA and ssDNA binding activities (see Appendix A). These active subcomplexes can be purified by conventional gel-filtration chromatography to identify

the position of the proteolytic cleavage, to allow for detailed characterization of the DNA binding activity (i.e. – is it sequence-specific and/or ATP dependent?), and to examine the other biochemical properties of the complexes (i.e. - ATP binding, ATP hydrolysis, ssDNA-induced ORC bending, and interactions with other proteins). Genetic deletion studies and *in vitro* proteolysis experiments are complementary approaches that together can generate data useful for the design of a more ideal candidate for crystallography. In addition, the partial complexes identified by these methods can be tested for all of the known biochemical properties of ORC to look for mutated complexes that are specifically defective in individual ORC functions. Such mutants are difficult to screen for directly but will be essential to demonstrating whether ORC activities such as ATP hydrolysis and ssDNA binding are important for *in vivo* function.

Understanding ORC Function in the Context of the Nucleus: Pre-RC Components and Cell-Cycle Regulated Kinases

In the cell, ORC functions in the presence of other proteins, specialized chromatin domains, and possibly specialized nuclear compartments. The biochemical characterization of ORC, however, has been performed in the absence of other cellular factors. Thus, understanding the role of ORC during replication initiation and as part of the mechanisms regulating replication will require an analysis of how other proteins, particular cell-cycle contexts, or the environments created by unique nuclear structures modulate ORC activities.

ORC is essential for the assembly of the pre-RC. ORC recruits Cdc6p to origins during G1, and direct interactions between these two proteins have been observed, both in the presence and absence of DNA (Liang et al., 1995; Appendix B). The recruitment of MCM proteins requires both Cdc6p and ORC, although *in vitro* interaction studies

have not been successful at determining whether MCMs bind Cdc6p and/or ORC directly (A. Schwacha, D. G. L. and S. P. B., unpublished observations). Closer to the initiation of replication, Cdc45p, the replicative polymerases and possibly other proteins are recruited to the origin. Any of these proteins could potentially modulate ORC function at key moments during the replication process.

ORC function must also be understood in the context of cell-cycle regulated kinases. The B-type cyclin dependent kinases have been shown to be required, not only for the entry into S-phase, but also to prevent the assembly of pre-RCs during S-, G2-, and M-phases. These kinases therefore play a key role in preventing re-replication within each cell-cycle. The mechanisms by which they act, however, are not yet know. Although Orc1p, Orc2p and Orc6p have been observed to be phosphorylated *in vivo* (S. P. Bell, unpublished observations) and the same three are *in vitro* substrates for Cdc28/Clb5 (R. J. Austin and S. P. B., unpublished data; Appendix B), it is not clear that phosphorylation is important for ORC function. Unpublished work from the laboratories of Joachim Li, Bruce Futcher and Bruce Stillman has shown that mutations of the consensus phosphorylation sites within Orc2p and Orc6p respectively do not lead to any observable phenotype *in vivo* (personal communications). Although ORC may not be regulated B-type Cdk activity, other components of the pre-RC are potential targets. Therefore, an examination of the effects of other replication proteins (including pre-RC components) on properties of ORC must also take into account the importance of appropriate cell-cycle kinase levels.

Another kinase required for DNA replication is Cdc7p, which is activated by its cyclin-like partner, Dbf4p. Rather than simply triggering the G1/S transition, Cdc7/Dbf4 is likely required in a local fashion to activate each origin throughout the course of S-phase (Bousset and Diffley, 1998; Donaldson et al., 1998). One-hybrid assays have

shown that Dbf4p is localized to the *ARS1* origin and likely interacts with ORC (directly or indirectly) since the researchers observed a correlation between mutations in *ARS1* that affected ORC binding and those that affected Dbf4p-origin recruitment in the one-hybrid assay. Therefore, rather than serving as a substrate for the Cdc7p kinase, ORC may serve to recruit it to origins (via an interaction with Dbf4p) and bring it into proximity with its true targets. An assay for direct interaction between ORC and Cdc7/Dbf4 has not yet been established and should be attempted.

Understanding ORC Function in the Context of the Nucleus: Specialized Sub-Nuclear Structures

The DNA in eukaryotic nuclei is packaged into highly ordered chromatin arrays. Thus, the function of ORC (and indeed any DNA binding protein) must be understood in the context of nucleosomes and nucleosome-remodeling activities. The yeast *ARS1* origin of replication has a unique chromatin structure with a nucleosome-free region at the origin flanked by precisely positioned nucleosomes (Thoma et al., 1984). The importance of this structure for origin function was suggested by experiments in which positioning nucleosomes over the origin reduced the copy number of plasmids, presumably by reducing origin activity (Simpson, 1990). *In vivo* and *in vitro* data from our lab has demonstrated that ORC and Abf1p, the transcription factor bound to the B3 element of *ARS1*, together function to generate this nucleosome free region and the adjacent positioned nucleosomes (J. R. Lipford and S. P. B., unpublished data). Chromatin can also affect properties of ORC. If nucleosomes are first deposited on DNA, ORC cannot bind origin DNA unless it is also co-incubated with a chromatin assembly and/or remodeling activity. These data suggest that, *in vivo*, ORC requires a nucleosome remodeling activity to compete with histones for origin binding.

Alternatively, the localization of ORC to unwound origins via its ssDNA binding activity may allow ORC to rapidly re-bind origins after they are replicated and before nucleosomes have an opportunity to bind. Since ORC appears to interact directly with histones (J. R. L. and S. P. B., unpublished data) and since ORC and chromatin can influence each other with respect to DNA binding, other properties of ORC assayed in the presence of naked DNA must be re-examined in the context of assembled nucleosomes.

In addition to regulated chromatin structure, eukaryotic nuclei show a high degree of global organization. For example, in cultured mouse and human cells, regions of the genome undergoing DNA replication and transcription are generally segregated into non-overlapping higher order domains (Wei et al., 1998). Additionally, the positions of individual loci within the nucleus may be controlled. Fluorescent *in situ* hybridization (FISH) combined with three-dimensional microscopy of interphase nuclei of *Drosophila* have shown that specific loci occupy different discrete nuclear subregions (Marshall et al., 1996). The constraint of individual loci to specific nuclear subregions is likely mediated by microtubules as treatment of yeast or *Drosophila* nuclei with nocodozole greatly increased the diffusion of labeled loci in real time (Marshall et al., 1997). The molecular mechanisms by which precise nuclear architecture is established and maintained are not yet defined (reviewed in Marshall et al., 1997); however, as research in this field progresses, the influence of nuclear substructure on the regulation of processes such as DNA replication can be addressed.

Concluding Remarks

As with most scientific research, my study of ORC architecture at origins of replication has addressed certain questions but has also suggested many more. The fact

that eukaryotic replication requires six polypeptides to serve the function of the initiator protein (when prokaryotes and viruses typically use one or two proteins) begs the simple question, what do each of the ORC subunits do? The finding that five of the six subunits are required for DNA binding suggests that at least one of the essential functions of Orc1p through Orc5p is origin binding. However, these subunits may also be required for other functions. For example, Orc1p is also required for ATPase activity, and it has recently been implicated in recruiting Cdc6p to origins (Wang et al., 1999). Orc6p is dispensable for both origin and ssDNA binding, yet it is essential for yeast cell viability. This subunit is therefore a prime candidate to test for interactions with other proteins recruited to origins, such as the Cdc7/Dbf4 kinase. In addition, neither the partial ORC complex lacking Orc6p nor ORC Lite has been tested for other biochemical activities of ORC. Since both complexes bind origins in an ATP-dependent manner, ATP binding is likely unaffected. However, the ssDNA-stimulated conformational switch and/or ATPase activity may be altered in the absence of Orc6p or the N-terminus of Orc1p. Interactions with Cdc6p and the ability to serve as a Cdc28/Clb5 substrate should also be examined.

My description of the arrangement of ORC subunits (along with future structural studies) will serve as an important foundation for understanding the architecture of the pre-RC. As protein-protein contacts between the components of the pre-RC are defined, a three-dimensional model of the positions of the proteins relative to ORC and the DNA can be generated. ORC is an asymmetric molecule that likely initiates pre-RC assembly in an assymetric manner (when pre-RCs are formed, *in vivo* DNase I assays at origins show an extended region of protection on only one side of the ORC footprint). At some point in the assembly of origin-associated complexes, however, this inherent asymmetry

must be translated into the assembly of two symmetric replication forks required for bi-directional replication.

Another key question concerns the activities of the pre-RC components: which protein (or combination of proteins) is required for origin opening and subsequent origin unwinding? ORC alone is unable to result in detectable distortion of origin DNA (R. J. Austin, D. G. L. and S. P. B., unpublished observations). Thus, either ORC must be modified by another protein to become active in origin melting or other proteins recruited to the origin serve this function. The MCM proteins are a favored candidate for a replicative helicase, however, no unwinding activity has been observed for purified yeast MCMs both in the presence and absence of ORC (A. Schwacha and S. P. B., unpublished observations). Either a different combination of pre-RC components (and the appropriate kinases) must be tested to observe origin unwinding, or perhaps a key component of the pre-RC still remains to be discovered.

These questions point towards the larger goal of reconstituting origin-dependent replication initiation in a completely cell-free system. Such an *in vitro* assay will allow for a powerful dissection of individual steps in the initiation pathway, as has been most elegantly demonstrated for the *E. coli* and phage replication systems. In combination with mutants of ORC that specifically inactivate one biochemical function, a cell free system will allow for the elucidation of the role of ORC activities such as ATP hydrolysis and ssDNA binding. Ultimately, such a system could then be combined with existing or developing experimental frameworks for studying chromatin structure and subnuclear organization to begin to understand DNA replication in its natural context, inside the nucleus.

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Appendix A

Regions of ORC Required for ssDNA Binding

I am grateful to members of the Bell laboratory for providing various reagents used in these experiments. ORC Lite was provided by Richard Klemm, the partial ORC complex lacking Orc1p and purified *Drosophila* Orc2p was provided by Richard Austin, and purified yeast RPA was provided by Anthony Schwacha.

RESULTS AND DISCUSSION

Orc6p and the N-terminus of Orc1p are not Required for ssDNA Binding

In Chapter II, I characterized the dsDNA binding properties of partial ORC complexes (lacking one subunit) and showed that Orc6p was the only subunit dispensible for this activity. These proteins were not fully purified and contained a small (40 kDa) contaminant with potent ssDNA binding activity, making it difficult to test these partial ORC complexes for ssDNA binding. The complex lacking the Orc6p subunit (the [-6] complex) was further purified on a mono-Q column to remove the contaminant. Two peak fractions were collected from this column. The fraction that eluted at a lower salt concentration (fraction 22) contained a [-6] complex with a truncated form of Orc1p that arose from proteolyis during the purification (data not shown) and this fraction also contained the small contaminant. The fraction that eluted at higher salt (fraction 25) contained the [-6] complex with a full-length Orc1p subunit and no contaminant.

These two mono-Q fractions were tested for origin DNA binding and ssDNA binding activity (Figure 1A). The full length ORC in fraction 25 reduced the mobility of both dsDNA containing *ARS1* (lane 4) and ssDNA (lane 13). The gel-shifted species had a faster mobility than wild-type ORC bound to dsDNA and ssDNA, respectively (lanes 7 and 16), consistent with a lower molecular mass due to the absence of Orc6p. To confirm that these gel-shifted species contained ORC, monoclonal antibodies directed against Orc4p (lanes 5, 8, 14 and 17) or against Orc2p (lanes 6, 9, 15 and 18) were added to the binding reactions and were found to cause a supershift. The partial ORC complex in fraction 22 likely contains a form of Orc1p with an N-terminal deletion. This protein was also able to bind dsDNA (lanes 1-3), but due to the presence of the small contaminant, ssDNA binding could not be assayed (lane 10-12). However, a similar form of ORC lacking Orc6p and the amino-terminal 214 amino acids of Orc1p was produced and

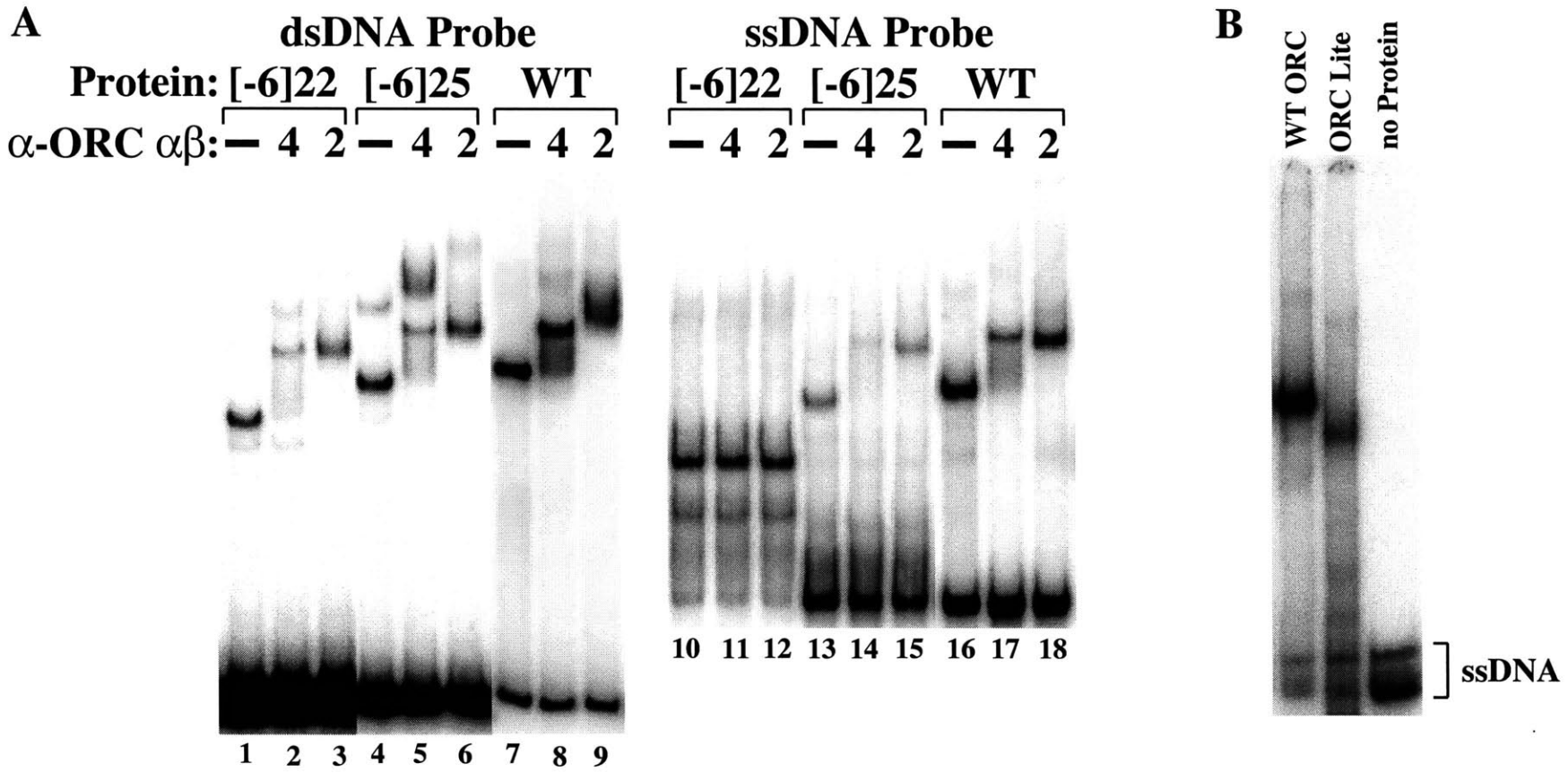

Figure 1. Orc6p and the N-terminus of Orc1p are not Required for ssDNA Binding. (A) Electrophoretic mobility shift assays of partial complexes lacking Orc6p (the [-6] complex) with *ARS1*-containing dsDNA (lanes 1-9) and ssDNA (lanes 10-18). Purification of the [-6] complex on a mono-Q column resulted in two peaks of ORC, one containing an N-terminal deletion of Orc1p (fraction 22; lanes 1-3 and 10-12) and one containing full-length Orc1p (fraction 25; lanes 4-6 and 13-15). Wild-type ORC was included as a comparison (lanes 7-9 and 16-18). Monoclonal antibodies directed against Orc4p (lanes 2, 4, 8, 11, 14 and 17) or against Orc2p (lanes 3, 6, 9, 12, 15 and 18) were included in the indicated reactions. The inability to supershift the gel-shifted species in the reactions containing fraction 22 and ssDNA (lanes 11 and 12) suggests that the putative protein-DNA is due to a contaminant and not to ORC. (B) Electrophoretic mobility shift assays of wild-type ORC and ORC Lite (lacking Orc6p and the amino-terminal 214 amino acids of Orc1p).

purified by R. D. Klemm in the lab (named "ORC Lite"; Chapter V) and was therefore tested for ssDNA binding activity (Figure 1B). The ability of ORC Lite to gel-shift ssDNA demonstrates that neither Orc6p nor the Orc1p N-terminus are required for ssDNA binding. This finding is consistent with previous data from our laboratory that showed that the N-terminus of Orc1p is required for transcriptional silencing but not DNA replication (Bell et al., 1995).

The C-terminal Region of Orc1p is Required for ssDNA Binding

To test the requirement of Orc1p for ssDNA binding, I compared purified Orc1p and a complex lacking Orc1p (provided by R. J. Austin) in an electrophoretic mobility shift assay (Figure 2). Titrations of these two proteins, as well as wild-type ORC, revealed that the complex lacking Orc1p is severely compromised for ssDNA binding. Since the N-terminus of Orc1p is not required for ssDNA binding, these data suggest that the C-terminal 700 amino acids contain a region of the protein important for this activity. Purified Orc1p is able to interact with ssDNA, albeit less efficiently than wild-type ORC. Unexpectedly, the gel-shifted species is significantly more retarded in its mobility than an ORC-ssDNA complex. If this apparent high molecular mass is due to multimerization of Orc1p, then the observation that the gel-shifted species is a single, sharp band suggests that a precise number of Orc1p molecules associate to bind ssDNA. Alternately, the gel-shift could result from a single copy of Orc1p binding a single ssDNA molecule but generating a particular structure that makes the protein-DNA complex unusually slow in its mobility.

Clearly, more experiments must be performed to examine this putative interaction between ssDNA and Orc1p. To demonstrate that Orc1p is responsible for the gel-shift, supershift experiments using Orc1p monoclonal antibodies should be

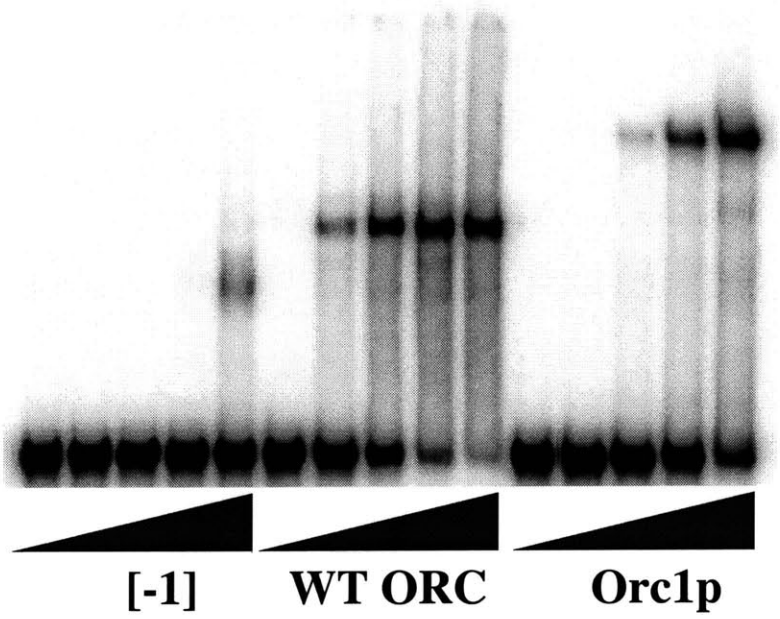

Figure 2. Orc1p is Required for ssDNA Binding. Electrophoretic mobility shift assays were performed using ssDNA and a titration of a complex lacking Orc1p (the [-1] complex), wild-type ORC or purified Orc1p. 16 fmol of ssDNA was incubated with increasing amounts of protein. Two-fold titrations of each protein were used, from 12 fmol of protein (a 0.75 fold excess of protein to DNA) to 192 fmol (a 12-fold excess of protein).

performed. Additionally, if the binding of Orc1p to ssDNA requires multimerization of the protein, electron microscopy of Orc1p and ssDNA can be used to estimate the number of Orc1p molecules present by estimating the molecular mass of the aggregate. Multimerization can also be addressed by altering the mass of Orc1p (either by deleting the N-terminus or by adding an epitope to increase its mass) and comparing its behavior in a mobility shift assay to that of either wild-type Orc1p or a mixture of wild-type and mutant Orc1p.

Southwestern Blot Analysis of ORC

Southwestern blot analysis, in which proteins immobilized on a nitrocellulose membrane are probed with radiolabeled DNA, has been used to analyze the human single-stranded DNA binding protein, hRPA (Wold et al., 1989). The authors showed that the largest subunit of RPA alone could bind ssDNA. To determine if any single ORC subunit had affinity for ssDNA, I performed a Southwestern blot using ORC, with *S. cerevisiae* RPA added as a positive control (Figure 3B). Consistent with published data, the largest subunit of RPA bound labeled ssDNA but not dsDNA. Among the ORC subunits, only Orc2p bound ssDNA. However, this interaction was not specific for ssDNA and occurred with similar affinity for non-origin containing dsDNA. Orc2p has a high theoretical isoelectric point (pI = 9.5). Thus, the observed interaction with both ssDNA and dsDNA may simply reflect a non-specific ionic interaction between positively charged regions of Orc2p with negatively charged nucleic acids. To test this hypothesis, I also examined *Drosophila* Orc2p, which has a lower isoelectric point (pI = 6). The *Drosophila* protein showed only a weak interaction with ssDNA and none with dsDNA (Figure 3B). The non-specific interaction of yeast Orc2p with nucleic acids does not occur when the protein is assembled into an ORC complex, as a complex

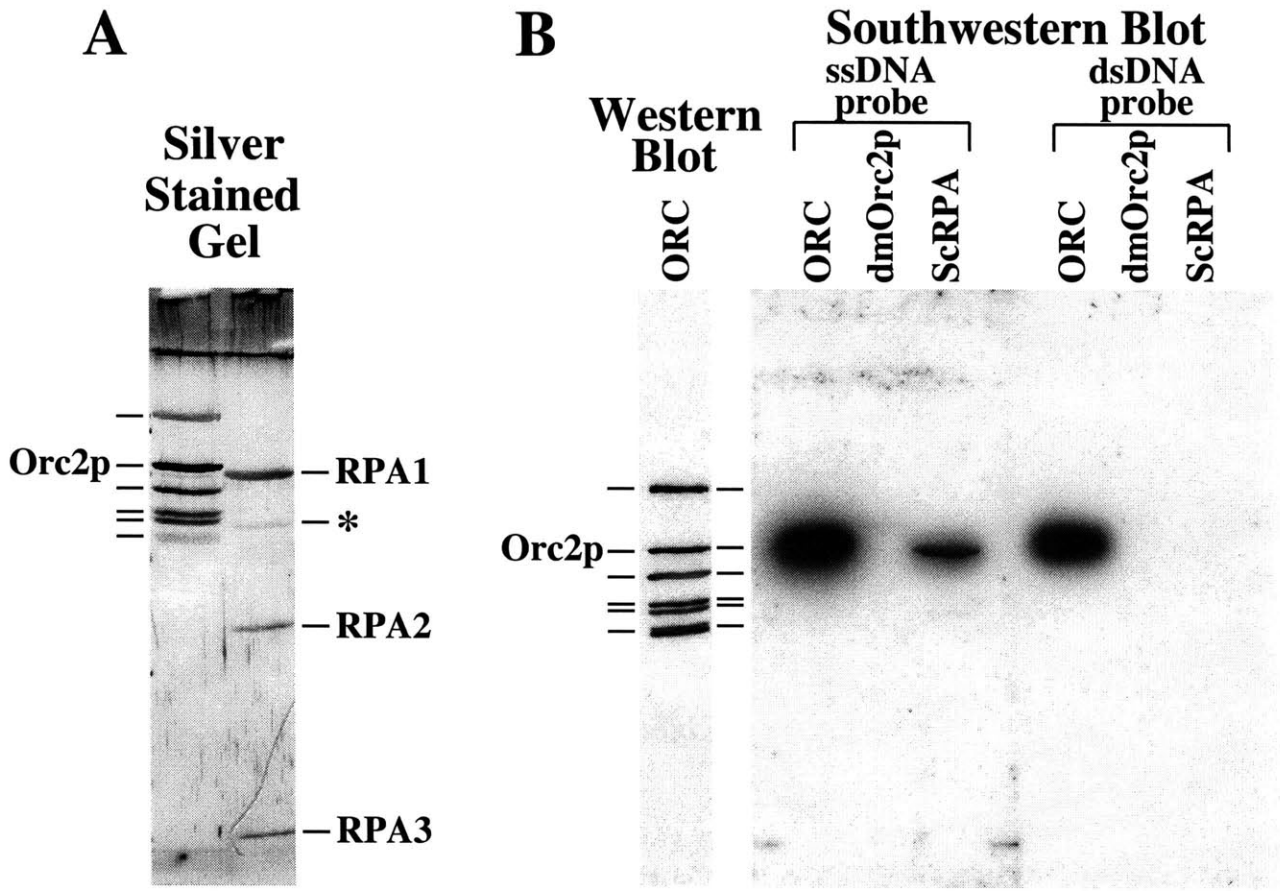

Figure 3. Southwestern Blot Analysis of ORC. (A) *S. cerevisiae* ORC and RPA were electrophoresed on a 10% SDS-PAGE and stained with silver. The asterisk represents a breakdown product of the RPA1 gene product. (B) Yeast ORC, yeast RPA and purified *Drosophila* Orc2p were electrophoresed on a 10% SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was cut into three portions and probed with anti-ORC antibodies (Western Blot, left panel), radiolabeled ssDNA (middle panel) or with radiolabelled non-origin dsDNA (right panel).

lacking Orc1p had neither dsDNA binding nor ssDNA binding activity (Chapter II and preceding section).

Attempts to Crosslink ORC Subunits to ssDNA

UV-induced crosslinking is a useful tool for identifying proteins that bind ssDNA (Philipova et al., 1996). I attempted to crosslink ssDNA to ORC using unmodified ssDNA, ssDNA modified with BrdU, and ssDNA modified with the high-efficiency crosslinking reagent 4'-AZPB (Chapter II). In all cases, no specific crosslinking of DNA to any ORC subunit was observed; either no subunit was crosslinked, or all six subunits were weakly crosslinked with equal efficiency (data not shown). Purified yeast RPA was included in these experiments as a positive control and showed the expected behavior (efficient crosslinking to the large subunit and weaker crosslinking to the middle subunit; data not shown).

Subcomplexes of ORC Generated by V8 Protease Digestion are Active for DNA Binding

In addition to the analysis of partial ORC complexes, another method for identifying the regions of ORC required for dsDNA and ssDNA binding involves limited proteolytic digestion to generate collections of smaller subcomplexes. ORC was incubated with V8 protease for various amounts of time, the reactions were stopped with PMSF, and the resulting digestion products were examined by SDS-PAGE and silver-staining and assayed for dsDNA and ssDNA binding activity (Figure 4). Digestion with V8 resulted in the generation of a number of gel-shift species with faster mobilities than untreated ORC bound to dsDNA (Figure 4B) or to ssDNA (Figure 4C), suggesting the formation of smaller subcomplexes of ORC capable of binding dsDNA and ssDNA. The

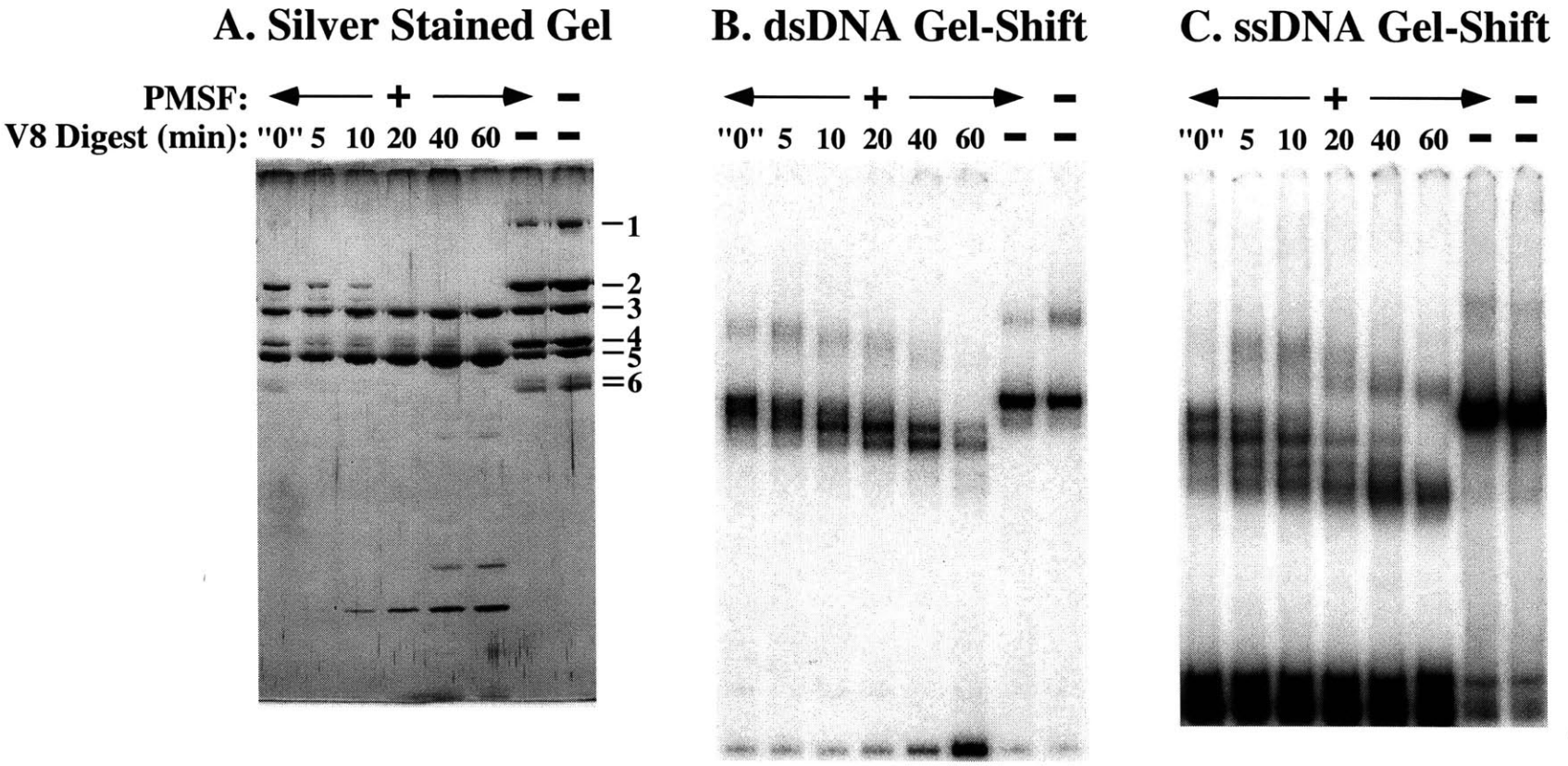

Figure 4. Limited V8 Protease Digests of ORC. ORC was incubated with V8 protease for the indicated amounts of time and the digests were stopped with PMSF. The reaction products were analyzed by SDS-PAGE and silver staining (A) or assayed for dsDNA binding (B) and ssDNA binding activities (C). The "0" minute digestion reaction contained PMSF prior to addition of V8 protease and yet, ORC was partially digested (Figure 4A, lane 1) indicating that the amount of PMSF used was insufficient for complete inactivation of the protease. The second-to-last lane in each panel includes ORC treated with PMSF in the absence of the protease and the last lane is untreated ORC.

sequence specificity and ATP-dependence of these DNA binding activities have not yet been examined. These putative subcomplexes of ORC can be purified by gel filtration chromatography and tested more rigorously for biochemical activities of ORC (including DNA binding, ATP hydrolysis, and interactions with Cdc6p). Additionally, a larger scale experiment should generate sufficient material to map the V8 cleavage sites. These experiments will provide information regarding the domain structure of ORC subunits and will facilitate the design of a minimal ORC-DNA complex suitable for X-ray crystallography (Chapter V).

EXPERIMENTAL PROCEDURES

Proteins

The [-6] ORC complex lacking Orc6p (described in Chapter II) was purified on a mono-Q column as described (Klemm et al., 1997; Lee and Bell, 1997). Peak fractions were identified by UV absorbance and by coomassie staining of samples electrophoresed on a 10% SDS-PAGE. Orc1p was expressed in insect cells and purified as described (Klemm et al., 1997; Lee and Bell, 1997), except that glycerol gradient sedimentation (Bell and Stillman, 1992) was used in place of gel filtration chromatography. ORC Lite (lacking Orc6p and the N-terminus of Orc1p) was provided by R. D. Klemm. The ORC complex lacking Orc1p was provided by R. J. Austin.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) using dsDNA and ssDNA were performed as described in Chapters II and IV. Unless otherwise stated, the *ARS1* containing dsDNA probe was an *EcoRI-HindIII* fragment of pARS1/WT end-labeled at the *EcoRI* site, and the ssDNA was obtained by melting this dsDNA fragment and purifying the labeled ssDNA as described in Chapter IV. EMSAs with mono-Q fractions of the [-6] complex were performed using 16.1 fmol of DNA, ~48 fmol of protein and 1 μg of pd(G-C) in a 10 μl reaction. For supershift experiments, antibodies SB67 (anti-Orc2p) and SB6 (anti-Orc4p) were diluted 1:50 in PBS, and 1 μl of diluted antibody was added to the 10 μl binding reaction 5 minutes after ORC was added to the DNA. The EMSA comparing ORC and Orc Lite was performed using 27 fmol of ssDNA and ~190 fmol of protein. The EMSA used to compare Orc1p with either a complex lacking Orc1p or wild-type ORC used the 295 nt ssDNA described in Chapter IV. 16 fmol of ssDNA was incubated with either 12, 24, 48, 96, or 192 fmol of protein.

Southwestern Blot Analysis

5.6 pmol of yeast ORC, 7.1 pmol of *Drosophila* Orc2p, and 10.6 pmol of yeast RPA were electrophoresed on a 10% SDS-PAGE and transfered to a nitrocellulose membrane. The membrane was cut into three strips. One strip was probed with anti-ORC antibodies as described (Lee and Bell, 1997). The other two strips were blocked in ORC binding buffer [100 mM Hepes-KOH (pH 7.6), 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, and 0.15 M KCl, 10 % (vol./vol.) glycerol, 0.01% (wt./vol.) NP-40, 1 mM DTT] containing 5% non-fat milk for 1 hour and rinsed four times in binding buffer. The membranes were then incubated with 6000 cps of either ssDNA (295 nt ssDNA described in Chapter IV) or non-origin dsDNA (a 360 bp *StuI-EcoRI* fragment of pARS1/WT that contains no matches to the ARS consensus sequence) for six hours at 4°C. The membranes were washed five times in binding buffer, dried, and exposed to film.

Limited V8 Protease Digests of ORC

4.4 pmol of ORC was incubated with 0.4 μg of V8 (in 16 μl final volume) at 27°C for 5, 10, 20, 40 or 60 minutes. 2 μl of 0.1 M PMSF (dissolved in isopropanol) was added to stop the reaction and samples were placed on ice. The "0" minute reaction contained PMSF prior to addition of V8 protease and was kept on ice for the duration of the experiment. 8 μl of each reaction was mixed with SDS-PAGE loading buffer and electrophoresed on a 10% SDS-PAGE. For gel-shift analysis, the equivalent of 190 fmol of ORC was incubated with 1 μg of pd(G-C) and 15 fmol of dsDNA or 27 fmol of ssDNA.

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Appendix B

Attempts to Characterize the ssDNA-Induced Conformational Change of ORC

I thank Richard Austin for providing polyclonal ORC antibodies, purified Cdc6p and purified Clb5p/Cdc28p.

INTRODUCTION

A change in the conformation of a protein can be inferred from changes in its mobility, in its susceptibility to proteolytic cleavage, and in its interactions with other proteins. To obtain independent evidence for an ssDNA-induced conformational switch (Chapter IV), I performed a number of assays to address these issues. The change in ORC conformation (as determined by EM) was stimulated by ssDNA and inhibited by dsDNA. Therefore, for all of the assays described below, I predicted that ssDNA and dsDNA would have opposite effects.

RESULTS AND DISCUSSION

Native Gel Mobilities of ORC-ssDNA and ORC-dsDNA Complexes

The ssDNA-induced bending of ORC should result in an altered mobility of ORC in a native polyacrylamide gel (Figure 1). I examined the mobility of ORC bound to radiolabeled dsDNA or ssDNA probes that were either both long (244 bp and 244 nt respectively) or both short (99 bp and 96 nt respectively). After electrophoresis, the positions of the radiolabeled DNA bound by ORC were determined by drying the gel and exposing it to film (Figure 1A), and the position of ORC was determined by Western blot analysis (Figure 1B). ORC migrated slowly in the gel in the absence of DNA, suggesting a low overall negative charge under these conditions, and the mobility of ORC was greatly improved when it was bound to DNA. When dsDNA and ssDNA of similar lengths were compared, the ORC-ssDNA complex had a faster mobility than the ORC-dsDNA complex. Therefore, ORC interacted with dsDNA and ssDNA in ways that are sufficiently different to result in distinguishable mobilities in a native gel. Although

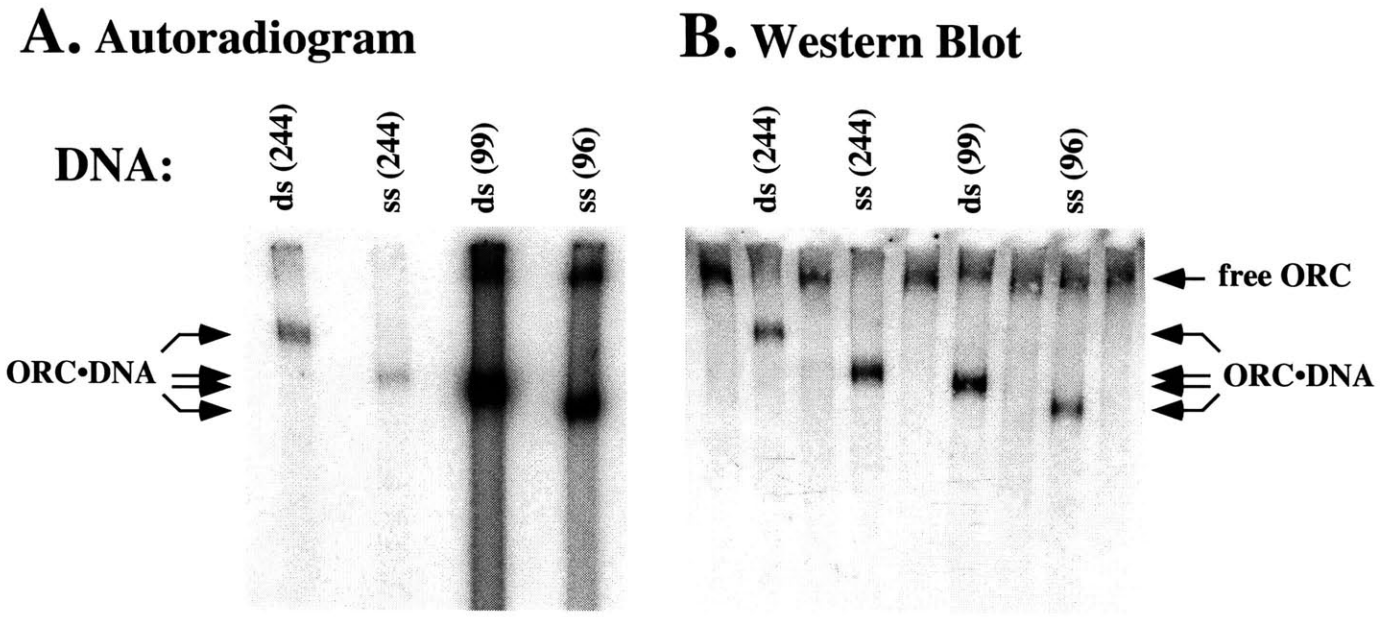

Figure 1. The Mobilities of ORC-ssDNA and ORC-dsDNA Complexes are Different. ORC was incubated with one of four radiolabeled DNA probes: a 244 bp dsDNA (ds 244), a 244 base ssDNA (ss 244), a 99 bp dsDNA (ds 99), or a 96 base ssDNA (ss 96), all in the presence of ATP. Reactions were electrophoresed on a native polyacrylamide gel and either analyzed by autoradiography to detect the radiolabeled DNA (A) or by Western blotting to determine the position of ORC in the gel (B). The ORC-ssDNA complex has a faster mobility when compared to an ORC-dsDNA complex (when the DNAs of similar lengths are compared), consistent with a more compact conformation of ORC when bound to ssDNA.

these data are consistent with a more compact structure of ORC in the presence of ssDNA, the mobility of a protein-DNA species is also dependent on the shape and flexibility of the nucleic acid. The difference in mobilities of the two different ORC-DNA complexes could also be due to the different electrophoretic properties of ssDNA and dsDNA. Therefore, this experiment does not definitively demonstrate a conformational change in ORC.

Limited Proteolysis of ORC

Limited proteolytic cleavage has been used to characterize conformational changes in proteins (Konigsberg, 1995). At low concentrations of protease, the enzyme typically cleaves proteins at exposed, flexible regions between domains to yield a characteristic cleavage pattern. Changes in the conformation of a protein can lead to changes in this cleavage pattern. If ssDNA bends ORC, then regions of ORC subunits may become more exposed and therefore hypersensitive to cleavage by the protease.

I compared the proteolytic cleavage patterns generated by trypsin and chymotrypsin with ORC alone and with ORC in the presence of ssDNA (Figure 2A). The addition of ssDNA did not increase the sensitivity of ORC to either protease. Instead, ORC became slightly more resistant to cleavage (see higher molecular weight bands for digests using 20 ng of trypsin or either amount of chymotrypsin). The increased resistance to proteolytic cleavage was also seen using V8 protease (Figure 2B). In these experiments, Western blots were performed to examine the stability of individual ORC subunits and proteolytic digests were performed using ORC alone, ORC with ssDNA, or ORC with dsDNA. Some subunits (Orc3p and Orc5p) were completely

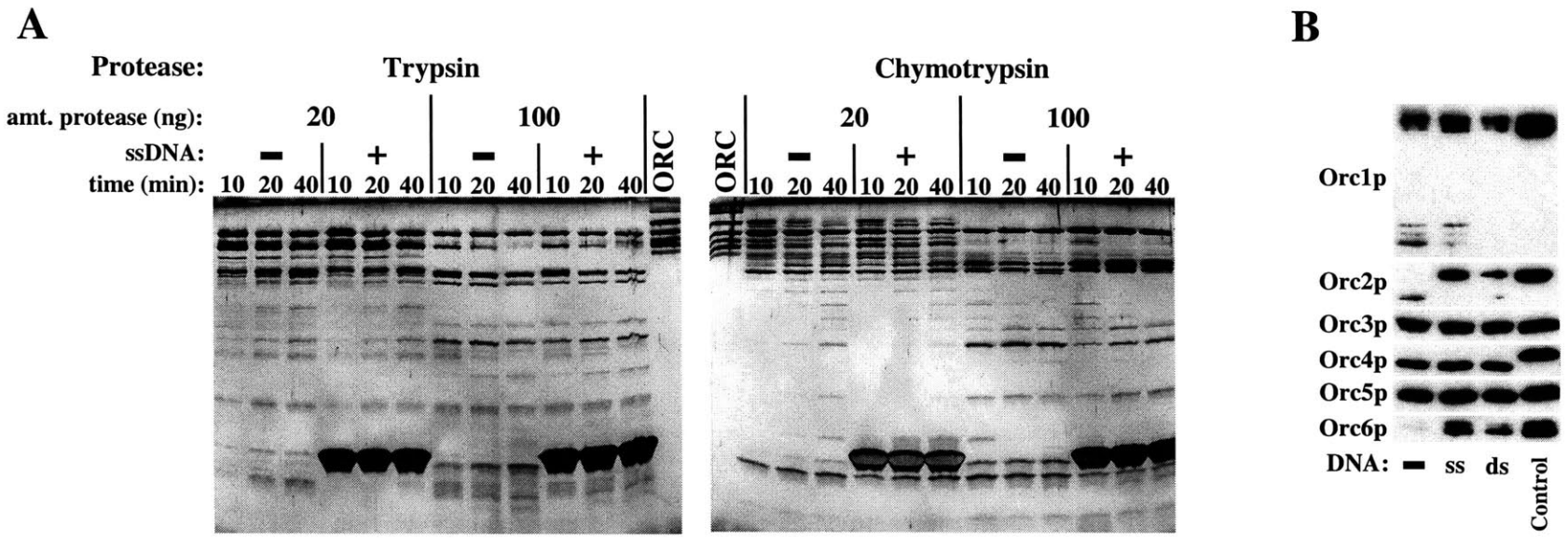

Figure 2. Limited Protease Treatment of ORC. (A) ORC was treated with trypsin (left panel) or chymotrypsin (right panel) in the presence or absence of ssDNA. Digests contained either 20 or 100 ng of protease as indicated and proceeded for 10, 20 or 40 minutes. The reaction products were electrophoresed on a 10% SDS-PAGE and stained with silver. Untreated ORC was also included to indicate the positions of the full-length subunits. (B) ORC was digested with V8 protease in the absence of DNA, in the presence of an 85 nt ssDNA (ss), or in the presence of a 99 bp origin containing dsDNA (ds). Digests were electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose and Western blotted with monoclonal antibodies directed against individual ORC subunits. The blot was stripped and reprobed multiple times. Untreated ORC (Control) was also included.

resistant to cleavage by V8 protease under these conditions. Orc4p was equally sensitive to digestion under all three conditions. However, in the case of Orc2p and Orc6p, the sensitivity of the subunits was altered in the presence of DNA. The addition of DNA increased the resistance of these subunits to digestion, with ssDNA protecting the ORC protein more effectively than dsDNA. The Orc1p subunit also shows increased protection in the presence of ssDNA but possibly increased sensitivity in the presence of dsDNA. The general decrease in proteolysis in the presence of ssDNA could be consistent with either a change in the conformation of ORC or with an inaccessibility of proteolytic cleavage sites due to the binding of DNA. In either case, the differences observed with ssDNA and dsDNA suggest that ORC binds these molecules in distinguishable ways.

Interactions with Other Proteins

If an ssDNA-induced conformational switch in ORC is required to remodel the pre-RC, then interactions of ORC with other proteins may be affected by ssDNA. Work from our lab and other labs has shown that ORC interacts directly with Cdc6p (R. J. Austin and S. P. B., unpublished observations; Liang et al., 1995; Wang et al., 1999). I determined whether ssDNA and dsDNA could differentially affect this interaction (Figure 3A). ORC was incubated with no DNA, with origin-containing dsDNA, or with ssDNA. Recombinant Cdc6p tagged with the hemagglutinin (HA) epitope was then added to each reaction. ORC was immunoprecipitated, and the resulting pellet was analyzed for the presence of Cdc6p. As had been previously observed, Cdc6p interacted directly with ORC (Figure 3A, lane 1). This interaction depended on both the presence of ORC (lane 4) and the addition of ORC antibodies (lane 5). Interestingly, the addition of either dsDNA (lane 2) or ssDNA (lane 3) strengthened the ORC-Cdc6p interaction.

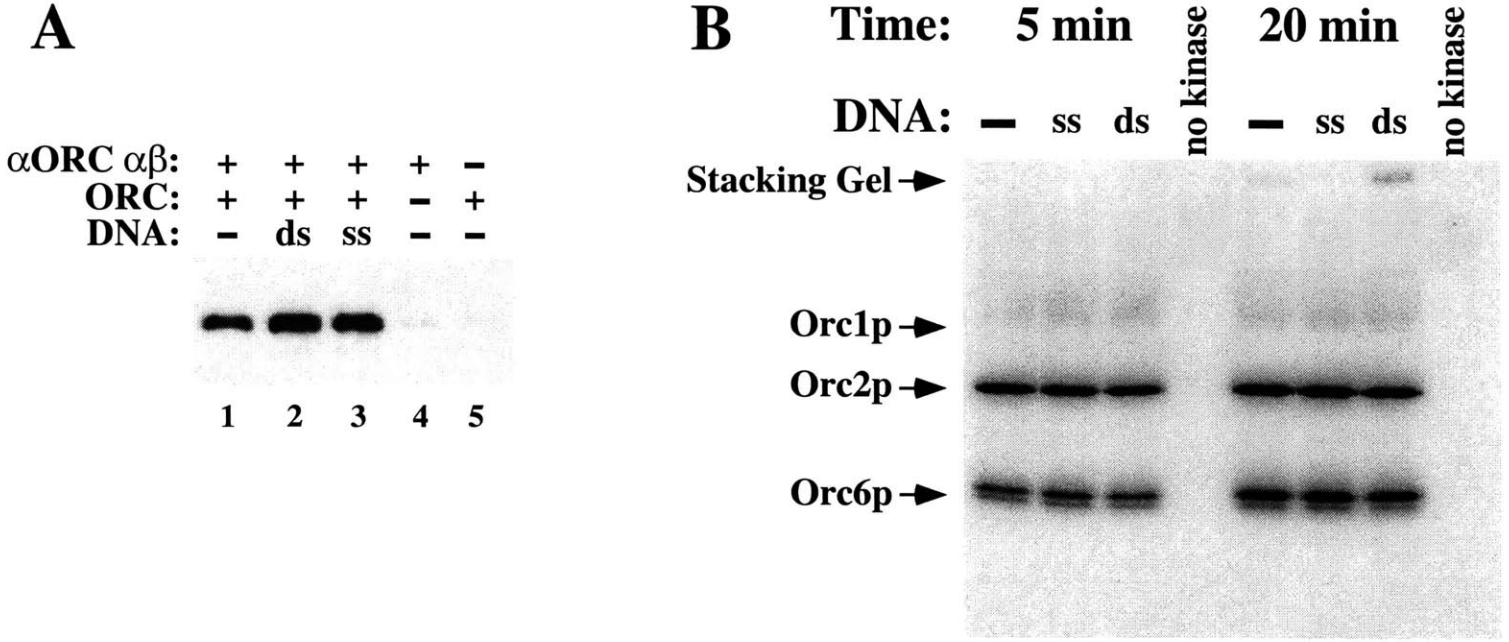

Figure 3. Interactions of ORC with Other Proteins are not Affected by ssDNA. (A) The ability of ORC to co-immunoprecipitate Cdc6p was determined in the absence of DNA (lane 1) or in the presence of ssDNA (lane 2) or origin-containing dsDNA (lane 3). HA-tagged Cdc6p was added to ORC-DNA binding reactions and ORC was immunoprecipitated using anti-ORC polyclonal antibodies. The precipitated proteins were electrophoresed on a 10% SDS-PAGE and analyzed by Western blotting using an anti-HA antibody to detect the tagged Cdc6p. In control reactions, either ORC or anti-ORC antibodies were omitted (lanes 4 and 5 respectively). (B) The ability of ORC to serve as a substrate for the Cdc28p/Clb5p kinase was determined in the absence of DNA or in the presence of ssDNA or origin-containing dsDNA. ORC (with or without DNA) was incubated with radiolabeled α-32P labeled ATP and purified Cdc28p/Clb5p for 5 minutes or 20 minutes as indicated and electrophoresed on a 10% SDS-PAGE. The gel was stained with silver, dried and exposed to film. The positions of ORC subunits as determined by silver staining are indicated to the left of the gel. Control reactions containing no kinase are also shown.

Therefore, the ssDNA-bound and the dsDNA-bound forms of ORC do not show differences in their interactions with Cdc6p.

ORC has been shown to be an *in vitro* substrate of Cdc28p (the yeast CDK) associated with the B-type cyclin, Clb5 (R. J. Austin and S. P. B., unpublished observations). If ssDNA alters the conformation of ORC, then the ability of ORC to serve as a substrate for this kinase may be altered. ORC alone, ORC incubated with ssDNA, and ORC incubated with origin-containing dsDNA were each mixed with purified Cdc28p/Clb5p and radiolabeled ATP (Figure 3B). Orc2p and Orc6p served as efficient CDK substrates, whereas Orc1p was weakly modified. Both the upper and lower bands of Orc6p were modified by Cdc28p/Clb5p, indicating that although the faster migrating form of Orc6p is not as heavily phosphorylated as the slower form, it is phosphorylated on at least one site. The addition of either ssDNA or dsDNA did not affect the modification of ORC subunits, arguing that the conformational change in ORC likely does not regulate the ability of Cdc28p/Clb5p to phosphorylate the complex.

EXPERIMENTAL PROCEDURES

Electrophoretic Mobility Shift Assay (EMSA)

The long dsDNA used in these assays (ds 244) was a 244 bp *EcoRI-HindIII* fragment of pARS1/WTA, and the long ssDNA (ss 244) was derived from the dsDNA by melting the fragment and purifying one of the strands of DNA (Chapter IV). The short dsDNA (ds 99) was a 99 bp PCR product containing the ACS and B1 elements. It was generated by using pARS1/WTA as a template and oligos DLX795-815 (tttatttaagtattgtttgtg) and DLX893-873 (atctttacatcttgttatttt) as PCR primers. The short ssDNA (ss 96) was the 96 nt ssDNA oligonucleotide described in Chapter IV. 10 μl binding reactions included 100 fmol of ds 244, 120 fmol of ss 244, and ~55 fmol each of ds 99 and ss 96. The amount of ORC added was 60 fmol for the ds 244 binding reaction and 100 fmol for all other reactions. Native gel mobility assays were performed as described in Chapter II and IV, except that the pH of the gel and running buffer was reduced from 8.5 to 7.0. Electrophoresis was performed at 200 V for 6 hours at 4°C. The gel was either dried for autoradiography or transferred to nitrocellulose for Western blot analysis as described (Chapter II).

Limited Protease Treatment of ORC

In a 15 ml reaction, 8 pmol of ORC was combined with 120 pmol of ssDNA (the 70 nt oligonucleotide described in Chapter IV) where indicated and incubated at room temperature for 10 minutes. 2 ml of PBS containing either 20 ng or 80 ng of trypsin or chymoptrypsin as indicated, were added to the ORC or ORC plus ssDNA reactions and incubated at room temperature. At 10 minute, 20 minute and 40 minute time points, 4.3 μl of the reaction was removed and added to 0.5 μl of 0.1 M PMSF (dissolved in

isopropanol). SDS-PAGE loading buffer was added, the reactions were electrophoresed on a 10% SDS-PAGE, and the gels were stained with silver.

For V8 protease digests, 10.5 µl reactions containing 4 pmol of ORC and ATP (35 µM final) were incubated with either no DNA, 14 pmol of ssDNA (the 70 nt oligonucleotide described in Chapter IV), or 12 pmol of dsDNA (the *ARS1*-containing ds 99 fragment described above for EMSAs). The binding reactions proceeded at room temperature. At 10 minute, 20 minute and 40 minute time points, 4.3 µl of the reaction was removed and added to 0.5 µl of 0.1 M PMSF (dissolved in isopropanol). SDS-PAGE loading buffer was added, and the reactions were electrophoresed on a 10% SDS-PAGE and transferred to nitrocellulose. The membrane was analyzed by Western blotting using the individual monoclonal antibodies described in Chapter II. The blot was stripped and reprobed multiple times. Only the 10 minute digest samples were shown in Figure 2B.

ORC-Cdc6p Co-Immunoprecipitation Assays

2.8 pmol of ORC were incubated with either 35 pmol of ssDNA (the 96 nt oligonucleotide described in Chapter IV), 36 pmol of dsDNA (the *ARS1*-containing ds 99 fragment described above for EMSAs), or no DNA in 50 µl of ORC binding buffer [100 mM Hepes-KOH (pH 7.6), 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, and 0.15 M KCl, 10 % (vol./vol.) glycerol, 0.01% (wt./vol.) NP-40, 1 mM DTT] containing 100 µM ATP. After a 10 minute incubation at room temperature, 4 µl of HA-tagged Cdc6p (provided by R. J. Austin) was added and the reaction continued for another 10 minutes. The reaction volume was increased to 200 µl with ORC binding buffer, and 2 µl of anti-yeast ORC polyclonal sera (provided by R. J. Austin) was added. The antibodies were allowed to bind ORC by incubating the reaction at 4°C for 1.5 hours with

constant mixing. 25 µl of protein-G Sepharose beads (a 1:1 vol./vol. slurry in 1X PBS) were added, and the binding reaction was allowed to continue for another hour at 4°C. The beads were then precipitated by centrifugation and the supernatant was removed. The beads were then washed 3 times in ORC binding buffer and 3 times in CHIP lysis buffer (50 mM HEPES/KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate). The beads were then boiled in SDS-PAGE loading buffer and the precipitated proteins were electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting using an anti-HA antibody to detect the tagged Cdc6p. In control reactions, ORC or antibodies were omitted as indicated.

Cdc28p/Clb5p Kinase Assays

12.5 µl reactions containing 2 pmol of ORC, 82 µM α-^{32}P labeled ATP (153.8 µCi/nmol), and 5 mM $MgCl_2$ were incubated with 4 pmol of ssDNA (the 96 nt oligonucleotide described in Chapter IV) or dsDNA (the *ARS1*-containing ds 99 fragment described above for EMSAs) as indicated. 0.2 µl of purified Cdc28p/Clb5p kinase (provided by R. J. Austin) was added and the kinase reactions were incubated at room temperature. After 5 minutes and 20 minutes, 5.5 ml of the reaction was removed and stopped with SDS-PAGE loading buffer. The proteins were electrophoresed on a 10% SDS-PAGE, stained with silver to determine the positions of the ORC subunits, and the gels were then dried and exposed to film.

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