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At least three distinct proteins are necessary for the reconstitution of a specific multiprotein complex at a eukaryotic chromosomal origin of replication

(*Saccharomyces cerevisiae*/eukaryotic nuclear origin/initiation complex)

HEATHER G. ESTES, BRETT S. ROBINSON, AND SHLOMO EISENBERG

Department of Microbiology, School of Medicine, The University of Connecticut Health Center, Farmington, CT 06030

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ABSTRACT **We have reconstituted *in vitro* a multistage assembly of a protein complex that specifically recognizes a yeast genomic origin of replication, the autonomously replicating sequence ARS*121*. The first step in the assembly was the interaction of the known origin-binding factor OBF1 and another factor, OBF2, with the ARS*121* origin of replication to form the OBF1–OBF2–origin complex. This complex was the substrate for the ATP-dependent binding of a third DNA-binding activity, the core binding factor, CBF. Binding of CBF to the origin, identified by the retarded mobility of the origin DNA fragment in agarose gels, required, in addition to ATP and the OBF1–OBF2–origin complex, a functional essential core nucleotide sequence. ARS*121* DNA containing mutations in the core, which inactivate the origin *in vivo*, did not sustain stable CBF binding, whereas ARS*121* DNA mutated outside the boundaries of the essential core, which has normal origin function, bound CBF as wild type. This tight, direct correlation between the ability of the origin to bind CBF and its function as an origin of replication *in vivo* strongly suggest that the multiprotein complex reconstituted *in vitro* has a key role in the initiation of DNA replication.**

DNA replication in eukaryotic cells is tightly regulated; replication of nuclear chromosomes initiates at the start of the S phase of the cell cycle and proceeds by multiple initiations along a chromosome, generating multiple replicons. *Saccharomyces cerevisiae* provides a convenient model system for studying DNA replication in eukaryotic cells since it is amenable to biochemical and genetic manipulations. Additionally, *S. cerevisiae* nuclear origins of replication have been isolated, enabling a molecular dissection of their structural organization. These origins, called autonomously replicating sequences (ARSs), were first isolated as DNA fragments that conferred on plasmids bearing them the ability to replicate autonomously in yeast (1). The ARSs incorporated in these plasmids function as origins of replication *in vivo* (2, 3). Some of these ARSs also function as origins in their natural chromosomal location (4–6).

Our laboratory has recently carried out a systematic molecular dissection by site-directed *in vitro* mutagenesis of a nuclear origin of replication, ARS*121*. This analysis has shown that the ARS*121* origin has a modular arrangement of three elements, one essential and two auxiliary, that together elicit maximal levels of origin function. The three elements are distinct and can be connected by random DNA without apparent loss of origin activity (6). Modular arrangement of DNA elements may be a general feature for yeast origins of replication since such sequence organization has been demonstrated for ARS*1* as well (7, 8).

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The mechanism and regulation of initiation of DNA replication at a yeast origin are not known. However, one of the auxiliary elements present in both ARS*121* and ARS*1* is the recognition site for binding the phosphoprotein OBF1 (9–11), also known as ABF1 and BAF1 (12–19). Site-directed *in vitro* mutagenesis has shown that in ARS*121* this element functions as an enhancer of DNA replication in a distance- and orientation-independent manner (20, 21). This observation has now been confirmed for ARS*1* as well (8), suggesting that the OBF1 enhancer may have a role in activating a wide spectrum of replicons in yeast. The other auxiliary domain identified in ARS*121*, ATR, is relatively A+T-rich and appears to interact synergistically with the OBF1 DNA binding site to enhance origin function (6). A similar domain is found in other ARSs, and it was suggested that this site is unwound during the initiation of DNA replication, analogously to the 13-mer repeats in *oriC* of *Escherichia coli* (22, 23).

The essential core sequence of ARS*121* comprises ≈35 base pairs (bp) and contains an 11-bp sequence resembling the ARS consensus, (A/T)TTTAT(A/G)TTT(A/T) (6). This region has a bipartite structure, one part sensitive to deletions and the other sensitive to both deletions and linker substitution mutagenesis that inactivate the origin. Therefore, the role of this element is central to the function of ARS*121* as an origin of replication.

The multitude of elements important for origin function has suggested that a multiprotein complex may comprise the initiation apparatus for DNA replication. Here we report a specific, *in vitro* reconstitution of a stable multiprotein complex at the ARS*121* nuclear origin, identified by the retarded mobility of the origin fragment in agarose gels. This complex may have a key role in the initiation of DNA replication in yeast.

MATERIALS AND METHODS

Growth Conditions. The *S. cerevisiae* strain used was TD5 (*MAT* **a**, *his4-712, ura3-52*). Cells were grown to 3–4 × 10^7 per ml in a yeast peptone/dextrose (YPD) medium (10) and then supplemented with hydroxyurea (6 mg/ml) to arrest them in S phase. After an additional 3 hr of incubation at 30°C the cells were harvested and treated as before (10).

Plasmids. The construction of the plasmids p19A121(ch+) and p19AB121 and the fs1, s4, and fs2 mutations in ARS*121* was described before (6, 21).

DNA Binding Assay. DNA containing the ARS*121* origin was excised from the plasmids by *Hin*dIII and *Eco*RI restriction enzymes and the termini of the excised DNA were labeled with [α-^{32}P]dATP by reverse transcriptase (9). DNA binding reaction mixtures containing 25 mM Tris acetate (pH 7.9), 5 mM $MgCl_2$, 0.8 mM ATP, 2.5–5 fmol of labeled ARS*121* origin DNA fragment, bovine serum albumin at 0.1

Abbreviation: ARS, autonomously replicating sequence.

mg/ml, 5 mM dithiothreitol, and an aliquot of a protein fraction were incubated for 10 min at room temperature. pUC19 DNA (4 μg) was then added to serve as a carrier for proteins that interact nonspecifically with DNA. After incubation on ice for an additional 30 min, the reaction mixtures were loaded on agarose gels for electrophoresis (9). DNA bands were visualized by autoradiography of dried gels (9).

RESULTS

Identification of ARS*121* Origin-Binding Activities in *S. cerevisiae* Extracts. Dissection of the ARS*121* origin of replication revealed a DNA element, OBF1 DNA binding site, which cooperated with a second auxiliary domain, ATR, in stimulating the essential core element in origin activity (6). These results suggested that an initiation protein may form a complex with the essential core and that this complex may be stabilized by other proteins such as OBF1, thus explaining the stimulatory role of OBF1 in DNA replication. To identify such a complex, we employed a direct DNA mobility-shift assay using as substrate the ARS*121*(ch^+) origin (see Fig. 6) containing the essential core sequence, ATR, and a fused, single synthetic OBF1 binding site (21).

Initially, we observed the formation of a specific protein–ARS*121*(ch^+) complex in extracts (fraction III) prepared from *S. cerevisiae* cells (Fig. 1, lane 12). Formation of the most retarded band, complex III, was dependent on the presence of ATP in the reaction. In the absence of ATP only the faster moving complex (complex II) was observed. To resolve these complexes, the yeast extracts were fractionated by DNA affinity using a DNA-cellulose resin originally prepared for the purification of the OBF1 protein (25). Three protein fractions were generated: a flowthrough and 0.4 M and 0.8 M KCl eluates (Fig. 1). Each one of these fractions was tested for the presence of DNA-binding proteins that interact with the ARS*121*(ch^+) origin. None of these fractions, separately, was able to generate the complexes observed in the crude extracts. As expected, OBF1 was identified in the 0.8 M KCl eluate. When incubated with the ARS*121*(ch^+) origin, this fraction formed a complex (complex I) indistinguishable from that formed with purified OBF1 (Fig. 1, lanes 2–5). Formation of this complex was inhibited by a competitor DNA containing the OBF1 binding site (data not shown), confirming that the binding activity in the 0.8 M KCl eluate was OBF1.

The 0.4 M KCl eluate contained a DNA-binding activity that formed two types of complexes with the ARS*121*(ch^+) origin. One of these, revealed when low levels of this protein fraction were added to the reaction mixture, migrated faster than complex I (Fig. 1, lane 6). Upon further addition of the 0.4 M KCl eluate, the labeled origin fragment was chased to a complex migrating slower than complex I (lanes 7 and 8). Thus, the 0.4 M KCl eluate may contain a unique DNA-binding activity that binds to ARS*121* at multiple sites. Alternatively, the two complexes formed may be a result of binding two distinct protein factors. Unlike the 0.4 and 0.8 M KCl eluates, the flowthrough protein fraction did not appear to contain a binding activity capable of forming a single well-defined stable protein–ARS*121*(ch^+) complex (lanes 9–11). Most of the origin DNA that was incubated with the flowthrough fraction appeared as a smear on the gel rather than a distinct tight band.

Reconstitution of a Multiprotein Complex at the ARS*121* Origin of Replication. Since the full origin DNA-binding activity was not recovered after fractionating the extracts, we decided to reconstitute this protein–ARS*121*(ch^+) complex from the protein fractions described in Fig. 1. As in Fig. 1, an OBF1–ARS*121*(ch^+) complex (complex I) was formed by incubating an aliquot of the 0.8 M KCl eluate with ARS*121*(ch^+) DNA (Fig. 2, lane 2). When the 0.4 M KCl eluate was added at increasing amounts to a reaction mixture containing the origin fragment and OBF1 (0.8 M KCl), a new protein–ARS*121*(ch+) complex (complex II) was observed on the gel (lanes 3–5). The activity that converts complex I to complex II was named OBF2.

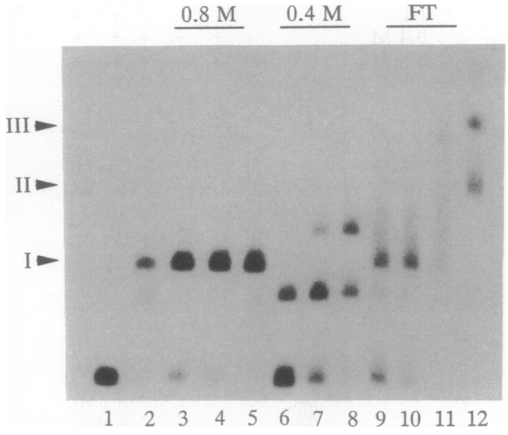

FIG. 1. ARS*121* origin-binding activities in fractionated extract of *S. cerevisiae* TD5. Cells were resuspended and disrupted by glass beads (24) and the cell extract was fractionated by DNA affinity essentially as before (10), with modifications. Fraction II (ammonium sulfate pellet) was resuspended in buffer A [50 mM Tris acetate, pH 7.9/10% (vol/vol) glycerol/1 mM EDTA/1 mM NaF/1 mM dithiothreitol/0.1 mM phenylmethanesulfonyl fluoride with leupeptin (0.3 μg/ml), pepstatin A (1.5 μg/ml), and aprotinin (2 μg/ml)] and dialyzed against buffer A/0.25 M KCl. The dialyzed protein fraction was then loaded onto a DEAE-cellulose column at 25 mg of protein per ml of bed volume. The flowthrough fraction (fraction III) was collected and the proteins were again precipitated by the addition of 1.5 volumes of a saturated ammonium sulfate solution. The ammonium sulfate precipitate was resuspended in buffer A at 25–50 mg/ml and dialyzed against buffer A/0.2 M KCl. The dialyzed protein solution was then applied to an OBF1-specific DNA-cellulose affinity column (25) at 25–30 mg of protein per ml of bed resin, and the column was washed with 2 bed volumes of buffer A/0.2 M KCl. This wash was then pooled with the flowthrough fraction and the proteins were precipitated by the addition of 1.5 volumes of a saturated ammonium sulfate solution. The ammonium sulfate pellet, referred to as the flowthrough fraction, was then resuspended in buffer A/0.2 M KCl and dialyzed against the same buffer. The 0.4 M and 0.8 M KCl washes were obtained by washing the DNA affinity resin with 5 bed volumes of buffer A/0.4 M KCl and buffer A/0.8 M KCl, respectively. Other details of this procedure will be published elsewhere. Each of the fractions (flowthrough, 0.4 M and 0.8 M KCl eluates) was tested for activities that bind specifically to the ARS*121*(ch^+) origin in the mobility-shift assay. Lane 1, no protein added; lane 2, purified OBF1 (0.8 ng; ref. 10); lanes 3–5, the 0.8 M KCl fraction (15, 30, and 45 ng); lanes 6–8, the 0.4 M KCl eluate (0.2, 0.4, and 0.8 μg); lanes 9–11, the flowthrough fraction (FT; 14, 28, and 56 μg); lane 12, fraction III (47 μg). Protein concentrations were determined by the Lowry assay. Arrows I, II, and III denote the OBF1–origin complex and the ATP-independent and ATP-dependent complexes formed in crude extracts (fraction III).

Since the OBF1–OBF2–ARS*121*(ch^+) complex did not comigrate with complex III shown in Fig. 1, we presumed that the flowthrough fraction might contain a DNA-binding protein that required for its stable interaction with the origin the prior binding of OBF1 and OBF2. Therefore, the flowthrough fraction was added at increasing amounts to a preformed OBF1–OBF2–ARS*121*(ch$^+$) complex, yielding a new distinct structure, complex III (Fig. 2, lanes 6 and 7). The DNA-binding activity that converts complex II to complex III was named CBF (core binding factor).

CBF was revealed only when complex II [OBF1–OBF2–ARS*121*(ch^+)] was used as substrate in the binding reaction. In the absence of either OBF1 or OBF2, CBF did not interact stably enough with the DNA to be identified by mobility-shift

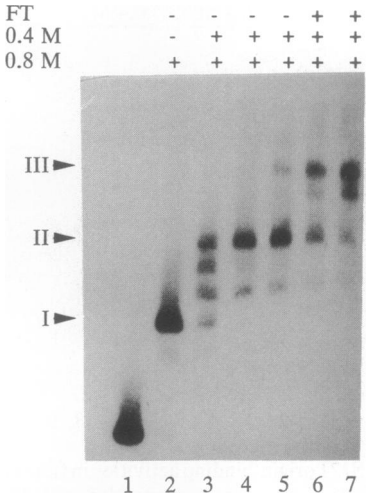

FIG. 2. Reconstitution of a multiprotein complex at the ARS*121*(*ch*$^+$) origin of replication, assayed by mobility shift. Lane 1, no protein added; lane 2, the 0.8 M KCl fraction (45 ng); lanes 3–5, the 0.8 M KCl fraction (45 ng) and the 0.4 M KCl fraction (0.2, 0.4, and 0.8 μg, respectively); lanes 6 and 7, the 0.8 M KCl (45 ng) and 0.4 M KCl (0.8 μg) fractions of the flowthrough fraction (FT; 28 and 56 μg, respectively). All seven reactions were preincubated for 10 min at room temperature in the absence of the flowthrough fraction. Then, where appropriate (reactions 6 and 7) the flowthrough fraction was added and the incubation continued for another 10 min. Arrows I, II, and III indicate the OBF1–ARS*121*(*ch*$^+$), OBF1–OBF2–ARS*121*(*ch*$^+$), and OBF1–OBF2–CBF–ARS*121*(*ch*$^+$) complexes, respectively. Full reconstitution of complex III (lanes 6 and 7) was also obtained when the 0.8 M KCl eluate fraction was replaced by highly purified OBF1 protein in the binding reaction (data not shown).

assay. When OBF1 was omitted from the reaction (Fig. 3, lane 4), most of the DNA migrated as the OBF2–ARS*121*(*ch*$^+$) complex shown in Fig. 1, lane 5, while upon omission of OBF2 the bulk of the DNA migrated as the OBF1–ARS*121*(*ch*$^+$) complex (Fig. 3, lane 3). In both cases some of the radioactively labeled DNA migrated as a diffused band emanating from the more intense bands, but no clear complex II or complex III was observed. We conclude that CBF requires the OBF1 and OBF2 proteins for its stable interaction with the origin DNA.

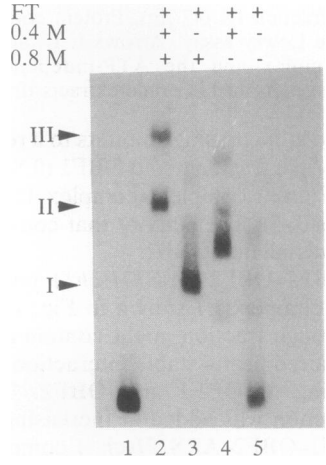

FIG. 3. CBF binding to the origin depends on OBF1 and OBF2. Lane 1, no protein added; lane 2, a complete reaction including all three protein fractions (as in Fig. 2, lane 6); lane 3, a complete reaction from which OBF2 (0.4 M KCl eluate) was omitted; lane 4, a complete reaction from which OBF1 (0.8 M KCl eluate) was omitted; lane 5, a reaction containing CBF (flowthrough fraction, FT) only.

CBF Binding to the Essential Core Requires ATP. An ATP requirement for a functional interaction of the DnaA protein with *oriC* of *E. coli* has been previously demonstrated (26). Anticipating such a possibility for the binding of a hypothetical yeast initiator protein to the ARS, we have included ATP in all the origin-binding reactions. To ascertain the requirement of ATP for the assembly of the multiprotein complex, the experiment described in Fig. 4 was performed. When ATP was omitted, complex II but not complex III was detected in a reaction containing all three protein fractions (Fig. 4, lane 3). Complex II was also formed in the absence of ATP by incubating the origin with just OBF1 and OBF2 (lane 2). As already shown in Fig. 3, when the three protein fractions were incubated with the origin DNA in the presence of ATP the assembly of complex III was observed (Fig. 4, lane 4). Addition of GTP substituted inefficiently for the requirement of ATP, since only a minor fraction of total DNA was identified as complex III in this reaction (lane 5). The ATP analog adenosine 5′-[γ-thio]triphosphate appeared to sustain the formation of complex III (lane 6), while a nonhydrolyzable ATP analog, adenosine 5′-[β,γ]-methylenetriphosphate, did not support the formation of this complex (lane 7). We do not know whether it is ATP hydrolysis or ATP binding that is necessary for the specific recognition of the origin by CBF. However, since the nonhydrolyzable ATP analog did not support the formation of complex III, it is possible that ATP hydrolysis may be required.

Binding of CBF Requires a Functional Essential Core Sequence at the ARS*121* Origin of Replication. The ARS*121* essential element is ≈35 bp long (including an 11-bp sequence resembling the canonical ARS consensus sequence) and is bipartite, with one part (≈12 bp) sensitive to deletions and the other (≈23 bp) sensitive to both deletions and linker substitution mutagenesis (6). To localize the binding of CBF, we used the cellular ARS*121* DNA (480 bp) (20) and ARS*121* DNA bearing mutations in the essential core nucleotide sequence as substrates in the binding reaction (Fig. 5*A*). One of these mutants contained a linker substitution mutation, fs1, at a location 5′ to the T-rich strand of the 11-bp ARS consensus-like sequence, outside the boundaries of the essential core domain. Another mutation, s4 (within the boundaries of the essential core), was a substitution mutation of the consensus-like sequence TGTTTTGTTTA, and fs2 was a linker substitution mutation located 3′ to this sequence.

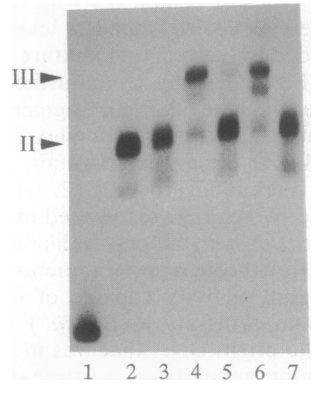

FIG. 4. Binding of CBF to the origin is ATP-dependent. Lane 1, no protein added; lane 2, OBF1 (0.8 KCl M eluate; 45 ng) and OBF2 (0.4 M KCl eluate; 0.8 μg) without ATP; lane 3, as in lane 2 except that 56 μg of CBF (flowthrough fraction) was also added; lane 4, as in lane 3 except that ATP was present; lane 5, as in lane 4 except that GTP was present in lieu of ATP; lane 6, as in lane 4 except that the adenosine 5′-[γ-thio]triphosphate was present in lieu of ATP; lane 7, as in lane 4 except that adenosine 5′-[β,γ-methylene]triphosphate was present in lieu of ATP.

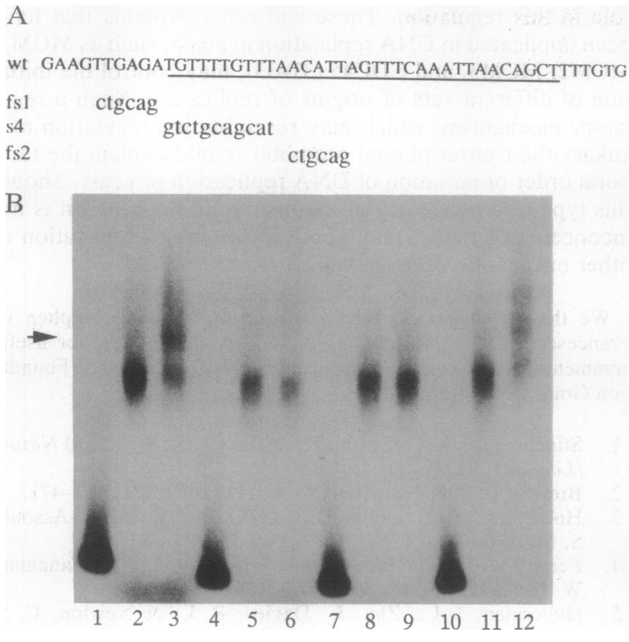

FIG. 5. CBF recognizes the essential core sequence. The specificity of CBF–ARS*121* interaction was tested by using the wild-type ARS*121* DNA and the DNA of three linker substitution mutations in and near the essential core nucleotide sequence. (*A*) Nucleotide sequence of a portion of the 480-bp ARS*121* DNA; the essential wild-type (wt) core nucleotide sequence is underlined. Location of the fs1, s4, and fs2 mutations (6, 21) is shown. (*B*) Results of DNA-binding reactions using the wild-type ARS*121* DNA (lanes 1–3), s4 DNA (lanes 4–6), fs2 DNA (lanes 7–9), and fs1 DNA (lanes 10–12). Lanes 1, 4, 7, and 10; no protein added; lanes 2, 5, 8, and 11, without ATP; lanes 3, 6, 9, and 12 with ATP. The reactions containing proteins included OBF1, OBF2, and CBF as in Fig. 2, lane 6. Arrow indicates the ATP-dependent complex.

All the DNA substrates, including the wild type and the mutant origins s4, fs2, and fs1, formed complex II in the absence of ATP when incubated with OBF1, OBF2, and CBF (Fig. 5*B*, lanes 2, 5, 8, and 11). This complex was also observed when the DNA was incubated with OBF1 and OBF2 alone (data not shown). The ATP-dependent CBF binding, however, was observed only with the wild-type and the fs1 DNA. In these cases, upon the addition of ATP a new retarded DNA band could be seen (lanes 3 and 12). The ATP-dependent DNA band was somewhat diffuse. This may be explained by the fact that ARS*121* contains two OBF1 DNA-binding sites of differing affinities (10). Binding of OBF1 to the low-affinity binding site may have produced an unstable complex resulting in its diffuse migration in the gel. In contrast to the wild-type ARS*121* and the fs1 mutant, the s4 and fs2 mutant origins did not sustain the formation of the ATP-dependent complex. Addition of ATP did not generate the additional complexes that were observed with the wild type ARS*121* and fs1 origin DNA (lanes 6 and 9).

These analyses indicated that CBF required for recognition a functional essential core nucleotide sequence. This recognition sequence should at least encompass the sites of the s4 and fs2 mutations. Previous work showed that the s4 and fs2 mutations inactivated the ARS*121* origin *in vivo* while the fs1 mutation had no effect on origin activity (6, 21). This observation correlates directly with the ability of CBF to interact with these origins. Such tight correlation strongly suggests that the reconstituted complex at this origin may have a key role in the initiation of DNA replication.

DISCUSSION

S. cerevisiae is an attractive model system for DNA replication studies in eukaryotic cells because nuclear origins of replication have been isolated and have been well defined. One of these, ARS*121* on chromosome X, was delineated in our laboratory (6, 20, 21). Here we have used a direct DNA binding assay (DNA mobility retardation in agarose gels) to identify proteins that interact specifically with the different elements of the ARS*121* origin. We have reconstituted a multistage assembly of a specific multiprotein–ARS*121* complex, using three protein fractions obtained by fractionating yeast extracts through an OBF1-specific DNA-cellulose affinity resin.

The precise mechanism of assembly of the multiprotein complex at the origin is not known. However, based on our results, certain steps in the assembly may be predicted (Fig. 6). The binding of the OBF1 and OBF2 proteins has to precede the binding of CBF. Although the scheme in Fig. 6 suggests that the first step in the assembly is the binding of OBF1 to the enhancer of the origin of replication, the possibility that OBF2 binds the origin first cannot be excluded. Since mutations in the essential core, which cover 23 bp of the core nucleotide sequence, did not inhibit OBF2 binding (Fig. 5), we suggest that OBF2 may interact with nucleotide sequences at the boundary of the core and ATR or the ATR domain itself. This is consistent with the observation that the enhancer site and the ATR cooperate synergistically

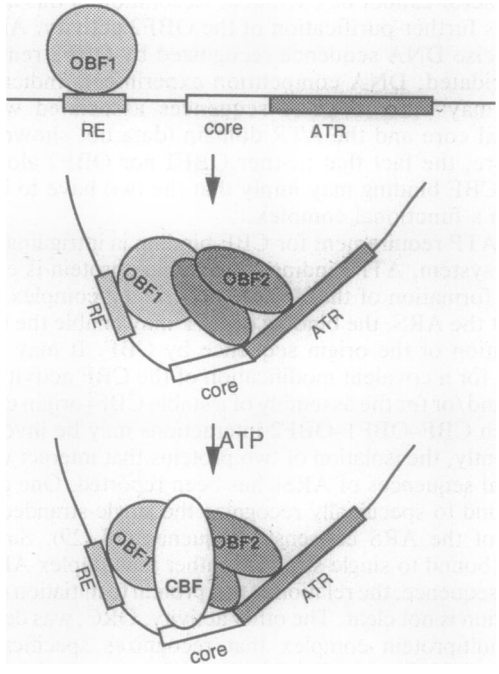

FIG. 6. Tentative scheme for the assembly of a hypothetical multiprotein initiation complex at the ARS*121*(*ch*$^+$) origin of replication (21). This origin includes the essential core element (≈35 bp) and the ATR domain (≈72 bp). The thin line connecting the synthetic OBF1 DNA binding site (RE) and the core represents 18 bp of a pUC19 polylinker sequence and ≈110 bp of ARS*121* DNA that separates the *Rsa* I restriction site from the essential core element (21). The scheme shows three distinct steps in the assembly of the complex. Initially OBF1 interacts with its recognition sequence, RE (replication enhancer). This is followed by the binding of OBF2. It is equally probable that OBF2 binds first, followed by OBF1. The scheme suggests that the binding of OBF2 is to the boundary of the core and ATR and the ATR element itself. This is supported by DNA competition and DNase I protection analyses (data not shown). The scheme also predicts the binding of at least two identical OBF2 complexes to the origin. However, as discussed before, OBF2 may consist of more than a single factor. The final step in this assembly is the ATP-dependent binding of CBF, an activity that forms a stable complex with a functional essential core domain. This stable interaction can occur only in the presence of OBF1 and OBF2 complexed to the DNA.

in stimulating the activity of the ARS*121* essential core (6). The final step in this complex assembly is the ATP-dependent binding of CBF to the ARS essential core domain. Since CBF does not bind stably to the naked origin fragment, we suggest that the interaction of OBF1 and OBF2 generates a conformational change at the origin, perhaps by bending the DNA, facilitating a tight binding of CBF to the essential core sequence. Alternatively, CBF may interact directly with the OBF1–OBF2 complex bound to the DNA via protein–protein interactions, which in turn may confer on CBF the ability to bind tightly to the essential core DNA. Further resolution and reconstitution of this multiprotein complex assembly should provide a better understanding of these mechanistic questions.

A role for OBF1 in the initiation of DNA replication has been inferred previously from the mutagenesis analysis of the OBF1 DNA binding site (6, 20, 21). The efficiency of the ARS*121* origin is drastically reduced in an *obf1/abf-1* temperature-sensitive mutant strain grown at semipermissive temperatures (27). The scheme described above suggests a more precise and direct role of OBF1 function in initiation of DNA replication; namely, OBF1 cooperates with OBF2 to promote the assembly of a stable multiprotein initiation complex at the origin.

OBF2 has been defined as the origin-binding activity that promotes the conversion of complex I to complex II (Fig. 2). At present the possibility that OBF2 consists of more than a single factor cannot be excluded. Resolution of this question requires further purification of the OBF2 activity. Although the precise DNA sequence recognized by OBF2 remains to be elucidated, DNA competition experiments indicate that OBF2 may bind to DNA sequences associated with the essential core and the ATR domain (data not shown). Furthermore, the fact that neither OBF1 nor OBF2 alone promotes CBF binding may imply that the two have to interact to form a functional complex.

The ATP requirement for CBF binding is intriguing. In the *E. coli* system, ATP binding to the DnaA protein is essential for the formation of the "open" prepriming complex at *oriC* (26). At the ARS, the binding of ATP may enable the specific recognition of the origin sequence by CBF. It may also be needed for a covalent modification of the CBF activity at the origin and/or for the assembly of a stable CBF–origin complex in which CBF–OBF1–OBF2 interactions may be involved.

Recently, the isolation of two proteins that interact with the essential sequences of ARSs has been reported. One of these was found to specifically recognize the single-stranded T-rich strand of the ARS consensus sequence (28, 29). Since this protein bound to single-stranded rather than duplex ARS consensus sequence, the relation of this protein to initiation of DNA replication is not clear. The other activity, ORC, was described as a multiprotein complex that recognizes specifically the ARS*1*, ARS*307*, and ARS*121* essential sequences in an ATP-dependent manner (30). This activity, which was identified by DNA footprinting, could not be detected by the DNA mobility-shift assay when subjected to gel electrophoresis (30). The CBF protein, however, was identified on the basis of its ability to form a stable complex with the origin that can be identified by the retarded mobility of the origin fragment in agarose gels. From this assay, we concluded that the substrate for CBF binding is not naked DNA but rather the OBF1–OBF2–ARS*121* origin complex. Since both activities recognize the essential sequence of a yeast origin of replication in an ATP-dependent manner, it is not inconceivable that the two are similar. However, since assays used to identify ORC and CBF were different, the complete identity of these activities is not certain.

The reconstitution of the multiprotein complex at the ARS*121* origin of replication has raised an intriguing possibility; namely, that initiation of DNA replication in yeast may be regulated at the level of the assembly of a stable multiprotein initiation complex at a specific origin of replication. Proteins such as OBF1 and OBF2 may have a direct and key role in this regulation. These and other proteins that have been implicated in DNA replication in yeast, such as MCM1, MCM2, MCM3, and CDC45 (31–33), may control the initiation of different sets of origins of replication. Such a regulatory mechanism, which may resemble the regulation of a eukaryotic transcriptional promoter, could explain the temporal order of initiation of DNA replication in yeast. Should this type of a regulatory mechanism hold for yeast, it is not inconceivable that it may apply to origins of replication of other eukaryotic cells as well.

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