Purification of a yeast protein that binds to origins of DNA replication and a transcriptional silencer

(autonomously replicating-sequence-binding factor/Saccharomyces cerevisiae/mating type regulation/site-specific DNA binding)

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ABSTRACT Two factors that interact specifically with the chromosomal replicator, autonomously replicating sequence 1 (ARS1), were identified in whole-cell yeast extracts. One of these proteins, ARS binding factor I (ABF-I), was purified to homogeneity as a polypeptide of 135 kDa. ABF-I binds within a region previously shown to be essential for the function of ARS1 when yeast are grown under certain nutritional conditions, and deletion analysis presented here suggests that the ARS1 domain containing the ABF-I-binding site, domain B, is a complex element composed of at least two subdomains. ABF-I also binds within an ARS at the HMR silent mating locus. This ARS functions as a cis-acting transcriptional silencer, and the ABF-I-binding site within this ARS is important for both ARS and silencer function.

Eukaryotic DNA replication initiates from multiple origins once and only once during a discrete part of the cell cycle S phase. Both the number of origins used and the temporal activation of origins during S phase can vary enormously in response to conditions such as developmental state (1, 2). The molecular mechanism by which eukaryotic chromosomal DNA replication initiates is at present not known; however, the direct demonstration that autonomously replicating sequences (ARS) in the yeast Saccharomyces cerevisiae act in vivo as origins of replication (3–5) provides an experimental system to study chromosomal DNA replication.

Sequence requirements for ARS function suggest that eukaryotic origins of replication are complex (6–10). All ARSs contain and absolutely require the ARS core consensus sequence (11) which alone is insufficient for efficient ARS activity. Sequences flanking the core are important for ARS function, and functional redundancy often appears in these flanking sequences. ARS1 is located at the 3' end of the TRP1 gene, and has been shown by deletion analysis to consist of three functional domains designated A, B, and C (6). Domain A contains the ARS core sequence and is essential for ARS1 function. Domain B is an 84-base-pair (bp) region located 29 bp 3' to the ARS core sequence and is extremely important for efficient ARS activity. Recently, this domain was shown to contain a binding site for a protein identified in crude extracts (11), which overlaps both a region of bent DNA (12) and a sequence conserved among many ARSs (13). Domain C is located further than 100 bp 3' to the ARS consensus sequence and has a smaller effect on ARS1 function. Domains B and C are functionally redundant because domain A will function with either domain B or C, although domain B appears to be more efficient in this capacity (6). Furthermore, deletion analysis within domain B indicates that it may contain distinct functional elements.

For example, a sequence within domain B that contains the binding site for the protein here described was essential for ARS function when galactose was the primary carbon source, but this sequence appeared less important when glucose was the primary carbon source (12).

Origin-specific DNA binding proteins are important components of most prokaryotic and eukaryotic viral replication systems (14–24). Interestingly, in addition to being required for DNA replication, they can often exert either positive or negative effects on transcription (24–29). Because specific DNA sequences are required for ARS function, proteins that bind specifically to these sequences probably have a role in eukaryotic DNA replication. In this paper we describe two DNA-binding factors that interact specifically with ARS1.

MATERIALS AND METHODS

Strains and DNAs. The yeast strain used for the identification and purification of ARS binding factors I and II (ABF-I and ABF-II) was a protease-deficient strain BJ1991 (Mata leu2 ura3 trp1 prb1-I122 pep4B) from R. Kostriken (Cold Spring Harbor Laboratory).

The Rsa I (nucleotide nt 927)–HindIII (nt 616), the Rsa I (nt 927)–Pst I (nt 827) and the Rsa I (nt 927)–HindIII (nt 734) fragments of ARS1 (see Fig. 5) were cloned into pUC19 (30) to yield pARS1.1, pARS1.2, pARS1.3, and pARS1.4.1, respectively. A pARS1.4.1 fragment containing ARS1 sequences was excised with EcoRI and HindIII for use in DNA-binding assays. Bal-31 deletions were generated in domain B from either the HindIII site in the plasmid vector polylinker (ΔH series) or the Bgl II site at nt 854 (ΔB series) of pARS1.4.1. The HindIII site in the ΔH series and the Bgl II site in the ΔB series were replaced with HindIII or Bgl II linkers, respectively. These domain-B deletions contained on a HindIII–Bgl II fragment were then used to replace wild-type domain B in pARS1.4.1. To invert domain B with respect to domain A (pARS1.4.1), the Bgl II (nt 854)–BamHI (pUC polylinker) fragment was cut out of pARS1.4.1, religated, and recut with Bgl II and BamHI to eliminate religation in the wild-type orientation before transformation. The pARS1.7 series was constructed by ligating random, total Sau3A-digested pBR322 into the Bgl II site of pARS1.4.1. The EcoRI–HindIII fragment containing wild-type domain A and mutated domain B was then inserted into yeast plasmid YRp14/CEN4 between the unique EcoRI and HindIII sites for use in the colony color assay described by Hieter et al. (31).

DNA-Binding Assay. DNA-binding reactions were formulated as described using 10–20 ng of labeled DNA probe (32) and subjected to electrophoresis in low-ionic-strength poly-

Abbreviations: ARS, autonomously replicating sequence; ABF-I and ABF-II proteins, ARS binding factors I and II; SBF-B, silencer binding factor B; HMR, ARS at E region of HMR silent mating locus; ΔH and ΔB, deletions generated from HindIII site or Bgl II site, respectively.

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acrylamide gels essentially as described (33). DNase I "foot-
printing" (34) was done as described (32) without added
competitor DNA. Recovery and renaturation of ABF-I from
NaDodSO4/polyacrylamide gels (35) was essentially by the
method of Hager and Burgess (36). Silver staining of Na-
DodSO4/polyacrylamide gels was by the method of Oakley
et al. (37).

Preparation of Extracts and Purification of ABF-I. Cells
were grown to late logarithmic phase at 30°C in yeast extract/
peptone/dextrose (YPD) medium, harvested by centrifuga-
tion, resuspended in cold phosphate-buffered saline, har-
vested again, and stored at -70°C. Cells from 36 liters of
yeast were thawed in 2-3 volumes of buffer X (50 mM Pipes
(piperazinediethanesulfonate)/10 mM sodium metabisulfite/1
mM phenylmethylsulfonyl fluoride/10 mM dithiothreitol/1
mM EDTA/20% (vol/vol) glycerol) and disrupted in a bead
beater (Biospec Products, Bartlesville, OK) with an equal
volume of acid-washed glass beads in 10 30-sec bursts.
Extracts were diluted to 0.2 M (NH4)2SO4 with 4 M stock.
After 30 min on ice, cell debris was removed by centrifugation
at 10,000 × g for 20 min. Conductivity was adjusted to 160
mM (NH4)2SO4 with buffer X and loaded onto a column of
heparin-Sepharose (38) (4 × 18 cm) in buffer X containing 160
mM (NH4)2SO4. The column was washed with 200 ml of
buffer X containing 160 mM (NH4)2SO4 and developed with a
1.5-liter linear gradient from 160 to 500 mM (NH4)2SO4 in
buffer X. ABF-I and ABF-II DNA-binding activities eluted at
≈300 mM (NH4)2SO4. Active fractions were combined, ad-
justed to 300 mM (NH4)2SO4, and loaded onto a column of
Blue Sepharose CL-6B, Pharmacia (2.2 × 6 cm) in buffer X
containing 300 mM (NH4)2SO4. The column was washed with
50 ml of buffer X containing 300 mM (NH4)2SO4. These
fractions contain all the ABF-I activity. The column was
subsequently washed with 50 ml of buffer X/1.0 M NaCl, and
ABF-II was eluted with 50 ml of buffer X/2.5 M NaCl/5%
Triton X-100. The ABF-I fraction was adjusted to 1.0 M
(NH4)2SO4 and loaded onto a phenyl-Sepharose CL-4B col-
umn (3 × 8 cm; Pharmacia) in buffer X containing 1 M
(NH4)2SO4. The column was washed with 50 ml of buffer X
containing 0.5 M (NH4)2SO4, and ABF-I was eluted with a
500-ml linear gradient from 0.5 to 0.0 M (NH4)2SO4 in buf-
fer X. ABF-I elutes in a broad peak centered at 400 mM
(NH4)2SO4. Active fractions were combined and dialyzed
against buffer X containing 50 mM NaCl. ABF-I was loaded
onto a denatured calf thymus DNA cellulose column (39) (2.2
× 5 cm) equilibrated in buffer X containing 50 mM NaCl.
The column was washed with 20 ml of buffer X containing 50
mM NaCl, and ABF-I was eluted at 160 mM NaCl with an 80-ml
linear gradient from 50 to 350 mM NaCl in buffer X. Active
fractions were dialyzed overnight against buffer X containing
500 mM NaCl and 30% (wt/vol) sucrose and loaded (5 ml)
on a Sephacryl S-300 column (2 × 90 cm; Pharmacia) in
buffer X containing 500 mM NaCl; the column was run at 10
ml/hr, and 2.5-ml fractions were collected. Active fractions
were dialyzed overnight against buffer X and loaded onto a
Mono Q 5/5 column (Pharmacia) in buffer X, washed with 3
ml of buffer X containing 80 mM NaCl, and eluted with a
10-ml linear gradient from 80 to 350 mM NaCl in buffer X. Active
fractions were combined, aliquoted, and stored at
-70°C.

RESULTS

ARSJ-Specific DNA-Binding Activities. ARS/ specific
DNA–protein complexes were identified after electrophores-

Fig. 1. Detection of ARSj-specific DNA–protein interactions
with a crude whole-cell extract by gel retention analysis. Whole-cell
yeast extract was titrated into DNA-binding reactions formulated
and processed as described. Labeled DNA was either the EcoRI-
HindIII fragment from pARSJ.4.1 that contains domains A and B of
ARSj (A) or the BamHl-Sal I fragment from pBR322 (B).

Fig. 2. Competition of the ABF-I–ARSj complex by other
ARSs. A partially purified fraction of ABF-I equivalent to the
denatured DNA cellulose fraction was added to reactions formu-
lated as described containing additional varying amounts of linear-
ized plasmids (from 3 to 8.5 kilobases in size); pARSJ.2 (c), pBR322
(0), YRp14/CEN4/histone H4 ARS (a), YRp14/CEN4/histone
H2B ARS (a), the HindIII fragment containing HMR in pBR322 (a),
and YEp213 (2-μm origin of replication) (c). Bands corresponding to
the ABF-I–ARSj complex were excised from gels after autoradiog-
raphy, and amounts of radioactivity in the complex were quan-
titated by liquid scintillation counting. Results are plotted as % of
ABF-I–ARSj complex without plasmid competitor.
mating type cassette (data not shown), only the HMR silent mating type cassette, which has ARS activity, could compete with ARSl efficiently and therefore must contain at least one ABF-I binding site. A high-affinity ABF-I binding site at HMR was subsequently localized with purified ABF-I (see below).

**Purification of ABF-I.** The purification procedure described in Materials and Methods was developed to purify ABF-I to homogeneity. ABF-I and ABF-II cochromatograph during the first step, heparin–agarose, which results in a 50- to 100-fold purification, and these activities were subsequently separated by Blue Sepharose chromatography. ABF-I was further purified as described above to yield ~5 µg of homogeneous protein from 36 liters (~600 g (wet weight) of cells) of yeast in late-logarithmic phase. Although accurate measurement of DNA-binding activity in the relatively crude fractions is difficult, we estimate roughly a 30,000- to 50,000-fold purification based on specific activity.

In Fig. 3 A and B a Mono Q column profile is shown. The elution of a 135-kDa polypeptide as judged by silver-stained NaDodSO4/PAGE (Fig. 3B) corresponds exactly with the elution of ARS1-specific DNA-binding activity (Fig. 3A), arguing strongly that the 135-kDa polypeptide is the active ABF-I protein. To demonstrate this point unambiguously, the 135-kDa polypeptide was recovered from a preparative NaDodSO4/polyacrylamide gel and renatured. The gel-purified and renatured 135-kDa polypeptide formed a complex with ARS1 DNA that migrated through low-ionic strength nondenaturing gels at an identical position to authentic ABF-I activity from the Mono Q fraction (Fig. 3C). Approximately 30% of the ABF-I activity could be recovered in pilot denaturation-renaturation experiments without gel isolation (Fig. 3C, lane 3), and at least half of that could be recovered after gel isolation of the 135-kDa polypeptide. Furthermore, plasmids containing ARS1 and HMR (Fig. 3C, lanes 8 and 11) but not vector (pBR322) or plasmids containing ARS2, HML, histone H4 ARS, histone H2B ARS or the 2-µm origin of replication (lanes 7, 9, 10, 12-14) could compete with labeled ARS1 DNA for binding of the renatured protein. Therefore, ABF-I-binding activity resides in the 135-kDa polypeptide.

**ABF-I-Binding Sites at ARS1 and HMR.** DNase I footprinting with purified ABF-I was used to locate and define the ABF-I-binding sites at ARS1 and HMR (Fig. 4). This analysis demonstrates that ABF-I binds to a single site in ARS1 at the distal end of domain B and also at a single site in the ARS associated with HMR E region (HMR(E)). In Fig. 4B, the location and nucleotide sequence of the ABF-I-binding sites at ARS1 and HMR(E) is depicted.

**Functional Analysis of Domain B.** The ABF-I-binding site at ARS1 is essential when yeast were grown in medium containing galactose (12). We have examined the effect of mutations within domain B on ARS1 activity on medium containing glucose. Plasmid stability was measured as percent of plasmid lost per generation using the colony color assay described by Hieter et al. (31) used to indicate ARS strength. These results are summarized in Fig. 5. Domains A and B in the absence of domain C (pARS1.4.1) are lost at a rate of ~10% per generation compared with 5% loss per generation for the wild-type ARS1 (data not shown). Deletion of domain B (pARS1.3) results in a plasmid that exhibits an extremely high degree of instability, confirming the crucial role of domain B in ARS1 function (6). The ΔH5 mutation, which deletes nt 754 and is unable to bind ABF-I (data not shown), exhibited a small, but significantly decreased stability, when compared with wild-type domains A and B. Because this decrease in stability is considerably less than the decrease exhibited by the complete deletion of domain B, this domain appears to have at least two definable elements. One element contains the ABF-I-binding site and under these conditions has a small role in ARS function, whereas the other element containing sequences between the ABF-I-binding site and domain A has a dominant role in ARS function. The internal deletions ΔB3 and ΔB5 that remove nucleotides 818–852 and 783–852, respectively, also have severe effects on ARS function. Although insertion or deletion of 4 bp at the Bgl II site separating domains A and B had no effect on ARS activity, either insertion of any random pBR322 DNA from 195 to 1080 bp into this site or inversion of domain B with respect to domain A at this site completely inactivated domain B. Domain B function is, therefore, both orientation- and position-dependent with respect to domain A.

**DISCUSSION**

Two ARS1-specific DNA-binding factors (ABF-I and ABF-II) have been identified in crude extracts. These proteins are
Biochemistry: Diffley and Stillman

Fig. 4. DNase I footprinting of ABF-I at ARS1 and HMR. (A) DNA fragments for footprinting were 5' end-labeled with polynucleotide kinase by standard procedures, and footprinting reactions were done as described with 5 ng of the Mono Q fraction of ABF-I. For footprinting ARS1, the EcoRl-HindIII fragment of pARS1.2 was used, 5' end-labeled at either the HindIII (ARS1 top) or EcoRI (ARS1 bottom) site. For footprinting at HMR, either pA82.6A173 (HMRE, top) or pA82.6A224 (HMRE, bottom) linker scanning mutants labeled at the unique Xho I linker insertion site were used (35). Alongside each footprint is a diagram of each region with sequence features aligned as on sequencing gel. Hatched region represents ABF-I-binding site. The three groups of gels at left are 5%, and the HMRE bottom gel (at right) is 12.5% 8 M urea sequencing gel. (B) Positions and base-pair sequences of the ABF-I-binding sites (shaded regions) at ARS1 and HMRE with sequence features of these two regions (depicted to scale). The functional A and B domains at ARS1, and the A, E, and B elements at HMRE were previously described (20, 37). Stippled regions, location of the ARS core consensus sequence at these two ARSs. Arrows, extent of the ABF-I footprint. For optimal comparison to the ARS1-ABF-I binding site, HMRE site should be inverted.

ARS1-specific by virtue of the fact that they form complexes with ARS1 DNA, whereas equivalent complexes are not formed with pBR322 DNA. One of these proteins, ABF-I, has been purified to homogeneity by conventional chromatography as a polypeptide of 135 kDa. The other protein, ABF-II, has been purified as a 21-kDa protein that interacts specifically at multiple sites in ARS1 and will be reported elsewhere.

Fig. 5. Analysis of domain B function. ARS1 derivatives were constructed as described and are depicted. The percent plasmid loss per generation was determined by the half-sector method described by Hieter et al. (31). Regions deleted are indicated by gaps, vector sequences are indicated by thin lines, functionally important domains of ARS1 (A and B) (6, 7) are shown as boxes, and other yeast sequences in ARS1 are denoted by thick lines. The ABF-I-binding site in domain B is indicated by a hatched box, and the position of inserts in the pARS1.7 series is denoted by the wavy line. Final number in plasmid names of pARS1.7 series indicates size of inserted DNA.

specifically with ARS1 DNA, whereas equivalent complexes are not formed with pBR322 DNA. One of these proteins, ABF-I, has been purified to homogeneity by conventional chromatography as a polypeptide of 135 kDa. The other protein, ABF-II, has been purified as a 21-kDa protein that interacts specifically at multiple sites in ARS1 and will be reported elsewhere.

Competition analysis with seven of the best-characterized ARSs revealed that ABF-I is not a universal ARS-binding factor. However, this protein binds specifically to both ARS1 and an ARS at the HMR silent mating type locus (HMRE) at sites that are important for the efficient function of these ARSs and suggests that ARS1 and HMRE are members of a functionally related subset of all ARSs. The ABF-I-binding site at ARS1 lies within domain B, an 84-bp region previously shown to be important for ARS1 function. Snyder et al. (12) showed by deletion analysis that the ABF-I-binding site, which overlaps a region of bent DNA, is essential for ARS function when the primary carbon source for growth is galactose. In agreement, we demonstrate that a similar deletion exhibits only a small effect on ARS1 function when the primary carbon source for growth is glucose, whereas deletion of the rest of domain B still has a profound effect on ARS1 function. Taken together, these results suggest that domain B is a complex sequence element, the function of which is both position and orientation dependent with respect to domain A and which is composed of at least two subdomains. Although a function of the ABF-I-binding site could be to regulate specifically ARS1 function under certain growth conditions, more probably all ARSs function less well on poor carbon sources, where the differences between "weak" and "strong" ARSs are more pronounced. The role of the ABF-I-binding site in function of the HMRE is more clear. Small insertion/deletions that remove the ABF-I-binding site from HMRE profoundly decrease mitotic stability of plasmids containing HMRE, perhaps by reducing efficient replication of these plasmids (40).

HMRE, besides being an ARS element, is also required for repression of transcription of the mating type information contained at this locus (41, 42). HMRE can silence transcription in a position- and orientation-independent manner, and the silencer can function on heterologous promoters (42). The HMRE region itself has been further divided into three functional elements, A, E, and B (40) and it is within one of these, element B, that ABF-I binds. Importantly, there is functional redundancy among these elements because deletion of any one has little or no effect on silencer function, whereas deletion of any two elements completely inactivates
the silencer (40). These three elements alone are sufficient for ARS activity, and the functional overlap between elements required for silencing and ARS activity is striking. Element A contains the ARS consensus sequence and is essential for ARS activity on small cloned fragments containing these three elements (40). Recently, Shore et al. (11) identified two proteins in crude extracts designated silencer binding factors B and E (SBF-B and SBF-E) that interact with elements B and E, respectively. SBF-B, in addition to binding at HMRE, also binds to ARS1 within domain B, and the binding sites for both ABE-I and SBF-B at these two ARSs appear to be equivalent, although the potential identity of ABE-I and SBF-B awaits further characterization of SBF-B. In addition to importance for HMRE ARS function (see above), the ABE-I/SBF-B binding site becomes essential for silencer function in the absence of either the A or E elements, suggesting that ABE-I may have a role in both DNA replication and repression of transcription. Although the molecular mechanism of silencing is not understood, it is intriguing that silencer activity is so closely correlated with ARS function, particularly since reestablishment of the repressed transcriptional state at the silent mating type loci depends upon a round of DNA replication (43).

Because the HMRE silencer can function at either the 5' or 3' end of genes on any promoter (including the TRP1 promoter) (42) and with the A and B elements alone (40), it is tempting to speculate that ARS1, by virtue of its similarity to HMRE, may have a role in repressing TRP1 transcription. As for other replication origins (21, 24, 40), the relationship between transcription and replication at TRP1–ARS1 may prove quite interesting.

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