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The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators

(DNA replication/*Saccharomyces cerevisiae*/initiation/DNA binding protein)

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ABSTRACT **Replicators are genetically defined elements within chromosomes that determine the location of origins of DNA replication. In the yeast *Saccharomyces cerevisiae*, the *ARS1* replicator contains multiple functional DNA elements: an essential A element and three important B elements—B1, B2, and B3. Functionally similar A, B1, and B2 elements are also present in the *ARS307* replicator. The B3 element binds a replication and transcription enhancer protein Abf1p, whereas the A element is required for binding the origin recognition complex (ORC). The function of the B1 and B2 elements remains to be defined. We have used a gel-based DNA binding assay to study the interaction between replicators and the putative initiator protein ORC. In addition to the established requirements for ATP and the A element for ORC–DNA interaction, the new data demonstrate that sequences in the B1 element are also important for ORC–DNA association. This conclusion is supported by DNase I footprint analyses and demonstrates that ORC binds to a bipartitite recognition element within the DNA. Furthermore, mutation of nucleotides in the B1 element suggests that this element has other functions in the initiation of DNA replication besides participating in the ORC–DNA interaction.**

Eukaryotic cells duplicate their genetic information for the next generation during the S phase of the cell division cycle. In general, two steps are required for a cell to duplicate its genome. First, a multiprotein complex is assembled at specific sites within the chromosome where DNA replication starts (the replication origins). Second, the enzymatic machinery that replicates the DNA is activated and replication forks move bidirectionally to synthesize the new DNA chains (1). The replication process is highly regulated at the initiation step. Studies in bacteria and with viruses that infect mammalian cells support the replicon model (2), which posits that DNA replication initiates at a specific site (origin) and this initiation is controlled by a cis-acting DNA sequence called the replicator (1–3). A replicator-specific DNA binding protein called the initiator facilitates the assembly of the enzymatic machinery at the replication origin to allow initiation of DNA replication to proceed.

The nature of replicators and the proteins that are involved in the initiation of DNA replication are the keys to understanding the mechanism and regulation of initiation of eukaryotic DNA replication. Autonomous replicating sequences (ARSs) were identified in the yeast *Saccharomyces cerevisiae* by their ability to promote the maintenance of extrachromosomal DNA (4–7). Subsequently, both replicators and origins of DNA replication in the chromosome were found to colocalize with a subset of these ARS elements (7). Fine structure analysis of the *ARS1* and *ARS307* replicators demonstrated that they contain multiple short functional elements (8–10). For example, *ARS1* contains an essential A element that harbors a perfect or close match to a degenerate 11-bp ARS consensus sequence (ACS). This conserved sequence is present in all ARS sequences (4, 7). *ARS1* also contains three distinct elements—B1, B2, and B3—adjacent to the A essential element (8). Mutations in any single B element reduce ARS activity, but combinations of mutations demonstrate that the B elements are collectively essential for replicator activity in both chromosomes and plasmids (11). The functions of the A and B3 elements have been defined. The B3 element is the binding site for the replication and transcription factor Abf1p (7, 8). The B3 element is found in some but not all of the ARSs and it functions in an orientation- and position-independent manner to enhance origin utilization (12, 13).

A multisubunit origin recognition complex (ORC) was identified as a candidate initiator protein using the DNase I footprint method and was shown to bind to *ARS1* and all other ARS sequences in an ATP-dependent manner (14, 15). ORC contains six subunits of 120, 72, 62, 56, 53, and 50 kDa. Genes encoding all six subunits have been cloned and found to be essential for cell viability (S. P. Bell, R. Kobayashi, and B.S., unpublished data; see refs. 15–18). Further genetic analyses suggest that ORC plays an important role in DNA replication (reviewed in ref. 7). The ORC DNase I protection pattern at ARS sequences extends over a 50-bp region that includes the A and B1 elements (9, 14). In addition, several DNase I-hypersensitive sites are induced in the DNA. These hypersensitive sites are found at 10-bp intervals, suggesting that the DNA may wrap around ORC (9, 14). Mutations in the A elements of *ARS1* and *ARS307* that reduce or abolish the replicator activity also reduce or abolish ORC protection. However, mutations in the B elements had no effect on the ORC footprint under previous test conditions (9, 14).

Despite a lack of obvious sequence similarity, the B1 and B2 elements are functionally conserved in *S. cerevisiae* replicators (9, 10), but how they contribute to initiation of DNA replication has yet to be determined. In this report, we demonstrate that the ATP-dependent ORC–ARS interaction involves, in addition to the A element, the B1 element. Furthermore, analysis of mutations in B1 suggests that this element has another function, possibly involved in attracting another DNA replication protein.

MATERIALS AND METHODS

Purification of ORC. Yeast whole cell extract was prepared from 400 g of BJ926 *S. cerevisiae*, and ORC was purified as described (15). The fractions containing ORC activity following glycerol gradient sedimentation were used in all experiments.

Purification of Abf1p. Whole cell extract was prepared from 400 g of BJ926 *S. cerevisiae*. Abf1p activity was determined by

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Abbreviations: ARS, autonomously replicating sequence; ACS, ARS consensus sequence; ORC, origin recognition complex.

a DNA binding assay using *ARS1* DNA as described (19). Buffers T and H have been described (14) and contained 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 μM pepstatin A, 0.1 mg of bacitracin per ml, and 2 mM dithiothreitol (DTT). The extract was applied to a 300-ml S-Sepharose column (5 × 12 cm; Pharmacia LKB) that was equilibrated with buffer H/0.1 M KCl (14). The column was washed with 500 ml of buffer H/0.1 M KCl and developed with a 1500-ml linear gradient of KCl (0.1–0.6 M) in buffer H. The active fractions eluting at ≈0.15 M KCl were pooled and dialyzed against buffer T/0.1 M KCl (14). Proteins were then loaded onto a 50-ml Q-Sepharose column (2.6 × 9 cm; Pharmacia LKB) that was equilibrated with buffer T/0.1 M KCl. The column was washed with buffer T/0.1 M KCl and developed with a 600-ml linear gradient of KCl (0.1–0.6 M) in buffer T. The active fractions eluting at ≈0.35 M KCl were pooled and dialyzed against buffer H/0.1 M NaCl. The protein sample was loaded onto a 4-ml sequence-specific DNA affinity column equilibrated in buffer H/0.1 M NaCl and prepared as described (14, 20) using the oligonucleotides 5′-CGAGGCAAGTGCCGTGCATAATGATGTGGGTGC-3′ and 5′-CCTCGGCACCCACATCATTATGCACGGCACTTG-3′. The column was washed successively with 0.1, 0.3, and 1 M NaCl in buffer H. This procedure yielded ≈200 μg of purified Abf1p.

Plasmids. The plasmids containing wild-type and mutant forms of *ARS1* have been described (8). Point mutations in the *ARS1* A and B1 elements were described (9, 14). The length of each *ARS1* DNA fragment is ≈190 bp. The wild-type and mutant plasmids of *ARS307* were described (9), and each *ARS307* DNA fragment is ≈200 bp.

DNA Binding Assays. The DNA probes were copied from plasmids by a standard 20 amplification cycle PCR using [γ-^{32}P]ATP-labeled universal sequencing primer and unlabeled reverse sequencing primer (New England Biolabs catalog nos. 1211 and 1201, respectively). After amplification, the DNA was isolated by PAGE. The competitor DNA for the DNA binding reactions, poly[d(AG)]-poly[d(CT)] (Pharmacia; average length, 1047 bp) was prepared in 10 mM Tris·HCl, pH 7.6/1 mM EDTA/0.1 M NaCl. A typical binding reaction mixture contained 25 mM Tris·HCl (pH 7.8), 5 mM $MgCl_2$, 0.8 mM ATP, 70 mM KCl, 2 mg of bovine serum albumin per ml, 5 mM DTT, 5% (vol/vol) glycerol, 0.1 mg of poly[d(AG)]-poly[d(CT)] per ml, 0.1 ng of labeled probe (200 cps), and the indicated amounts of ORC and/or Abf1p. The protein–DNA binding reaction mixture was incubated in a 25°C water bath for 10 min and then on ice for 10 min. The reaction sample was then loaded onto a 3.5% polyacrylamide gel containing 0.5× TBE (0.045 M Tris borate, pH 8.3/0.001 M EDTA) (polyacrylamide/bisacrylamide, 29:1), and 0.5× TBE was used as the gel running buffer. The gel was run for 8 hr at a constant 120 V at 4°C and dried and autoradiographed.

DNase I protection assays were performed under similar conditions, except that the amount of probe used for each reaction was 0.02 ng of 5′-end-labeled DNA probe (40 cps) and thus the amount of competitor DNA was adjusted to 0.03 mg/ml. The DNase I digestion reaction was for 1 min at room temperature and the DNA products were analyzed by denaturing PAGE as described (14).

RESULTS

A Gel Mobility Shift Assay for ORC Binding to DNA. The candidate initiator protein, ORC, was first identified by a DNase I protection assay by its ability to bind yeast replicators in an ATP-dependent manner (14). At both *ARS1* and *ARS307*, the ACS within the A element was essential for ORC to bind DNA and, under the conditions used previously, mutations in all three B elements had no effect on ORC footprint protection (9, 14). ORC also protects the B1 element from DNase I digestion, suggesting that ORC might interact specifically with *ARS1* in this region. Another frequently used assay for proteins binding to DNA is the gel mobility-shift assay; however, earlier studies failed to detect complex formation between ORC and *ARS1* DNA by this assay, even in the presence of ATP (14). This discrepancy between the gel mobility-shift assay and the DNase I protection assay caused us to reexamine the interaction between ORC and ARS sequences. Using modified assay conditions, the ORC–ARS association was detected by the gel-based assay and two factors were found to be important for its success. One factor is the use of high concentrations of the competitor DNA poly[d(AG)]-poly[d(CT)], which eliminated nonspecific ORC–DNA interaction and the other factor was the inclusion of bovine serum albumin, which increased the signal of ORC–ARS interaction (data not shown).

Using glycerol gradient-purified ORC and a wild-type *ARS1* probe, we observed that ORC reduced the mobility of a double-stranded *ARS1* probe during PAGE (Fig. 1*A*). Consistent with the complex being formed by ORC and *ARS1*, this activity required the presence of ATP (Fig. 1*A*, lanes 1–3). A linker mutation in the A element (nucleotides 858–865), which abolished ARS activity *in vivo*, also abolished the ORC–ARS association (lane 4). Furthermore, the activity causing the ATP-dependent mobility shift cofractionated over a number

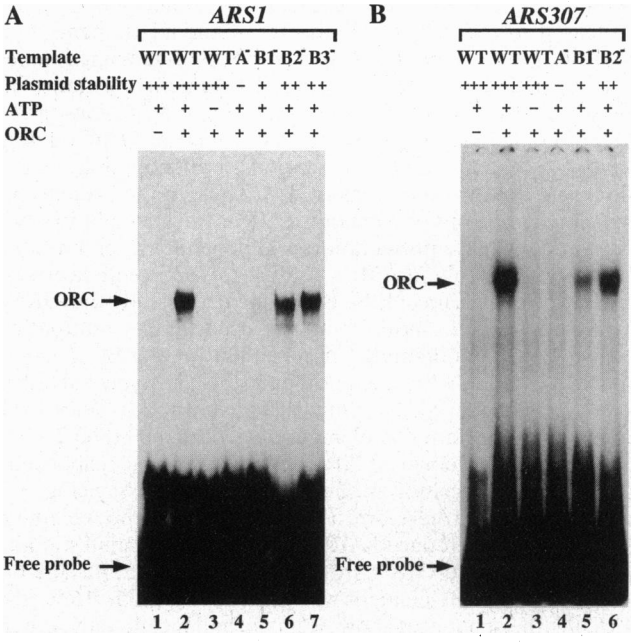

FIG. 1. ORC–ARS interaction observed by gel mobility-shift assay. (*A*) ORC binds to *ARS1*. Gel mobility-shift DNA binding reactions were done as described except ATP was omitted from the reaction mixture in lane 2. Lane 1, no protein; lanes 2–7, 2.5 ng of glycerol gradient-purified ORC. Templates are labeled on the top of each set of reactions and probes were made from the *ARS1* wild-type and mutant plasmids (8); nucleotides 858–865 (A⁻), nucleotides 835–842 (B1⁻), nucleotides 802–808 (B2⁻), and nucleotides 756 and 758 (B3⁻). Plasmid stability [measured as described (8)] of each construct is indicated above each set of reactions: +++, >30%; ++, 6–10%; +, 1–3%; −, defective for high-frequency transformation. (*B*) ORC binds to *ARS307*. Reaction conditions were as described above. Lane 1, no protein; lanes 2–6, 4.5 ng of glycerol gradient-purified ORC. ATP was omitted from the reaction mixture in lane 2. Template plasmids for each probe are labeled above each lane. Probes were made from the *ARS307* wild-type and mutant plasmids described (9); nucleotides 186–193 (A⁻), nucleotides 163–165 (B1⁻), and nucleotides 124–131 (B2⁻). Plasmid stability of each construct is indicated above each set of reactions: +++, >24%; ++, 10–15%; +, 1–3%; −, defective for high-frequency transformation. Positions of free probe and complex formed by ORC and ARS are indicated by arrows.

of column chromatographic steps with ORC activity measured by DNase I protection (data not shown).

While consistent with the previous footprint studies that linker mutations in elements B2 and B3 had no effect on ORC–*ARS1* complex formation, under the altered DNA binding conditions a linker mutation in the B1 element greatly reduced the ORC–ARS association (Fig. 1*A*, lanes 5–7). Similar results were obtained with mutants in another well-characterized chromosomal replicator, *ARS307*, which consists of three elements—A, B1, and B2 (Fig. 1*B*). ORC bound to *ARS307* and this binding required an intact A element and the presence of ATP (Fig. 1*B*, lanes 1–4). ORC–*ARS307* association was also sensitive to a linker scan mutation in the *ARS307* B1 element (Fig. 1*B*, lane 5) but was unaffected by a mutation in the *ARS307* B2 element (Fig. 1*B*, lane 6). Mutation of the *ARS307* B1 element reduced, but did not eliminate, the ORC–ARS interaction, and a linker scan mutation introduced in between the A and B1 elements had no effect on ORC DNA binding (data not shown).

DNase I Protection Analysis of ORC–DNA Interactions. Under conditions similar to those used for the gel retardation assay, we reexamined the DNase I protection analysis on wild-type and mutant *ARS1* plasmids with purified ORC (Fig. 2). Compared to the wild-type *ARS1* (lanes 1 and 2), a mutation in the A element that abolished ARS activity also abolished the ORC footprint pattern (lanes 3 and 4; see refs. 14 and 15). Mutation of the *ARS1* B1 element eliminated nuclease protection by ORC over the A and B1 elements, but, interestingly, the hypersensitive site in B1 still remained (lanes 5 and 6). This demonstrates that the B1 element contributes to the specificity of ORC binding to the DNA. Mutations in the B2 or B3 elements of *ARS1* had no effect on ORC binding (lanes 8–11), consistent with previous results obtained under different reaction conditions (9, 14). Thus, in the presence of high concentrations of competitor DNA, the gel mobility-shift assay and nuclease protection reveal dependence on both the A and B1 elements for ORC binding to ARS sequences.

Multiple Functions of the B1 Element. To study the ORC–ARS interaction in more detail, we used the gel retardation assay to analyze point mutations in either the A or B1 elements of *ARS1* (Fig. 3). In these experiments, the wild-type and linker scan mutations served as controls (Fig. 3 *A* and *B*, lanes 1–3). Point mutations in the A element nucleotide 860 (860 T → C or T → G) that abolished ARS activity *in vivo* also abolished ORC–*ARS1* association in the gel retardation assay (Fig. 3*A*, lanes 4 and 5). Furthermore, a point mutation in the A element (860 T → A) that reduced ARS activity in the plasmid stability assay also reduced ORC–*ARS1* association (Fig. 3*A*, lane 6).

Although the B1 element was identified in both *ARS1* and *ARS307*, the sequences show little resemblance to each other. We noticed previously two essential A nucleotides, 839A and 838A, in the *ARS1* B1 element located 18 and 19 bases from the end of the ACS (nucleotides 857–867), respectively. Similarly, two important A nucleotides in the *ARS307* B1 element are located 17 and 18 bases from the end of the ACS (nucleotides 182–192) (9). Interestingly, mutation of the 839A nucleotide (839 A → C and A → G) in the *ARS1* B1 element (Fig. 3*B*, lanes 4 and 5) and the 3-bp linker mutation (nucleotides 163–165) that alters the two A nucleotides in the *ARS307* B1 element (Fig. 1*B*, lane 5) all reduced ORC binding. Thus, despite a lack of overall sequence similarity, the B1 elements present in *ARS1* and *ARS307* seem to contain conserved A nucleotides that are required for efficient plasmid replication *in vivo* and for sequence-specific DNA binding by ORC *in vitro*.

Another point mutation in the *ARS1* B1 element (838 A → G), which resulted in even lower plasmid stability than point mutations at nucleotide 839 (9), surprisingly had no effect on ORC binding (Fig. 3*B*, lane 6). Nucleotide 838 is further away from the ACS than nucleotide 839, and it might be required for recognition of this region of the replicator by a protein other than ORC.

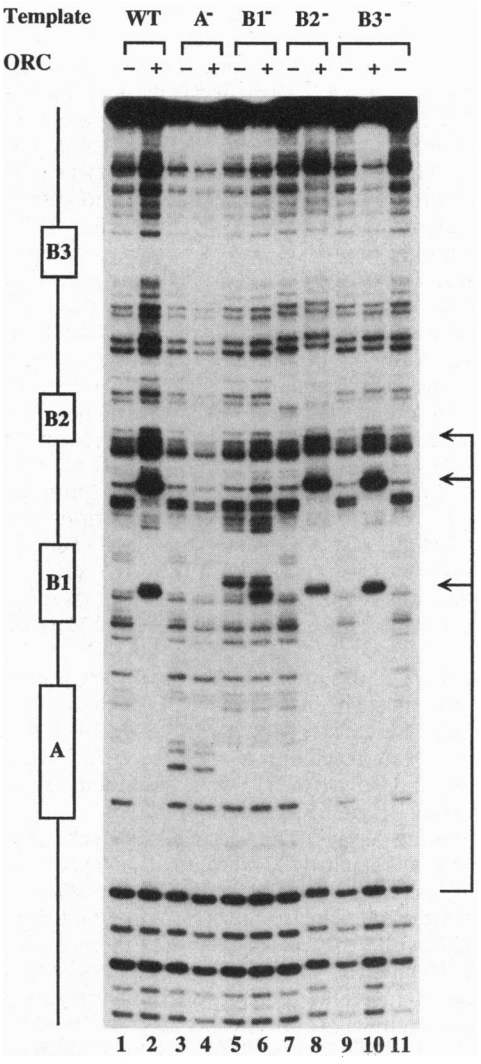

FIG. 2. ORC–*ARS1* interaction observed by a DNase I footprint assay. The T-rich strand of the A element was labeled by using a 5′-labeled sequencing primer in the PCRs to generate the probe. Templates for these PCRs were as described in Fig. 1 legend, and they are indicated above each lane. DNase I protection assay was performed as described. Lanes 1, 3, 5, 7, 9, and 11, no protein; lanes 2, 4, 6, 8, and 10, 50 ng of ORC. Positions of elements identified in *ARS1* are indicated on the left. Sites of enhanced DNase cleavage and protection are indicated by arrows and vertical line, respectively, on the right.

Abf1p and ORC Binding to *ARS1*. The transcription factor Abf1p binds to the B3 site at *ARS1* and stimulates replicator activity, but the mechanism of enhancement by Abf1p is unclear (reviewed in ref. 7). One possibility is that Abf1p facilitates ORC binding to the A element through protein–protein interactions. To test this, a series of titrations of ORC and Abf1p were performed to determine whether ORC and Abf1p bind to *ARS1* in a cooperative manner (Fig. 4). Addition of either ORC or Abf1p alone to the wild-type ARS1 probe caused a single gel-shifted band (Fig. 4, lanes 2–5 and lanes 6–9, respectively). In the presence of both ORC and Abf1p, we observed a slower migrating complex (Fig. 4, ORC + ABF1). As expected, the slowest migrating complex is sensitive to mutations in the A, B1, and B3 elements but not in the B2 element (Fig. 4, lanes 14–17). Under these conditions, however, the majority of complex formed was by either ORC alone or Abf1p alone. Only a small fraction of complex had both ORC and Abf1p coexisting on *ARS1* DNA. Thus, DNA

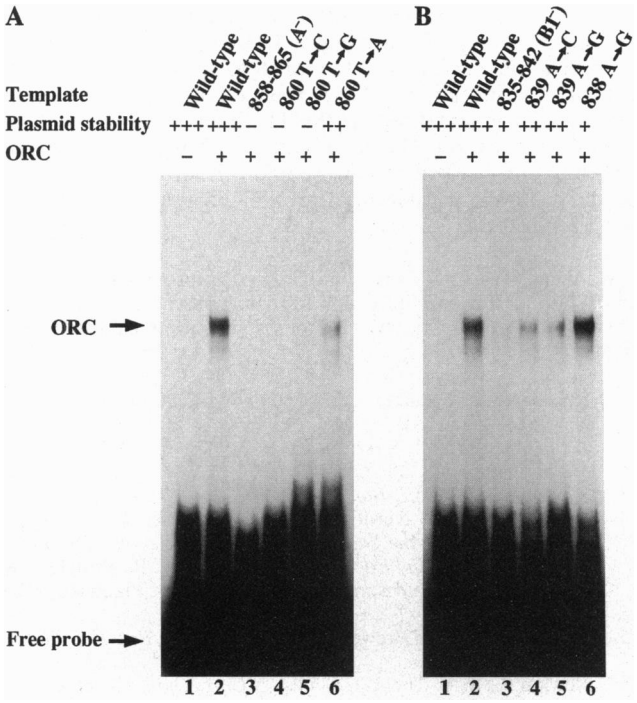

FIG. 3. Effect of point mutations in *ARS1* A and B1 elements. (*A*) Point mutations in *ARS1* ACS abolish or reduce ORC binding. Templates used were described (8, 14), and they are labeled above each lane. Lane 1, no protein; lanes 2–5, 2.5 ng of ORC. Plasmid stability of each construct is indicated above each set of reactions: +++, >30%; ++, 10%; −, defective for high-frequency transformation. (*B*) Some point mutations in the *ARS1* B1 element reduce ORC binding. Templates for generating the probe have been described (8, 9), and they are labeled above each lane. Lane 1, no protein; lanes 2–5, 2.5 ng of ORC. Plasmid stability of each construct is indicated above each set of reactions: +++, >30%; ++, 6–10%; +, 1–3%.

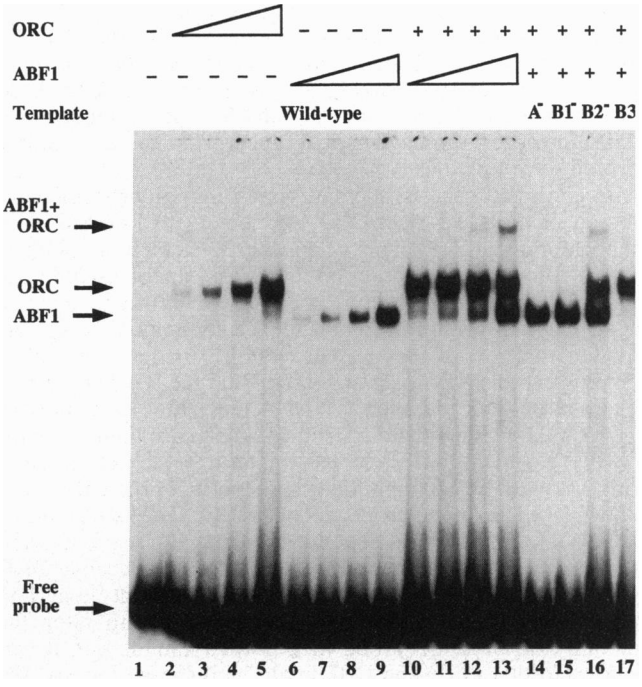

FIG. 4. ORC and Abf1p bind to *ARS1* independently. *ARS1* wild-type and mutant templates are as described in Fig. 1 legend and they are indicated above each lane. Lane 1, no protein; lanes 2–5, 6, 12, 24, and 48 ng of ORC, respectively; lanes 6–9, 0.3, 1.2, 2.4, and 9 ng of Abf1p, respectively; lanes 10–13, 48 ng of ORC and 0.3, 1.2, 2.4, and 9 ng of Abf1p, respectively; lanes 14–17, 48 ng of ORC and 9 ng of Abf1p.

recognition events by ORC and Abf1p are most likely independent of each other.

DISCUSSION

The yeast *S. cerevisiae* provides an attractive system to study the mechanism of initiation of eukaryotic DNA replication primarily because replicators have been isolated and genetically defined. From the limited number of replicators that have been studied in detail (8–10, 13), an emerging picture seems to be that the replicators are modular in nature, with an essential A element and two or three other elements that collectively are also essential. The A element that contains the ACS is found in every ARS sequence and is the principal ORC recognition sequence (14), but, from the data presented here, it is clear that the B1 element also plays a critical role in establishing a stable ORC–DNA interaction.

In the presence of low levels of competitor DNA, the ORC–DNA interaction can be observed in the absence of specific base pairs in the B1 region (9, 14). Under these conditions, however, it is necessary to have DNA adjacent to the A element since ORC will not bind to short duplex DNAs that contain only the A element (S. P. Bell, H.R., and B.S., unpublished data). In the presence of higher concentrations of competitor DNA, the B1 element in both *ARS1* and *ARS307* is necessary for efficient binding of ORC to the DNA and formation of a stable DNA–protein complex. In the absence of a functional B1 element, however, a DNase I-hypersensitive site is still observed in the B1 region, even though the ORC-dependent protection over the A element is absent. This suggests that in the absence of the B1 element, ORC still interacts with the DNA, but that it cannot form a stable DNA–protein complex. Furthermore, specific base pairs are required in the B1 element for ORC to bind tightly to the DNA when nonspecific competitor DNA is present. These same base pairs are also required for efficient ARS-dependent plasmid DNA replication *in vivo* (9).

The A residues in the B1 element that are required for ORC binding to DNA are conserved between *ARS1* and *ARS307* and they lie approximately the same distance from the ACS in each replicator (9). Apart from these nucleotides, there is little nucleotide sequence conservation between the two B1 elements. Despite this lack of apparent sequence similarity, the two B1 elements are functionally interchangeable (9).

It is not clear how the initiation of eukaryotic DNA replication is regulated. *In situ* genomic footprint analysis of the chromatin structure of *ARS1* suggested that ORC is bound to the replicator DNA through most or all of the cell cycle (21, 22). This further suggests that initiation of DNA replication is not controlled by the binding of ORC to the replicator at the G_1/S-phase boundary. Thus, what confines replication to S phase of the cell cycle remains obscure. Although some phosphorylation sites have been found in ORC, the importance of these phosphorylation sites has yet to be determined. It also remains to be determined whether the composition of the ORC protein complex changes throughout the cell cycle.

Also observed by genomic footprinting *in situ* is a prominent DNase I-hypersensitive site within the *ARS1* B1 element (22). The appearance of this hypersensitive site is dependent on cell cycle progression from G_1 into S phase. Thus, to ensure that DNA replication occurs only in S phase it is possible that the B1 element also serves as a docking site for a protein that in turn regulates initiation of DNA replication (3, 22). Our observation that there is a mutation in the *ARS1* B1 element (838 A → G) that does not affect ORC–DNA binding but affects DNA replication suggests that the B1 element may bind to another protein in a sequence-specific manner, perhaps in cooperation with ORC. There are several candidates for such

a protein in *S. cerevisiae*, including Cdc6p, Cdc7p, Dbf4p, Mcm2p, Mcm3p, and Cdc46p [see reviews by Rowley *et al.* (7) and Stillman (3)]. Genetic analysis has linked some of these proteins that function early in S phase to ORC (S. Loo, T. A. Fox, J. Rine, R. Kobayashi, B.S., and S. P. Bell, unpublished data); however, the biochemical functions of these candidate proteins are unknown.

In contrast to the B1 element, point mutations in the core region of the A+T-rich *ARS1* B2 element had no effect on replicator activity (9). This makes the B2 element an unlikely target for protein binding, although we cannot exclude the possibility that it binds to a protein with relaxed sequence specificity. It was reported that the B2 element is susceptible to denaturation in the presence of the single-strand DNA binding protein RPA (23), suggesting that the B2 element may play a structural role, such as a DNA unwinding element (24).

An ACS binding protein fraction called core binding factor (CBF) was identified previously by an agarose gel mobility-shift assay (25). Although the exact identity of the CBF is not known, it shares some properties with ORC. The DNA binding ability of both proteins is dependent on the ACS sequences and the presence of ATP in the reaction mixture. Because ORC was originally identified by a DNase I footprint assay and under those conditions previously had failed to alter the mobility of an *ARS1* probe in a polyacrylamide gel, it was suggested that ORC and CBF might be different factors. We report here that ORC can form a complex in a gel with replicator DNAs, suggesting that ORC might be the active factor in the CBF fraction. The dependence of the CBF fraction on other extract fractions for binding to DNA (25) may reflect different DNA binding conditions or the use of partially fractionated extracts versus purified proteins.

The requirement for both the A and B1 elements, but not sequences in between these two, for stable ORC interaction with replicators demonstrated that ORC has a bipartite DNA recognition site. In this respect, the ORC DNA recognition elements are similar to the highly divergent, bipartite RNA polymerase recognition elements at the −35 and −10 region *Escherichia coli* promoters (26). ORC interaction with the replicator may also be similar to the interaction between the simian virus 40 (SV40) T antigen and the SV40 origin of DNA replication. T antigen, a known initiator protein, binds to its principal sequence-specific recognition element (site II or PEN) and independently to an inverted repeat sequence (IR) in the SV40 replicator, causing structural distortion of the IR sequence in an ATP-dependent manner (27). The *ARS1* A and B1 elements show functional similarity to the SV40 PEN (site II) and IR elements, respectively. The A and B1 elements in both *ARS1* and *ARS307* have at least 9 nucleotides of DNA or one turn of the double helix in between them. Since purified ORC consists of six protein subunits, it is possible that two different protein subunits interact with the two recognition sites. Other ORC subunits may function by interacting with proteins required for the initiation of DNA replication, much like the TATA box binding protein and its associated transcription activating factors facilitate sequence-specific initiation of transcription at eukaryotic RNA polymerase II promoters (28).

The binding of purified ORC and Abf1p to the DNA does not appear to be cooperative. Furthermore, since other transcriptional activators can replace Abf1p to enhance DNA replication at ARS1 (8), ORC and Abf1p probably do not interact directly. Abf1p binding to DNA most probably locates an activation surface on the protein at the replicator to enhance the initiation of DNA replication, perhaps by maintaining a chromatin structure that facilitates efficient initiation of DNA replication. The gel mobility-shift assay for ORC and Abf1p binding described here should further facilitate studies on the cell cycle regulation of protein–protein interactions at yeast replicators.

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