Structure–function analysis of the DNA binding domain of *Saccharomyces cerevisiae* ABF1

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Received March 21, 1995; Revised and Accepted June 21, 1995

ABSTRACT

To localize the DNA binding domain of the *Saccharomyces cerevisiae* Ars binding factor 1 (ABF1), a multifunctional DNA binding protein, plasmid constructs carrying point mutations and internal deletions in the ABF1 gene were generated and expressed in *Escherichia coli*. Normal and mutant ABF1 proteins were purified by affinity chromatography and their DNA binding activities were analyzed. The substitution of His61, Cys66 and His67 respectively, located in the zinc finger motif in the N-terminal region (amino acids 40–91), eliminated the DNA binding activity of ABF1 protein. Point mutations in the middle region of ABF1, specifically at Leu353, Leu360, Leu399, Tyr403, Gly404, Phe410 and Lys434, also eliminated or reduced DNA binding activity. However, the DNA binding activity of point mutants of Ser307, Ser496 and Glu649 was the same as that of wild-type ABF1 protein and deletion mutants of amino acids 200–265, between the zinc finger region and the middle region (residues 323–496) retained DNA binding activity. As a result, we confirmed that the DNA binding domain of ABF1 appears to be bipartite and another DNA binding motif, other than the zinc finger motif, is situated between amino acid residues 323 and 496.

INTRODUCTION

Eukaryotic promoters are regulated by the synergistic action of general transcription factors (GTFs), which form pre-initiation complexes at the TATA box, and activator (repressor) proteins, which bind elsewhere in the promoter (1). This synergistic control by GTFs and activator or repressor proteins determines the timing, the location and the level of gene expression and thus affects development and cell differentiation (1).

In the yeast *Saccharomyces cerevisiae* a number of transcriptional activator proteins have been characterized genetically and biochemically. The activator proteins can be broadly subdivided into two groups. The first group, which includes GAL4, GCN4 and so on, is present at low intracellular concentration and is

specifically required for the expression of only a limited number of genes. Members of the second group, such as ABF1, RAP1, CPF1 and REB1, are present at high intracellular concentration, are essential for cell growth and modulate transcription of various genes involved in many unrelated metabolic activities (2). Although these latter proteins often regulate transcription, they also act on DNA replication origins, telomeres and the mating type silencer and may carry out their diverse roles by affecting chromatin (2). Eukaryotic transcription factors typically consist of separate DNA binding domains and activation domains (3). DNA binding motifs include the zinc finger motif, the helix–turn–helix (HTH)/homeodomain, the basic/leucine zipper (bZIP) and others (4–10).

ABF1 protein specifically binds to double-stranded DNA with the consensus sequence 5'-RTCRYNNNNNACG-3', which is present within the UAS of many unrelated genes and also at many chromosomal origins of replication (ARSs) (11). At its binding site within the UAS of genes ABF1 weakly activates transcription in some cases and in other cases acts as a silencer, for instance at the silent mating type loci, *HMR* and *HML* (12–15). Accumulated evidence suggests that the ABF1 protein may also influence replication efficiency (16). The gene encoding ABF1 has been cloned from *S.cerevisiae* and sequenced (17). It encodes a 731 amino acid protein with a predicted molecular weight of 81.7 kDa. This is considerably less than the estimated size, 130 kDa, from SDS–PAGE (17). The ABF1 gene is essential for growth of *S.cerevisiae* (17).

Studies of various ABF1 mutant proteins suggested that all of the sequences responsible for specific DNA binding lie within the N-terminal two-thirds of the ABF1 protein (amino acid residues 1–530) (16,18). Within the N-terminal region of ABF1 (amino acid residues 40–91) there is an atypical zinc finger motif that has been implicated in DNA binding by mutation analysis and by demonstration of a requirement for zinc ion in binding assays (17–19). This limited region does not appear to be sufficient, however, leading to the conclusion that the ABF1 protein may contain an as yet uncharacterized DNA binding motif in addition to the zinc finger motif.

Here we report a novel DNA binding motif (amino acid residues 323–496) in the ABF1 protein and demonstrate a

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Figure 1. (A) Diagram of the pMAHW construct containing the *ABF1* gene. The number 4 refers to the position of the amino acid in the *ABF1* sequence. The *malE* gene (encoding MBP) was fused to the fourth codon of the *ABF1* gene. (B) Location and DNA binding activity of *ABF1* derivatives studied in this work. X indicates the mutation site. +++ represents DNA binding activity of wild-type *ABF1*. ++ represents lower DNA binding activity than wild-type *ABF1*. + represents very weak DNA binding activity. - represents no DNA binding activity. The blank box represents the middle region of the *ABF1* protein in which a DNA binding motif other than the zinc finger motif exists.

potential role for this sequence in DNA binding by gel retardation assay of several deletion and point mutants.

MATERIALS AND METHODS

Construction of expression vectors and generation of the *ABF1* mutants

The 4.5 kb *Bgl*III fragment of the *ABF1* gene from YEp24-3a (12) was cloned into the *Bam*HI site of M13mp18. This plasmid is referred to as M13mp18ABF1. It contains the 5' upstream region as well as the coding region of the *ABF1* gene. We introduced a *Hind*III restriction site at the beginning of the protein coding region, by using the mutagenesis method of Kunkel (20) with the DEL oligonucleotide (5'-CACGACTAAGCTTTCCATCCTT-GTGT-3'). The product of mutagenesis is called M13mp18ABF1H. An MBP (maltose binding protein)-ABF1

fusion construct was made by ligating the 3.2 kb *Hind*III fragment of M13mp18ABF1H into the *Hind*III site in vector pMAL c2 (New England BioLabs). The resulting plasmid, pMAHW, contains MBP fused to the fourth amino acid of *ABF1* (Fig. 1). Deletion and point mutants were generated by the method of Kunkel (20). Each mutant was characterized by DNA sequencing. The amino acids affected in each *ABF1* mutants are shown in Table 1 and Figure 1.

Expression and purification of the MBP-ABF1 fusion protein and its derivatives in *Escherichia coli*

Escherichia coli strain Y1090 [*supF hsdR araD139 Δlon ΔlacU169 rpsL trpC22::Tn10 (tet^r) pMc9*] was transformed with pMAHW encoding the MBP-ABF1 fusion protein and the mutant *ABF1* derivatives. The cells were induced with IPTG as recommended by New England BioLabs. After induction with

IPTG cells were harvested by centrifugation, column buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) was added and they were sonicated with an ultrasonicator for 1 min on ice. Bacterial lysates were incubated with amylose resin on ice for 30 min and washed with a 5-fold excess of column buffer, with respect to harvest cell volume. After washing the MBP-ABF1-bound amylose resin was incubated with column buffer containing 10 mM maltose for 30 min on ice. After centrifugation, the supernatant containing the MBP-ABF1 protein was treated with Factor Xa at 4°C for 17 h. The proteins were used for gel shift assays and other experiments

Western blot analysis

Partially purified MBP-ABF1 fusion proteins were electrophoresed by 7.5% SDS-PAGE. The proteins were transferred to nitrocellulose membrane. The membrane was blocked by incubation for 1 h in 5% non-fat dry milk in PBST (1.3 mM KH₂PO₄, 4.3 mM Na₂HPO₄, 2.7 mM KCl, 137 mM NaCl, 0.03% Tween 20). The membrane was then incubated for 2 h with an anti-ABF1 monoclonal antibody, mAb2E7E9 (17), supplied by Dr J. Campbell, or an anti-MBP polyclonal antibody, purchased from NEB Co., then washed three times in PBST. The membrane was incubated for 2 h with an anti-mouse horseradish peroxidase-conjugated secondary antibody (for mAb2E7E9) or anti-rabbit horseradish peroxidase-conjugated secondary antibody (for the anti-MBP polyclonal antibody), washed three times with PBST

and incubated for 5–10 min in horseradish peroxidase reaction solution (0.06% chloronaphtol, 20% methanol, 0.06% H₂O₂ in phosphate-buffered saline).

Gel retardation assay

Partially purified MBP-ABF1 protein was digested with Factor Xa at 4°C. DNA binding assays were performed as follows. One microgram of ABF1 protein was incubated with 0.25 ng ³²P-end-labeled B3 element double-stranded oligonucleotide (5'-GAATTCATTTCTTAGCATTTTTGACGAAATTTG-3') and a ³²P-end-labeled dimer of a double-stranded oligonucleotide (5'-TCGAGTCGCTGGTCACGAG-3') containing the ABF1 binding site in the yeast ADH1 coding region (21) in 20 µl binding buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 5% glycerol) on ice for 30 min. After the incubation, 20 ml of reaction mixture was loaded onto a 6% polyacrylamide gel containing 2% glycerol and 1× TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Before electrophoresis, pre-running was performed at 100 V for 1 h. Electrophoresis was performed at 33 mA and stopped when the bromophenol blue (BPB) dye was located 3 cm from the bottom of the gel. Bands were visualized by autoradiography. We used the B3 element and an oligonucleotide containing the ABF1 binding site in the yeast ADH1 coding region as competitors and salmon sperm DNA as non-specific competitor.

Table 1. Nucleotide and amino acid changes in the mutants

Mutant	Nucleotide affected	Nucleotide change	Amino acid change
Zinc finger motif region			
H61A	181	C→G	His61→Ala
C66R	196	T→C	Cys66→Arg
H67D	199	C→G	His67→Asp
K69E	205	G→A	Lys69→Glu
Middle region			
L353R	1057, 1058	C→A, T→G	Leu353→Arg
L360R	1081 1082	T→A, T→G	Leu360→Arg
L35360R	1057, 1058	C→A, T→G	Leu353→Arg
	1081, 1082	T→A, T→G	Leu360→Arg
Y403D	1204	T→G	Tyr404→Asp
Y403H	1204	T→C	Tyr404→His
K434T	1298	A→C	Lys434→Thr
L399E	1489, 1490	T→G, T→A	Leu399→Glu
G404K	1504, 1505	G→A, G→A	Gly404→Lys
F410P	1522, 1523	T→C, T→C	Phe410→Pro
S307A	1213	T→G	Ser307→Ala
S496A	1780, 1781	A→G, G→C	Ser496→Ala
C-terminal mutant			
E649K	1945	G→A	Asp609→Lys
Deletion mutant			
Δ200/249	D601–747		
Δ200/265	D601–796		

Southwestern blotting analysis

First, MBP-ABF1 fusion protein and Factor Xa-treated MBP-ABF1 protein were electrophoresed by 7.5% SDS-PAGE. Aliquots of 10 µg protein/lane were loaded on 0.7 mm thick protein minigels. The proteins in the electrophoresed gel were then electrophoretically transferred onto a nitrocellulose membrane and subsequently renatured on the membrane by treatment with and withdrawal of a protein denaturant, such as 6 M guanidinium chloride. The nitrocellulose membrane was incubated with Z buffer (25 mM HEPES-KOH, pH 7.6, 100 mM KCl, 12.5 mM MgCl₂, 10 µM ZnSO₄, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 0.25% bovine serum albumin) containing ³²P-labeled B element for 30 min and washed with Z buffer to remove non-binding probes. The nitrocellulose membrane was exposed to X-ray film.

RESULTS

DNA binding properties of the bacterially produced wild-type and mutant ABF1 proteins

ABF1 was mutagenized and MBP-ABF1 fusions were constructed as described in Materials and Methods. MBP-ABF1 fusion proteins purified through an amylose resin affinity column were analyzed by SDS-PAGE and protein blotting. As a result of fusion of the 43 kDa MBP and 130 kDa ABF1 the full-length MBP-ABF1 protein migrates as an ~170 kDa product (Fig. 2A). Bands appearing between 170 and 43 kDa most likely result from specific proteolysis of the ABF1 portion of the proteins, because only proteins containing the complete MBP bind to amylose resin (Fig. 2A). As illustrated in Figure 1, pMAHW provides the recognition site for restriction protease Factor Xa between MBP and ABF1. Treatment of the MBP-ABF1 fusion protein with Factor Xa at 4°C for 17 h resulted in loss of the 170 kDa MBP-ABF1 fusion protein band and generation of the expected 43 and 130 kDa bands (Fig. 2A). Western blot analysis using a monoclonal antibody generated against ABF1 confirmed that the 170 kDa protein was a MBP-ABF1 fusion protein. In contrast, the ABF1 protein in a crude extract of yeast had the mobility of the 130 kDa product (Fig. 2C). All proteins arising from point mutations were expressed at similar levels and had mobilities corresponding to 170 kDa (Fig. 2C and D). Two internal deletion mutant proteins were also expressed and had slightly increased electrophoretic mobilities compared with the MBP-ABF1 protein (Fig. 2C). Thus the proteins are sufficiently stable for analysis.

The monoclonal antibody bound weakly to the G404K mutant and did not appear to bind to the F410P mutant (Fig. 2D), suggesting that the epitope is not available to react with the antibody in these particular mutants. It is possible that the monoclonal antibody recognition site may be located in the 404-410 region of the ABF1 protein or that the structure of the G404K and F410P mutant proteins may be perturbed. However, Western blot analysis using an anti-MBP polyclonal antibody showed that the MBP-G404K and MBP-F410P mutants bound to the antibody as efficiently as the MBP-ABF1 wild-type protein (Fig. 2E). This result confirms that the G404K and F410P mutants were expressed as efficiently as the wild-type protein.

The ability of ABF1 produced by digestion of the MBP-ABF1 fusion with Factor Xa to bind specifically to its recognition site was tested in gel retardation assays using the synthetic oligo-

Figure 2. SDS-PAGE, Western blotting analysis and Southwestern blotting analysis of ABF1 fusion proteins and ABF1 derivatives purified by amylose resin affinity chromatography. (A) SDS-PAGE analysis of 5 µg purified wild-type ABF1 fusion protein (lane 1) and 5 µg fusion protein digested by Factor Xa (lane 2). The full-length MBP-ABF1 fusion proteins are indicated by a black arrow. The ABF1 protein digested by Factor Xa is indicated by an open arrow. The MBP protein digested by Factor Xa is indicated by a black arrowhead. (B) Southwestern blotting analysis of 5 µg purified wild-type ABF1 fusion protein (lane 1) and 5 µg fusion protein digested by Factor Xa (lane 2). (C) Western blotting analysis of wild-type and mutant proteins. Proteins were separated on 7.5% SDS-polyacrylamide gels. The ABF1 protein from yeast is indicated by a black arrow. The MBP-ABF1 fusion protein derivative expressed in *E. coli* is indicated by an open arrow. The anti-ABF1 monoclonal antibody was used in this experiment. Lane 1, 20 µg yeast crude extract; lanes 2-10, 1 µg MBP-ABF1 wild-type and mutant proteins expressed in *E. coli* and purified on amylose resin: wild-type, Δ200/249, Δ200/265, H61A, C66R, H67D, K69E, L353R and L360R respectively. (D) Lanes 1-11, wild-type MBP-ABF1, L35360R, K434T, Y403D, Y403H, E649K, S307A, S496A, L399E, G404K and F410P respectively. The concentration of protein loaded onto 7.5% SDS-polyacrylamide gels is as in (C). (E) Western blotting analysis of wild-type ABF1, G404K and F410P, expressed in *E. coli*, using the anti-MBP polyclonal antibody. Lanes 1 and 2, G404K and F410P respectively. The concentration of each mutant protein loaded to 7.5% SDS-polyacrylamide gels is 1 µg. Lane 3, 0.5 µg wild-type MBP-ABF1 protein was loaded onto 7.5% SDS-polyacrylamide gels.

nucleotide B3 element of ARS1 as probe. Gel retardation experiments using the B element of ARS1 and the ABF1 binding site in the ADH1 coding region as probe and specific competitor respectively were also performed in accordance with the method described above. ABF1 formed a specific complex with DNA fragments of the B element of ARS1 and the ABF1 binding site in the ADH1 coding region respectively, as judged by competition binding studies (Fig. 3). However, comparison of the gel retardation pattern of ABF1 expressed in *E.coli* and intact ABF1 protein present in yeast showed that their protein-DNA complexes migrated differently in the gel retardation assay (Fig. 3). To confirm that it was not the degraded product but the full-length ABF1 expressed in *E.coli* that bound to DNA, we performed a Southwestern blotting analysis. The result showed that full-length MBP-ABF1 protein bound to the B element at the 170 kDa position and ABF1 protein digested with Factor Xa bound to the B element at the 130 kDa position on 7.5% SDS-PAGE (Fig. 2B). We presume that the degree of post-translational modification in yeast was different from in *E.coli*. The DNA binding affinity of MBP-ABF1 protein was higher than that of ABF1 protein digested by Factor Xa on ice for 17 h. Thus the expression system is suitable for analyzing the ABF1 mutants.

His61, Cys66, His67 and Lys69 residues in the atypical zinc finger motif of ABF1 are important for DNA binding

Previously ABF1 protein was recognized to have a metal binding motif in its N-terminal region spanning amino acids 40–91 (16,18). This metal binding motif is different from the C4, H4 and C2H2 type zinc finger motifs that exist in other eukaryotic transcription factors, in that it contains a single finger. Three cysteine and three histidine residues, which could coordinate with a zinc ion, occur in the atypical zinc finger motif of ABF1 (amino acid residues 40–91). The cysteine residues are located at positions 49, 66 and 71 and the histidine residues are at 57, 61 and 67. Amino acids 49, 57 and 71 have been shown previously to be important for specific DNA binding (16,18).

To investigate whether other cysteine or histidine residues within this region of ABF1 play an important role in DNA binding, four proteins carrying the mutations H61A, C66R, H67D and K69E respectively were prepared. As expected, these mutant proteins could not bind to DNA specifically (Fig. 3C). We also performed gel shift assays under conditions of increased mutant protein concentration (at 20-fold the wild-type protein concentration). In spite of the increased protein concentration, these mutant proteins did not bind to DNA specifically (Fig. 5). These data suggest that the His61, Cys66, His67 and Lys69 residues in the atypical zinc finger motif of ABF1 are important for DNA binding and confirm that although this unusual zinc finger motif is not found in other known eukaryotic transcription factors, it is indispensable for DNA binding by ABF1.

The middle region of ABF1 is also important for DNA binding

Previous deletion analysis of the *ABF1* gene suggested that the entire N-terminal two-thirds of the ABF1 protein (residues 1–530), not simply the zinc finger motif, is necessary to retain DNA binding activity (18). We have therefore searched for additional DNA binding motifs. ABF1-like proteins which possess the ability to carry out all essential ABF1 functions in

Figure 3. DNA-protein binding analysis of ABF1 derivatives in the zinc finger motif. Competition DNA-protein binding analysis with double-stranded oligonucleotides corresponding to the ARS1 B element and the ABF1 binding site in the yeast ADH1 coding region (20). (A) Gel retardation analyses were performed with an α - 32 P-labeled ARS1 B element oligonucleotide in the presence of increasing amounts of a dimer of double-stranded oligonucleotide containing the ABF1 binding site in the yeast ADH1 coding region as competitor. Lane 1, α - 32 P-labeled free ARS1 oligonucleotide (0.25 ng); lane 2, binding mixture containing wild-type ABF1 protein (500 ng) and α - 32 P-labeled ARS1 oligonucleotide (0.25 ng); lanes 3 and 4, binding mixture containing 50- and 200-fold molar excesses of unlabeled ADH1 element respectively; lane 5, binding mixture containing 20 μ g yeast extract and α - 32 P-labeled ARS1 oligonucleotide (0.25 ng). (B) Gel retardation analyses were performed with an α - 32 P-labeled dimer oligonucleotide of the ADH1 coding region containing the ABF1 binding site in the presence of increasing amounts of ARS1 B element as competitor. Lane 1, α - 32 P-labeled free ADH1 element oligonucleotide (0.25 ng); lane 2, binding mixture containing wild-type ABF1 protein (500 ng) and α - 32 P-labeled ADH1 element oligonucleotide (0.25 ng); lanes 3 and 4, binding mixture containing 50- and 200-fold molar excesses of unlabeled ARS1 B element oligonucleotide respectively; lane 5, binding mixture containing 20 μ g yeast extract and α - 32 P-labeled ADH1 element oligonucleotide (0.25 ng). (C) Competition analysis using ARS1 B element oligonucleotide as probe and competitor. Lane 1, free α - 32 P-labeled ARS1 oligonucleotide (0.25 ng); lane 2, binding mixture containing wild-type ABF1 protein (500 ng) and α - 32 P-labeled ARS1 oligonucleotide (0.25 ng); lanes 3–7, binding mixture containing 2-, 5-, 10-, 20- and 50-fold molar excesses of unlabeled ARS1 B element oligonucleotide respectively; lanes 8–11, binding mixture containing each of the mutant proteins H61A, C66R, H67D and K69E respectively. The black arrowhead indicates the DNA-ABF1 complex expressed in *E.coli*. The blank arrowhead indicates the DNA-ABF1 complex which exists in yeast. Non-specific complex is indicated by the black arrow.

Figure 4. (A) DNA-protein binding analysis of internal deletion mutants. Lane 1, α - 32 P-labeled free ARS1 oligonucleotide (0.25 ng); lane 2, binding mixture containing wild-type ABF1 protein (1 μ g) and α - 32 P-labeled ARS1 oligonucleotide (0.25 ng); lane 3, Δ 200/249 ABF1 protein (3 μ g); lane 4, Δ 200/265 ABF1 protein (3 μ g). (B) DNA-protein binding analysis of ABF1 derivatives in the middle region. Lane 1, α - 32 P-labeled free ARS1 oligonucleotide (0.25 ng); lane 2, binding mixture containing wild-type ABF1 protein (1 μ g) and α - 32 P-labeled ARS1 oligonucleotide (0.25 ng); lanes 3–8, binding mixture containing each of the mutant proteins L353R, L360R, L353/360R, K434T, Y403D and Y403H respectively. (C) DNA-protein binding analysis of ABF1 derivatives in the middle region of ABF1. Lane 1, α - 32 P-labeled free ARS1 oligonucleotide (0.25 ng); lane 2, binding mixture containing wild-type ABF1 protein (1 μ g) and α - 32 P-labeled ARS1 oligonucleotide (0.25 ng); lanes 3–8, mutant proteins E649K, S307A, S496A, L399E, F410P and G404 K respectively.

Figure 5. DNA-protein binding analysis of increased concentrations of ABF1 derivatives which lost DNA binding activity. The experiment was performed as described in Methods and Materials, except the concentration of mutants protein was increased to 10 μ g. Lane 1, free α - 32 P-labeled ARS1 B element; lane 2, 0.5 μ g wild-type ABF1; lanes 3–14, H61A, C66R, H67D, K69E, L353R, L360R, K434T, L399E, G404K, F410P, Y403D and Y403H respectively.

S.cerevisiae have been identified in *Kluyveromyces lactis* and *K.marxianus* (22,23). Homology between the three proteins falls in two regions, first between amino acids residues 9 and 91 of *S.cerevisiae* ABF1 (*K.lactis* and *K.marxianus* ABF1 residues 4–91) and, second, between *S.cerevisiae* ABF1 residues 323 and 439 (*K.lactis* ABF1 residues 132–257 and *K.marxianus* ABF1 residues 151–277) (22). Thus *S.cerevisiae* ABF1 residues 91–323 are absent in *K.lactis* and *K.marxianus* ABF1. Thus residues 91–323 are probably not involved in DNA binding by ABF1 and we were led to predict that the putative additional DNA binding motif probably falls in the middle region of the ABF1 protein (residues 323–530). As a preliminary test of this prediction two internal deletion mutants, Δ 200/249 and Δ 200/265, were generated and expressed in *E.coli*. They were used to survey DNA binding activity. As anticipated, proteins carrying these internal deletions bind to the B3 element DNA (Fig. 4A).

Having confirmed that the amino acids spanning positions 200–265 are not necessary for binding to DNA, we deduced that the putative additional DNA binding motif is located between residues 265 and 530 and that the DNA binding domain of ABF1 is bipartite, consisting of the atypical N-terminal zinc finger and the additional region between positions 265 and 530. To survey amino acids in this latter region that might be involved in DNA binding we generated the following point mutants: S307A, L353R, L360R, L353/360R, L399E, Y403D, Y403H, G404K, F410P, K434E, K434T, S496A and C-terminal mutant E649K. These mutant proteins were analyzed by gel retardation assay. As shown in Figure 4B and C, mutants S307A, S496A, E649K exhibited weaker DNA binding activity than wild-type ABF1 protein, while remnant mutants could no longer specifically bind to DNA. The results suggest that Leu353, Leu360, Leu399, Tyr403, Gly404, Phe410 and Lys434 are amino acid residues

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
20	L	E	E	<u>L</u>	<u>A</u>	Q	K	<u>Y</u>	<u>G</u>	<u>M</u>	T	K	S	G	<u>L</u>	<u>E</u>	N	F	L	V	RepA
396	L	D	I	<u>L</u>	<u>M</u>	Q	R	<u>Y</u>	<u>G</u>	<u>L</u>	T	A	E	D	<u>E</u>	<u>E</u>	K	R	L	L	ABF1(S. C)
214	L	D	I	<u>I</u>	<u>K</u>	Q	R	<u>Y</u>	<u>G</u>	<u>L</u>	T	E	A	E	<u>M</u>	D	K	K	M	L	ABF1(K. L)
234	I	D	I	<u>I</u>	<u>K</u>	Q	R	<u>Y</u>	<u>G</u>	<u>L</u>	T	D	A	E	<u>M</u>	D	K	K	M	L	ABF1(K.M)
17	Q	A	E	<u>L</u>	<u>A</u>	Q	K	<u>V</u>	<u>G</u>	<u>I</u>	T	Q	Q	S	<u>I</u>	E	Q	L	E	N	434Rep
17	Q	T	E	<u>L</u>	<u>A</u>	T	K	<u>A</u>	<u>G</u>	<u>A</u>	L	Q	Q	S	<u>I</u>	Q	L	I	E	A	434CroA
33	Q	E	S	<u>V</u>	<u>A</u>	D	K	<u>M</u>	<u>G</u>	<u>M</u>	G	Q	S	G	<u>V</u>	G	A	L	F	N	Lamda RepN
16	Q	T	K	<u>T</u>	<u>A</u>	K	D	<u>L</u>	<u>G</u>	<u>V</u>	T	Q	S	A	<u>I</u>	N	K	A	I	H	Lamda CroH
169	R	Q	E	<u>I</u>	<u>G</u>	E	I	<u>V</u>	<u>G</u>	<u>C</u>	S	R	E	T	<u>V</u>	G	R	I	L	K	CAP
68	Q	R	E	<u>L</u>	<u>K</u>	N	E	<u>L</u>	<u>G</u>	<u>A</u>	G	I	A	T	<u>I</u>	T	R	G	S	N	Trp Rep
6	T	T	D	<u>V</u>	<u>A</u>	R	L	<u>A</u>	<u>G</u>	<u>V</u>	S	T	Q	T	<u>V</u>	S	R	V	V	N	Lac Rep
33	Q	R	S	<u>L</u>	<u>L</u>	K	A	<u>L</u>	<u>K</u>	<u>I</u>	S	H	V	S	<u>V</u>	S	Q	W	E	R	Dic A
22	M	K	D	<u>V</u>	<u>A</u>	L	K	<u>A</u>	<u>K</u>	<u>V</u>	S	T	A	T	<u>V</u>	S	R	A	L	M	CytR
				#	-			#	G	#					#						HTH consensus

Figure 6. Comparison of the putative HTH motif of ABF1 with several bacterial transcription factors that contain HTH motifs. Identical amino acids between sequences are shown in bold type. Identical stereochemical constraints to HTH amino acid residues are shown by underlining. The hydrophobic or possibly weakly polar residue is marked by #. The Gly residue is represented by G. The residue containing a non-branched side chain is marked -. The amino acid sequences were taken from Lawrence *et al.* (27) and Pabo and Sauer (32).

involved in DNA binding. The result of a gel shift assay performed under conditions of increased mutant protein concentration (20-fold the wild-type protein) showed that mutants Y403H and G404K at high protein concentration specifically bound DNA (Fig. 5). The evidence described above determined that the secondary DNA binding domain of ABF1 protein is situated within amino acid residues 323–496.

DISCUSSION

Assuming that the mutations we have introduced into ABF1 do not induce gross structural alterations in the protein, our results support the notion that ABF1 is composed of two distinct DNA binding motifs: one is a single potential zinc finger motif in the N-terminal region, the other is an unknown DNA binding motif in the middle region. Although we cannot rigorously exclude the possibility that the conformation of at least some of the mutant proteins produced in *E. coli* and partially purified was perturbed, resulting in their inability to bind to DNA, this interpretation is unlikely, because an undegraded protein could be easily detected by gel retardation assay in every case.

It is interesting, in view of our proposal that the DNA binding site of ABF1 is bipartite, that the DNA recognition site also seems to consist of two separate conserved domains, raising the question of whether the structure of the protein is reflected in the structure of the recognition site. The consensus recognition site for the ABF1 protein is 5'-RTCRYNNNNNACG-3', where R, Y and N represent a purine, a pyrimidine and any base respectively (11). Thus the conserved TC region is separated from the conserved ACG region by degenerate bases (11). The bipartite binding site may also underlie the recently described ability of ABF1 to bend DNA at multiple sites upon binding (24).

While considerable evidence suggesting the existence of some kind of metal binding/DNA binding motif in ABF1 has accumu-

lated (17–19), the putative helix–turn–helix motif in the middle region of the ABF1 protein which we describe here has not been previously recognized.

Although a SWISS-PROT protein database search revealed that there is homology between the putative helix–turn–helix consensus motif, similar to that of RepA protein, which is known to control plasmid copy number in *Lactobacillus planetarium* (25), and the amino acid sequence from positions 396 to 416 of ABF1, the calculated HTH score of ABF1 was negative (–1.4 SD, compared with RepA 2.95 SD), according to the method of Dodd and Egam (26) (a threshold of 2.5 SD has been proposed for an HTH DNA binding motif) (Fig. 6). This discrepancy may be caused by the presence of amino acids (M400, D409, F410 and E411) which are rare in the master set sequence for HTH motif detection.

The HTH detection method of Dodd *et al.* has achieved some success in making several predictions that were later confirmed and that have also aroused controversy (27). A previous report on LasR, which is a known intracellular receptor-type transcription factor, showed that although its SD score is low (0.4 SD), it contains the putative HTH DNA binding motif (28). Therefore, even if the SD score of the putative HTH motif of ABF1 is also low (–1.4 SD), a putative HTH motif of ABF1 of *S. cerevisiae* and *K. lactis* is consistent with the stereochemical constraints on the HTH consensus (29). The possibility of a putative HTH motif existing in ABF1 may be high. The rules are as follows: (i) the ninth residue must be a Gly (G); (ii) the fourth and fifteenth residues are completely buried within the protein and the eighth and tenth residues are almost buried (all four of these residues should be hydrophobic or possibly weakly polar); (iii) the helical third to seventh and fifteenth to twentieth residues are unlikely to be proline; (iv) the fifth residue should not have a branched side chain (29). Several *abf1* mutants were constructed based on these constraints.

In keeping with the above rules, the Y403D mutant in which we substituted the polar amino acid Asp for Tyr403 of ABF1 (eighth residue of the HTH motif) could not bind to DNA (Fig. 4C). In contrast, substitution of His, which is weakly polar, for Tyr403, resulted in a protein that could bind weakly to DNA. Leu399, Gly404 and Phe410, corresponding to residues 4, 9 and 15 of the consensus HTH motif, were also changed to other amino acids. A L399E, G404K, F410P mutant did not bind to DNA (Fig. 4C). The conserved ninth HTH residue Gly of mutant G404K was changed to Lys, violating rule (i) of the HTH motif. However, the G404K mutant also weakly bound to DNA under conditions of increased protein concentration (Fig. 5). This can be explained by the fact that in other HTH motifs, such as CytR and DcA (28,30,31), the ninth residue of the HTH motif is Lys instead of Gly. Therefore, the charged amino acids, violating constraint (ii) of the HTH motif, of mutants L399E and F410P were substituted for the hydrophobic amino acids which existed as the fourth and fifteenth residues of the HTH motif. The F410P mutant placed Pro in helix 2 of the HTH motif and as predicted binding was disrupted. The results of these mutant studies are consistent with the ABF1 protein containing an HTH motif in the middle region.

The presence of leucine residues at every seventh position in a peptide leads to the coiled coil configuration, often involved in protein-protein interaction and homodimer and heterodimer formation. Leu353, Leu360 and Leu367 of ABF1 do not contribute to dimerization, because ABF1 proteins do not form dimers. We think that the Leu residues play a role in interaction with other proteins (the transcription initiation complex). It is possible that L353 and L360, which fall outside the secondary DNA binding motif of ABF1, are nevertheless important for conformation stabilization, because DNA binding was lost in the L353R and L360R mutants. The presence of a basic region which contains four Lys amino acid residues at positions 431–434 of ABF1 is also important for ABF1 binding to DNA. Basic amino acids are necessary for proteins to recognize DNA, which has a negative charge. In mutant E649K the mutated amino acid residue lies outside the 530 amino acid region and this mutation does not affect DNA binding (Fig. 4C). This confirms the mutation analysis of Halfter *et al.*, which showed that the C-terminal region of ABF1 is not necessary for DNA binding activity (18).

ACKNOWLEDGEMENTS

This work was supported in part by the Korea Science and Engineering Foundation (grant 91-0500-06-01-3) and the SRC [the Research Center for Cell Differentiation (94-4-2)]. This work was also supported in part by US Public Health Service grant GM25008 to Judith L. Campbell.

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