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

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In many organisms, the replication of DNA 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, we have investigated the architecture of the Saccharomyces cerevisiae origin recognition complex (ORC) bound to yeast origins of replication. Determination of DNA residues important for ORC-origin association indicated that ORC interacts 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 requires the coordinate action of five of the six ORC subunits. Protein-DNA cross-linking 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.

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 assembly of a multiprotein replication machine. Studies of the replication of bacteria, phages, and the genomes of viruses infecting eukaryotes have established that a protein called the initiator binds to the cognate origin of DNA replication in a sequence-specific manner. Once bound, initiator proteins often participate in other aspects of replication initiation; for instance, they facilitate origin unwinding and recruit other replication proteins to the origin (for a review, see reference 3). Detailed analyses of initiator proteins bound to their cognate origins have been important in the determination of how these proteins function during replication initiation. Our aim was to extend this type of analysis to a putative eukaryotic chromosomal initiator protein bound to an origin of DNA replication.

The strongest candidate for a eukaryotic initiator protein is the origin recognition complex (ORC) (7). ORC was first identified in the yeast Saccharomyces cerevisiae as a six-subunit complex that specifically binds to origin sequences in vitro in the presence of ATP. 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 (5, 6, 30, 32). ORC-DNA binding is required for the essential function of ORC, since mutations in origin sequences that reduce or eliminate origin function also reduce or eliminate ORC binding in vivo (1, 1a). In addition, most conditional mutations in ORC genes lead to decreased origin usage (18, 31) and decreased origin binding in vivo (1, 1a). A role for ORC as an initiator protein is further supported by its conservation in multiple eukaryotes. Proteins with amino acid sequence similarity to yeast ORC subunits have been identified in numerous other eukaryotes (for a review, see reference 17), and complexes of these ORC-like proteins have been purified from Xenopus laevis and Drosophila melanogaster (19, 38). More importantly, ORC-related proteins are required for DNA replication in Schizosaccharomyces pombe, Xenopus, and Drosophila cells (28; for a review, see reference 17).

Studies of proteins associated with yeast origins of DNA replication in vivo have demonstrated the existence of multiple ORC-dependent protein-DNA complexes (1a, 15, 40, 42a). During S phase, G2, and M phase, a postreplicative complex (post-RC) that produces a DNase I protection pattern similar to that seen in vitro with purified ORC is observed (7, 15). As cells cross the M phase-to-G1 boundary, however, additional proteins involved in replication, including Cdc6p and the MCM proteins, are recruited to origins to assemble a pre-RC (1a, 13, 40, 42a). At the G1-to-S transition, a third complex is formed that includes at least one DNA polymerase; this is referred to as the RC (1a). Studies of chromatin-associated proteins required for Xenopus DNA replication have demonstrated a clear order of assembly. ORC-related proteins bound to chromatin recruit X. laevis Cdc6p, and the resulting complex is required for the recruitment of X. laevis MCM proteins (14, 37, 38). Studies in S. cerevisiae suggest a similar order of assembly (1a, 42a). Thus, ORC forms the foundation for the assembly of critical higher-order origin structures that change during the cell cycle.

The best-defined eukaryotic origins of replication are those of S. cerevisiae. These elements were first identified as genomic DNA sequences capable of supporting the autonomous replication of episomal DNA (autonomous replicating sequences [ARSs]) (23, 42). Many of these elements were subsequently shown to act as origins of replication in their normal chromosomal context (for a review, see reference 34). Yeast origins are modular in nature and contain an 11-bp 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) (4). ARS1, the first well-characterized origin, has three such elements (B1, B2, and B3 [see Fig. 7]). In vitro and in vivo, DNase I protection assays of ARS1 demonstrated that ORC...
protects approximately 50 bp of DNA that include the ACS and B1 sequences (7, 16, 40). These two elements direct ORC-DNA binding at ARS1 and at ARS307 (35, 39) and together represent the smallest functional region of either origin. We will refer to this minimal region required for ORC-DNA binding and origin function as the core origin.

We have a general understanding of the DNA sequence requirements for the association of ORC with origins (7, 36, 39), 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. (i) How does ORC interact with DNA? In particular, we would like to determine which of the approximately 50 protected base pairs are important for ORC-DNA binding, how the structure of origin DNA is affected by interaction with ORC, and which of the six essential ORC proteins are required for DNA binding. (ii) How do ORC subunits interact with each other? ORC is a preassembled complex in the absence of DNA, and we want 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. (iii) How does the ORC interact with other proteins? In addition to genetic and physical interactions with the CDC6 gene product (31), ORC subunits show multiple genetic interactions with other essential genes required for DNA replication (for a review, see reference 17). 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 this study, we have 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, we have used analysis of ORC complexes lacking one of the six subunits and protein-DNA cross-linking 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.

MATERIALS AND METHODS

Plasmids and competitor DNA. pDL01, used in modification-interference and missing-contact assays, was prepared by inserting the following sequence into the BgIII fragment of pARS1/858–865 (which encodes a minimal ORC binding site that contains 74 bp of ARS1 sequence, including the ACS and B1 elements). This DNA is bound by ORC in a manner similar to the binding of 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 BglII-HindIII fragment of pARS1/858–865 (which has a linker substitution of the B2 element) (these plasmids were previously described in reference 33). Plasmid stability assays were performed as described previously (6). Plasmids pARS1/WT and pARS1/a b2 were used as templates for production of wild-type and ACS-negative, B2-negative competitor DNA. Competitor DNA was linearized by digestion with BglII and with universal forward and reverse sequencing primers, and PCR products were purified on a 2% agarose gel (1× Tris-borate-EDTA [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, 1× 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 previously (27) except that 10 mM dithiothreitol, 5 mM EGTA, 0.22 ng of radiolabeled probe (300 cps), and 12 ng of ORC. Binding reactions were incubated for 10 min at room temperature. The ARS1 probe used in the mobility shift assay was digested by pARS1/WT with EcoRI, 5′-end labeling with the Klenow fragment of DNA polymerase I, and digestion with HindIII. The labeled 244-bp EcoRI-HindIII fragment was purified as described above. All ORC mobility shift assays were performed as described previously (36), except that gels and running buffers included 80-µg/ml BSA and gels were run at 4°C for 4 h at 200 V.

Modification-interference and missing-contact assays. Chemical modifications of labeled pDL01 DNA was carried out as follows. Dithiol pyrocatechol (DEPC) carboxethylolation was performed essentially as described previously (22), except that end-labeled DNA was heated for 5 min at 90°C prior to incubation with DEPC, 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 reanneal by slowly cooling the suspension to room temperature. Deionization of ARS1 DNA was performed as described previously (12). KmO4 modification was carried out as described previously (44), except that treatment with KmO4 was performed for 15 min at room temperature. Reannealing of melted DNA was performed as described above, except that incubation with hydrazine was carried out for 30 min at room temperature. Phosphate backbone ethylation with ethylisothiourea (EUN) was performed as described previously (21).

Prior to separation of bound and unbound DNA molecules, an aliquot of each modified DNA sample was reserved for chemical cleavage (see input samples [I] in Fig. 1A). 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 mixture. Bound and unbound DNA molecules were separated by electrophoresis in a mobility shift protocol. DNA was separated by electrophoresis in a 5% native acrylamide gel. The gel was dried and then 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; pARS1/WT, pARS1/a b2, and pARS1/858–885 for wild-type and mutant ARS1 fragments). Oligonucleotides were designed to add XbaI sites to both ends of the following regions amplified from each ARS: nucleotides −69 to +145 of ARS305 (24), 285 to 498 of ARS121 (45), and 240 to 445 of ARS1 (44). PCR reactions were carried out for 25 cycles in the presence of 0.2 µM [α-32P]dATP. The PCR products were digested with XbaI 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 of buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM MgCl2, 0.2 µM [α-32P]dATP, and a 50-fold molar excess of unlabeled competitor DNA (containing either the wild-type ARS1 sequence or the ARS1/a b2 sequence). One hundred fifty nanograms of ORC was added where indicated, and all reaction mixtures contained 1 ng of BSA. Circularization of origin probes was performed as described previously (26), using 56 NEB U of T4 DNA ligase per reaction. Samples were removed at various times, reactions were stopped, and reaction products were electrophoresed on a 20-µm-long 5% native acrylamide gel (1:60 acrylamide/bisacrylamide, 1× TBE) for 80 V. Circular DNA samples were distinguished from linear molecules by their resistance to exonuclease III.
digestion. Gels were either dried and exposed to film or exposed to Molecular Dynamics PhosphorImager screens for quantitation by ImageQuaNT software.

**Protein-DNA cross-linking.** The production of 4'-azidophenacyl bromide (4'-AZPB) UV cross-linking probes combined methods described by Bell and Stillman (7) and Yang and Nash (47). The EcoRI-HindIII fragment of pARS1/WTA was subcloned into M13mp18 and M13mp19 replicative forms which had previously been cut with the same enzymes, and single-stranded DNAs were produced for use as templates for ARS1 cross-linking 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-nucleotide universal primer and a second oligonucleotide were annealed to single-stranded templates. Extension using T4 DNA polymerase in the presence of α-32P-CTP or α-32P-GTP and labeled [α-32P]ATP or [α-32P]TTP resulted in the incorporation of one or two thiosphate nucleotides and several radioabeled nucleotides immediately following the second primer. The extension was chased with an excess of unlabelled, unmodified nucleotides to complete DNA synthesis. The resulting double-stranded circles were precipitated, and 4'-AZPB was coupled to the incorporated thiosphate nucleotides as described previously (47). Free 4'-AZPB was removed by using a 1-ml G-50 spin column. The purified DNA was digested with EcoRI and SalI to remove any modified or labeled dimeric thiosphate nucleotides incorporated following the universal primer, and the liberated restriction fragment was gel purified. DNA fragments for bromodeoxyuridine (BrdU) cross-linking were synthesized as described previously (2), using the same single-stranded DNA templates as the 4'-AZPB cross-linking probes; they were then digested with EcoRI and SalI and gel purified.

**Orc-ORC binding conditions for cross-linking experiments were as described above, with the following changes. The amount of purified protein added per binding reaction was increased twofold for 4'-AZPB cross-linking, and the amount of ARS1/a 2 competitor was increased three and fivefold for BrdU and 4'-AZPB cross-linking, respectively. Reaction mixtures were transferred to a microtiter plate and irradiated with a 254-nm light source (model UVG-54; UVP) at a distance of 1 cm for either 2 min (4'-AZPB cross-linking) or 30 min (BrdU cross-linking). Cross-linked proteins were treated with DNase I (Worthington) and micrococcal nuclease (Worthington) as described previously (7), precipitated with trichloroacetic acid, and resolved on sodium dodecyl sulfate–10% polyacrylamide gels. Gels were silver stained, dried, and exposed to film. We had previously reported that Orc1p did not cross-link to ARS1 (27); however, we subsequently discovered that the micrococcal nuclease used to digest DNA after cross-linking 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 cross-linking became observable.

**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 the DNA by adding an adduct. 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 a region of the DNA that is important for association with protein. Since in the case of ORC-DNA binding we found that modification-interference and missing-contact experiments identified similar residues (Fig. 1), henceforth either the addition of an adduct or the removal of a base will be termed a modification of a residue. In these assays, ARS1 DNA fragments end labeled on either the top or bottom strand were modified with one of five reagents prior to ORC-DNA binding (see the legend to Fig. 1A for a description of the 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 inhibited 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 (Fig. 1A and highlighted residues in Fig. 1B). ORC-DNA binding was extremely sensitive to modification of residues 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 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 that does not involve ORC-DNA binding (36). The modification data also exhibited strand-specific differences. Modification of the top strand consistently affected ORC-DNA binding to a greater extent than did 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. The most striking strand-specific differences were 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, we mutated residues 852 to 854, changing AGA to either GAG or CTC. Both mutations were tested to determine how they affected plasmid stability and ORC-DNA binding in vitro (Table 1). The AGA-to-GAG mutant had no detectable defect in vivo or in vitro, whereas the AGA-to-CTC substitution mutation resulted in a 50% 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 lead to transversion mutations in the important AGA (33). Thus, this region contributed to ARS1 function and appeared to show a preference for purines on the top strand and for pyrimidines on the bottom strand.

**ORC bends DNA at some, but not all, origins.** Because some initiator proteins induce DNA bending at origins as a prerequisite to DNA unwinding (9, 11), we investigated whether ORC bends yeast origin DNA. A radiolabeled ARS1 fragment with compatible cohesive ends was incubated with DNA ligase, and the rate of formation of circular monomers, either with or without ORC, was monitored (Fig. 2A). In the presence of ORC, the rate of formation of circular monomers was stimulated threefold; 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 and mutant competitor DNAs (Fig. 2A; compare lanes 9 to 12 with lanes 5 to 8) and by experiments using a DNA-bending template containing a mutated ORC binding site (Fig. 2A, lanes 13 to 24). To determine if bending of origin DNA was a general property of ORC, two other ARS elements were examined. ARS127 and ARS305 are both active chromosomal origins of replication (24, 46). Although ORC bound strongly to these origins (data not shown), neither was bent by ORC (Fig. 2B). Thus, bending of DNA by ORC occurs at ARS1 but is not a general property of ORC interaction with origins of replication.

**Orc6p is not required for DNA binding.** To determine the roles of individual ORC subunits in ORC-DNA binding, we produced six different mutant ORC complexes, each missing a different ORC subunit (referred to as partial ORC complexes). Insect cells that infected with baculoviruses expressing five of the six ORC polypeptides, and these mutant ORC complexes were partially purified. The integrity of each of the six partial complexes was examined by determining the ORC subunits present in the partially purified fractions. In two cases, the omission of one subunit clearly compromised complex in-
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 (Fig. 3B). 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 (Fig. 3A) manner. This protein-DNA complex is likely to contain all five of the ORC proteins present in the partial-complex fraction, as all five subunits coimmunoprecipitate and antibodies to three of the subunits (Orc1p, Orc2p, and Orc4p) can supershift the partial ORC-DNA complex (data not shown). 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 (Fig. 3A), 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 subassembly of polypeptides.

**Organization of ORC subunits at ARS1.** The partial complex experiments described above indicate that Orc1p, Orc2p,

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**TABLE 1. Effect of mutations in Region 852 to 854 of ARS1**

| Plasmid        | Sequence (850–855)  | Plasmid loss rate per generation (% ± SD) | Defect in mobility shift activity |
|----------------|--------------------|-----------------------------------------|----------------------------------|
| ARS1/WTA       | AAAGAT             | 6 ± 0.5 (6)                             | None                             |
| ARS1/GAG       | AAAGAT             | 4.3 ± 0.9 (11)                          | None                             |
| ARS1/CTC       | AAAGAT             | 10.8 ± 1.1 (6)                          | 2.4-fold                         |
| ARS1/844–850   | AAAGAT             | 13.3 ± 2.3 (3)                          | ND†                              |
| ARS1/835–842   | AAAGAT             | 27.9 ± 1.7 (3)                          | 13-fold                          |

* Sequences differing from the wild-type sequence are in boldface type.
* Numbers in parentheses indicate the number of independent experiments.
* Linker substitutions of the B1 element, previously described (33).
* ND, not determined.
Orc3p, Orc4p, and Orc5p are 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, we performed UV protein-DNA cross-linking studies. In these experiments, we generated a series of 10 ARS1 cross-linking probes that were modified by addition of a photoreactive azido group on one or two specific phosphate residues. The azido group is coupled to thiophosphate groups in the DNA backbone through the use of the reagent 4′-AZPB, which constrains the photoreactive group to a distance of approximately 10 Å from the phosphate backbone. Proteins cross-linked to a particular modified residue are therefore within this distance, and we will describe such proteins as being in close proximity to the DNA at this site. The portion of ARS1 examined using this set of 10 probes is 92 bp long and includes the entire region protected from DNase I digestion by ORC (Fig. 4B).

FIG. 2. 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 were converted to circular monomers, linear dimers, and linear dimers. The three electrophoretic species of linear dimers were the result of a weak intrinsic bend in the B3 element (removal of this DNA bend by the use of a B3 linker substitution did not affect the ORC-induced binding of ARS1 [data not shown]). The rates of circular monomer formation in the presence (lanes 5 to 8 and 9 to 12) and absence (lanes 1 to 4) of ORC were tested. ORC-ARS1 binding and ligation reactions were carried out in the presence of a 50-fold excess of unlabeled competitor DNA containing either a wild-type ARS1 binding site (lanes 9 to 12) or a mutant ARS1 binding site (lanes 5 to 8). A radiolabeled DNA probe containing a mutated ARS1 binding site (the ARS1/a1b22 mutation) was also tested (lanes 13 to 24). (B) Circular monomer formation for ARS305 (left graph) and ARS121 (right graph) in the presence (●) and in the absence (□) of ORC, 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). The amount of circular monomer produced at each time point was expressed as the percentage of total counts in each lane.
and Orc3p are colocalized to the entire ACS and to DNA that extends leftward toward the B1 element (probes E, F, G, and I); they are the only ORC subunits cross-linked to residues located between the ACS and B1 that contribute to ARS1 function (probes F and G) (Fig. 4). Because Orc4p and Orc5p have similar electrophoretic mobilities and are difficult to resolve, we modified Orc4p by fusing an additional 168 amino acids, derived from the Sp1 transcription factor, to its N terminus (Fig. 5A). This modified ORC4 protein migrated to a position above that of the Orc2p subunit. Cross-linking was performed with a wild-type ORC complex and a complex containing the modified Orc4p, and only one cross-linked protein exhibited altered mobility, allowing unambiguous identification of Orc4p and Orc5p (Fig. 5B). In the wild-type ORC complex, Orc4p is cross-linked strongly to the ACS (Fig. 4A, probe H) and more weakly to sequences to the right of the ACS (probe J). Orc5p is the only subunit cross-linked to the region of B1 important for ORC-DNA binding (Fig. 4A, probe D), and Orc6p is cross-linked 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 cross-linked to only one region of the DNA, Orc2p, Orc4p, and Orc5p are each cross-linked to two distinct regions of ARS1, with no cross-linking to the intervening sequence (Fig. 4A; for a summary, see Fig. 7). Orc2p, Orc4p, and Orc5p are cross-linked to sites on the DNA separated by 78, 54, and 35 bp, respectively. Cross-linking of subunits to two distinct regions of DNA could be explained by ORC subunits having an elongated shape, by the existence of multiple complexes or individual subunits bound at each origin, or by the occurrence of DNA bending at the origin. Based on comparison with protein-DNA cross-linking 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 cross-linkers at positions analogous to those tested for ARS1 (determined relative to the ACS). Four cross-linking

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**FIG. 3. 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 lacking Orc1p through Orc6p are in the first 12 lanes (left to right), and purified wild-type ORC is shown in the last two lanes as a control. For each ORC complex, DNA binding reactions were performed 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 polyacrylamide-10% sodium dodecyl sulfate gel and transferred to nitrocellulose. Wild-type ORC is shown in the last lane. ORC proteins were identified by incubation of blots with monoclonal anti-ORC antibodies.

**FIG. 4. 4'-AZPB cross-linking of ORC subunits to ARS1.** (A) UV cross-linking of ORC subunits to 10 ARS1 fragments (probes A through J), each incorporating a photoreactive cross-linker at a distinct site. Purified ORC was incubated with each probe in the presence (+) or absence (−) of ATP and irradiated with UV light. Gels were silver stained to determine the positions of ORC subunits, dried, and exposed to film. In all cases, the observed protein-DNA cross-linking was ATP dependent. Cross-linking to ORC subunits was also UV dependent and sensitive to competition by a wild-type ARS1 fragment (data not shown). (B) Positions of cross-linking nucleotides in ARS1 probes A through J. Arrows identify the modified positions; the phosphate coupled to the photoreactive cross-linker is 5' to the residue indicated. Each probe has either one or two modified bases as indicated. Outlined residues between positions 839 and 868 are as described in the legend to Fig. 1B.
An interesting difference between ARS1 and ARS305 is that the Orc5p subunit is only cross-linked to one discrete site at ARS305 (it is present only at B1) and not to multiple sites as in the case of ARS1. To determine if the other subunits that cross-linked to more than one region of ARS1 (Orc2p and Orc4p) were also localized to only one region of ARS305, we examined sequences beyond the core ARS305 binding site. ARS305 cross-linking probes analogous to ARS1 probes A and B that cross-linked Orc2p and/or Orc4p, as well as a third probe that modified a nearby residue on the opposite strand, were synthesized (Fig. 6B). These three ARS305 probes showed no evidence of specific cross-linking (data not shown).

Thus, these cross-linking 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 do differ.

**ORC subunits in the major groove of ARS1.** Because the cross-linking 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 cross-linking to ARS1 was performed. DNA fragments that incorporated BrdU and radiolabeled nucleotides into either the top or the bottom strand of ARS1 were generated and cross-linked to ORC. DNA fragments modified on either strand efficiently cross-linked to Orc2p and Orc4p (Fig. 8). Orc1p cross-linked to the bottom strand of ARS1, although weak Orc1p cross-linking to the top-strand was occasionally observed (data not shown). BrdU cross-linking 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, that specifically recognize a region of DNA approximately 30 bp in length. We found that the coordinate action of five of the six ORC subunits is required for this interaction (Fig. 9). Recognition of critical DNA residues at two genetically defined re-
regions of origins (the ACS and B1-like elements) is likely to be mediated by nonoverlapping ORC subunits or groups of subunits. Although we have no evidence of direct DNA contact by Orc5p, the failure to cross-link any other ORC subunit to the B1 regions of both ARS1 and ARS305 strongly 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 cross-linked 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 manners in which ORC interacts with its binding site at different yeast origins of replication are similar. A comparison of nine yeast origins showed that the four most highly conserved residues outside of the ACS fall within regions of ARS1 that strongly inhibit ORC-DNA binding when chemically modified (residues A839, A840, A852, and A854 [Fig. 9]). These residues also fall within regions required for efficient origin function in origins whose structures have been well characterized. The residues at positions 839 and 840 of ARS1 are conserved in the B1-like elements of ARS305 (24) and ARS307 (35, 43), and residues at positions 852 and 854 of ARS1 fall within the box 3' element of ARS305 (24), the extended A element of ARS307 (35, 43), and the extended core region of ARS121 (46). The similar organizations of ORC subunits over the core binding site at ARS1 and ARS305 also argue that features of ORC-origin binding are conserved (Fig. 7) 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 cross-linked to a region within the core origin, Orc2p, Orc4p, and Orc5p are cross-linked to a second site in the flanking DNA. This interaction with two distinct regions of DNA is consistent with one of the following interpretations: (i) Orc2p, Orc4p, and Orc5p are elongated proteins; (ii) multiple copies of Orc2p, Orc4p, and Orc5p are present in each ORC complex; (iii) multiple ORC complexes bind to ARS1; or

![Figure 7](image-url)

**FIG. 7.** Summary of ORC subunit arrangement at ARS1 and ARS305. ORC subunits cross-linked 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. The unlabeled arrows along ARS305 represent the positions of cross-linkers on probes tested for which no sequence-specific or ATP-dependent ORC cross-linking was detected.

![Figure 8](image-url)

**FIG. 8.** BrdU cross-linking at ARS1. ARS1 fragments incorporating BrdU into either the top strand or the bottom strand were incubated with purified ORC. Cross-linking was performed as described in the legend to Fig. 4A, with (+) or without (−) ATP as indicated.
and (ii) Individual 4
the complex (Fig. 3B, partial complexes lacking Orc3p and Orc5p, respectively),
pensable for specific ORC-DNA binding. Physical interactions between Orc2p
Orc4p. Orc5p is likely to contact important residues in B1, and Orc6p is dis-
demed by the combined major-groove interactions of Orc1p, Orc2p, and
heads. Protein-DNA contacts are made primarily with the top strand of origins
six of nine origins is shown as an uppercase letter (residue A840). Hypothesized
residues that are conserved in seven of nine yeast chromosomal origins of
uppercase letters. Outside of the ACS, boldface uppercase letters represent
chemical modification strongly interferes with ORC-DNA binding are indicated,
ARS1
but not at
ARS305
(Fig. 2B). Thus, the association of ORC
DNA elements. ORC, however, is a heteromultimer which
and/or origin DNA binding.
(iv) the DNA at ARS1 is bent such that distant regions of the
DNA are in close proximity to the same polypeptide. Although
the differences in protein-DNA cross-linking observed at ARS1
and ARS305 could be explained by differences in stoichiometries of ORC or ORC subunits bound at these two origins, this
interpretation is unlikely, as ORC-DNA complexes migrate
arily when ARS1 and ARS305 DNA fragments are used in
electrophoretic mobility shift assays (data not shown). We fa-
vor DNA bending as the most likely explanation for the dis-
crepancy in cross-linking results, an interpretation that is con-
sistent with the ability of ORC to induce DNA bends at ARS1
but not at ARS305 (Fig. 2B). Thus, the association of ORC
subunits with the core origin is likely to be similar at all origins,
but higher-level interactions like DNA bending may strongly
fluence cross-linking 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 coop-
eratively to two adjacent binding sites for origin function in vivo (20). The crystal structure of the EBNA1 dimer bound to
DNA has been resolved, and modeling of two dimers bound to
two adjacent binding sites has been performed (8). Because the
DNA binding sites are separated by only 3 bp, two dimers
cannot co-occupy these adjacent sites unless the DNA in be-
tween them is distorted to prevent collision of the proteins.
Typically, initiator proteins are homomultimers that make
multiple protein-DNA contacts by interacting with repeated DNA
elements. ORC, however, is a heteromultimer 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., DNA unwinding).

The observation that ORC is much more sensitive to mod-
ifications of the top strand of ARS1 than it is to bottom-strand
modifications 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
DNA binds to its origin, it induces
melting of adjacent DNA (the repeated 13-mer site) in a pro-
cess called open complex formation (11). In the open complex,
DNA preferentially interacts with one of the single strands of
the unwind 13-mer region (11, 25). The simian virus 40
initiator, T antigen, forms a double-hexamer structure encir-
cling two DNA elements that are subsequently unwind or
untwisted (for a review, see reference 10). Although the T-
antigen hexamers encircle both strands of the DNA in these
distorted regions, each hexamer contacts only one strand (41).
Since ORC is bound to origins throughout most of the cell
cycle, ORC-DNA binding is unlikely to be sufficient for origin
unwinding. We therefore imagine three possible models
whereby preferred interaction with one strand of DNA may be
utilized by ORC. (i) ORC is responsible for initial DNA un-
winding 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. (ii) Another protein(s) recruited to origins
performs the unwinding function, but ORC stabilizes the
melted region of the duplex by binding to one of the single
strands. (iii) ORC is passive in the unwinding process but uses
single-stranded DNA binding to remain associated with origins of
replication after they are unwind. 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 provided information
regarding ORC subunit arrangement within the complex
(Fig. 9). The 4'-AZPB cross-linking studies identified ORC
subunits in close proximity to the same modified DNA residue,
and these results are consistent with the subunit organization
that we derive from partial-complex experiments. The compo-
sition of the partial complex lacking Orc5p suggests that Orc4p
and Orc5p physically contact each other (Fig. 3B), and 4'-
AZPB cross-linking demonstrated that these two subunits can
be cross-linked to the same DNA site (Fig. 4A, probe J). Simi-
larly, a complex lacking Orc3p is also deficient in Orc2p
(Fig. 3B), and both subunits are cross-linked by a number of
ARS1
and ARS305 probes (Fig. 4A, probes E, F, I; Fig. 6A,
probe 1).

UV cross-linking studies can only describe the arrangement
of ORC subunits in the presence of DNA. However, in the
absence of DNA, ORC is a preassembled complex, and we
believe that the conformation and relative positions of its sub-
units may be different when the 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 γ-S-ATP (7,
27). 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 (27). Such coordinate
action is likely to be mediated by allosteric changes within the
complex. Thus, we are interested in determining how the rel-
ative positions of ORC subunits 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 un-
derstanding must ultimately be expanded to incorporate vari-
ous cell cycle contexts and the effects of proteins whose asso-
ciation with ORC is cell cycle regulated. These initial studies
will provide a foundation for understanding 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 (15). How the asymmetry inherent in the ORC-origin complex is ultimately translated into the assembly of two symmetric replication forks at origins of bidirectional DNA replication remains to be understood.

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REFERENCES
1. Aparicio, O., and S. P. Bell. Unpublished data.
2. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1994. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
3. Baker, T. A., and A. Kornberg. 1994. DNA replication, 2nd ed. W. H. Freeman and Company, New York, N.Y.
4. Bell, S. P. 1995. Eukaryotic replicators and associated protein complexes. Curr. Opin. Genet. Dev. 5:162–167.
5. Bell, S. P., R. Kobayashi, and B. Stillman. 1993. Yeast origin recognition complex functions in transcription silencing and DNA replication. Science 262:1844–1849.
6. Bell, S. P., J. Mitchell, J. Leber, R. Kobayashi, and B. Stillman. 1995. The multi-domain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. Cell 83:563–568.
7. Bell, S. P., and B. Stillman. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multi-protein complex. Nature 357:128–134.
8. Bochkarev, A., et al. 1996. Crystal structure of the DNA-binding domain of the Epstein-Barr virus origin-binding protein, EBNA1, bound to DNA. Cell 84:791–800.
9. Borowiec, J., and J. Hurwitz. 1988. Localized melting and structural changes in the SV40 origin of replication induced by T-antigen. EMBO J. 7:3149–3158.
10. Borowiec, J., J. Mitchell, J. Leber, R. Kobayashi, and B. Stillman. 1995. The multi-domain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. Cell 83:563–568.
11. Bramhall, D., and A. Kornberg. 1988. Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the E. coli chromosome. Mol. Cell. Biol. 8:743–755.
12. Brunselle, and R. Schleif. 1987. Missingcontact probing of DNA-protein interactions. Proc. Natl. Acad. Sci. USA 84:6673–6676.
13. Cochener, J. H., S. Piatti, C. Santocana, K. Nasmyth, and J. F. Difley. 1996. An essential role for the Cdc6 protein in the pre-replicative complexes of budding yeast. Nature 379:180–182.
14. Coleman, T. R., P. B. Carpenter, and W. G. Dunny. 1996. The Xenopus Cdc6 protein is essential for the initiation of a single round of DNA replication and MCM binding to chromatin. Curr. Biol. 6:1416–1425.
15. Rowles, A., et al. 1996. Interaction between the origin recognition complex and the replication licensing system in Xenopus. Cell 87:287–296.
16. Rowles, A., J. H. Cock, J. Hurwood, and J. F. Difley. 1995. Initiation complex assembly at budding yeast replication origins begins with the recognition of a bipartite sequence by limiting amounts of the initiator, ORC. EMBO J. 14:2631–2641.
17. Santocana, C., and J. F. X. Difley. 1996. ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in Saccharomyces cerevisiae. EMBO J. 15:6671–6679.
18. SenGupta, D. J., and J. A. Borowiec. 1994. Strand and face: the topography of interactions between the SV40 origin of replication and T-antigen during the initiation of replication. EMBO J. 13:982–992.
19. Stinchcomb, D. T., K. Struhl, and R. W. Davis. 1979. Isolation and characterization of a yeast chromosomal replicator. Nature 282:39–43.
20. Truss, M., G. Chalepakis, and M. Beato. 1990. Contacts between steroid hormone receptors and thymines in DNA: an interference method. Proc. Natl. Acad. Sci. USA 87:7180–7184.
21. Walker, S. S., S. S. C. Francesconi, and S. Eisenberg. 1990. A DNA replication enhancer in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 87:4665–4669.
22. Yang, S., and H. Nash. 1994. Specific photocrosslinking of DNA-protein complexes: identification of contacts between integration host factor and its target DNA. Proc. Natl. Acad. Sci. USA 91:12183–12187.