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Molecular cloning of a gene encoding an *ARS* binding factor from the yeast *Saccharomyces cerevisiae*

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ABSTRACT **We report the isolation of the gene for origin binding factor 1 (OBF1) from the yeast *Saccharomyces cerevisiae* by screening a yeast genomic DNA library in λgt11 with an *ARS*-specific oligonucleotide probe. One recombinant encoded a fusion protein of ≈180 kDa that bound *ARS*-specific oligonucleotide probes *in vitro*. The restriction map of this gene was determined after isolation of the complete gene by screening a yeast genomic DNA library in YEp24. Characterization of the gene for OBF1 by pulsed-field gel electrophoresis and Northern and Southern blot analyses demonstrated that (*i*) the gene is located in chromosome IV, (*ii*) the gene is a single-copy gene, (*iii*) the mRNA is ≈3.8 kilobases, which could code for an ≈130-kDa polypeptide, consistent with the reported size of OBF1. An antibody, affinity-purified using the lysogen-encoded fusion protein, specifically detected an ≈130-kDa polypeptide in yeast extract. The isolation of the gene for OBF1 should allow further analysis of the mechanism of action of this protein *in vitro* and *in vivo*.**

Sequence-specific DNA binding factors are involved in the control of various cellular processes, such as control of gene expression and DNA replication (1–9). Eukaryotic DNA replication initiates from multiple origins. In yeast, a large number of isolated DNA sequences, termed autonomously replicating sequences (*ARS*s), have been shown to be the origins of chromosomal DNA replication *in vivo* (10–13). Several putative factors have been identified that bind to various *ARS*s and related sequences. Shore *et al.* (7) and Buchman *et al.* (2) have demonstrated sequence-specific DNA binding in domain B of *ARS1* by a cellular protein. They have identified specific DNA binding sites in various yeast DNA fragments for two yeast DNA binding factors: *ARS* binding factor I and general regulatory factor I. Eisenberg *et al.* (14) and Francesconi and Eisenberg (15) have reported the identification and purification of origin binding factor 1 (OBF1) that binds to a sequence in the telomeric *ARS120*, *ARS131C*, and *ARS131L*. It also binds *ARS1*, somewhat weaker.

To further explore the roles of various *ARS* binding factors in DNA replication and gene expression, we have cloned and characterized the gene for OBF1.

MATERIALS AND METHODS

Strains, Bacteriophage and Plasmid Libraries, and Antisera. *Saccharomyces cerevisiae* genomic DNA library in λgt11 was a gift from Michael Snyder of Yale University and in YEp24 was a gift from Marian Carlson of Columbia University. *S. cerevisiae* strain BJ2168 was from the yeast genetic stock center, University of California at Berkeley. Antiserum was prepared against partially purified OBF1 protein and immunoaffinity-purified using either gel-purified 130-kDa OBF1 protein or recombinant λ bacteriophage-encoded fusion protein.

Synthetic Oligonucleotides. The oligonucleotides used in this study were as follows: EI, 5′-GATCCAATACATCATAAAATACGAACGA-3′, derived from *HMRE-ARS* (2); F1, 5′-GATCCAAGTGCCGTGCATAATGATGTGGG, derived from the OBF1 recognition sequence in *ARS120* (14); and other oligonucleotides were as described (2). Oligonucleotides EI and F1 bind to OBF1 or OBF1-like factors in the yeast nuclear extract (25). The oligonucleotides used for screening the yeast genomic library were catenated [mean length of 200 base pairs (bp)] and were labeled by nick-translation using [α-^{32}P]dATP and dCTP to a specific activity of 1×10^8 cpm/μg. Oligonucleotide probes used in mobility-shift DNA binding assays were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase.

Screening of Yeast Genomic DNA Library. The yeast genomic library in λgt11 was screened using *in situ* sequence-specific DNA binding activity by a modification of the methods of Hoeffler *et al.* (8), Singh *et al.* (16), and Vinson *et al.* (17). Screening was carried out with catenated and nick-translated oligonucleotide F1. Lysogens of the isolated recombinants were prepared by the protocol of Snyder *et al.* (18). Induction and preparation of fusion proteins were carried out by the method of Singh *et al.* (16). The extracts were dialyzed against a buffer containing 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20% (vol/vol) glycerol, pepstatin A (5 μg/ml), leupeptin (5 μg/ml), and 0.02% Nonidet P-40 for 1–2 hr at 4°C, divided into portions, and stored frozen at −80°C until further use. YEp24 yeast genomic DNA library was screened with the radiolabeled 3.2-kilobase (kb) insert DNA from the AA2 recombinant phage (5, 19).

Mobility-Shift DNA Binding Assays. The mobility-shift DNA binding assays were carried out as described (2).

Northern, Southern, and Contour-Clamped Homogeneous Electric Field (CHEF) Analysis. Northern and Southern blot analyses were carried out following published methods (19). CHEF analysis was carried out in a Bio-Rad CHEF apparatus using yeast plugs prepared from yeast strain BJ2168. The smaller chromosomes that did not hybridize to the probe were allowed to migrate out of the gel to resolve the larger chromosomes.

DNase I Footprinting Analysis. DNase I footprinting analysis was carried out with the 237-bp *Hin*dIII–*Bgl* II fragment of *ARS1*, ^{32}P-labeled at the *Bgl* II site, as described by Buchman *et al.* (2).

RESULTS

Isolation and Characterization of Origin Binding Factor Recombinants from Yeast DNA Libraries. We have screened 5×10^5 yeast genomic DNA clones in λgt11 vector containing

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Abbreviations: OBF1, origin binding factor 1; CHEF, contour-clamped homogeneous electric field.

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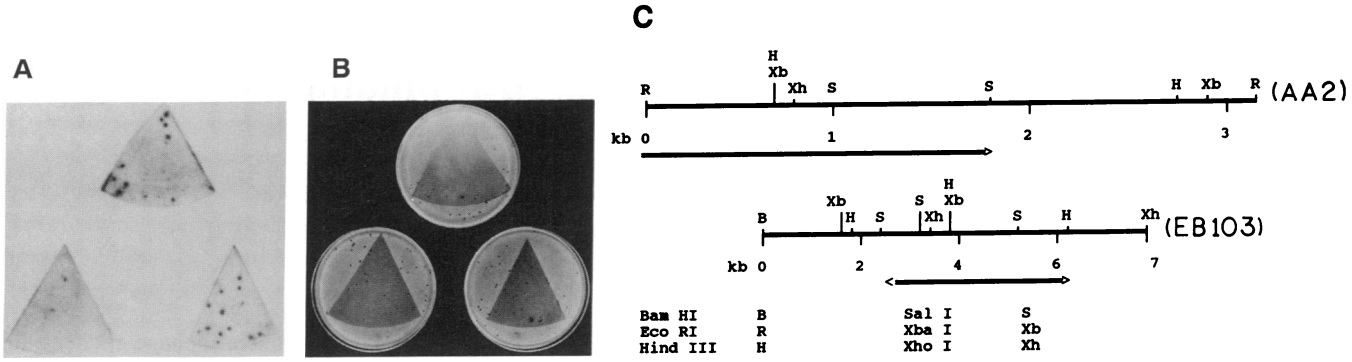

FIG. 1. Screening of bacteriophage colonies with sequence-specific oligonucleotides. Autoradiogram (*A*) and plates (*B*) containing bacteriophage colonies from the third screening of two positive clones (at the lower right and top for clones AA1 and AA2, respectively) and one negative clone (at the lower left) are shown. (*C*) Restriction maps of the 3.2-kb insert DNA of the AA2 bacteriophage clone and the 12-kb insert DNA of EB103 plasmid. The putative location and the direction of transcription are indicated by the arrow.

2.5×10^5 independent recombinants using sequence-specific oligonucleotide binding as the screening method (8, 16, 17). Nine clones were isolated in the initial screening and two of these nine clones were found to be repeatedly positive in three consecutive screenings. The results of the third screening with two positive clones and one negative clone are shown in Fig. 1 *A* and *B*. Both of these clones produced positive signals when screened with oligonucleotide F1 or EI. These two positive clones (AA1 and AA2) were identical in the insert DNA size (3.2 kb) and restriction map. The restriction map of the AA2 clone is shown in Fig. 1*C*. The 3.2-kb insert DNA was used to probe a yeast genomic DNA library in YEp24 and three independent clones were isolated. Each contained the entire putative gene for OBF1 and its 5′ and 3′ adjacent sequences. The restriction map of a 6.8-kb DNA fragment containing the complete gene for OBF1 and its 3′ and 5′ regions (EB103) is shown in Fig. 1*C*.

Northern blot analysis of yeast poly(A)$^+$ RNA with a radiolabeled 3.2-kb insert DNA indicated that a single ≈3.8-kb mRNA hybridized to the probe (Fig. 2*A*). Thus, the likely size of the gene product would be ≈130 kDa. The size of the encoded protein is consistent with the reported size of OBF1 (15). Southern blot analysis with yeast chromosomal DNA digested with various restriction enzymes is shown in Fig. 2*B* and indicates that this gene is probably a single-copy gene. Pulsed-field CHEF gel electrophoresis of yeast chromosomes is shown in Fig. 2*C*. Hybridization of a nitrocellulose blot of this gel probed with the 3.2-kb insert DNA is shown in Fig. 2*C*. Hybridization was observed only with chromosome IV and the hybridization appeared unambiguous. A band was observed at the origin that was due to yeast cells not completely lysed by Zymolase.

Isolation and Analysis of the Recombinant Fusion Protein. Extract of the induced lysogen containing the fusion protein was prepared from clone AA2 by a standard protocol (16). The fusion protein on Western blot was analyzed using a commercial β-galactosidase antibody. The result of the Western blot analysis is shown in Fig. 2*D*. The fusion protein from the lysogen appeared to be degraded rapidly even in the presence of various protease inhibitors at high concentration. The highest band observed in the Western blot was ≈180 kDa. Therefore, the isolation recombinant coded for ≈60 kDa of the *ARS* binding factor. The highest visible band (≈180 kDa) in lane 2 (band I) is presumably the undegraded phage-

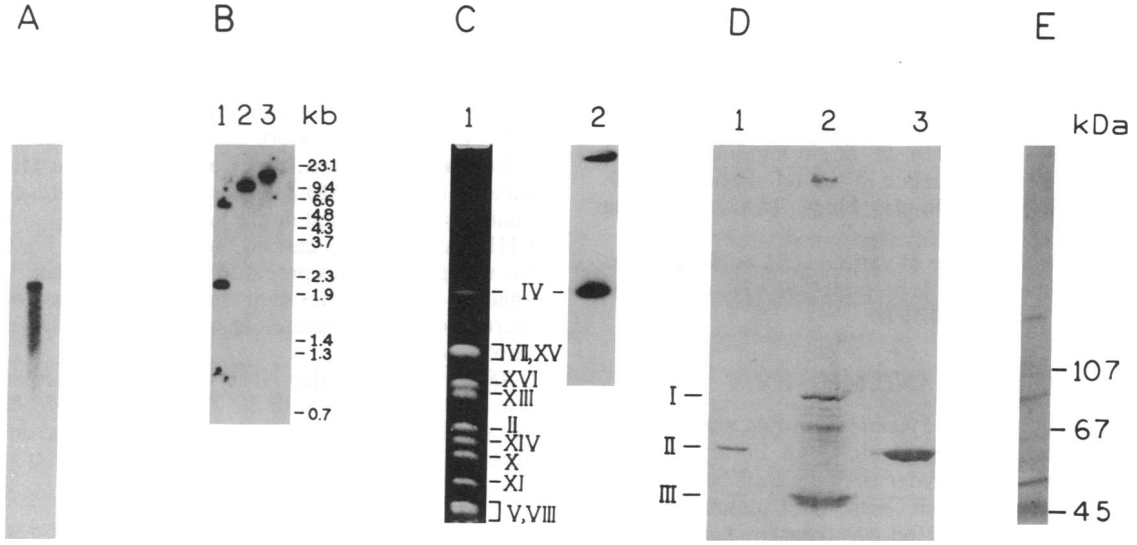

FIG. 2. Analysis of the gene and its gene products. (*A*) Northern blot analysis of yeast poly(A)$^+$ RNA probed with the radiolabeled 3.2-kb AA2 insert DNA. The size of the top band was ≈3.8 kb. (*B*) Southern blot analysis of yeast genomic DNA digested with *Hin*dIII (lane 1), *Eco*RI (lane 2), or *Bam*HI (lane 3) probed with the radiolabeled 3.2-kb AA2 insert DNA. (*C*) Ethidium bromide-stained pulsed-field CHEF gel of yeast chromosomes (lane 1) and its Southern blot (lane 2). Chromosome locations are indicated in the middle. (*D*) Western blot of extracts using anti-β-galactosidase antibody. Lanes: 1, purified β-galactosidase protein (2 μg); 2, AA2 extract (20 μg); 3, λgt11 lysogen (BNN97) extract (20 μg). (*E*) Western blot of yeast extract (50 μg) using AA2 fusion protein affinity-purified antibody. The protein markers were phosphorylase b, bovine serum albumin, and ovalbumin, from the top of the gel.

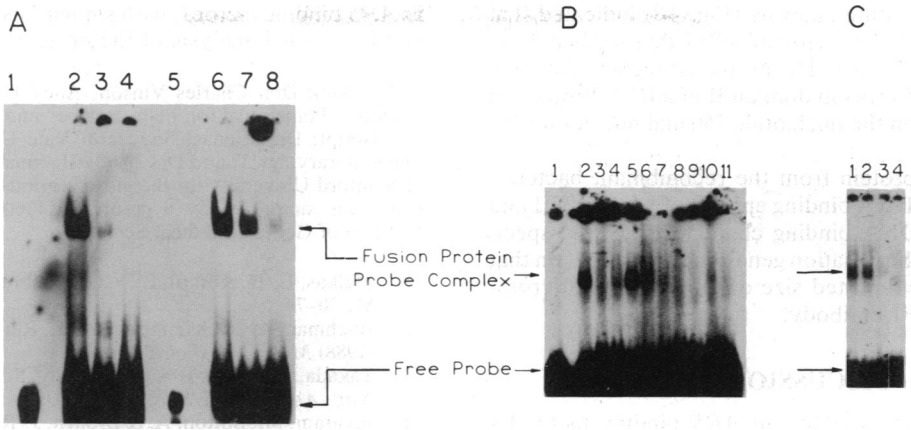

FIG. 3. Binding of oligonucleotides EI and F1 by the AA2 bacteriophage-encoded fusion protein. (*A*) Lanes 1–4 contained 0.2 ng of labeled oligonucleotide F1, 200 ng of crude fusion protein (except lane 1), 200 ng of poly(dI·dC), and oligonucleotide competitors as follows: none (lane 2), 10 ng of EI (lane 3), or 10 ng of F1 (lane 4). Lanes 5–8 contained 0.2 ng of oligonucleotide EI, 200 ng of crude fusion protein (except lane 5), 200 ng of poly(dI·dC), and oligonucleotide competitors as follows: none (lane 6), 10 ng of EI (lane 7), or 10 ng of F1 (lane 8). (*B* and *C*) Binding of oligonucleotide F1 to the crude fusion proteins of AA2-positive clone (lanes 2–6 in *B* and 1–4 in *C*) and a negative clone (lanes 7–11 in *B*) was challenged with various oligonucleotides. (*B*) Lanes 1–11 contained 0.2 ng of labeled oligonucleotide F1, 400 ng of fusion proteins (except lane 1), 250 ng of poly(dI·dC), and oligonucleotide or DNA competitors as follows: none (lanes 2 and 7), 20 ng of EI (lanes 3 and 8), 20 ng of F1 (lanes 4 and 9), 20 ng of EII (lanes 5 and 10), or 20 ng of CEN (lanes 6 and 11). (*C*) Lanes 1-4 contained the same components as lane 2 in *B* and competitors as follows: 20 ng of CEN (lane 1), 20 ng of EII (lane 2), 20 ng of mtIa (lane 3), or 2 ng of 237-bp fragment of *ARS1* (lane 4).

encoded fusion protein. β-Galactosidase from the λgt11 lysogen and purified β-galactosidase migrated identically (band II) and the protease-degraded fusion protein in lane 2 is shown as band III. The protease degradation resulted in some variations in the DNA binding with different batches of the extract. Western blot analysis (Fig. 2*E*) of yeast extract using antibody affinity-purified with the AA2 recombinant fusion protein detected a 130-kDa protein. Smaller polypeptides, presumably protease-degraded forms of OBF1, were also observed. The fusion protein cross-reacted strongly with an affinity-purified OBF1 antibody (data not shown). Antibody, after affinity purification using AA2 fusion protein, detected an ≈130-kDa polypeptide in the yeast extract (Fig. 2*E*). Two additional polypeptides of lower molecular mass were also detected that appeared to be protease-degraded forms of OBF1, because levels of these low molecular mass bands increased with storage of the extract.

Binding of the Fusion Protein to OBF1-Specific Oligonucleotides. The DNA binding properties and specificities of the fusion protein in the extract were analyzed by mobility-shift DNA binding assays using synthetic and native DNA sequences. Binding of the fusion protein to labeled oligonucleotides EI and F1 is shown in Fig. 3*A*. The fusion protein bound to both oligonucleotides with high specificity. In addition, oligonucleotide EI or F1 could inhibit the binding of the other to the fusion protein. To determine the specificity of binding, we analyzed other oligonucleotides as competitors of oligonucleotide F1 binding and the result is shown in Fig. 3*B*. Oligonucleotides EI and F1 acted as specific competitors of binding but oligonucleotides CEN and EII, specific for the centromere binding protein (20) and general regulation factor I (2), respectively, did not inhibit the binding. However, the binding of the fusion protein to oligonucleotide F1 was inhibited by a 237-bp fragment containing the domain B of *ARS1* (Fig. 3*C*). Oligonucleotide mtIa, a mutant form of the EI sequence (2), weakly inhibited the binding. Thus the fusion protein binding to oligonucleotides EI and F1 appeared to be sequence-specific.

Binding of the Fusion Protein to Domain B of *ARS1*. The fusion protein tightly bound a 237-bp *Hin*dIII–*Bgl* II fragment of *ARS1* that contains domain B. A titration of fusion protein binding is shown in Fig. 4*A*. The binding was carried out in the presence of 1.5 μg of poly(dI·dC) and 50 ng of oligonucleotide CEN. The binding of the *ARS1* fragment by the fusion protein appeared to be highly specific. At a higher level of extract a larger complex was detected, which is probably due to protein–protein interaction involving the fusion pro-

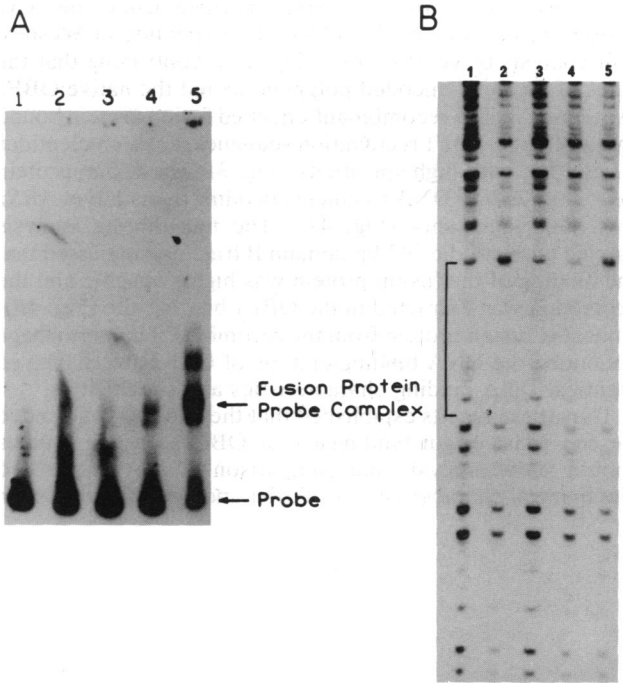

FIG. 4. Binding of domain B of *ARS1* to the AA2 bacteriophage-encoded fusion protein. (*A*) Binding of the fusion protein was carried out with a 237-bp fragment of *ARS1*. The binding reaction contained 5000 cpm of labeled DNA fragment, 500 ng of poly(dI·dC), 50 ng of oligonucleotide CEN (as nonspecific inhibitor), and crude fusion protein as follows: none (lane 1), 500 ng (lane 2), 1.0 μg (lane 3), 2 μg (lane 4), or 5 μg (lane 5). (*B*) DNase I footprint analysis of fusion protein binding to *ARS1*. The reaction mixture contained 25,000 cpm ^{32}P-labeled 237-bp *ARS1* fragment, 2 μg of poly(dI·dC), 50 ng of oligonucleotide CEN, and crude binding protein as follows: no protein (lane 1), 5 μg of yeast nuclear extract (lane 2), 5 μg of BNN97 extract (lane 3), or 2 and 5 μg of AA2 extract, respectively (lanes 4 and 5). The digestion was carried out with 40 ng of DNase I for 60 sec at room temperature. The bracketed region indicates the OBF1 binding site in *ARS1*.

tein. DNase I footprinting analysis (Fig. 4*B*) indicated that fusion protein protected a region of *ARS1* that was similarly protected by OBF1 of yeast. The protected region (shown in the bracket in Fig. 4*B*) was in domain B of *ARS1*. Protection was observed between the nucleotide 750 and nucleotide 780 of *ARS1* (21, 22).

Thus, the fusion protein from the recombinant bacteriophage contained the DNA binding epitope of yeast OBF1 and displayed identical DNA binding characteristics and specificities. In addition, the isolation gene coded for a protein that corresponded to the reported size of OBF1 (15) and cross-reacted with an OBF1 antibody.

DISCUSSION

We have isolated the gene for an *ARS* binding factor by screening a yeast genomic DNA library in λgt11 with synthetic recognition sequences of OBF1. We have isolated the complete gene by screening a yeast genomic DNA library in YEp24 plasmid and the restriction map of the gene is shown in Fig. 1*C*. Northern blot analysis with an isolated 3.2-kb insert DNA of the AA2 clone indicated that the mRNA is ≈3.8 kb, which could encode a protein of ≈130 kDa. The reported size of the purified OBF1 is ≈130 kDa (15). Southern blot analysis of restriction enzyme-digested yeast chromosomal DNA (Fig. 2*C*) indicated that the gene is a single-copy gene. Pulsed-field CHEF electrophoresis of yeast chromosomes and Southern blot analysis indicated that this gene is located in chromosome IV.

A polyclonal antibody, affinity-purified using the AA2 fusion protein, detected a 130-kDa polypeptide in Western blot analysis of yeast extract (Fig. 2*E*), confirming that the sizes of the gene-encoded polypeptide and the native OBF1 are identical. The recombinant-encoded fusion protein bound two synthetic OBF1 recognition sequences, oligonucleotides EI and F1, with high specificity (Fig. 3). The fusion protein bound the 237-bp DNA fragment encoding domain B of *ARS1* with high specificity (Fig. 4*A*). The footprinting analysis carried out with the 237-bp domain B fragment indicated that the binding of the fusion protein was highly specific and the protection was extracted in the OBF1 binding site (Fig. 4*B*). Thus, the fusion protein from the recombinant bacteriophage contained the DNA binding epitope of OBF1 and displayed identical DNA binding characteristics and specificities.

Thus these results established that the isolated gene coded for the yeast origin binding factor OBF1. Further studies should allow analysis and comparison of the genetic and biochemical characteristics of OBF1 and other factors, such as *ARS* binding factor I, with similar DNA binding properties and mutational analysis of the protein (23, 24).

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