ARS Binding Factor I of the Yeast *Saccharomyces cerevisiae* Binds to Sequences in Telomeric and Nontelomeric Autonomously Replicating Sequences

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We have analyzed various autonomously replicating sequences (ARSs) in yeast nuclear extract with ARS-specific synthetic oligonucleotides. The EI oligonucleotide sequence, which is derived from *HMRE-ARS*, and the F1 oligonucleotide sequence, which is derived from telomeric *ARS120*, appeared to bind to the same cellular factor with high specificity. In addition, each of these oligonucleotides was a competitive inhibitor of the binding of the other. Binding of the ARS binding factor (ABF) to either of these oligonucleotides was inhibited strongly by plasmids containing *ARS1* and telomeric TF1-ARS. DNase I footprinting analyses with yeast nuclear extract showed that EI and F1 oligonucleotides eliminated protection of the binding site of ARS binding factor I (ABFI) in domain B of *ARS1*. Sequence analyses of various telomeric (*ARS120* and TF1-ARS) and nontelomeric ARSs (*ARS1* and *HMRE-ARS*) showed the presence of consensus ABFI binding sites in the protein binding domains of all of these ARSs. Consequently, the ABFI and ABFI-like factors bind to these domain B-like sequences in a wide spectrum of ARSs, both telomeric and nontelomeric.

Sequence-specific DNA binding has been shown to be involved in the control of DNA replication and transcription in both procaryotic and eucaryotic systems (1–9, 15–17, 27–31, 34–38). The sequence-specific DNA binding of cellular factors presumably controls the initiation of DNA replication in these origin sites in a fashion similar to that observed with *Escherichia coli*, simian virus 40, and bacteriophage lambda DNA replication (1, 2, 15, 16, 22, 27, 33). In *E. coli*, the DnaA protein binds to specific sequences at the origin of DNA replication (*oriC*) and allows initiation of chromosomal DNA replication (22). The genome of the yeast *Saccharomyces cerevisiae* contains a large number of autonomously replicating sequences (ARSs), which appear to be the origins of chromosomal DNA replication (8, 19, 24–26, 32). Buchman et al. (9) have performed a detailed analysis of yeast proteins that bind to various ARS elements such as *ARS1*, *ARS2*, 2- μ m plasmid ARS, and the *HIS3-DED1* gene, as well as to various regulatory sequences in *HMRE*, *HMRI*, and *HMLI*. Buchman et al. (9) have identified specific binding sites in various yeast DNA fragments for two DNA binding factors: ARS binding factor I (ABFI) and general regulatory factor I. These studies have established a consensus binding sequence, 5'-TATCATTNNNNACGA-3', for ABFI.

Eisenberg et al. (18) and Francesconi and Eisenberg (20) have reported the identification and purification of origin binding factor 1 (OBF1) that binds to sequences in the telomeric *ARS120*, *ARS131C*, and *ARS131L*. Although there are a large number of ARSs in *S. cerevisiae*, it remains speculative whether there are a number of ABFs in *S. cerevisiae* and, if so, how many such factors exist. Therefore, we have explored the probable ABFI-like ABFs with synthetic oligonucleotides and various ARSs. It was necessary to analyze ABFI-like proteins directly with nuclear extract, rather than with purified ABFI or OBF1 protein, so that competition experiments may allow discrimination be-

tween similar factors. Buchman et al. (9) used a similar strategy for the identification and analysis of ABFI and general regulatory factor I and their numerous binding sites in *S. cerevisiae*. The use of well-defined synthetic oligonucleotides eliminated probable binding by other putative factors such as ARS binding factor II (ABFII; 14).

MATERIALS AND METHODS

Chemicals, reagents, and enzymes. All chemicals used to prepare buffers were of analytical reagent grade and were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) or J. T. Baker Chemical Co. (Phillipsburg, N.J.). Radioisotopes were purchased from Amersham Corp. (Arlington Heights, Ill.). All restriction enzymes were obtained from LKB-Pharmacia, and T4 polynucleotide kinase was obtained from U.S. Biochemicals.

Buffers. Buffer A contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 50 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μ g each of pepstatin A and leupeptin per ml. Buffer B contained 6.7 mM Tris hydrochloride (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA. Buffer C contained 50 mM Tris hydrochloride (pH 8.0), 10% glycerol, 100 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 1 mM dithiothreitol, and 2 μ g each of leupeptin and pepstatin A per ml.

Oligonucleotides. We synthesized oligonucleotides corresponding to the sequences shown in Table 1 in a BIO-SEARCH CYCLONE DNA synthesizer. The oligonucleotides were further purified by electrophoresis on a 20% polyacrylamide–8 M urea gel (21 by 50 by 0.4 cm).

Plasmids and DNA probes. M13-ARS, containing an 837-base-pair (bp) *HindIII*-*EcoRI* fragment of *ARS1*, was subcloned in M13SK phagemid (Stratagene, La Jolla, Calif.) as previously described (3). Construction of TF1-ARS and TF5-ARS plasmids was as previously described (10, 11). The *HindIII*-*BglII* fragment containing domain B of *ARS1* was isolated from M13-ARS by restriction enzyme digestion. The

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TABLE 1. Sequences of oligonucleotides used in this study

Oligo-nucleotide	Sequence ^a	Reference
EI	GATC CAAT ACAT CATA AAAT ACGA	9
EII	GATC TTAT ATTG CAAA AACC CATC AACC TTG	9
CEN	AATT AAAA TAGA TCAC GTGA TCTA TTTT	6
mtIa	CAAT ACAT CATA AAAA TACG AACG ATC	9
F1	GATC CAAG TGCC GTGC ATAA TGAT GTGG G	18

^a One strand of each oligonucleotide is shown in 5' to 3' at left to right direction as shown in the references.

substrates for footprinting were generated by asymmetric labeling of the *Hind*III-*Bgl*II fragment with T4 polynucleotide kinase and [γ -³²P]ATP.

Yeast nuclear extract. Yeast nuclear extract was prepared from strain BJ2168 (Yeast Genetic Stock Center, University of California, Berkeley, Calif.) by the method of Bram and Kornberg (5) as modified by Buchman et al. (9). The concentration of the dialyzed protein was 2 to 10 mg/ml in buffer C.

Gel retardation DNA binding assays (21, 23). The binding reactions were carried out in a 25- μ l final volume of 0.5 $\times$ buffer A containing the indicated amount of yeast nuclear extract, 200 pg of labeled oligonucleotide (0.1 μ Ci/ng), and 500 ng of poly(dI $\cdot$ dC). The binding reactions were allowed to proceed for 15 min at room temperature, after which 2 μ l of 0.1% bromophenol blue in 0.5 $\times$ buffer A was added. The mixtures were immediately loaded on a 4 to 8% gradient polyacrylamide gel containing 1 $\times$ buffer B. Electrophoresis was carried out at 30 mA for 1.5 h at ambient temperature in buffer B. After the gels were electrophoresed, they were dried and autoradiographed with Kodak XAR-5 film. In competition experiments, the competitor DNA was added to the extract and incubated for 5 min at room temperature prior to the addition of the labeled probe.

In binding studies involving domain B of *ARS1* (237-bp *Hind*III-*Bgl*II fragment), the binding was carried out as described above, except that 1.5 μ g of poly(dI $\cdot$ dC) and 50 ng of CEN oligonucleotide (6, 12) were added as nonspecific inhibitors, and the bound DNA was separated from free DNA by electrophoresis in a 1.5% agarose gel in 0.5 $\times$ TBE (45 mM Tris hydrochloride, 45 mM boric acid, 1 mM EDTA [final pH, 8.3]) at a constant current of 75 mA for approximately 1.5 h at 4°C. The gel was dried and autoradiographed as described above.

DNase I footprinting analysis. Binding reactions were performed with the 237-bp fragment of *ARS1* domain B essentially as described in gel retardation DNA binding assays with 20,000 cpm of ³²P-labeled (at the *Bgl*II site) fragment per reaction. Digestion of the protein-DNA complexes with DNase I, electrophoresis, and autoradiography was carried out as previously described (9).

RESULTS

Binding of EI and F1 oligonucleotide sequences in the yeast nuclear extract. The sequences of various synthetic oligonucleotides used to study the binding of ABFs are shown in Table 1. The sequence of the EI oligonucleotide was derived from *HMRE-ARS*, as described by Buchman et al. (9), and

FIG. 1. F1 oligonucleotide binding and challenge with EI and F1 oligonucleotides. Gel retardation DNA binding assay was carried out as described in Materials and Methods with 2 μ g of yeast nuclear extract (except in lane 1), 1.5 μ g of poly(dI $\cdot$ dC), and 200 pg of ³²P-labeled F1 oligonucleotide. Competitor oligonucleotides in the reaction mixture in each lane were as follows. Lane 1, No extract; lanes 2 through 12, 2 μ g of yeast nuclear extract and competitors as follows. Lane 2, None; lanes 3 through 7, EI oligonucleotide (0.5, 1, 2, 5, and 10 ng, respectively); lanes 8 through 12, F1 oligonucleotide (0.5, 1, 2, 5, and 10 ng, respectively).

the sequence of the F1 oligonucleotide was derived from the OBF1 binding sequence in *ARS120*, as described by Eisenberg et al. (18). Specific binding of EI and F1 oligonucleotides by cellular proteins was observed in yeast nuclear extract (Fig. 1 and 2). The binding was not inhibited by 1,000-fold excess of poly(dI $\cdot$ dC) or sheared calf thymus DNA (data not shown). However, the binding was inhibited by unlabeled EI or F1 oligonucleotides (Fig. 1). Titrations of F1 oligonucleotide binding with unlabeled EI or F1 oligonucleotides indicated that the patterns of inhibition with either of them were comparable. The binding of the EI oligonucleotide by an ABF in the yeast nuclear extract was specific, as demonstrated by a mobility shift DNA binding analysis (Fig.

FIG. 2. EI oligonucleotide binding and challenge with EI and F1 oligonucleotide. Assays were carried out as described in the legend to Fig. 1, except that 200 pg of ³²P-labeled EI oligonucleotide was substituted for F1 oligonucleotide, and competitors were added as follows. Lane 1, No competitor; lanes 2 through 6, EI oligonucleotide (0.5, 1, 2, 5, and 10 ng, respectively); lanes 7 through 11, F1 oligonucleotide (0.5, 1, 2, 5, and 10 ng, respectively).

TABLE 2. Comparison of putative ABFI binding sequences in ARSs^a

ARSs	Binding sequences
<i>ARSI</i>	CCATTTTCTTAGCATTTTGGACGAAATT-3'
	:
El oligonucleotide	GATCCAATACATCATAAAATACGAACGA-3'
	: :
F1 oligonucleotide	TTAACCACATCATTATGCAGGCCACTTG-3'
	: : : : : : : : : : : : : : : : : : : :
<i>ARS120</i>	CTCGAGGCACCCACATCATTATGCACGGCACTTGC-3'
	: : : : : : : : : : :
TF1- <i>ARS</i>	ATGCACTCGCATCATTATGCACGGCTTGC-3'
Consensus ^b	TATCATNNNNACGA-3'

^a Symbols: |, homology with the consensus sequence; :, homology within the sequences.

^b Consensus refers to the consensus ABFI binding sequence proposed by Buchman et al. (9).

2). The binding was inhibited by either unlabeled EI or F1 oligonucleotide, and the patterns of inhibition were identical to that observed with F1 oligonucleotide binding (Fig. 1). Thus, it appeared from these experiments that the binding and the inhibition patterns of EI and F1 oligonucleotides in the yeast nuclear extract were identical (Fig. 1 and 2) and that the EI and F1 oligonucleotides were probably binding to the same ABF at the same binding site. It is important to note that the F1 oligonucleotide sequence is significantly homologous (as discussed in detail below) to the consensus ABFI binding sequence (Table 2), as determined by Buchman et al. (9). Moreover, the F1 oligonucleotide has a higher degree of homology to the consensus sequence than does the EI oligonucleotide (Table 2). As a result, the F1 oligonucleotide was at least as effective as, if not more effective than, EI in binding to the ABF, as well as in the inhibition of binding of the EI oligonucleotide.

Inhibition of binding of EI and F1 oligonucleotides by various ARS sequences. The specificity of binding of EI and F1 oligonucleotides to the ABF was examined by competition experiments with oligonucleotides that bind to other known cellular DNA binding factors (5, 9), such as EII and CEN oligonucleotides for general regulatory factor I and centromere binding protein, respectively (Table 1). Neither the EII oligonucleotide nor the CEN oligonucleotide (a highly AT-rich sequence) inhibited either F1 or EI oligonucleotide binding to the ABF (Fig. 3) when present at an 100-fold excess. The mtIa oligonucleotide, having a single point mutation in the EI sequence (9), somewhat inhibited the binding but much less efficiently than did the EI and F1 oligonucleotides. At an 1,000-fold excess (200 ng), nonspecific inhibition with various oligonucleotides was observed.

Inhibition of binding of EI and F1 oligonucleotides by *ARS1* and TF1-*ARS*. The binding of ABF to either of the EI or F1 oligonucleotides was inhibited by both 10 ng and 25 ng of M13-*ARS1* or TF1-*ARS* (10, 11) plasmids (Fig. 4A and B). Binding to either of these oligonucleotides was not inhibited by the M13SK phagemid (an *E. coli* phagemid) or by the TF5-*ARS* plasmid. Therefore, the binding of ABF to intact *ARS1* or TF1-*ARS* appeared to be far stronger than that of the EI and F1 oligonucleotides. In addition, titrations of *ARS1* and TF1-*ARS* in EI and F1 oligonucleotide binding indicated that the binding of ABF to TF1-*ARS* was even stronger than to *ARS1*. However, TF5-*ARS*, a deletion fragment of TF1-*ARS* with significantly lower ($\leq 20\%$) *ARS* activity, did not inhibit the binding.

Comparison of putative ABFI binding domains in telomeric

FIG. 3. Challenge of F1 oligonucleotide binding by various oligonucleotides. Assays were carried out as described in the legend to Fig. 1. (A) Lane 1, no extract; lanes 2 through 8, 4 μ g of yeast nuclear extract and competitors as follows. Lane 2, None; lane 3, 5 ng of mtla oligonucleotide; lane 4, 5 ng of CEN oligonucleotide; lane 5, 5 ng of EII oligonucleotide; lane 6, 5 ng of F1 oligonucleotide; lane 7, 5 ng of EI oligonucleotide; lane 8, 2 ng of 237-bp *HindIII-BglII* fragment of *ARS1*. (B) Lane 1, No extract; lanes 2 through 10, 4 μ g of yeast nuclear extract with competitors as follows. Lane 2, None; lane 3, 20 ng of EII oligonucleotide; lane 4, 200 ng of EII oligonucleotide; lane 5, 20 ng of mtla oligonucleotide; lane 6, 200 ng of mtla oligonucleotide; lane 7, 20 ng of F1 oligonucleotide; lane 8, 200 ng of F1 oligonucleotide; lane 9, 20 ng of CEN oligonucleotide; lane 10, 200 ng of CEN oligonucleotide.

and nontelomeric ARSs. TF1-ARS contains a sequence, 5'-CATCATTATGCACGGC-3', located between nucleotides 816 and 830 (10, 11) that is remarkably homologous (underscored) to the core ABFI binding sequence proposed by Buchman et al. (9). This 16-bp sequence is also the central core of the F1 oligonucleotide and the OBF1 binding region of *ARS120* (Table 2). TF5-ARS, generated by an approximately 0.82-kilobase deletion of TF1-ARS, retains the consensus ARS core sequence (5'-TTTTATGTTT-3') located between nucleotides 1036 and 1048 (11). However, the deletion removed at least part of the consensus ABFI binding sequence (10, 11). This deletion resulted in the diminution of the in vivo ARS activity of this telomeric ARS to one-fifth that of TF1-ARS (10, 11; L. Button, personal communication). Consequently, the inability of the TF5-ARS to compete with the binding of EI and F1 oligonucleotides was likely due to partial deletion of ARS, and these results correlate well with the transformation efficiency of ARS. Therefore, the binding of ABF to ARS is not essential for minimal ARS function in the case of TF1-ARS. Rather, ABFI binding acts as an enhancer of ARS activity in vivo.

Inhibition of ABFI binding to domain B of *ARS1* by EI and F1 oligonucleotides. Buchman et al. (9) has shown that the ABFI binding site in *ARS1* is in domain B, which is contained within a 237-bp *HindIII-BglII* fragment (9). Binding of ABFI in the yeast nuclear extract to this fragment is shown by mobility shift gel analysis (Fig. 5). When this binding was challenged with EI and F1 oligonucleotides, both of the oligonucleotides acted as inhibitors of ABFI binding to

FIG. 4. Inhibition of EI and F1 oligonucleotide binding by *ARS1* and non-*ARS* plasmids. Assays were carried out as described in the legend to Fig. 1, except that 200 pg of 32 P-labeled F1 oligonucleotide was used in panel A and 200 pg of 32 P-labeled EI oligonucleotide was used in panel B. (A and B) Lane 1, No extract; lanes 2 through 8, 4 μ g of yeast nuclear extract and competitors as follows. Lane 2, None; lane 3, 10 ng of M13-*ARS1*; lane 4, 25 ng of M13-*ARS1*; lane 5, 10 ng of TF1-*ARS*; lane 6, 25 ng of TF1-*ARS*; lane 7, 10 ng of TF5-*ARS*; lane 8, 25 ng of TF5-*ARS*; lane 9, 10 ng of M13SK phagemid; lane 10, 25 ng of M13SK phagemid.

domain B of *ARS1*. These binding studies were carried out in the presence of poly(dI · dC) and CEN oligonucleotide as nonspecific inhibitors. Consequently, the observed inhibition of *ARS1* binding by EI and F1 oligonucleotides was highly sequence specific.

DNase I footprinting analysis of domain B of *ARS1*. In order to delineate the effects of EI and F1 oligonucleotides on the binding of ABFI to the 237-bp domain B of *ARS1*, we carried out DNase I footprinting analyses of the binding and competitions by EI, F1, and CEN oligonucleotides (Fig. 6).

FIG. 5. Inhibition of ABFI binding to domain B of *ARS1*. Assays were carried out as described in the legend to Fig. 1, except that the reaction contained 1 ng of 32 P-labeled (at the *Bgl*II site) 237-bp *Hind*III-*Bgl*II fragment of *ARS1*, 1.5 μ g of poly(dI · dC), 50 ng of CEN, and 4 μ g of yeast nuclear extract. Electrophoresis was carried out in a 1.5% agarose gel at 4°C. Lane 1, No extract; lanes 2 through 8, yeast nuclear extract and competitors as follows. Lane 2, No competitor; lane 3, 10 ng of F1 oligonucleotide; lane 4, 20 ng of F1 oligonucleotide; lane 5, 50 ng of F1 oligonucleotide; lane 6, 10 ng of EI oligonucleotide; lane 7, 20 ng of EI oligonucleotide; lane 8, 50 ng of EI oligonucleotide.

FIG. 6. DNase I footprinting analysis of ABFI binding to domain B of *ARS1*. DNase I footprinting analysis was carried out as described in Materials and Methods with the 237-bp *Hind*III-*Bgl*II fragment of *ARS1* labeled at the *Bgl*II site. The bracketed region at the right of the lanes indicates the protected region. Each reaction contained 20,000 cpm of labeled DNA, 1 μ g of yeast nuclear extract (except in lane 1), and 1.5 μ g of poly(dI · dC). Lane 1, No extract; lanes 2 through 5, yeast nuclear extract and competitors as follows. Lane 2, No competitor; lane 3, 25 ng of F1 oligonucleotide; lane 4, 25 ng of EI oligonucleotide; lane 5, 25 ng of CEN oligonucleotide.

ABFI in the yeast nuclear extract (Fig. 6, lane 2) produced a footprint similar to that described by Diffley and Stillman (14) and Buchman et al. (9). The DNase I protection of this region in *ARS1* was challenged with 25 ng each of F1 (Fig. 6, lane 3), EI (Fig. 6, lane 4), and CEN (Fig. 6, lane 5) oligonucleotides. Both EI and F1 oligonucleotides effectively eliminated the protection of the ABFI binding site, as was observed with the yeast nuclear extract, and the pattern was comparable with that observed with unprotected DNA (Fig. 6, lane 1). In contrast, CEN oligonucleotide, a nonspecific oligonucleotide, did not inhibit the protection of this region.

DISCUSSION

We have analyzed the sequence-specific DNA binding characteristics of ABFs in *S. cerevisiae* to telomeric and nontelomeric *ARS*s through a variety of competition experiments with sequence-specific synthetic oligonucleotides and plasmids containing *ARS1* and type X-telomeric TF1-*ARS* (from chromosome III; 10, 11). The binding of EI and F1 oligonucleotides to cellular proteins was highly specific (Fig. 1 and 2). The factor(s) that bound these two oligonucleotides migrated identically in the mobility shift DNA binding assay. EI and F1 oligonucleotides could compete in binding of either one in a comparable manner (Fig. 1 and 2). Therefore, these two oligonucleotides were indistinguishable in the mobility shift DNA binding assays and competition experiments. The binding of the F1 oligonucleotide appeared to be somewhat stronger than that of the EI nucleotide and, as a result, was a stronger inhibitor. Both *ARS1* and TF1-*ARS*

competed efficiently in the binding of either of these oligonucleotides to the ABF(s). On the other hand, TF5-ARS, an approximately 0.82-kilobase deletion mutant of TF1-ARS (11), and a bacterial plasmid (M13SK) without any ARS did not compete at all.

DNase I footprinting analysis of *ARS1* by ABFI in the yeast nuclear extract demonstrated a protected region similar to that delineated by Diffley and Stillman (14). The EI and F1 oligonucleotides appeared to compete effectively for this protected region and eliminated the protection (Fig. 6). On the other hand, the nonspecific CEN oligonucleotide did not inhibit ABFI protection. Therefore, the EI and F1 oligonucleotides bound to ABFI and blocked ABFI binding to *ARS1*.

Comparison of the putative ABF binding sequences in telomeric and nontelomeric ARSs (Table 2) showed that all of these ARSs have significant homology with the consensus ABFI binding sequence proposed by Buchman et al. (9). It is interesting to note that the two telomeric ARSs that we analyzed showed a higher degree of homology with the consensus sequence than did *ARS1* or *HMRE-ARS*. This sequence analysis indicated that, contrary to earlier belief, ABFI and OBF1 both bind to the same consensus sequence that has been defined as the consensus ABFI binding sequence (9). It should be pointed out that the purification of the OBF1 protein was carried out by affinity chromatography on immobilized DNA that essentially contained multiple copies of this consensus ABFI binding sequence (20).

These results, taken together, suggested that the EI and F1 oligonucleotides bound to the same or similar ABFs. Our studies demonstrated that the two previously reported ABFs, ABFI and OBF1, were indistinguishable as far as their DNA binding properties and sequence specificities are concerned. However, the possibility of the existence of a family of such ABFs could not be eliminated at the present time. As far as the identity between ABFI and OBF1 is concerned, Francesconi and Eisenberg (20) indicated that the purified OBF1 protein also bound to *ARS1* and *HMRE-ARS*; thus, the possibility exists that the ABFI and OBF1 proteins are similar. This proposal is strongly supported by the results presented in this report.

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