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ABF1 Is a Phosphoprotein and Plays a Role in Carbon Source Control of *COX6* Transcription in *Saccharomyces cerevisiae*

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Previously, we have shown that the *Saccharomyces cerevisiae* DNA-binding protein ABF1 exists in at least two different electrophoretic forms (K. S. Sweder, P. R. Rhode, and J. L. Campbell, J. Biol. Chem. 263: 17270–17277, 1988). In this report, we show that these forms represent different states of phosphorylation of ABF1 and that at least four different phosphorylation states can be resolved electrophoretically. The ratios of these states to one another differ according to growth conditions and carbon source. Phosphorylation of ABF1 is therefore a regulated process. In nitrogen-starved cells or in cells grown on nonfermentable carbon sources (e.g., lactate), phosphorylated forms predominate, while in cells grown on fermentable carbon sources (e.g., glucose), dephosphorylated forms are enriched. The phosphorylation pattern is affected by mutations in the *SNF1-SSN6* pathway, which is involved in glucose repression-derepression. Whereas a functional *SNF1* gene, which encodes a protein kinase, is not required for the phosphorylation of ABF1, a functional *SSN6* gene is required for its dephosphorylation. The phosphorylation patterns that we have observed correlate with the regulation of a specific target gene, *COX6*, which encodes subunit VI of cytochrome *c* oxidase. Transcription of *COX6* is repressed by growth in medium containing a fermentable carbon source and is derepressed by growth in medium containing a nonfermentable carbon source. *COX6* repression-derepression is under the control of the *SNF1-SSN6* pathway. This carbon source regulation is exerted through domain 1, a region of the upstream activation sequence UAS_6 that binds ABF1 (J. D. Trawick, N. Kraut, F. Simon, and R. O. Poyton, Mol. Cell Biol. 12:2302–2314, 1992). We show that the greater the phosphorylation of ABF1, the greater the transcription of *COX6*. Furthermore, the ABF1-containing protein-DNA complexes formed at domain 1 differ according to the phosphorylation state of ABF1 and the carbon source on which the cells were grown. From these findings, we propose that the phosphorylation of ABF1 is involved in glucose repression-derepression of *COX6* transcription.

Recently, a class of abundant sequence-specific DNA-binding proteins has been identified in *Saccharomyces cerevisiae*. These proteins bind in the promoters of many genes as well as functionally diverse elements such as transcriptional silencers, origins of DNA replication (autonomously replicating sequences [ARSs]), and centromeres and telomeres, suggesting that they regulate a number of important nuclear processes. One such protein, ARS-binding factor 1 (ABF1), was initially identified by its interactions with regions at ARS1 and the *HMRE* silencer/ARS that are required for optimal ARS activity (4, 14, 38, 41). Subsequent studies in a number of laboratories have shown that ABF1 (also called SBF-B, TAF, SUF, GF1, and BAF1) specifically recognizes the motif RTCRYBNNNNACG (R = A or G, Y = C or T, and B = A, G, or T) at many sites in the yeast genome (2, 4, 5, 12, 13, 16, 22, 23, 27, 43). Depending on their context, these binding sites have been shown to be important in transcriptional activation or repression and plasmid segregation as well as in ARS activity. On the basis of these findings, it has been proposed that ABF1 plays a role in the coordination of gene expression with DNA synthesis and cell division (17).

ABF1 appears to regulate transcription in a complex promoter-specific manner. At the *HMRE* silencer, an ABF1 binding site (B element), in combination with other regulatory sequence elements, represses the transcription of the adjacent *HMR* locus (2, 27). However, when tested in a heterologous promoter, the B element was capable of weak transcriptional activation (2, 5). In addition, this element could combine with other weak activation sequences to form a strong constitutive activator (5). The role of the ABF1 binding site as a constitutive upstream activation sequence (UAS) has been demonstrated directly for several genes (13, 22, 23). ABF1 also plays a role in carbon source-regulated transcriptional control of some genes. One of these is *COX6*, the gene encoding subunit VI of the cytochrome *c* oxidase (33, 48). *COX6* is repressed by glucose (44, 49, 50), activated by heme (45), and down-regulated in the absence of a mitochondrial genome (19). Study of the *COX6* promoter has localized most of this regulation to UAS_6, located between −256 and −340 bp upstream of its initiation codon (44). Linker scanning mutagenesis of UAS_6 has revealed an ABF1 binding site, in domain 1 of UAS_6, that is important for glucose repression-depression (43). Another glucose-regulated gene with an ABF1 binding site is *ENO2* (3). In contrast to *COX6*, transcription of the *ENO2* gene is induced in cells grown in glucose in comparison with a nonfermentable carbon source (10). Analysis of the *ENO2* promoter has shown that an ABF1 binding site can play a role in glucose-regulated expression of this gene in certain promoter deletions (3, 10). Finally, Herruer et al. (24) found that an ABF1 binding site upstream of the glucose-inducible gene encoding

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ribosomal protein S33 appears to facilitate efficient transcription of this gene during changing nutritional conditions. How a single DNA-binding factor can mediate such a range of transcriptional effects is not known. However, given the complexity of the promoters that use ABF1, it seems likely that specific interactions between ABF1 and other DNA-binding factors (or other regulatory proteins) are responsible, at least in part, for these effects.

It is also possible that ABF1 exists in different functional states. Sweder et al. (41) showed that purified ABF1 consists of at least two electrophoretically distinct polypeptides, both capable of binding DNA specifically. Furthermore, preliminary experiments on the products of the *ABF1* gene suggested that the heterogeneity was the result of protein modification of a single polypeptide (34). Other transcription factors exhibit similar heterogeneity which appears to arise from differential phosphorylation (26, 30, 31, 40). Of particular interest is the yeast transcription factor GAL4, whose phosphorylated state is regulated by carbon source (30). These changes in turn correlate with expression of the GAL4-regulated *GAL* and *MEL* genes, suggesting that the phosphorylation of GAL4 makes it competent to activate transcription (30, 31). We have investigated whether protein phosphorylation affects ABF1 function in a similar manner. We demonstrate here that ABF1 is a phosphoprotein with at least four different states of phosphorylation. In addition, the level of ABF1 phosphorylation is regulated by growth conditions and correlates with the expression of one of its target genes, *COX6*. Finally, we show that different patterns of protein-DNA complexes, containing ABF1, are formed at domain 1 of the *COX6* promoter, depending on the phosphorylation state of ABF1.

MATERIALS AND METHODS

Yeast strains and growth conditions. The *S. cerevisiae* strains used were JM43 (*MATα leu2-3 leu2-112 his4-580 ura3-52 trp1-289*) (11), MCY1093 (*MATahis4-539 lys2-801 ura3-52*), MCY1595 (*MATα his4-539 lys2-801 ura3-52 snf1Δ3*) and MCY1097 (*MATα ade2-101 lys2-801 ura3-52 ssn6-1*) (7, 49), and MCY1640 (*MATa ade2-101 lys2-801 ura3-52 ssn6-1 snf1-28*). For the preparation of unlabeled extracts, yeast cells were grown at 30°C in liquid YP medium (1% yeast extract, 2% Bacto Peptone) supplemented with either 8% glucose (repressing conditions) or 2% lactate (derepressing conditions). All liquid cultures were grown with shaking (200 rpm) and harvested when cells were in late logarithmic or early stationary phase (4×10^7 to 5×10^7 cells per ml) unless otherwise indicated. We also used strain PEP4D (*MATa/α his1/+ trp1/+ prc1-126/prc1-126 pep4-3/pep4-3 prb1-1122/prb1-1122 can1/can1*) for the initial characterization of the effects of media on ABF1 phosphorylation. For nitrogen starvation, PEP4D cells were grown in YNB medium [containing, per liter, 1.6 g of Difco yeast nitrogen base without amino acids and ammonium sulfate, 1 g of $(NH_4)_2SO_4$, 10 g of succinic acid, 6.7 g of NaOH, and 20 g of glucose] to an A_{595} of 0.5 (mid-log phase), harvested by filtration, resuspended to an A_{595} of 0.5 in YNB-N medium (YNB medium without ammonium sulfate), and grown at 30°C for 12 h. Cells arrested with an unbudded morphology.

Preparation of cell extracts and DNA binding assays. Cell extracts were prepared according to the method of Arcangioli and Lescure (1), with the modifications described by Trawick et al. (43). Cells (25-ml cultures) were harvested by centrifugation and washed once with buffer A [200 mM Tris-Cl (pH 8.0), 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM Na_2EDTA, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10% (wt/vol) glycerol] by resuspension and centrifugation. The pelleted cells were resuspended in 1 ml of buffer A and chilled on ice. An equal volume of glass beads was added, and the cells were disrupted by vigorous shaking with a Vortex Genie mixer set on the highest setting. Cells were shaken for a total of 5 min, spread out over five 1-min intervals separated by a 1-min incubation on ice. The disrupted cell suspensions were incubated on ice for 30 min and then centrifuged at 9,000 × *g* for 60 min. Following centrifugation, the supernatant was removed carefully to a 30-ml Corex tube, and an equal volume of buffer B [20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 8.0), 5 mM Na_2EDTA, 4 M $(NH_4)_2SO_4$] was added. The turbid mixture was incubated on ice for 30 min and centrifuged at 9,000 × *g* for 15 min. The pellet was resuspended in 0.2 ml of buffer C (20 mM HEPES [pH 8.0], 1 mM Na_2EDTA, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20% glycerol), and samples were stored at −70°C. Prior to use, the protein concentration was determined by the method of Lowry et al. (28), using bovine serum albumin (BSA) as a standard.

DNA binding assays were performed as follows. Ten micrograms of whole cell extract protein was brought to 25 μl (final volume) with TM buffer (25 mM Tris-HCl [pH 7.9], 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.05 M KCl). Then 5 μg of poly(dI-dC)–poly(dI-dC) (Pharmacia) was added as a nonspecific competitor DNA, and the final volume was adjusted to 50 μl with distilled water. These extracts were incubated for 20 min at room temperature with 1 to 2 ng of a ^{32}P-labeled 32-bp double-stranded oligonucleotide corresponding to domain 1 DNA sequences (between −306 and −281 bp upstream of the *COX6* translational initiation codon [43]). This double-stranded oligonucleotide probe was constructed by annealing two gel-purified single-stranded oligonucleotides (43) (synthesized by Operon Technologies, Alameda, Calif.). The double-stranded oligonucleotide was gel purified, and 10 to 20 ng of it was end labeled by incubation in 15 μl of 50 mM Tris-Cl (pH 7.8)–5 mM $MgCl_2$–10 mM β-mercaptoethanol–10 μg of BSA per ml–20 μM dCTP–20 μM dGTP–20 μM dTTP–50 μCi of [α-^{32}P]dATP (300 Ci/mmol)–0.5 to 1 U of Sequenase (United States Biochemical) for 15 min at 30°C. The end-labeling reaction was stopped by the addition of 100 μl of TE buffer (20 mM Tris-Cl, 1 mM Na_2EDTA [pH 7.8]), and the entire mixture was passed through a G-50 spun column. The void volume was collected, ethanol precipitated, and brought up in 100 μl of distilled water for use in gel retardation assays. Prior to electrophoresis, 10 μl of 6× Ficoll load dyes (15% Ficoll 400, 0.1% zylanol blue, 0.1% bromophenol blue) was added to the extract. Gel retardation assays were done on 4% polyacrylamide gels as described previously (43). After autoradiography, the radioactivity of the unbound and bound DNA present in the various retarded complexes was determined by using an AMBIS radioanalytic imaging system (Automated Microbiology Systems, San Diego, Calif.).

Acid phosphatase treatment of cell extracts. An ammonium sulfate suspension of potato acid phosphatase (grade 1; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was pelleted by centrifugation (10 s) at full speed in an Eppendorf Microfuge and then resuspended in 10 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.0). For gel retardation studies, 10 μg of cell extract protein was added to the desired amount of acid phosphatase, and the mixture was adjusted to a final volume of 20 μl with 10 mM

PIPES (pH 6.0). For Western immunoblot analysis, 100 μg of cell extract protein was added to acid phosphatase, and the mixture was adjusted to a final volume of 80 μl with 10 mM PIPES (pH 6.0). As indicated, the amount of acid phosphatase added was between 0.05 and 1.5 U for gel retardation analysis and 10 U for Western blot analysis. All incubations were at room temperature for 60 min unless otherwise indicated. The acid phosphatase reaction was stopped by adjusting the digest to 330 mM Na_2HPO_4 (pH 7.9) and incubation on ice. To examine the effects of phosphate on extracts and on acid phosphatase-produced effects, extracts were adjusted to 330 mM Na_2HPO_4 (pH 7.9) prior to incubation. Control reactions, lacking acid phosphatase, were performed as described above except that acid phosphatase was replaced by 10 mM PIPES (pH 6.0).

In vivo labeling with $^{35}SO_4$ and immunoprecipitation. For in vivo labeling with [^{35}S]sulfate, yeast strain JM43 was grown in a low-sulfate medium containing, per liter, 1 g of yeast extract, 0.01 g of Na_2SO_4, 0.5 g of NaCl, 0.7 g of $MgCl_2 \cdot 6H_2O$, 0.4 g of $CaCl_2$, 0.005 g of $FeCl_3$, 1 g of KH_2PO_4, 0.8 g of NH_4Cl, 0.1 g each of the amino acids histidine, tryptophan, and leucine, 0.025 g of uracil, and 80 g of glucose. Cells were inoculated into 30 ml of medium at 0.2×10^7 cells per ml, grown overnight with [^{35}S]sulfate (20 μCi/ml, 566.14 mCi/mmol; NEN Research Products), and harvested in stationary phase (4×10^7 to 5×10^7 cells per ml). Cell extracts were prepared as described above. Samples were prepared for immunoprecipitation as follows. Aliquots of cell extract (20 μl) were incubated for 10 min on ice with 50% (wt/vol) trichloroacetic acid (TCA) and then centrifuged for 10 min at 10,000 rpm. The TCA pellet was quickly rinsed by the addition of 200 μl of 2 M Tris base and then solubilized by heating for 15 min at 37°C in 200 μl of sample buffer (50 mM Tris-HCl [pH 6.8], 5 mM EDTA, 4% [wt/vol] sodium dodecyl sulfate [SDS], 0.02% [wt/vol] bromophenol blue) (39). Samples were diluted fivefold in TNET (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) and centrifuged for 20 min at 10,000 rpm. The volume of the supernatant was adjusted to 5 ml with TNET, and the mixture was incubated at room temperature for 1 h in the presence of 120 μl of glutaraldehyde-fixed *Staphylococcus aureus* cells (Immunoprecipitin; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The mixture was centrifuged for 10 min at 10,000 rpm, and the supernatant was incubated overnight in the presence of 30 μl of either preimmune antiserum or ABF1 antiserum and then for 1 to 2 h in the presence of 120 μl of Immunoprecipitin. The mixture was centrifuged as described above, and the pellet was washed seven times with TNET. The immunoprecipitated proteins were resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer.

Partial purification of ABF1. Extracts (100 μl, containing about 2 mg of protein), prepared from cells labeled in vivo with [^{35}S]sulfate, were loaded onto a 0.5-ml heparin-agarose column (Sigma, St. Louis, Mo.) equilibrated in buffer C (200 mM HEPES [pH 8.0], 5 mM EDTA, 20% [vol/vol] glycerol, 7.5 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride) containing 0.05 M $(NH_4)_2SO_4$. The column was washed with 2 ml of the same buffer. Proteins that remained bound were eluted with a step gradient from 0.1 to 0.6 M $(NH_4)_2SO_4$ in buffer C. Each step consisted of 3 ml of a 0.1 M increment. UAS_G-specific DNA-binding activity eluted in fractions between 0.2 and 0.4 M $(NH_4)_2SO_4$. These fractions were pooled, and proteins were TCA precipitated. Samples were then processed for immunoprecipitation as described above except that a first incubation with the preimmune serum was performed in TNET buffer before the first incubation with Immunoprecipitin.

In vivo labeling with $^{32}P_i$ and immunoprecipitation. For in vivo labeling with $^{32}P_i$, yeast strain JM43 was grown in SSP, a low-phosphate medium containing, per liter, 1 g of phosphate-depleted yeast extract, 0.5 g of NaCl, 0.7 g of $MgSO_4 \cdot 7H_2O$, 0.4 g of $CaCl_2$, 0.005 g of $FeCl_3$, 1 g of KCl, 0.01 g of KH_2PO_4, 0.8 g of $(NH_4)_2SO_4$, 0.1 g each of histidine, leucine, and tryptophan, 0.025 g of uracil, and 80 g of glucose. Phosphate-depleted yeast extract was made by incubating a solution (1 g/liter) of Difco yeast extract at room temperature with 0.01 M $MgSO_4$ and 0.148 M NH_4OH for 30 min to precipitate phosphate. The precipitated phosphate was eliminated by filtration, and the filtrate was adjusted to pH 5.8 with concentrated HCl. The remaining components of the growth medium were added to the filtrate and sterilized. The cells were precultured in this low-phosphate medium overnight. They were then inoculated into fresh medium at 0.2×10^7 cells per ml and grown overnight with the isotope $^{32}P_i$ (0.5 mCi/ml, 8,500 Ci/mmol; NEN Research Products). Cells (2 ml) were harvested, resuspended in 200 μl of buffer C, lysed by addition of 60 μl of 1.85 M NaOH and 7.5% β-mercaptoethanol, and incubated for 10 min on ice as described previously (52). Proteins were then precipitated by adjusting the solution to 10% TCA. Samples were processed for immunoprecipitation as described above except that the last pellet of Immunoprecipitin was resuspended in sample buffer diluted in 1 ml of TNET buffer, and a second immunoprecipitation was performed as described above with a monoclonal antibody (25 μl) raised against purified ABF1 (34). The immunoprecipitated proteins were resuspended in SDS-PAGE sample buffer.

Phosphatase treatment of immunoprecipitated proteins and purified ABF1. Immunoprecipitated proteins, solubilized in SDS-PAGE sample buffer, were precipitated by the addition of 4 volumes of cold acetone and incubated 30 min on ice in the presence of 50 μg of BSA per ml. The sample was then centrifuged at 4°C for 10 min at 10,000 rpm, and the acetone-precipitated proteins were resuspended in 20 μl of buffer C and diluted to 50 μl with 10 mM PIPES (pH 6.0). The mixture was incubated for 60 min at room temperature in the presence or absence of 0.3 U of acid phosphatase. The reaction was stopped by adjusting the mixture to 0.1 M Na_2HPO_4 (pH 7.9) and then diluting it with 25 μl of sample buffer. Phosphatase treatment of purified ABF1 was performed as follows. A 0.2-μg sample of ABF1 protein, purified by chromatography on an oligonucleotide affinity column (41), was incubated with 0.5 U of acid phosphatase in 10 mM PIPES (pH 6.0) for 15 min at 37°C. In control reactions, 0.1 M Na_2HPO_4 (pH 7.0) was added prior to the start of the incubation. The reactions were stopped by the addition of SDS loading buffer. The samples were analyzed by SDS-PAGE and immunoblotted with an anti-ABF1 monoclonal antibody as described below.

Electrophoresis and immunoblotting. Prior to electrophoresis, samples were solubilized in 20 μl of sample buffer (25 mM Tris-Cl [pH 6.8], 2 mM Na_2EDTA, 2% [wt/vol] SDS, 10% [wt/vol] glycerol, 1% [wt/vol] β-mercaptoethanol, 0.002% bromophenol blue) by incubation at room temperature for 5 to 10 min. SDS-PAGE was performed in a discontinuous buffer system (39) that contained the following buffer components: upper and lower reservoir buffer, 25 mM Tris–0.192 mM glycine–2 mM Na_2EDTA–0.1% SDS; stacking gel buffer (pH 8.0), 0.125 M Tris-Cl–0.1% SDS; and separating gel buffer (pH 8.5), 0.375 M Tris-Cl–0.1% SDS. Electrophoresis was carried out in a 2-cm-long stacking gel

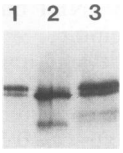

FIG. 1. Effect of acid phosphatase on the electrophoretic properties of purified ABF1. ABF1 (lane 1) was purified as described by Sweder et al. (41) and incubated with acid phosphatase in the absence (lane 2) or presence (lane 3) of 0.1 M Na_2HPO_4, an inhibitor of phosphatase activity, as described in Materials and Methods. Samples were then analyzed by SDS-PAGE and Western immunoblotting with a monoclonal antibody to ABF1.

and a 12-cm-long separating gel. Both gels were 0.75 mm thick. Electrophoresis was at 20 mA through the stacking gel and at 35 mA through the separating gel. The stacking gel contained 4.5% acrylamide, 0.12% bisacrylamide, 0.08% *N,N,N',N'*-tetramethylethylenediamine, and 0.08% ammonium persulfate, and the separating gel contained either 7.5% acrylamide–0.2% bisacrylamide or 10% acrylamide–0.27% bisacrylamide, 0.05% *N,N,N',N'*-tetramethylethylenediamine, and 0.05% ammonium persulfate. Two types of electrophoretic systems, low resolution and high resolution, were used. For low-resolution SDS-PAGE, samples were run on 7.5% acrylamide gels until the tracking dye reached the bottom of the gel. For high-resolution gels, prestained molecular weight standards (28,000 to 180,000) from Sigma were coelectrophoresed with the samples of interest in an adjacent lane (60 μg per lane). Electrophoresis was run until the 46,500-molecular-weight standard migrated off the bottom of the gel. After SDS-PAGE, the resolved proteins were electrophoretically transferred to nitrocellulose sheets as described previously (42). ABF1 was detected by incubation with a polyclonal antibody (34) (dilution of 1:1,000) and alkaline phosphatase-conjugated goat anti-rabbit antibody, using a Protoblot AP kit (Promega, Madison, Wis.).

For examining the effects of acid phosphatase on yeast cell extract protein, BSA, ovalbumin, and casein samples were run on 12% acrylamide gels and stained with a solution of 0.1% Coomassie blue, 50% methanol, and 10% acetic acid.

Preparation of antisera. Polyclonal antiserum and the monoclonal antibody 6C11G-4 were prepared in rabbits and mouse hybridoma cells, respectively. Their characterization was described previously (34).

RESULTS

ABF1 is a phosphorylated protein. Purified ABF1 protein consists of at least two electrophoretic forms (41). To determine whether this electrophoretic heterogeneity is attributable to phosphorylation, we tested whether the electrophoretic mobilities of these forms are affected by treatment with phosphatases and whether ABF1 can be labeled in vivo with [^{32}P]phosphate. For the first experiment, we treated purified ABF1 protein with acid phosphatase in the presence or absence of phosphate, a phosphatase inhibitor. As expected, purified ABF1 runs as a doublet of bands on an SDS–7.5% polyacrylamide gel (Fig. 1A, lane 1). The faster-migrating form has an apparent molecular mass of 130 kDa, while the more slowly-migrating form has an apparent molecular mass of 135 kDa. Incubation of the purified protein with acid phosphatase shifts the ratio of these two forms; the amount of the 130-kDa form is increased while the amount of the 135-kDa form is diminished (lane 2). Incubation with phosphate, an inhibitor of acid phosphatase, partially inhibits this conversion of the 135-kDa form to the 130-kDa form (lane 3). These results are consistent with the hypothesis that ABF1 is a phosphoprotein and that the upper band is more highly phosphorylated than the lower band. Unlike acid phosphatase, alkaline phosphatase had no effect on the relative amounts of the two forms of ABF1 (data not shown). The fainter bands seen below the ABF1 doublet in lanes 2 and 3 may be the result of a small amount of ABF1 proteolysis during incubation with acid phosphatase. It is noteworthy that even here, a single band is observed in the acid phosphatase-treated sample (lane 2), while a doublet is observed in the sample incubated with both acid phosphatase and phosphate (lane 3), supporting the hypothesis that the doublet observed for either intact or partially proteolyzed ABF1 is due to differential phosphorylation.

To provide more direct evidence that ABF1 is a phosphoprotein, we tested whether it could be labeled with [^{32}P]phosphate in vivo. This was done by growing cells in the presence of ^{32}P and then subjecting extracts from these cells to immunoprecipitation with polyclonal anti-ABF1 serum, an antiserum that has been shown previously to bind ABF1 on immunoblots (34). Before doing this experiment, we established that this antiserum would immunoprecipitate ABF1 protein from crude cell extracts. To do so, we grew JM43 cells in [^{35}S]sulfate to radiolabel the ABF1 polypeptide itself. The extract from the ^{35}S-labeled cells was separated into two portions. One portion was incubated with either preimmune serum or anti-ABF1 serum. The other portion was loaded onto a heparin-agarose column to partially purify ABF1 as described previously (41). The eluant from this column was then subjected to immunoprecipitation with the anti-ABF1 serum and analyzed by SDS-PAGE. From Fig. 2A, it is clear that the anti-ABF1 serum (lane 2) precipitated a number of proteins compared with preimmune serum (lane 1). It is also clear, however, that the anti-ABF1 serum recognized a doublet of proteins that migrate with the same apparent molecular masses (i.e., 130 and 135 kDa) as observed for the purified ABF1 protein shown in Fig. 1. When the immunoprecipitated proteins were incubated with acid phosphatase, the upper band in this doublet disappeared and the lower band increased in intensity (Fig. 2A, lane 3). It is interesting that acid phosphatase had little if any effect on the other bands that were immunoprecipitated. This latter observation is important because it renders unlikely the possibility that the added acid phosphatase facilitates proteolysis in these preparations. From the apparent molecular masses of the doublet, its reactivity with anti-ABF1 serum, and its behavior when treated with acid phosphatase, we conclude that it is authentic ABF1. Further support for this conclusion comes from the fact that it is greatly enriched after heparin-agarose chromatography (Fig. 2B).

Having established that authentic ABF1 is immunoprecipitated from crude extracts by this polyclonal antiserum, we could use it to determine whether it is radiolabeled when JM43 cells are grown in $^{32}P_i$. Extracts were prepared from ^{32}P-labeled cells and immunoprecipitated exactly as they were from the ^{35}S-labeled cells discussed above. From Fig. 2B, it is clear that a ^{32}P-labeled protein (lane 3) that comigrates with a partially purified ^{35}S-labeled ABF1 protein (lane 2) is present in these immunoprecipitates. Because the ^{32}P-labeled band is broader than the ^{35}S-labeled doublet, it is difficult to determine whether both electrophoretic forms of ABF1 are labeled. Nevertheless, from its apparent size (135 kDa), comigration with partially purified ABF1, and cross-reactivity with anti-ABF1 serum, it is clear that this phos-

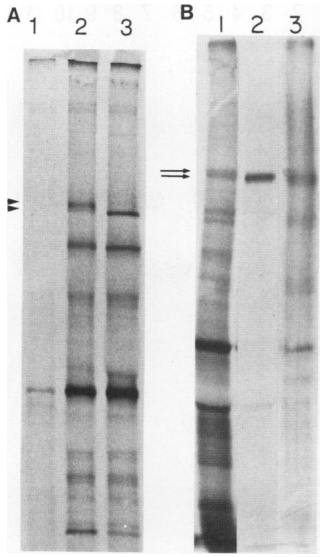

FIG. 2. Labeling of ABF1 with $^{32}PO_4$ in vivo. (A) Anti-ABF1 serum precipitates ABF1 from crude extracts. JM43 cells were labeled overnight with [^{35}S]sulfate (20 μCi/ml) in SSG (low-sulfate) medium supplemented with 8% glucose. Extracts were prepared, and samples were subjected to immunoprecipitation with a preimmune serum (lane 1) or with a polyclonal antibody raised against ABF1 (33) (lanes 2 and 3) as described in Materials and Methods. The immunoprecipitated proteins were incubated for 60 min at room temperature in the absence (lane 2) or presence (lane 3) of 0.3 U of acid phosphatase. (B) JM43 cells were labeled overnight with $^{32}P_i$ (0.5 mCi/ml) and lysed with NaOH and β-mercaptoethanol, and extract proteins were TCA precipitated as described in Materials and Methods. The protein pellet was resuspended and immunoprecipitated with a polyclonal antibody raised against ABF1 (33) (lane 3). For comparison, the precipitated proteins were coelectrophoresed with a ^{35}S-radiolabeled immunoprecipitated ABF1 before (lane 1) or after (lane 2) heparin-agarose chromatography. The polyclonal anti-ABF1 antibody and [^{35}S]sulfate-labeled extracts that were used for the experiment shown in panel A were used. Samples for each experiment were analyzed by SDS-PAGE on low-resolution polyacrylamide gels as described in Materials and Methods.

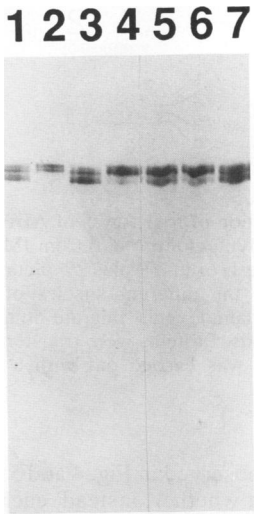

FIG. 3. Effects of growth media on ABF1 phosphorylation. Cell extracts from strain PEP4D were prepared and analyzed for the presence of ABF1 by immunoblotting with a monoclonal antibody against ABF1 as described previously (33). Unless indicated, the cells were grown to mid-log phase at 30°C. The cells were grown in YNB medium (lane 1), YNB-N (nitrogen starvation) medium (lane 2), YP plus 2% glucose (lane 3), YP plus 2% glucose to saturation density (lane 4), YP plus 2% glycerol and 2% lactate (lane 5), YP plus 2% ethanol (lane 6), and YP plus 2% ethanol to mid-log phase followed by addition of 2% glucose for 2 h (lane 7). SDS-PAGE was performed as described in the legend to Fig. 1.

phoprotein is ABF1. Moreover, this phosphoprotein is also precipitated by a monoclonal antibody to ABF1 (data not shown).

The phosphorylation of ABF1 is affected by growth conditions. To determine whether the phosphorylation of ABF1 is a regulated process, we examined the effects of growth conditions on the ratio of the phosphorylated forms of ABF1. From Fig. 3, it is clear that the pattern of bands seen is affected by the growth medium. On this gel, the pattern of bands seen is slightly different from those in Fig. 1 and 2. In Fig. 3, the two closely migrating doublets that are observed are due to splitting of the 135- and 130-kDa forms during electrophoresis. In other experiments with these samples, the 135- and 130-kDa forms were not resolved into doublets but gave the same pattern as in Fig. 1. The 135-kDa doublet is enriched when cells are grown on low-nitrogen growth media (Fig. 3, lane 2) and in medium with nonfermentable carbon sources (lanes 5 and 6). In contrast, the 130-kDa doublet is enriched in cells grown on glucose under repressing conditions (lane 3). Cells grown in a mixture of repressing and derepressing carbon sources have equal amounts of both 135- and 130-kDa doublets (lanes 1 and 7). Cells grown to stationary phase on glucose medium (lane 4) show a preponderance of the 135-kDa doublet instead of the 130-kDa doublet, probably because *S. cerevisiae* cells produce ethanol from glucose and use ethanol as a carbon source during the latter stages of growth in glucose batch cultures like those used here (20). Consequently, the last carbon source used by the cells in this experiment should have been ethanol. This view is consistent with the fact that the pattern observed for these cells is essentially identical to that seen for ethanol-grown cells (lane 6).

Together, these results suggest that the more highly phosphorylated, more slowly migrating forms of ABF1 are enriched when cells are grown on low-nitrogen media or in media with nonfermentable carbon sources.

Identification of four phosphorylation states of ABF1. To further analyze the phosphorylation of ABF1, we used an SDS-PAGE protocol that gave enhanced resolution. This protocol allowed us to resolve ABF1 into four distinct bands (Fig. 4). As observed with the split ABF1 doublets in Fig. 3, the more slowly migrating bands 1 and 2 are enriched in derepressed cells, whereas the faster-migrating bands 3 and 4 are enriched in repressed cells. After acid phosphatase treatment (Fig. 5), there is an accumulation of band 4 and, to a lesser extent, band 3; bands 1 and 2 completely disappear (Fig. 5, lane 4). These effects of acid phosphatase are prevented by the addition of phosphate, an acid phosphatase inhibitor (lane 5). Together, these results lead us to conclude that band 4 is either dephosphorylated or minimally phosphorylated and bands 1, 2, and 3 are more highly phosphorylated. This interpretation is consistent with the migration of other phosphoproteins on SDS-polyacrylamide gels. For example, the phosphorylated forms of mammalian casein (36) and both GAL4 (31) and heat shock factor (40) from *S. cerevisiae* migrate more slowly in SDS-PAGE than do their dephosphorylated counterparts. It is not known whether

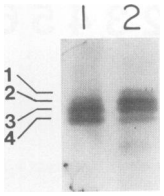

FIG. 4. Identification of four forms of ABF1. Shown is a Western blot of extracts (100 μg of protein) from JM43 cells grown in YP plus 8% glucose (lane 1) or in YP plus 2% lactate (lane 2). To obtain better resolution of the different species of ABF1, we let the SDS–7.5% polyacrylamide gels migrate longer as described in Materials and Methods. Proteins were transferred to nitrocellulose, and immunoblotting was carried out with a polyclonal antibody raised against ABF1.

each of the bands observed in Fig. 4 and 5 represents a single species of ABF1 or whether, instead, each band is a mixture of multiply phosphorylated species of ABF1. Resolution of this question will require a detailed analysis of phosphorylation sites themselves. However, to facilitate discussion, we will refer to the bands as phosphorylation states.

It is not clear whether the apparent reduction in amount of anti-ABF1-reactive protein observed after treatment of cell extracts with acid phosphatase or acid phosphatase plus phosphate (Fig. 5) is attributable to partial proteolysis of ABF1 or to its diminished cross-reactivity with anti-ABF1 serum. To examine the possibility that acid phosphatase alone or in combination with cell extract promotes general proteolysis, we examined the effects of extract, acid phosphatase, and extract plus acid phosphatase on the protein substrates ovalbumin, casein, and BSA. From Fig. 6, it is clear that the addition of acid phosphatase alone or together with cell extract does not promote the proteolysis of either ovalbumin (lanes 6 to 11) or casein (lanes 12 to 17). As expected, however, acid phosphatase does lead to the dephosphorylation, and hence faster mobility, of casein (lanes 13 and 16). This effect is inhibited by phosphate and unaffected by the presence of the extract (lanes 14 and 17). Finally, the addition of acid phosphatase to the extract alone does not lead to noticeable degradation of extract protein or the BSA carrier in the acid phosphatase preparation itself (lanes 3 to 5). In light of these findings, it seems unlikely that

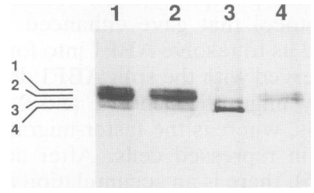

FIG. 5. Effects of acid phosphatase on the four forms of ABF1. Extracts (70 μg of protein) from JM43 cells grown in YP plus 2% lactate were incubated in the presence or absence of acid phosphatase plus or minus 330 mM sodium phosphate for 60 min at room temperature as described in Materials and Methods. Samples were electrophoresed on SDS–7.5% polyacrylamide gels as for Fig. 4, transferred to nitrocellulose, and blotted with a polyclonal anti-ABF1 antiserum. Lanes: 1, untreated lactate extract; 2, lactate extract plus 330 mM sodium phosphate; 3, lactate extract plus 1 U of acid phosphatase; 4, lactate extract plus 330 mM sodium phosphate and 1 U of acid phosphatase. Although the conditions for electrophoresis and blotting were the same as for Fig. 4, there is slightly poorer resolution of bands 2 and 3.

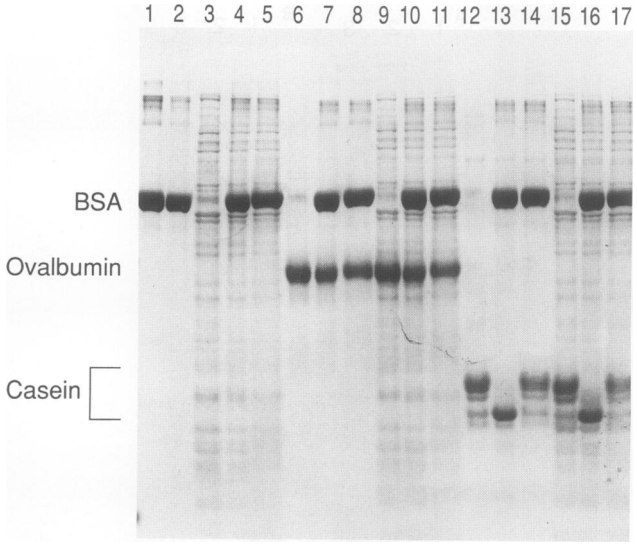

FIG. 6. Evidence that acid phosphatase does not lead to proteolysis of cell extracts or standard proteins. Fifteen micrograms of ovalbumin (lanes 6 to 11) or human casein, a phosphoprotein (lanes 12 to 17), was treated with 1 U of acid phosphatase plus or minus 330 mM sodium phosphate in the absence (lanes 6 to 8 and 12 to 14) or presence (lanes 9 to 11 and 15 to 17) of 14 μg of cell extract from cells grown in YP plus 8% glucose. Cell extract (14 μg) itself (lane 3) was also incubated with acid phosphate (lane 4) or acid phosphatase plus 330 mM sodium phosphate (lane 5) for 60 min at room temperature. Samples were dissociated in SDS-PAGE sample loading buffer for 30 min at 37°C and boiled for 2 min before aliquots were electrophoresed on SDS–12% polyacrylamide gels. After electrophoresis, the gel was stained with Coomassie blue. Lanes: 1, BSA; 2, acid phosphatase (containing carrier BSA); 3, extract; 4, extract plus acid phosphatase; 5, extract plus acid phosphatase and sodium phosphate; 6, ovalbumin; 7, ovalbumin plus acid phosphatase; 8, ovalbumin plus acid phosphatase and sodium phosphate; 9, ovalbumin plus extract; 10, ovalbumin plus extract and acid phosphatase; 11, ovalbumin plus extract, acid phosphatase, and sodium phosphate; 12, casein; 13, casein plus acid phosphatase; 14, casein plus acid phosphatase and sodium phosphate; 15, casein plus extract; 16, casein plus extract and acid phosphatase; 17, casein plus extract, acid phosphatase, and sodium phosphate.

the loss of anti-ABF1-reactive protein is attributable to generalized proteolysis activated by the addition of acid phosphatase to cell extracts. Therefore, we propose that the apparent reduction in the amount of anti-ABF1-reactive protein observed after acid phosphatase treatment results most likely from diminished cross-reactivity of dephosphorylated ABF1 with anti-ABF1 serum. Of course, we cannot rule out the possibility that acid phosphatase also activates an ABF1-specific protease.

The phosphorylation states of ABF1 are affected by the *SNF1-SSN6* pathway. Previously, we have found that *SNF1*, a gene that encodes a protein kinase (7), and *SSN6* (6), a gene whose product acts epistatically to *SNF1*, are required to derepress *COX6* transcription when cells are shifted from glucose-repressing to derepressing conditions (49). We have also shown that this regulation is affected through an ABF1 binding site in UAS_6 (43). We therefore examined the possibility that ABF1 is a target of the *SNF1-SSN6* pathway by analyzing the phosphorylation states of ABF1 in *snf1* and *ssn6* mutants. In glucose-repressed cells of $SNF1^+$ $SSN6^+$ (MCY1093) and *snf1* $SSN6^+$ (MCY1595) strains, the level of expression of *COX6* is low, but it is high in $SNF1^+$ *ssn6*

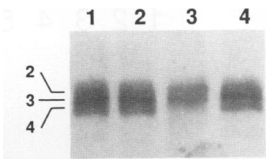

FIG. 7. *SNF1-SSN6* pathway dependence of ABF1 phosphorylation. Samples (100 μg) of extracts from *SNF1*$^+$ *SSN6*$^+$ (lane 1), *snf1 SSN6*$^+$ (lane 2), *SNF1*$^+$ *ssn6* (lane 3), and *snf1 ssn6* (lane 4) cells grown in YP plus 8% glucose were subjected to SDS-PAGE and immunoblotting as described in the legend to Fig. 4.

(MCY1097) and *snf1 ssn6* (MCY1640) strains (49). Immunoblots of extracts from these strains grown on repressing media reveal multiple forms of ABF1 (Fig. 7). As with JM43, the predominant forms of ABF1 in repressed cells are states 3 and 4. State 2 is present but in lower amounts than in derepressed cells. The relative amounts of phosphorylation states 2, 3, and 4 are similar or identical in *SNF*$^+$ *SSN6*$^+$, *snf1 SSN6*$^+$, and *snf1 ssn6* strains (Fig. 7, lanes 1, 2, and 4, respectively). These results suggest that the SNF1 protein kinase per se is not involved in phosphorylation of ABF1. In contrast, the *SNF1*$^+$ *ssn6* strain (lane 3) has bands representing phosphorylation states 2 and 3 but not state 4. Insofar as state 4 is the least phosphorylated state of ABF1, these findings suggest that a functional *SSN6* gene is essential for dephosphorylation of ABF1 in the presence of a functional *SNF1* gene. Interestingly, in the absence of a functional *SNF1* gene, *SSN6* is not essential for this dephosphorylation.

UAS_6 DNA-binding proteins are phosphorylated. In a previous study, we showed that the major protein binding sites within UAS_6 of *COX6* lie in domain 1, between −281 and −306 bp upstream of the *COX6* initiation codon (43). This domain includes an ABF1 binding site and is important for glucose repression-derepression of *COX6*. It forms different numbers of DNA-protein complexes in extracts from cells grown in repressing or derepressing carbon sources (43) (Fig. 8, lanes 1 and 7). With both extracts, a major complex (band C) and a minor complex (band N) are observed. The amount of band N is variable from preparation to preparation. In extracts from repressed cells, two major complexes (bands D and E) in addition to band C are observed.

Because of the differences in ABF1 phosphorylation in repressing and derepressing carbon sources, we were interested in whether the differences in the protein complexes formed at domain 1 could be attributed, at least in part, to protein phosphorylation, particularly that of ABF1. To address this question, extracts prepared from cells grown in YP with 2% lactate (derepressing conditions) or in YP with 8% glucose (repressing conditions) were incubated for 60 min with increasing amounts of phosphatase prior to incubation with labeled DNA and gel electrophoresis mobility shift assays. Treatment of either extract with alkaline phosphatase had no effect on the banding pattern (data not shown). However, treatment with acid phosphatase led to the appearance of new bands. At low concentrations (0.05 or 0.1 U), acid phosphatase treatment of the derepressed extract gave two new bands (D and E) compared with the pattern seen in the untreated control (Fig. 8; compare lanes 1 with lanes 2 and 3). This new pattern is similar to that observed with untreated extracts from glucose-repressed cells (compare lane 2 or 3 with lane 7). With increasing amounts of acid phosphatase, the intensity of bands D and E increased substantially and a new complex, band F, was

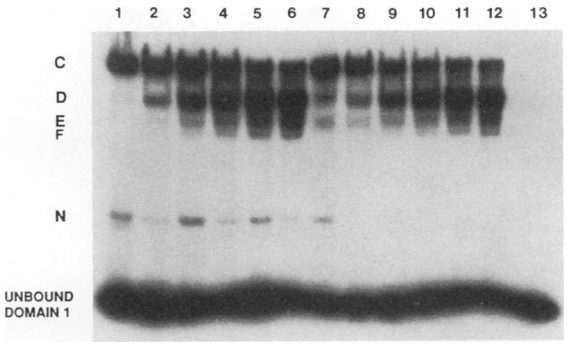

FIG. 8. Evidence that acid phosphatase treatment of cell extracts alters the gel shift positions of complexes that form between domain 1 of UAS_6 and proteins from crude yeast cell extracts. Aliquots (10 μg of protein) of extracts prepared from JM43 cells grown in YP plus 2% lactate (derepressed) (lanes 2 to 6) and YP plus 8% glucose (repressed) (lanes 8 to 12) were incubated with increasing concentrations of acid phosphatase (in the presence of protease inhibitors) for 60 min at room temperature as described in Materials and Methods. Reactions were stopped by adjusting each mixture to a final concentration of 330 mM Na_2HPO_4, and gel electrophoresis mobility shift assays were carried out with a ^{32}P-end-labeled 32-bp oligonucleotide probe whose sequence corresponds to domain 1. The intensity of band N was variable for reasons that are not known. Lanes: 1 and 7, no acid phosphatase; 2 and 8, 0.05 U; 3 and 9, 0.1 U; 4 and 10, 0.5 U; 5 and 11, 1 U; 6 and 12, 1.5 U; 13, unbound DNA. C, D, E, and F refer to the retarded complexes.

formed. The appearance of these new bands was accompanied by a decrease in the intensity of band C. Treatment of the repressed cell extract with acid phosphatase led to a substantial increase in bands D and E and the appearance of band F at high concentrations of acid phosphatase (lanes 8 to 12). In addition, treatment of this extract with acid phosphatase led to the disappearance of band N (compare lanes 7 and 12). The patterns observed at the highest concentration of acid phosphatase are similar for both extracts.

The decrease in radioactivity associated with band C is correlated with the increase in radioactivity in bands D, E, and F in both repressed and derepressed extracts (Fig. 9). It

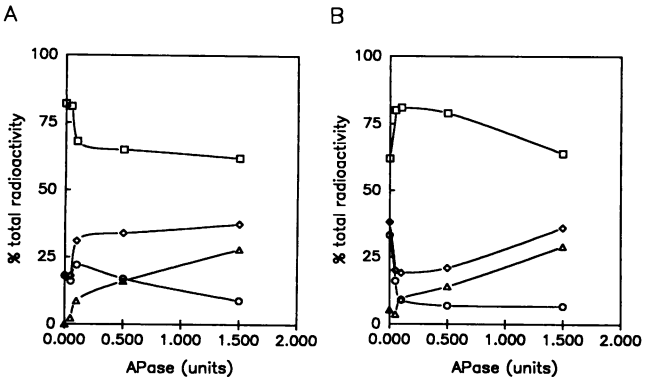

FIG. 9. Quantitation of the radioactivity present in the protein-DNA retarded complexes after treatment with different concentrations of acid phosphatase. The radioactivity in band C, bands D, E, and F, and unbound DNA in Fig. 8 was determined by using an AMBIS radioanalytic imaging system. Each graph presents the percentage of total radioactivity (bound and unbound) in band C (○), bands D, E, and F (△), total bound DNA (◇), and unbound DNA (□). (A) Derepressed extracts; (B) repressed extracts.

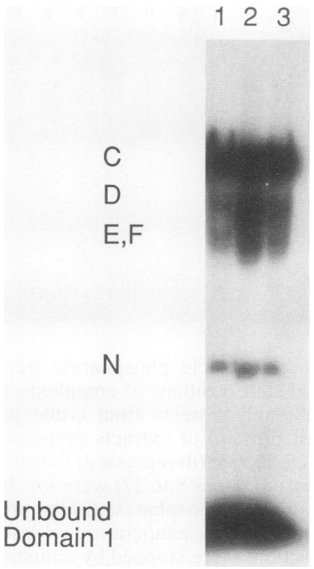

FIG. 10. Evidence that phosphate inhibits the effects of acid phosphatase on the gel shift positions of DNA-protein complexes that form with UAS_6 domain 1 DNA. An extract prepared from cells grown in YP plus 8% glucose was treated with 1 U of acid phosphatase (lane 2), 330 mM Na_2HPO_4 (lane 1), or 1 U of acid phosphatase plus 330 mM Na_2HPO_4 (lane 3) for 60 min at room temperature and subjected to gel mobility shift electrophoresis as for Fig. 7.

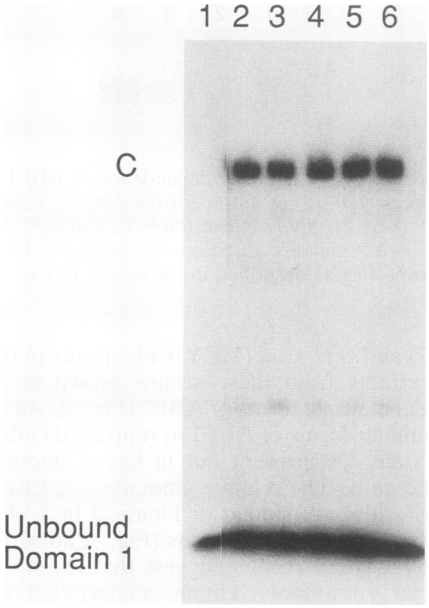

FIG. 11. Evidence that incubation of cell extracts at room temperature does not alter gel shift patterns. Aliquots (7 μg of protein) of extracts from JM43 cells grown in YP plus 2% lactate were incubated at room temperature for various times prior to gel mobility shift electrophoresis as for Fig. 8. Times of incubation were 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), and 60 min (lane 5).

is unlikely that the decrease in band C or the increase in bands D, E, and F is due to the presence of proteases in the phosphatase preparation, because incubation with 1.5 U of acid phosphatase and Na_2HPO_4, an inhibitor of acid phosphatase, inhibited this conversion (Fig. 10). Moreover, bands D, E, and F are not observed in extracts from derepressed cells or when extracts from repressed cells are treated with alkaline phosphatase, nor are bands D, E, and F observed after prolonged incubation of derepressed extracts at room temperature (Fig. 11).

The amount of radioactivity associated with each band in Fig. 8 is quantitated in Fig. 9. From these data, it is clear that treatment of the derepressed extracts (Fig. 9A) with acid phosphatase leads to an increase in the ratio of bound radioactivity to unbound radioactivity. This finding suggests that more protein is binding to the probe in the dephosphorylated extracts. In contrast, treatment of the repressed extract (Fig. 9B) with acid phosphatase leads to a decrease in the ratio of bound to unbound radioactivity at low phosphatase concentrations but to no change at higher phosphatase concentrations. These findings indicate that the relationship between the loss of band C and the appearance of bands D, E, and F after phosphatase treatment is not a simple one. For the derepressed extract, it appears that the radioactivity in bands D, E, and F comes both from band C and from the binding of additional proteins, which did not bind in extracts that were not phosphatase treated. For the repressed extract, it appears that the additional radioactivity associated with bands D, E, and F comes entirely from band C and not from the binding of additional proteins. Together, these results suggest that bands D, E, and F contain dephosphorylated forms of proteins in band C, that the proteins in band C compete with those in bands D, E, and F for binding to domain 1, and that the level of phosphorylation of proteins in band C affects its ability to compete.

ABF1 is present in most UAS_6-protein complexes. As mentioned above, ABF1 binds to domain 1. To determine whether ABF1 is present in all of the retarded complexes produced from repressed, derepressed, and phosphatase-treated extracts, we used a polyclonal anti-ABF1 serum. As observed previously for binding to ARS1 (34), the anti-ABF1 antiserum shifted the position of each protein-DNA complex formed in extracts from repressed cells up to the top of the gel (Fig. 12, lanes 2 to 4). Similarly, anti-ABF1 serum shifted band C as well as the bands formed after acid phosphatase treatment of derepressed extracts (lanes 7 to 9 and 14) to a more slowly migrating complex or to the top of the gel. A monoclonal antibody, 6C11G-4 (34), raised against ABF1, also shifted the position of each retarded complex formed in repressed conditions (data not shown). Preimmune serum did not change the mobility of any of the complexes (lanes 5 and 10). Interestingly, an additional retarded complex (band D′) was observed in the presence of the specific antibody, in both repressed and derepressed conditions, after treatment of cell extracts with acid phosphatase (lanes 8, 9, and 14). This complex could not be shifted even at high antibody concentrations and migrates differently from band D when the gel is run longer (data not shown). Band N is not affected by the presence of anti-ABF1 antiserum in either repressed or derepressed conditions. Therefore, it probably does not contain ABF1.

Together, these results permit three conclusions. First, ABF1 binds domain 1 in both repressed and derepressed cells. Second, ABF1 is a component of at least four of the retarded complexes (bands C, D, E, and F) that form with domain 1. Third, another protein-DNA complex, D′, can bind domain 1 after treatment of cell extracts with acid phosphatase.

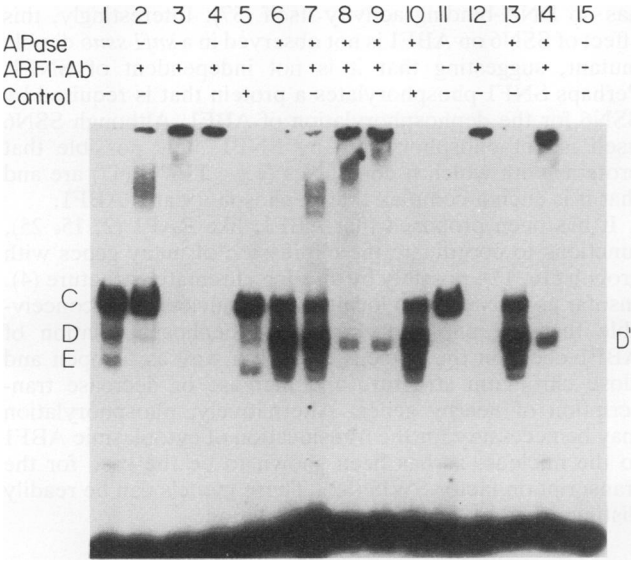

FIG. 12. Recognition by an antiserum raised against ABF1 of all of the retarded complexes formed with UAS_6 domain 1. Ten micrograms of extract protein, prepared from JM43 cells grown either in YP plus 8% glucose (lanes 1 to 10) or in YP plus 2% lactate (lanes 11 to 14), was incubated for 60 min at room temperature in the absence (−) or presence (+) of 1 U of acid phosphatase (APase). The reaction was stopped by addition of Na_2HPO_4. Then 1 μl of preimmune antiserum (lanes 5 and 10) or 0.2 μl (lanes 2 and 7), 1 μl (lane 3 and 8), or 2 μl (lanes 4, 9, 12, and 14) of anti-ABF1 serum (ABF1-Ab) (34) was added to the mixture, and samples were incubated with the radiolabeled probe for 20 min at room temperature before gel analysis. Lane 15, unbound DNA. C, D, E, and D' refer to the retarded complexes. D' migrates at a different position than does D after more prolonged electrophoresis.

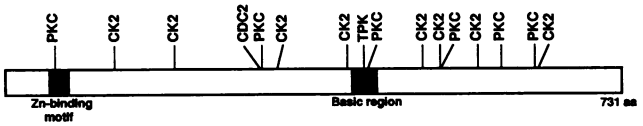

FIG. 13. Potential sites in ABF1 for protein kinases. The sequence of ABF1 was searched for potential phosphoacceptor recognition sites of protein kinases described previously (18, 28). The bar represents the 731-amino-acid (aa) ABF1 protein sequence; indicated above the bar are the positions of serine/threonine residues that may serve as substrates for cyclic AMP-dependent protein kinase (TPK), protein kinase C (PKC), casein kinase II (CK2), and $p34^{cdc2}$ protein kinase (CDC2); the shaded boxes are regions of ABF1 that are important for DNA binding (33a).

DISCUSSION

The results of this study show that (i) ABF1 is a phosphoprotein, (ii) phosphorylation of ABF1 changes in response to growth conditions, and (iii) the change in phosphorylation of ABF1 is correlated with a change in expression of *COX6*, one of its target genes, and with the patterns of ABF1-containing protein-DNA complexes that are formed with domain 1 of the *COX6* promoter. Together, these findings implicate the phosphorylation of ABF1 in the mediation of glucose repression and derepression of *COX6* transcription.

Two types of experiments were used to show that ABF1 is a phosphoprotein: (i) analysis of apparent molecular weight by SDS-PAGE after treatment with acid phosphatase and (ii) in vivo labeling with $^{32}P_i$. A recent study, published while this report was in review, also has reported that OBF1 (a pseudonym for ABF1) is a phosphoprotein and that it is phosphorylated on threonine and serine residues (21). We have shown here that both purified and ^{35}S-radiolabeled immunoprecipitated ABF1 protein migrate in SDS-PAGE as multiple species. By using a high-resolution SDS-PAGE system, it is possible to recognize as many as four ABF1 protein bands in crude cell extracts from derepressed cells and three bands in extracts from repressed cells. Treatment of these extracts with acid phosphatase converted these bands into one major band and a minor band; these two comigrate with the two bands of highest mobility in either extract. These findings indicate that the more slowly migrating bands are phosphorylated forms of ABF1 and that the fastest-migrating band is either less phosphorylated or unphosphorylated ABF1. Three aspects of these findings are of interest. First, it is likely that ABF1 is multiply phosphorylated like many proteins (35), including other yeast transcription factors (e.g., GAL4, ADR1, and heat shock factor) (9, 31, 40). Although we do not know how many species of ABF1 are present in each band, their different mobilities in SDS-PAGE strongly suggest that the different bands represent populations of ABF1 with different numbers of phosphates. It is noteworthy that ABF1 is rich in phosphoacceptor amino acid residues (102 Ser and Thr) and that at least 15 of these are found at consensus phosphorylation sites for known protein kinases (Fig. 13). Second, it is clear that ABF1 exists in mixed states of phosphorylation. This finding raises the possibility that the different states or sites of phosphorylation play different roles in the functions of ABF1. For example, it is conceivable that some sites are important for the transcriptional activation function (5) of ABF1, while other sites are important for its transcriptional repression (4, 14) or DNA replication (4, 14, 41) functions. Third, the ratios of the different phospho forms of ABF1 are affected by environmental conditions. This finding indicates that the phosphorylation of different sites within ABF1 is regulated differentially in response to growth conditions and suggests that the phosphorylation of ABF1 is under the control of more than one kinase cascade system.

By examining *COX6*, a target gene for ABF1, it has been possible to demonstrate a direct correlation between the phosphorylation of ABF1 and transcriptional activation. At least two environmental conditions (growth on nonrepressing carbon sources and growth to stationary phase in glucose batch cultures) that favor phosphorylated forms of ABF1 also produce enhanced transcription of *COX6* (44, 49, 50). The effects of glucose repression and derepression on *COX6* transcription are also correlated with the patterns of protein-DNA complexes that form at domain 1, the ABF1 binding site of the *COX6* promoter. Although it is not known how many polypeptides are present in these retarded protein-DNA complexes, it is clear that ABF1 is present in each of these complexes and that the multiple complexes observed in repressed extracts can be generated from derepressed extracts by treatment with acid phosphatase. Moreover, the conversion of band C to bands D, E, and F correlates with the dephosphorylation of ABF1 under the same conditions. It is unlikely that bands D, E, and F are proteolytic products of band C for a number of reasons. First, bands D, E, and F are not observed in derepressed extracts, even after prolonged incubation at room temperature (Fig. 11), but can be generated in these extracts by treatment with acid phosphatase (Fig. 8). Second, the patterns of bands formed after

treatment of repressed and derepressed extracts with acid phosphatase are essentially the same (Fig. 8), and the amount of bound DNA after phosphatase treatment of either extract reaches the same plateau (Fig. 9). Third, the conversion of band C to bands D, E, and F by acid phophatase is inhibited by added phosphate (Fig. 10), an inhibitor of phosphatase but not proteases.

At the moment, there are two possible models to explain the existence of the multiple protein-DNA complexes that bind to domain 1 of the *COX6* promoter. First, they may represent different hetero-oligomeric proteins composed of ABF1 and other transcription factors (either DNA-binding proteins or polypeptides that simply bind to ABF1 through protein-protein interactions). Second, they may result from different conformations of ABF1 or different-size ABF1 homo-oligomers, brought about by differences in phosphorylation. The first model predicts that the size and complexity of the multimers would be reflected in their gel electrophoretic mobilities; i.e., band C would be the largest complex and band F would be the smallest complex. The second model predicts that the mobility of each protein-DNA complex results from either the conformation of ABF1 or its state of oligomerization. In either model, the phosphorylation of ABF1 could affect assembly of the oligomer, binding of ABF1 to domain 1, ABF1 activity, or all three. Currently available data do not enable us to decide between these models; doing so will require purification and characterization of each complex.

Previous gel mobility shift assays with ABF1 binding sites from other genes have revealed the presence of a single complex for some genes or multiple complexes at the same binding site for others (14, 22, 25, 41). In a recent study, Holland et al. (3, 25) compared the DNA-binding activities that bind to an ABF1 site in front of the *ENO2* gene in cells grown in glucose and in glycerol-lactate. Interestingly, they found a single complex in glucose-grown cells and multiple complexes of faster mobility in glycerol-lactate-grown cells. These authors proposed that the faster-migrating complexes may correspond to proteolytic breakdown products of ABF1 or to unrelated proteins that bind to the ABF1 binding site (25). The data presented here provide an alternative explanation for this observation. Perhaps these faster-migrating complexes result from differential phosphorylation of ABF1. Insofar as *ENO2* transcription is induced some 20-fold in glucose-grown cells relative to its level in glycerol-lactate-grown cells (10), this interpretation would propose that a dephosphorylated ABF1 promotes transcription of *ENO2*. What is puzzling, however, is that in glucose-grown cells, there is only a single complex with the *ENO2* ABF1 binding site (25) but multiple complexes with the *COX6* ABF1 binding site. One way to reconcile these differences is to propose that these complexes actually contain hetero-oligomeric proteins (according to the first hypothesis discussed above), composed of ABF1 as well as other polypeptides, and that the phosphorylation of ABF1 affects the type or amount of these proteins that bind ABF1.

Previous studies have shown that glucose repression of *COX6* transcription is mediated by domain 1 (43) and that its derepression from glucose requires the *SNF1-SSN6* pathway (50). Results presented here have revealed that ABF1 phosphorylation is not dependent on *SNF1* but, surprisingly, that ABF1 dephosphorylation is affected by *SSN6*. This finding raises the possibility that SSN6, a phosphoprotein itself (37), either is a phosphatase or activates a phosphatase. It is also consistent with the recent observation that although SSN6 is a repressor of transcription of many yeast genes, it has no DNA-binding activity itself (37). Interestingly, this effect of SSN6 on ABF1 is not observed in a *snf1 ssn6* double mutant, suggesting that it is not independent of SNF1. Perhaps SNF1 phosphorylates a protein that is required by SSN6 for the dephosphorylation of ABF1. Although SSN6 itself is not phosphorylated by SNF1, it is possible that proteins with which it complexes (e.g., TUP1) (47) are and that it is such a complex that dephosphorylates ABF1.

It has been proposed that ABF1, like RAP1 (2, 15, 25), functions to coordinate the expression of many genes with growth (16, 17), possibly by altering chromatin structure (4). Insofar as SSN6 is also located in the nucleus, it is conceivable that the phosphorylation and dephosphorylation of ABF1 occur in the nucleus in such a way as to open and close chromatin structure and increase or decrease transcription of nearby genes. Alternatively, phosphorylation may be necessary for the translocation of cytoplasmic ABF1 to the nucleus, as has been shown to be the case for the transcription factor SWI5 (32). These models can be readily distinguished by further experimentation.

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