Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11569–11574, October 1996
Biochemistry

The Ku-like protein from *Saccharomyces cerevisiae* is required *in vitro* for the assembly of a stable multiprotein complex at a eukaryotic origin of replication

(Ku homolog/initiation complex)

NADER SHAKIBAI, VIJAY KUMAR, AND SHLOMO EISENBERG*

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

Communicated by Mary J. Osborn, University of Connecticut Health Center, Farmington, CT, August 1, 1996 (received for review June 14, 1996)

ABSTRACT **We have previously shown that three distinct DNA-binding activities, in crude form, are necessary for the ATP-dependent assembly of a specific and stable multiprotein complex at a yeast origin of replication. Here we show the purification of one of these DNA binding activities, referred to as origin binding factor 2 (OBF2). The purified protein is a heterodimer composed of two polypeptides with molecular mass values of 65 and 80 kDa as determined by SDS/PAGE. Purified OBF2 not only binds DNA but also supports the formation of a protein complex at essential sequences within the ARS*121* origin of replication. Interestingly, OBF2 binds tightly and nonspecifically to both duplex DNA and single-stranded DNA. The interaction with duplex DNA occurs at the termini. N-terminal sequencing of the 65-kDa subunit has revealed that this polypeptide is identical to the previously identified HDF1 peptide, a yeast homolog of the small subunit of the mammalian Ku autoantigen. Although the potential involvement of Ku in DNA metabolic events has been proposed, this is the first requirement for a Ku-like protein in the assembly of a protein complex at essential sequences within a eukaryotic origin of replication.**

DNA replication in eukaryotes must be regulated at multiple levels during the cell cycle. A critical point of control is expected to occur at the initiation of replication, at the start of the S phase. *Saccharomyces cerevisiae* serves as a convenient model system for studying replication initiation in eukaryotic cells because (*i*) it is amenable to genetic and biochemical analysis and (*ii*) origins of replication have been isolated and are well-defined. Although a number of candidate genes have been identified that satisfy criteria for functioning at the start of S phase, the mechanism of replication initiation remains unknown. As in the case of prokaryotic and viral replication systems, elucidation of the mechanism of initiation of eukaryotic DNA replication is expected to depend on the identification and isolation of critical trans-acting proteins that interact specifically with important DNA elements at the origin of replication.

Nuclear origins of replication, called autonomously replicating sequences (ARSs), were first isolated as DNA fragments that confer 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). A subset of these ARSs were also shown to be origins in their native chromosomal context (4–8).

Recent molecular dissection of several ARSs has shown that origins are composed of multiple functional domains (6, 9–11). These origins appear to consist of an essential core sequence (ECS) containing an 11-bp [(A/T)TTTAT(A/G)TTT(A/T)] ARS consensus sequence and auxiliary elements flanking the core. In ARS*121* these elements, which can be connected by random DNA without apparent loss of origin activity, interact to elicit maximal origin function (6). One of the DNA elements flanking the essential core sequence in several ARSs is the binding site for the multifunctional ABF1 protein (6, 7, 9). We have shown that this site functions as an enhancer of DNA replication in an orientation and distance independent manner (12). Another element, shared by all ARSs, is relatively (A+T)-rich and is located 3′ to the T-rich strand of the ARS consensus sequence. This element has been proposed to have a role in the unwinding of the origin during initiation (13). We have shown that in ARS*121* the correct positioning of the (A+T)-rich element relative to the essential core is critical for origin activity (6).

The modular arrangement of domains important for origin function raised the possibility that multiple protein factors interact with these elements. We have previously reported a partial resolution and *in vitro* reconstitution of a multiprotein complex at the ARS*121* origin of replication (14). One of the three DNA-binding activities required for complex assembly contained the known ABF1 protein. The other two have been designated origin binding factor 2 (OBF2) and core binding factor (CBF). We have observed that CBF could bind stably to the ARS DNA only in the presence of ABF1 and OBF2. Moreover, the binding of CBF required a functional ECS of ARS*121* as well as ATP. A mutation in the ECS that inactivated the origin did not sustain stable CBF interaction. The requirement for a functional ECS element suggests a role for the assembled complex in the initiation of DNA replication. In addition, the binding of CBF resembled the DNA-binding properties of the ORC protein. ORC, a protein consisting of six nonidentical subunits, has been shown to interact with sequences at the core of several ARSs (15).

To elucidate the mechanism and the biochemical significance of the *in vitro* assembly of a stable multiprotein-ARS complex, we have undertaken the task of resolving and reconstituting this complex from purified proteins. Here we report the purification and identification of OBF2. We show that OBF2 is a heterodimer that binds both duplex and single-stranded DNA (ssDNA). We also show that the purified protein together with purified ABF1 stabilizes the binding of CBF to the origin. Finally, the biochemical and physical characterization of the purified OBF2 shows that it is identical to the HDF protein. HDF is the putative yeast homolog of the mammalian Ku autoantigen (16). Thus, our findings strongly suggest that the Ku protein may have an important role in the initiation of DNA replication at eukaryotic origins of replication.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ARS, autonomously replicating sequence; ECS, essential core sequence; CBF, core binding factor; OBF, origin binding factor; ssDNA, single-stranded DNA.

*To whom reprint requests should be addressed.

MATERIALS AND METHODS

Yeast Strains. The *S. cerevisiae* strain used was BJ926 (*α/a prb1-1122/prb1-1122 prc1-407/prc1-407 pep4-3/pep4-3can1/can1 gal2/gal2 his1/+ +/trp*).

Preparation of Nuclear Extract. Cells were grown in a New Brunswick Scientific fermenter in 20-liter batches of yeast extract/bacto-peptone/2%dextrose to a final optical density of 4 at 600 nm. Wet cell paste (750 g) was processed for nuclear proteins essentially as described before (14). Cells were disrupted by glass beads in a large BioSpec Bead Beater in buffer C. Cytoplasmic contents were cleared by centrifugation and the nuclear pellet was resuspended and homogenized in buffer C + 1.25 M NaCl and 10% glycerol. Extracted proteins were precipitated by ammonium sulfate (0.4 g to each ml solution). The ammonium sulfate pellet of the nuclear extract was frozen and kept at −80°C. Subsequent steps are described in the legend to Fig. 1.

Plasmids. The construction of the plasmids p19A121(ch+), p19AB121, pCORE was previously described (6, 12). Plasmids containing 16 complete copies of ARS*121* (p19AB121-16), and 32 copies of the ARS*121* essential core sequence (pCORE-32) were constructed by the method described before (17).

Radioactively Labeled DNA Substrates. The DNA substrate for mobility shift assays was prepared as follows: Plasmid p19A121(ch+), containing the ARS*121(ch+)* origin was digested with *Hin*dIII and *Eco*RI. The digest was end-labeled with [α-^{32}P]dATP and [α-^{32}P]TTP using Sequenase. Labeled DNA was subjected to PAGE and the 290-bp substrate fragment was isolated and purified from the gel. The synthetic oligonucleotide used for the ssDNA binding reactions was a 78 base fragment from the T-rich strand of ARS*121* encompassing sequences from nucleotide 375-452, as described (12). The synthetic, single-stranded oligonucleotide substrate was labeled with T4 polynucleotide kinase using [γ-^{32}P]ATP, and subsequently purified from polyacrylamide gels.

DNA Competitors. Covalently closed, relaxed pUC19 plasmid was prepared by topoisomerase I (GIBCO/BRL) treatment. Relaxation was tracked by ethidium bromide-agarose gels. The relaxed pUC19 DNA was isolated from the agarose gels. Linear pUC19 DNA, cut at a single site, was generated by *Xmn*I digestion, followed by isolation and purification of the linearized fragment from agarose gels. DNA competitions with other linear DNA was performed using a *Hae*III digest of pUC19 DNA. Single-stranded phage ϕX174 DNA was purchased from New England Biolabs.

DNA-Binding Assay. DNA mobility shift assays in agarose gels were used to test the binding of proteins to the ARS*121(ch+)* origin, essentially as described before (14). To track the OBF2 activity during the purification, binding reactions were done in a 40-μl reaction volume using 2.5 fmol of radioactively labeled ARS*121(ch+)* DNA. Assays were performed in the presence of 0.1–1.0 μg of pUC19 plasmid DNA as nonspecific carrier. Nonspecific carrier DNA was also added to DNA-binding reactions containing CBF. These reactions contained 3.0 μg of total DNA in the mixture and were kept on ice for an additional 10 min. When more than one DNA-binding protein was used in an assay, all proteins were added simultaneously prior to the incubation at room temperature. All DNA-binding mixtures were in an ionic environment equivalent to 200 mM KCl.

DNA competition experiments were performed by adding the relevant competitor DNA with the labeled substrate prior to the addition of the DNA-binding proteins.

Preparation of ABF1 and CBF. The ABF1 protein was isolated as described (18). The CBF used was prepared as follows: the flow through fraction from the ABF1 DNA-cellulose resin prepared as described in Fig. 1 was loaded onto an hydroxyapatite column (2.6 mg protein per ml resin)

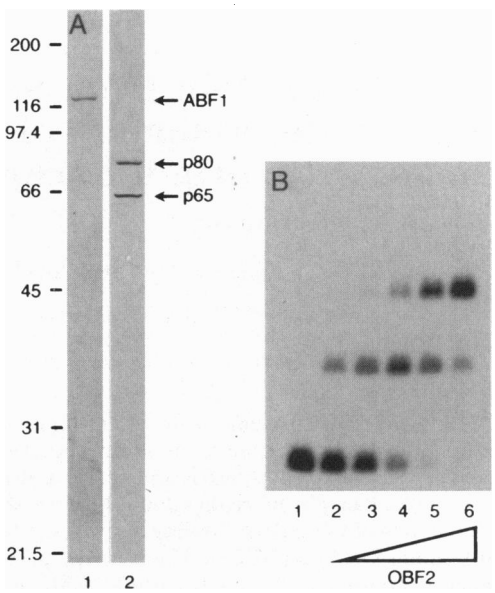

FIG. 1. Purification of OBF2 was accomplished as follows: A protein pellet of the nuclear extracts was resuspended in buffer A and dialyzed against the same buffer to a conductivity equivalent to 0.25 M KCl. The dialysate was loaded onto a DEAE-cellulose column (25 mg protein/ml resin) equilibrated with buffer A + 0.25 M KCl. The flow through and a column wash with buffer A + 0.25 M KCl (1.5 column volume) were pooled as a single fraction. Proteins were first precipitated from this fraction with ammonium sulfate (0.4 g to each ml) at 4°C and then resuspended in buffer A. The resuspended fraction was dialyzed against buffer A to a conductivity equivalent to 0.15 M KCl. After dialysis, ATP (1.0 mM, final concentration) was added and the dialysate was loaded onto a p19AB121-16 DNA-cellulose column (3.0 mg protein/ml resin). This column, which was equilibrated with buffer A + 0.15 M KCl, 1.0 mM ATP, was washed with the same buffer (1 column volume) followed by a wash with buffer A + 0.2 M KCl, 1.0 mM ATP (4 column volume). Proteins that bound tightly to this DNA-cellulose resin were eluted by a wash with buffer A + 1 M KCl. This protein fraction was first diluted with buffer A to a conductivity equivalent to 0.25 M KCl and then loaded onto a DNA-cellulose column containing multiple copies of the ABF1 binding site (2.5 mg protein/ml resin). Protein fractionation on this column was as previously described (14). Fractions containing OBF2 were diluted with buffer A to a conductivity equivalent to 0.2 M KCl and loaded onto SP-Sepharose (10 mg protein/ml resin) equilibrated in the same buffer. The flow through and a column wash with buffer A + 0.2 M KCL (1.5 column volume) were pooled as a single fraction and loaded directly onto a denatured calf thymus DNA-cellulose column (0.35 mg protein/ml resin). The column was first washed with buffer A + 0.2 M KCl and then followed with a wash with buffer A + 0.5 M KCl (5 column volume). Fractions containing OBF2 were pooled and concentrated through a P-30 Centricon filtration unit. The concentrated fraction was loaded on a Superose-12 (FPLC) filtration column in multiple batches. Peak fractions containing OBF2 were pooled and concentrated again as described above. The concentrated fraction was loaded on a 20–40% glycerol gradient (5.0 ml) in buffer B. Centrifugation was performed at 1°C in an SW 50.1 rotor at 50,000 rpm for 36 hr. Fractions (200 μl) were collected from the bottom of the tube. The final yield of the OBF2 protein was 15–20 μg/750 g cell paste. Since the assay of OBF2 was not quantitative at the early, crude stages of the purification, an accurate determination of OBF2 recovery after each purification step has not been possible. *A* shows the profile of silver-stained ABF1 and OBF2 on 10% (wt/vol) SDS/PAGE. The arrows to the right mark the positions of the ABF1 protein and the p80 and p65 subunits of OBF2. Marks to the left denote the position of molecular mass markers expressed in kDa. Lane 1 shows the purified ABF1 (50 ng) (18). Lane 2 shows the migration of the purified OBF2 (glycerol gradient fraction). Molecular mass standards were (in kDa): myosin, 200; β-galactosidase, 116; phosphorylase b, 97.4; BSA, 66; ovalbumin, 45; carbonic anhydrase, 31; and trypsin inhibitor, 21.5. *B* shows binding of purified OBF2 to the ARS*121(ch+)* DNA fragment analyzed by a DNA mobility shift assay. Lane 1 shows the mobility of the DNA substrate. Lanes 2–6 represent a titration of OBF2 with ≈0.5, 1, 2, 4, and 8 ng of the purified protein (*A*, lane 2), respectively.

equilibrated with buffer A + 1 mM potassium phosphate, 250 mM KCl (pH 7.9) followed by a wash with buffer A + 100 mM potassium phosphate (pH 7.9), 150 mM KCl. The CBF activity was eluted with buffer A + 400 mM potassium phosphate.

DNA Affinity Resins. DNA-cellulose resins were prepared by mixing the appropriate DNA with cellulose powder essentially as described before (17). The ARS*121* DNA-cellulose resin was prepared using the p19AB121-16 plasmid DNA. The ABF1 DNA affinity resin was described before (14). ssDNA-cellulose resin was prepared by using denatured calf thymus DNA.

Protein sequencing. Fractions from the Superose-12 column containing the OBF2 activity were subjected to SDS/PAGE 4% (wt/vol) acrylamide stacking and 7.5% (wt/vol) acrylamide resolving gels. Electrophoresis was performed using the Bio-Rad minicell for 40 min at 180 V. Electroblotting onto polyvinylidene difluoride membranes was performed by a method previously described (19). The Coomassie blue stained band corresponding to the 65-kDa subunit of the OBF2 protein was excised from the polyvinylidene difluoride membrane and processed for N-terminal sequencing using standard procedures. Protein sequence analysis was performed by the W. M. Keck Foundation, Biotechnology Resource Lab, Yale University (New Haven, CT).

Buffers. Buffer A contained 25 mM Tris·acetate, pH 7.9, 1 mM EDTA, and 10% glycerol. Buffer B contained 25 mM potassium phosphate buffer, pH 7.9/1 mM EDTA/0.5 M KCl. Buffer C contained 100 mM Tris·acetate, pH 7.9/50 mM KAc, pH 7.5/10 mM $MgSO_4$/30% glycerol. In addition, all buffers were supplemented with either 20 mM 2-mercaptoethanol or 1 mM dithiothreitol and a protease inhibitors cocktail described before (18).

RESULTS

Purification of the OBF2 Activity. We have previously identified a DNA-binding activity, referred to as CBF, that recognizes *in vitro* the ECS element of the ARS*121* origin of replication (14). The stable binding of CBF to the origin, identified by electrophoretic mobility shift assay, required ATP and two additional DNA-binding activities. One of these activities was identified as the ABF1 protein while the other, referred to as OBF2, was defined as an activity able to supershift an ABF1-ARS*121* DNA complex. In Fig. 1*A* we describe the purification of OBF2.

In addition to supershifting the ABF1-ARS DNA complex, OBF2 exhibited a characteristic mobility shift pattern on its own (14). This pattern of two shifted bands shown in Fig. 1*B* was maintained throughout the purification. Thus, both shifts are a function of the same protein. The binding of OBF2 to naked ARS*121* DNA, as shown in Fig. 1*B*, was used as a standard assay for its purification. In addition, fractions containing the OBF2-binding activity were tested for their ability to both supershift the ABF1-ARS DNA and for their requirement in the formation of a stable CBF-origin DNA complex.

Purification of OBF2 involved DNA affinity and conventional ion exchange chromatography. DNA affinity included three different DNA-cellulose resins. First, crude extracts were fractionated on a DNA affinity resin containing ARS*121* DNA sequences to isolate OBF2, ABF1 and CBF as a DNA bound fraction. This DNA bound fraction was then fractionated on a resin containing ABF1 recognition sequences to separate the three DNA-binding activities, essentially as described before (14). When we realized that OBF2 could interact with ssDNA, a ssDNA cellulose matrix was also included in the purification. Fractionation on the ssDNA-cellulose column was preceded by ion exchange chromatography on SP-Sepharose as described in Fig. 1. The final step in the purification was sedimentation through a glycerol gradient.

The rate of sedimentation of the OBF2 DNA-binding activity on glycerol gradients was similar to that of aldolase (data not shown). In addition, OBF2 migrated close to aldolase on a Superose 12 filtration column (data not shown), suggesting that the apparent molecular mass of the native protein is around 150 kDa. Analysis of the purified OBF2 by SDS/PAGE revealed the presence of two polypeptides of molecular masses of 65 and 80 kDa (Fig. 1*A*, lane 2). Both co-sedimented precisely with the OBF2 DNA-binding activity. Hence, these results suggest that the OBF2 protein exists as a heterodimer in solution.

OBF2 Binds to Termini of Duplex DNA. The DNA substrate used in the binding assays was ARS*121(ch+)*. This DNA contains the ARS*121* ECS, the (A+T)-rich domain flanking the core on the 3′ side of the ARS consensus sequence T-rich strand, and the replication enhancer (ABF1-binding site) 5′ to the core (12).

As shown in Fig. 1*B*, two shifted bands in an agarose gel were observed when the DNA was incubated with OBF2. To test whether OBF2 recognizes a specific nucleotide sequence within the ARS*121* origin, we have performed DNA competition experiments described in Fig. 2. In these experiments the amount of OBF2 used was sufficient to shift most of the DNA substrate to the second most retarded position (Fig. 2*A*, lane 2). Radioactively labeled ARS *121(ch+)* substrate was incubated with OBF2 in the presence of increasing levels of competing superhelical p19AB121-16 plasmid containing 16 copies of ARS*121* DNA. Only a minor competition for binding OBF2 was observed (Fig. 2*B*). This result was comparable to the competition with the control superhelical pUC DNA (Fig. 2*C*). Thus, it appeared that the ability of OBF2 to bind the ARS*121(ch+)* DNA was independent of internal sequences and that the binding may have occurred at the termini of the linear DNA substrate.

Indeed, as shown in Fig. 3*D*, only linear plasmid DNA competed effectively for OBF2. Relaxed circular DNA did not compete, indicating that OBF2 binding is a function of free DNA ends (Fig. 2*E*). The binding to the termini was further confirmed by DNA footprinting analysis. We observed that OBF2 protects 15–20 nucleotides at the ends of the linear

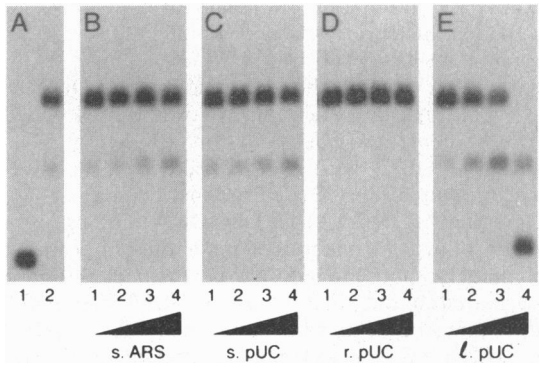

FIG. 2. OBF2 binding to duplex DNA analyzed by DNA competition. The DNA used in the mobility shift assays was the radioactively labeled ARS*121(ch+)* substrate. (*A*) Lane 1 shows the mobility of the DNA substrate. Lane 2 shows the mobility of the substrate in the presence of 10 ng of OBF2. (*B*) Lanes 1–4 represent DNA competitions for OBF2 binding by supercoiled p19AB121-16 plasmid DNA. The molar ratio of competitor DNA to labeled substrate DNA in lanes 1–4 is 2.9, 5.8, 12, and 23, respectively. (*C*) DNA competitions with supercoiled pUC19 plasmid are shown. The molar ratio of competitor to substrate DNA in lanes 1–4 is 11, 22, 45, and 91, respectively. (*D*) Competition reactions with relaxed, covalently closed, circular pUC19 DNA are shown. The molar ratio of competitor to substrate DNA in lanes 1–4 is 9.2, 23, 46, and 91, respectively. (*E*) DNA competitions with linearized pUC19 DNA are shown. The molar ratio of competitor to substrate DNA in lanes 1–4 is 0.9, 1.8, 3.6, and 7.3, respectively. Individual reactions in *B–E* contain 10 ng of OBF2.

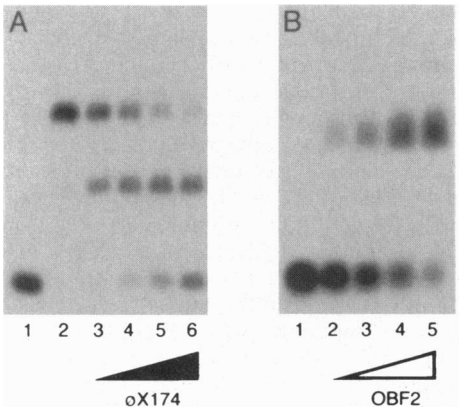

FIG. 3. OBF2 interacts with ssDNA. DNA binding analysis was performed by the mobility shift assay. The DNA used in the binding assays described in *A* was the radioactively labeled ARS*121(ch+)* substrate. Lane 1 shows the mobility of the substrate. Lane 2 shows the mobility of the substrate in the presence of 10 ng of OBF2; lanes 3–6 represent binding reactions as in lane 2 except that single-stranded φX174 competitor DNA was added. The molar ratio of competitor to substrate DNA in lanes 3–6 is 1.9, 4.8, 12, and 30, respectively. *B* shows direct binding by OBF2 to a ssDNA substrate as analyzed by the electrophoretic mobility shift assay. The DNA substrate used was a synthetic 78-nucleotide-long oligonucleotide. Lane 1 shows the mobility of the ssDNA substrate; lanes 2–5 show a titration of OBF2 in the ssDNA binding reactions. The amount of OBF2 in lanes 2–5 is ≈1.2, 2.5, 5, and 10 ng, respectively.

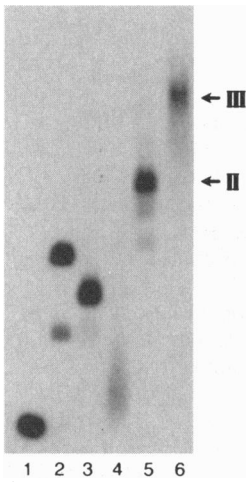

FIG. 4. ABF1, OBF2, and CBF are required for the assembly of a multiprotein-ARS*121ch(+)* origin DNA complex. The analysis of the assembly was performed by mobility shift assays. Lane 1 shows the mobility of the DNA substrate. Lane 2 shows the mobility of the substrate with purified OBF2 (8 ng). Lane 3 shows an assay of purified ABF1 (15 ng). Lane 4 is DNA incubated with a CBF fraction (1.6 μg). Lane 5 represents DNA incubated with ABF1 (15 ng), and OBF2 (8 ng). Lane 6 represents DNA incubated with ABF1 (15 ng), OBF2 (8 ng), and CBF (1.6 μg). Arrows to the right mark the position of the ABF1-OBF2-DNA complex (complex II) and the ABF1-OBF2-CBF-DNA complex (complex III).

DNA from DNase I digestion (data not shown). Since any linear DNA could bind OBF2, we conclude that OBF2 binding to the free duplex DNA ends is sequence independent.

OBF2 Binds Tightly to ssDNA. Although supercoiled plasmid DNA did not compete effectively for OBF2, some loss of OBF2 binding to the origin was observed in the presence of high levels of a supercoiled DNA (Fig. 2*C*). In contrast, no detectable competition for binding OBF2 was seen when relaxed circular DNA was used as a competitor (Fig. 2*D*). Since melted regions may be present in the supercoiled DNA, we examined the possibility that OBF2 could interact with ssDNA. Direct mobility shift assays of a synthetic, single-stranded oligonucleotide revealed complex formation between OBF2 and the ssDNA substrate (Fig. 3*B*). Unlike the binding to duplex DNA termini, the interaction with ssDNA is probably with internal sequences since covalently closed φX174 phage DNA competed effectively for OBF2 (Fig. 3*A*).

Identity of OBF2 with the Ku-Like Protein HDF. The physical structure of OBF2 (heterodimer comprised of 65- and 80-kDa subunits, Fig. 1*A*) and its DNA binding properties (discussed above) strikingly resemble the properties of the mammalian Ku autoantigen. We performed N-terminal sequence analysis of the small subunit of OBF2. This analysis has revealed that the N-terminal sequence of the p65 OBF2 subunit (MRPVTNAFGN) matches that of the *S. cerevisiae* polypeptide Hdf1. Hdf1 is the small subunit of a *S. cerevisiae* protein, HDF, that binds DNA termini nonspecifically (16). The large subunit of HDF migrates as an 80-kDa band on SDS/PAGE (16) and thus probably represents the large subunit of OBF2. Hence, these results have established the identity of OBF2 and HDF, the yeast Ku-like protein.

OBF2 is Required for the *in Vitro* Assembly of a Specific and Stable Multiprotein-DNA Complex at ARS121. Previously we have used an electrophoretic mobility shift assay to assemble *in vitro* a multiprotein-ARS*121* DNA complex. This ATP-dependent complex, termed complex III, required three partially fractionated DNA-binding activities (14). In Fig. 4, lane 6, we show that both OBF2 and ABF1, purified to near homogeneity (Fig. 1*A*), can substitute for two of the three crude DNA-binding activities in the formation of complex III. The third protein used, CBF, was a partially purified fraction. As has previously been described (14), the OBF2 and ABF1 proteins together formed complex II (Fig. 4, lane 5) with the substrate DNA. In contrast, CBF alone could not sustain a stable complex with the ARS DNA (Fig. 4, lane 4).

The assembly of complex III was DNA sequence dependent (Fig. 5). DNA competition experiments have shown that plasmid DNA, pCORE-32, containing multiple copies of the ARS*121* ECS element effectively inhibited formation of complex III, while pUC DNA had no effect on the assembly of the complex (Fig. 5 *B* and *C*). It is the binding of CBF that was inhibited by the ARS*121* ECS elements since the product of the competition was complex II containing ABF1 and OBF2 (Fig. 5*C*).

The requirement of the Ku-like activity, OBF2, for complex III formation was readily apparent by a titration of OBF2 into ABF1 and CBF containing reactions (Fig. 6). Origin DNA incubated with OBF2 and CBF alone appeared as a broad smear upon electrophoresis in an agarose gel (Fig. 6*A*, lane 2). A similar broad smear of the DNA was observed when the radioactively labeled substrate DNA was incubated with ABF1 and CBF (Fig. 6*A*, lane 3). Addition of increasing amounts of OBF2 to the binding mixtures containing ABF1 and CBF resulted in the appearance of a tight DNA band at the position of complex III (Fig. 6*A*, lanes 4–7). Moreover, the formation of this complex was inhibited by the addition of linear DNA, which specifically binds OBF2 (Fig. 6*B*, lanes 1–4). These results clearly demonstrate that the formation of a stable CBF-origin complex depends on the binding of the Ku-like protein OBF2 to the substrate DNA.

DISCUSSION

Identification and isolation of proteins that specifically interact with an origin of replication is critical for understanding the mechanism of DNA replication initiation. In this paper we have reported the purification of OBF2, a DNA-binding activity previously shown to be required for the *in vitro* assembly of a stable multiprotein complex at the ARS*121* origin of replication. Physical characterization of the protein

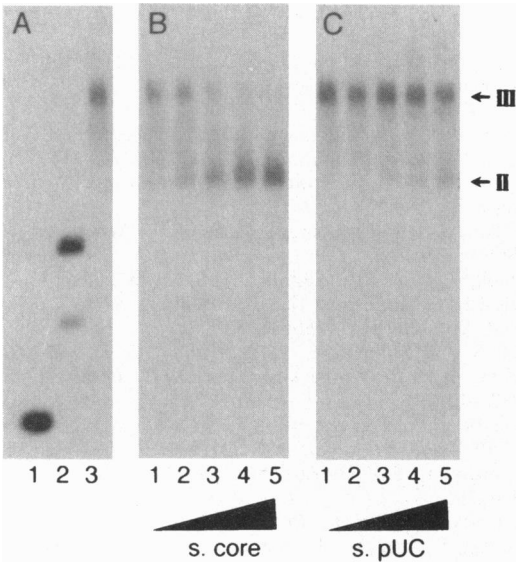

FIG. 5. Assembly of complex III is DNA sequence-dependent. (*A*) Lane 1 shows the mobility of the DNA substrate. Lane 2 represents DNA incubated with OBF2 (10 ng). Lane 3 shows a complex III reaction as in Fig. 4, lane 6. *B* represents DNA competition experiments. DNA binding reactions described in lane 3 of *A* were supplemented with increasing amounts of supercoiled pCORE-32 DNA. The molar ratio of competitor to substrate DNA was 1, 2, 4, 8, and 16, respectively. (*C*) show a similar DNA competition except that the competitor DNA used was supercoiled pUC19. The molar ratio of competitor to substrate DNA was 46, 91, 182, 365, and 730, respectively. Arrows to the right indicate the position of complexes II and III.

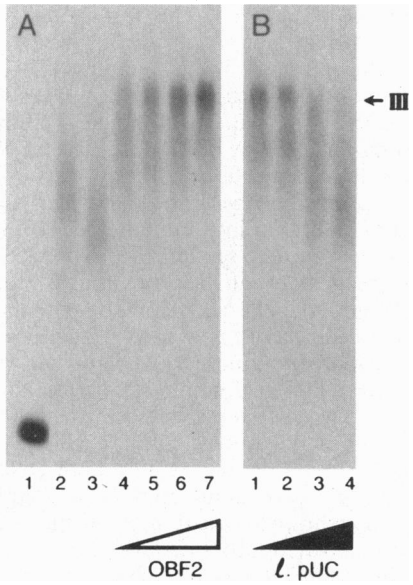

FIG. 6. Stable interaction of CBF with the origin DNA requires the OBF2 protein. DNA mobility shift assays. (*A*) lane 1 shows the mobility of the ARS*121(ch+)* DNA. Lane 2 represents DNA incubated with CBF (1.6 μg) and OBF2 (8 ng). Lane 3 shows an assay of CBF (1.6 μg) with ABF1 (15 ng). Lanes 4–7 show a titration of OBF2 into a CBF and ABF1 containing reaction. The levels of OBF2 in lanes 4–7 are 1, 2, 4, and 8 ng, respectively. (*B*) shows the effect of competing linear DNA (*Hae*III digest of pUC19 DNA) on the formation of complex III. The molar ratio of competitor fragments to substrate DNA in lanes 1–4 are 0.7, 1.4, 2.8, and 5.6, respectively. The arrow to the right indicates the position of complex III.

suggested that OBF2 is a heterodimer comprised of two subunits with apparent molecular mass values of 65 and 80 kDa. Surprisingly, in our attempts to localize the site on the DNA recognized by OBF2 we observed that the protein bound tightly to both duplex DNA termini and ssDNA. These results and the N-terminal amino acid sequence determination of the p65 subunit indicated that OBF2 is identical to the yeast Ku-like protein, HDF.

The Ku protein was first identified as an autoantigen recognized by sera from patients with autoimmune disorders such as scleroderma and lupus erythematosus (20–22). The mammalian Ku is a heterodimer that binds to duplex termini (23, 24). It serves to both recruit and activate the p350 DNA-dependent protein kinase at the site of double-stranded DNA breaks (25–27). At these sites Ku and the p350 proteins were shown to be important for mediating repair functions and DNA rearrangements *in vivo* (28). Ku was also shown to interact specifically with a recognition site in the long terminal repeat of the mouse mammary tumor virus DNA to recruit p350 and repress transcription of a reporter gene (29).

The HDF protein is believed to be the yeast homolog of the mammalian Ku (16). Although no homolog of the p350 kinase has been found in yeast, genetic evidence has been reported to suggest an *in vivo* role for HDF in DNA repair and the metabolism of telomeric sequences (30–32). Furthermore, genetic evidence has also suggested a role for HDF in DNA replication. Disruption of the gene encoding the small peptide of HDF confers a temperature-sensitive cell division cycle phenotype on cells (16). Cells arrest at the restrictive temperature with a dumbbell shaped morphology and accumulate a >2C DNA content. This phenotype is consistent with a loss of some aspect of DNA replication control at elevated temperature—potentially unregulated replication initiation. Our finding that OBF2 is required *in vitro* for the assembly of a complex at a replication origin suggests that HDF may participate directly in the formation or establishment of a regulatable nucleoprotein complex involved in the initiation of DNA replication.

The *in vitro* assembly of the multiprotein complex at ARS*121* suggests a role for OBF2 in facilitating a stable interaction of CBF with the origin DNA. This function of OBF2 appears to be accomplished in concert with ABF1 since it also is required for a stable CBF-DNA interaction. Because not all *S. cerevisiae* origins were found to contain an ABF1 binding site, however, it is possible that under certain conditions OBF2 alone may sustain stable CBF binding. The fact that the assembly of the complex described in this paper seems to depend on (*i*) ABF1, a factor known to enhance replication *in vivo* and (*ii*) DNA sequences essential for origin function lends further support to the idea that the reconstituted complex has a role in initiation of DNA replication.

The key interaction in this assembly is the ATP-dependent binding of CBF to the origin's ECS element. Although we have not purified the CBF to homogeneity, we believe that CBF is ORC-related. ORC is thought to play a direct role in the initiation of replication at yeast origins, although an enzymatic activity has yet to be shown for ORC. Besides a common requirement for ATP in interacting with ARS sequences, both CBF and ORC appear to have a similar native size, as noted by sedimentation through glycerol gradients (data not shown). Thus, CBF and ORC may share common subunits. The complete identity of CBF and ORC, however, should await the complete purification of CBF. It is also worth noting that *in vivo*, in addition to the ORC, other proteins such as CDC46, DBF4, and CDC6 may act at the origin of replication (33–38).

The mechanism of the *in vitro* assembly of the multiprotein-origin complex in our system is not known. It is clear that CBF on its own cannot form a stable complex with the DNA *in vitro*. This is based on the finding that CBF is unable to shift and form a tight band with the origin DNA upon electrophoresis in agarose gels (Figs. 4–6). To explain how OBF2 and ABF1 facilitate the stable CBF-origin interaction, two possible mech-

anisms should be considered. The first model presumes no direct protein–protein interactions within the fully formed *in vitro* complex. It supposes that the binding of OBF2 and ABF1 to the ARS establishes a structural change in sequences at the essential core that stabilize CBF binding. This possibility is testable and may shed light on the nature of DNA contacts made by the ORC-like protein CBF.

The second possibility presumes that either ABF1 and/or OBF2 protein could loop into the core region. Stable interaction of CBF with the core sequences may only be maintained if contacts are made between the proteins involved. The ability of OBF2 to stabilize the interaction of CBF with the origin may be analogous to the recruitment and regulation of the p350 DNA-dependent protein kinase by Ku in higher organisms. In the case of OBF2, its function may be specific for the origin, since its "partner," CBF, binds specifically to origin sequences. These two possibilities are not mutually exclusive, since stable CBF binding to the origin may require both DNA structural changes and protein–protein interactions. Resolution of these possibilities will require reconstitution of the system with a homogeneously purified CBF.

Since double-stranded DNA breaks are not an expected feature of replication initiation in *S. cerevesiae,* binding of OBF2 to the termini in the *in vitro* system may be a fortuitous condition for localizing the protein to the DNA. The localization of OBF2 to the origin *in vivo* may occur by a different mechanism. Our finding that OBF2 binds ssDNA suggests an alternative possibility for complex formation *in vivo*. OBF2 may interact with sequences that are partially unwound or that are in a different conformation to that of simple linear duplex DNA. The mammalian Ku protein has been shown to exhibit such binding characteristics (24). If OBF2 serves to establish or interact with an initiation complex, then it may do so through primary contacts with structural determinants at the origin. It is also interesting to note that the purified mammalian Ku protein has been shown to exhibit ssDNA-dependent ATPase and helicase activity *in vitro* (38, 39). This is an obviously relevant activity for a protein that may serve a biochemical function at a replication origin.

We thank Todd Upton for the purification of the ABF1 protein. This work was supported by the National Institutes of Health Grant GM46760-03.

1. Stinchcomb, D. T., Struhl, K. & Davies, R. W. (1979) *Nature (London)* **282,** 39–43.
2. Brewer, B. J. & Fangman, W. L. (1987) *Cell* **51,** 463–471.
3. Huberman, J. L., Spotila, D. L., Nawotka, K. A., El-Assouli, S. M. & Davies, R. W. (1987) *Cell* **51,** 473–481.
4. Ferguson, B. M., Brewer, B. J., Reynolds, A. E. & Fangman, W. L. (1991) *Cell* **65,** 507–515.
5. Huberman, J. L., Zhu, J., Davies, R. L. & Newlon, C. S. (1988) *Nucleic Acids Res.* **16,** 6373–6383.
6. Walker, S. S., Malik, A. K. & Eisenberg S. (1991) *Nucleic Acids Res.* **19,** 6255–6262.
7. Rivier, D. H. & Rine, J. (1992) *Science* **256,** 659–663.
8. Desphande, A. M. & Newlon, C. S. (1992) *Mol. Cell. Biol.* **12,** 4305–4313.
9. Marahrens, Y. & Stillman, B. (1992) *Science* **255,** 817–823.
10. Theis, J. F. & Newlon, C. S. (1994) *Mol. Cell. Biol.* **14,** 7652–7659.
11. Huang Ruea-Yea & Kowalski, D. (1996) *Nucleic Acids Res.* **24,** 816–823.
12. Walker, S. S. & Eisenberg, S. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4665–4669.
13. Umek, R. M. & Kowalski, D. (1988) *Cell* **52,** 559–567.
14. Estes, H. G., Robinson, B. S. & Eisenberg, S. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11156–11160.
15. Bell, S. P. & Stillman, B. (1992) *Nature (London)* **357,** 128–134.
16. Feldman, H. & Winnacker, E. L. (1993) *J. Biol. Chem.* **268,** 12895–12900.
17. Eisenberg, S., Francesconi, S. C., Civalier, C. & Walker, S. S. (1990) *Methods Enzymol.* **182,** 521–529.
18. Francesconi, S. C. & Eisenberg, S. (1989) *Mol. Cell. Biol.* **9,** 2906–2913.
19. Matsudara, P. (1987) *J. Biol. Chem.* **262,** 10035–10038
20. Mimori, T., Akizuki, M., Yamagata, H., Inada, S., Yoshida, S. & Homma, M (1981) *J. Clin. Invest.* **68,** 611–620.
21. Reeves, W. H. (1985) *J. Exp. Med.* **161,** 18–39.
22. Yaneva, M. & Busch, H. (1986) *Biochemistry* **25,** 5057–5063.
23. Mimori, T. & Hardin, J. A. (1986) *J. Biol. Chem.* **261,** 10375–10379.
24. Paillard, S. & Strauss, F. (1991) *Nucleic Acids Res.* **19,** 5619–5624.
25. Gottlieb, T. & Jackson, S. (1993) *Cell* **72,** 131–142.
26. Suwa, A., Hirakata, M., Takeda, Y., Jesch, S. A., Mimori, T. & Hardin, J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6904–6908.
27. Dvir, A., Peterson, S. R., Knuth, M. W., Lu, H. & Dynan, W. S. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11920–11924.
28. Troelstra, C. & Jaspers, N. G. J. (1994) *Curr. Biol.* **4,** 1149–1151.
29. Griffin, W., Torrance, H., Rodda, D. J., Prefontaine, G. G., Pope, L. & Hache, R. J. G. (1996) *Nature (London)* **380,** 265–268.
30. Siede, W., Friedl, A. A., Dianova, I., Eckardt-Schupp, F. & Friedberg, E. C. (1996) *Genetics* **142,** 91–102.
31. Mages, G. J., Feldmann, H. M. & Winnacker, E.-L. (1996) *J. Biol. Chem.* **271,** 7910–7915.
32. Porter, S. E., Greenwell, P. W., Ritchie, K. B. & Petes, T. D. (1996) *Nucleic Acids Res.* **24,** 582–585.
33. Yan, H., Gibson, S. & Tye, B. K. (1991) *Genes Dev.* **5,** 944–957.
34. Henessy, M. K., Clark, D. C. & Botstein, D. (1990) *Genes Dev.* **4,** 2252–2263.
35. Li, J. J. & Herskowitz, I. (1993) *Science* **262,** 1870–1874.
36. Dowell, S. J., Romanowski, P. & Diffley, J. F. X. (1994) *Science* **265,** 1243–1246.
37. Liang, C., Weinreich, M. & Stillman, B. (1995) *Cell* **81,** 667–676.
38. Christ, C. & Tye, B. K. (1991) *Genes Dev.* **5,** 751–763.
39. Cao, Q. P., Pitt, S., Leszyk, J. & Baril, E. F. (1994) *Biochemistry* **33,** 8548–8557.
40. Tuteja, N., Tuteja, R., Ochem, A., Taneja, P., Huang, N. W., Simoncsitis, A., Susic, S., Rahman, K., Marusic, L., Chen, J., Zhang, J., Wang, S., Pongor, S. & Falaschi, A. (1994) *EMBO J.* **13,** 4991–5001.