Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4089–4093, May 1991
Biochemistry

The multifunctional protein OBF1 is phosphorylated at serine and threonine residues in *Saccharomyces cerevisiae*

(phosphoserine/phosphothreonine)

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Communicated by Mary Jane Osborn, January 30, 1991

ABSTRACT **We have purified a DNA replication enhancer-binding protein, OBF1, from yeast cells grown in a medium containing ^{32}P-labeled orthophosphate. The purified ^{32}P-labeled protein comigrated on polyacrylamide gels with OBF1 bands identified by immunoblotting with anti-OBF1 antibodies. Furthermore, trypsin treatment of the ^{32}P-labeled OBF1 revealed several phosphorylated peptides, suggesting that OBF1 is multiply phosphorylated *in vivo*. Incubation of phosphorylated peptides with calf intestinal phosphatase liberated the radiolabel as free phosphate, indicating a phosphoester linkage. Acid hydrolysis of the tryptic peptides revealed ^{32}P label comigrating with phosphoserine; some of it, however, was also identified as phosphothreonine. Using anti-OBF1 antibodies, we cloned the *OBF1* gene from a λgt11 yeast expression library. The DNA sequence of the isolated gene and its overexpression in yeast indicated that OBF1 is identical to ABF-I and BAF1 proteins, believed to have a role in transcriptional repression and activation. Therefore, we suggest that OBF1 is a multifunctional protein, acting in transcription and replication, and that these activities are regulated by phosphorylation.**

Eukaryotic nuclear DNA replication initiates at the start of the S phase. Initiation occurs at multiple, presumably distinct, sites along a chromosome, generating multiple replicons. *Saccharomyces cerevisiae* provides a convenient model system for studying DNA replication in eukaryotic cells because origins of replication, amenable to biochemical and genetic manipulations, have been identified. These origins, called autonomously replicating sequences (ARSs), were first isolated as DNA fragments that confer on the plasmids bearing them an ability to replicate autonomously in yeast (1). These ARSs incorporated into plasmid DNA function as origins of replication *in vivo* (2, 3). In addition, at least some of these ARSs also function as origins of replication in their natural location, the yeast chromosomes (4, 5). Studies to define the functional elements of an origin of replication revealed that all ARSs share a common 11 base pairs (bp), WTTTATRTTTW (where W = A or T and R = G or A), the core consensus sequence essential for origin of replication function (6). However, in naturally occurring ARSs nucleotide sequences on either side of the core consensus are required for optimal ARS activity, although the amount and the sequence of the flanking DNA vary depending on the ARS (6).

To gain a deeper insight into the functional elements of an origin of replication, we embarked upon a search for proteins that interact directly with DNA sequences present in the ARS. We isolated a protein, OBF1, that binds specifically to a DNA sequence found in a broad spectrum of origins, including the single-copy ARS*121* (7). Purified OBF1 appeared on SDS/polyacrylamide gels as two closely migrating bands of 123 and 127 kDa (7). Both peptides have similar physical properties, such as migration in gel filtration and sedimentation on glycerol gradients, and both form a tight isolatable complex with ARS DNA. Because of these similarities, we suggested that the two peptides are related and probably differ by a posttranslational modification.

Studies on the function of the OBF1 DNA-binding recognition sequence indicated that it acts as an enhancer of DNA replication (8, 9). Other studies reported the identification of DNA-binding factors ABF-I, SUF, TAF, GF1, and BAF1, which share with OBF1 a common DNA-binding motif TCN_7ACG, first identified by Buchman *et al.* in 1988 (10). This motif is not restricted to ARSs alone; it was also found at sites that regulate transcription. In fact, DNA-binding sites containing the above motif have a role in transcriptional activation of the genes adjacent to a ty2 element (11), the ribosomal gene *TCM1* (12), the genes involved in mitochondrial biogenesis (13), and the *YPT1* (ras-like GTP-binding protein) and *TUB2* (β-tubulin) genes (14). Of these factors mentioned above only BAF1 and ABF-I, in addition to OBF1, were purified to near-homogeneity (14–17). Furthermore, recent isolation of the genes encoding ABF-I and BAF1 proteins indicated that the two are identical (18–20).

Here we report the isolation of the *OBF1* gene* and show that it is identical to ABF-I/BAF1, and we demonstrate that OBF1 is multiply phosphorylated *in vivo*.

MATERIALS AND METHODS

Strains and Growth Conditions. *S. cerevisiae* strain Y294 (*MAT*a; *leu2-3 -112; ura3-52; trp1; Δhis3*; Gal$^+$; *cir*$^+$) was provided by David Dorsky (University of Connecticut Health Center, Farmington, CT). Selective growth was in SD (synthetic dextrose) medium (8), containing 2% raffinose instead of dextrose. *S. cerevisiae* strain CYY2 has been described (7). *Escherichia coli* Y1090, used as the host for λgt11 library, was obtained from Clontech.

λgt11 Yeast Genomic Library Screening and *OBF1* Gene Cloning. A yeast genomic library in λgt11 (Clontech) was screened with a 1:250 dilution of a polyclonal rat anti-OBF1 antiserum (7), according to manufacturer's recommendations. A positive plaque, clone λgt11(81), was purified to homogeneity; its DNA served as a source for the 4061-bp yeast insert, which was excised by the *Eco*RI restriction endonuclease and then ligated into pUC19 plasmid DNA. The nucleotide sequence of the insert was determined by the Sanger primer-extension method, using double-stranded DNA as substrate.

Isolation of the OBF1 N-Terminal Portion by Using PCR. The 5′ terminus of the *OBF1* gene was isolated by PCR from

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Abbreviations: ARS, autonomously replicating sequence; nt, nucleotide(s).

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M63578).

genomic yeast DNA. Two oligonucleotide primers were prepared in a Cyclone DNA synthesizer (Biosearch): (*i*) 5′-AGATCTAGAGTCAACGCTTTTCAT-3′, corresponding to nucleotides (nt) 15–29 in the sequence described for the ABF-I-encoding gene (18); this oligonucleotide also included the restriction sites *Bgl* II and *Xba* I at the 5′ end. (*ii*) 5′-TACCGCTGTCATTACCATGAG-3′, corresponding to nt 1534–1554 of the yeast insert isolated from λgt11(81) (see Fig. 2*B*). Yeast genomic DNA used as substrate-template in the PCR reaction was isolated from the CYY2 strain. The 1.3-kilobase (kb) fragment was amplified as described (21).

Overexpression and Purification of OBF1. The 3.2-kb *Bgl* II fragment, containing the whole open reading frame of the *OBF1* gene, including ≈100 nt of the region 5′ to the initiation codon and 900 nt 3′ to the termination of the open reading frame, was cloned into the *Bam*HI site of the pMH101 expression vector (22) to yield the plasmid pMHOBF1. This maneuver placed the gene under control of the inducible *Gal1* promoter. The yeast strain Y294 was transformed by pMH-OBF1 as before (8). A culture of cells harboring pMHOBF1 was first grown in a YPR medium (1% yeast extract/2% Bacto-peptone/2% D-raffinose) and then induced with galactose. After 4 hr of growth at 30°C, cells were harvested, resuspended, and stored frozen at −90°C, as described (7). OBF1 protein was purified as before except for the following modification. Instead of dialysis, fraction II was desalted by gel filtration on a Bio-Gel P-6 column equilibrated with buffer F (7). Desalted fraction II was applied directly to a Bio-Rex 70 column equilibrated in buffer F. OBF1-binding activity was eluted with buffer F plus 250 mM NaCl. Fractions active in the DNA-binding assay were pooled, applied to the specific DNA-cellulose column, and fractionated as described previously (7). Fractions containing purified OBF1 were frozen in liquid nitrogen and stored at −90°C.

^{32}P-Labeling of OBF1 *in Vivo*. Cells harboring the pMH-OBF1 plasmid were grown in yeast/Bactone-peptone/D-raffinose medium (YPR) and were radioactively labeled with ^{32}P during galactose induction, as described (23). Two hours after induction at 30°C, the cells were harvested and processed as described above.

Electrophoresis on TLC-Cellulose Plates. Purified ^{32}P-labeled OBF1, embedded in a polyacrylamide gel, was treated with trypsin as described (24), except that an equal molar amount of trypsin inhibitor was added upon completion of digestion. Before analysis by electrophoresis on TLC plates, peptides liberated from the polyacrylamide gel were treated again with trypsin to insure complete digestion. Acid hydrolysis of the tryptic digest was as before (24). Aliquots of 1–3 μl (200–500 cpm) of either a tryptic digest or acid hydrolysate were spotted on cellulose MN300 TLC glass plates (20 × 20 cm, Analtech) and subjected to electrophoresis in a chilled TLC electrophoresis chamber at 2°C, 35 mA for 1–2 hr. Two different buffer systems were used to separate both tryptic peptides and amino acids: (*i*) 0.5% pyridine/5% acetic acid, pH 3.5 and (*ii*) 7.5% acetic acid/2.5% formic acid, pH 2.0. *O*-Phospho-L-serine, *O*-phospho-L-threonine, and *O*-phospho-L-tyrosine were obtained from Sigma.

RESULTS

Cloning of OBF1. Polyclonal antibodies prepared against OBF1 purified to near homogeneity (7) were used to screen a λgt11 yeast genomic library as described. In the initial screen we identified four clones, only one of which, λgt11(81) (Fig. 1), reacted positively with the antibodies upon purification of the phage to homogeneity (data not shown). This clone contained a yeast DNA insert of ≈4 kb, which was excised by digesting the λgt11(81) clone DNA with *Eco*RI restriction endonuclease. After cloning the insert into the

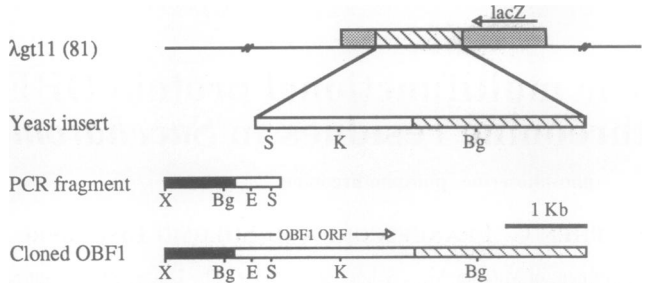

FIG. 1. Schematic presentation of the λgt11(81) clone containing the *OBF1* gene. Clone λgt11(81) was isolated from a λgt11 yeast genomic library on its reaction with anti-OBF1 antibodies. The clone contained a yeast DNA insert (cross-hatched box) of ≈4 kb at the *Eco*RI site of the *lacZ* gene (stippled box), which had an open reading frame in the direction indicated by the arrow. The nucleotide sequence of the yeast insert was determined as described. An *OBF1* open reading frame (open box) of 602 amino acids was located at one end of the insert proceeding inward (see arrow) in the opposite direction to that of *lacZ*. The N-terminal portion of the *OBF1* gene was obtained by PCR of yeast genomic DNA as described. The intact gene was reconstructed by joining the PCR fragment and the yeast insert of the λgt11(81) at the *Stu* I site. Bg, E, K, S, and X; restriction sites *Bgl* II, *Eco*RI, *Kpn* I, *Stu* I, and *Xba* I, respectively.

pUC19 plasmid, the nucleotide sequence of the yeast insert was determined. Instead of the expected LacZ–OBF1 fusion protein, the DNA sequence revealed a translational open reading frame of 1.7 kb that started at the *Stu* I end of the insert and proceeded inward in an inverted orientation relative to the open reading frame of *lacZ* (Figs. 1 and 2). This open reading frame encoded 602 amino acids of a sequence identical to a truncated portion, lacking 129 N-terminal amino acids of the published *ABF-I* and *BAF1* genes. To reconstruct the gene, a DNA fragment containing the N-terminal segment of the gene and its upstream sequences was isolated from yeast genomic DNA, using the PCR (*Materials and Methods*, Figs. 1 and 2). This fragment (Fig. 2*A*) was joined to the cloned *OBF1* DNA moiety (Fig. 2*B*) at the *Stu* I site, yielding the intact *OBF1* gene. Amino acid sequence of the intact gene, deduced from the DNA sequence, is identical to the one published for ABF-I and differs from BAF1 at several positions indicated in the legend to Fig. 2. The source for these differences in the amino sequence of ABF-I/OBF1 and BAF1 is not clear. However, because yeast has only one copy of the BAF1/ABF-I/OBF1-encoding gene, one has to presume that these alterations are tolerated *in vivo* because BAF1 is a functional protein (25).

OBF1 Overexpression in Yeast. To assess whether the cloned gene encodes the previously isolated OBF1 protein, we cloned the gene into a yeast expression vector, pMH101, under control of the *Gal1* promoter to yield plasmid pMH-OBF1. Expression of the cloned gene in yeast was done as described in Fig. 3. To quantitate the level of OBF1 overproduction, extracts were prepared from cells harboring the pMHOBF1 plasmid. Increased amounts of extracts from cells induced with galactose were electrophoresed on SDS/polyacrylamide gels and then subjected to immunoblot analysis (Fig. 2). As a reference, extracts were also prepared from uninduced cells and cells lacking the pMHOBF1 plasmid. From the results described in Fig. 3, we have estimated that the induction by galactose caused ≈40-fold overproduction of OBF1. A direct DNA band-shift assay also indicates at least 40-fold overexpression (data not shown). It is worth noting in Fig. 3 that (*i*) in crude extracts the multiform OBF1 appearance on SDS/polyacrylamide gels in galactose-induced and uninduced cells is identical and (*ii*) all forms of OBF1 appear to be induced by galactose. The *OBF1* gene was also cloned into an *E. coli* expression vector under control of the λpl promoter. When expressed in *E. coli*, the OBF1

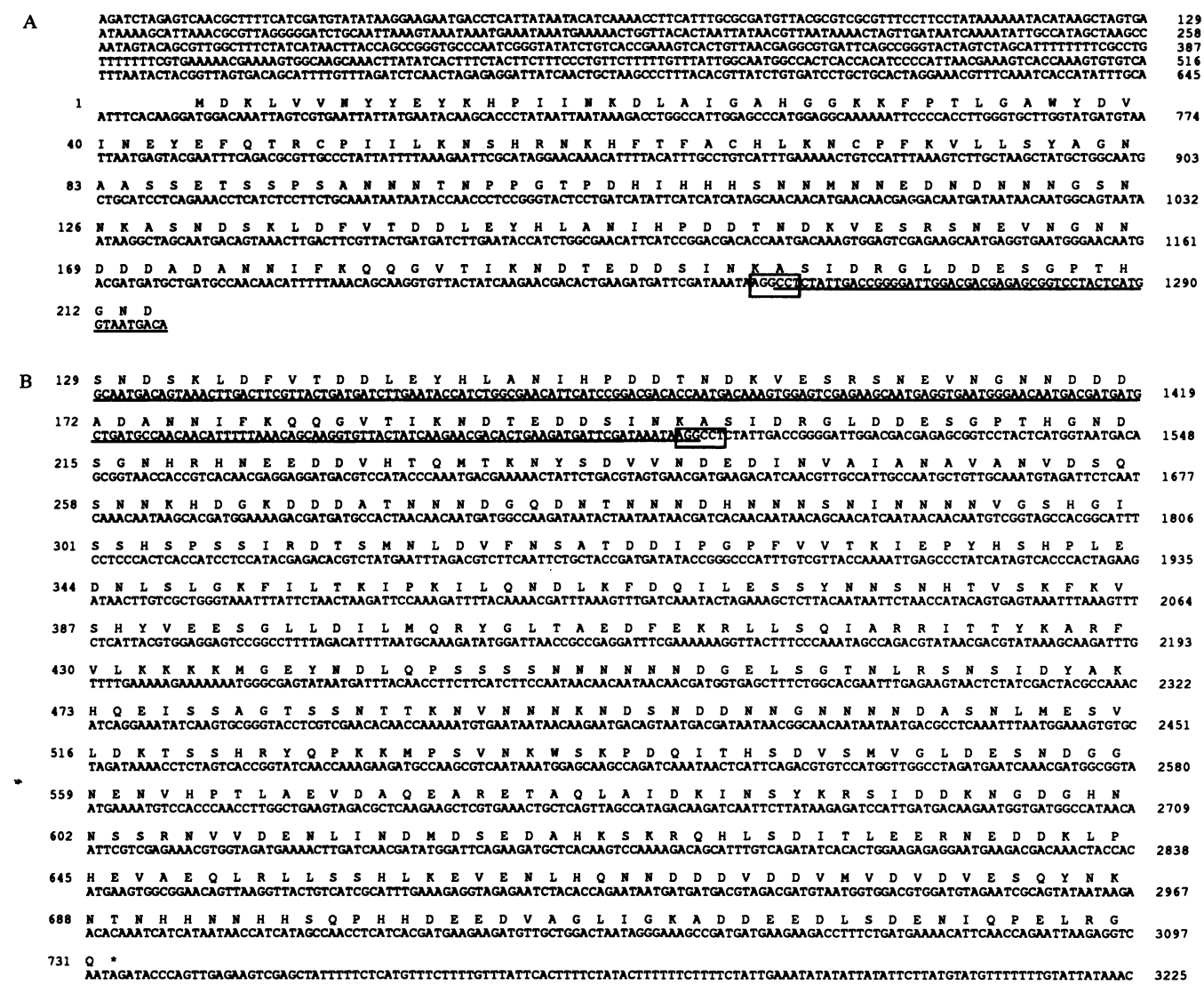

FIG. 2. Nucleotide sequence of *OBF1*. Sequencing of DNA was done as described. (*A*) Nucleotide sequence of the fragment obtained by PCR. (*B*) Nucleotide sequence of the portion of the yeast insert representing *OBF1*. Underlined sequences in both fragments, which are partially overlapping, were deleted when the two DNA chains were joined by ligase at the *Stu* I site indicated by the open box. The amino acid sequence inferred from the nucleotide sequence is identical to ABF-I and differs from BAF1 in the following positions: 125 (asparagine instead of lysine), 128 (alanine instead of valine), 148 (isoleucine instead of threonine), 279 (threonine instead of asparagine), 280 (asparagine instead of threonine), 303 (histidine instead of arginine), and 690 (asparagine instead of threonine).

protein migrated in SDS/polyacrylamide gel as a single band of 123 kDa identified by anti-OBF1 antibodies (data not shown). These results confirmed that the cloned gene encodes the protein originally identified as OBF1.

OBF1 Is Multiply Phosphorylated. We had previously observed that the OBF1 isolated from yeast has a multimeric appearance on polyacrylamide gels in contrast to the protein expressed in *E. coli*, which migrates as a single band (data not shown). Similar observations were reported for ABF-I (19), suggesting that the protein undergoes posttranslational modifications in yeast. It has been shown that phosphorylated proteins may appear as closely migrating bands upon electrophoresis in polyacrylamide gels (25). To test the possibility that OBF1 is phosphorylated, pMHOBF1-containing cells were grown in the presence of ^{32}P-labeled inorganic phosphate during galactose induction. OBF1 was then purified as described (*Materials and Methods*), including the specific DNA-affinity chromatography step (7). An aliquot of purified OBF1 was subjected to electrophoresis on an SDS/polyacrylamide gel, and the results were autoradiographed. After autoradiography the polyacrylamide gel was analyzed by immunoblotting with anti-OBF1 antibodies. The protein bands, visualized by anti-OBF1 antibodies, were also clearly labeled by ^{32}P (Fig. 4, lanes 2 and 3). Surprisingly, all bands seen by the immunoblot analysis appear phosphorylated, suggesting that OBF1 exists *in vivo* as a heterogeneous collection of differentially phosphorylated polypeptides. This notion was further substantiated by treating the phosphorylated OBF1 with trypsin, as described in Fig. 5*A*. Upon electrophoresis of the trypsin digest on TLC-cellulose plates, although two of the ^{32}P-labeled peptides were more abundant than the others, the label clearly comigrated with several distinct peptides. All radioactive label was liberated as free phosphate when the peptides were treated with calf intestinal phosphatase, indicating that the phosphate was linked to the protein via a phosphoester linkage (Fig. 5 *A* and *B*).

OBF1 Is Phosphorylated at Serine and Threonine Residues. To determine the amino acid residues phosphorylated, a tryptic digest of the ^{32}P-labeled OBF1 was prepared as described in Figs. 4 and 5. The tryptic peptides were then acid hydrolysed, and the hydrolysate was analyzed by electrophoresis on TLC-cellulose plates (Fig. 5*B*). A major portion of the radioactive label comigrated with a phosphoserine marker, identified by staining with ninhydrin. A minor frac-

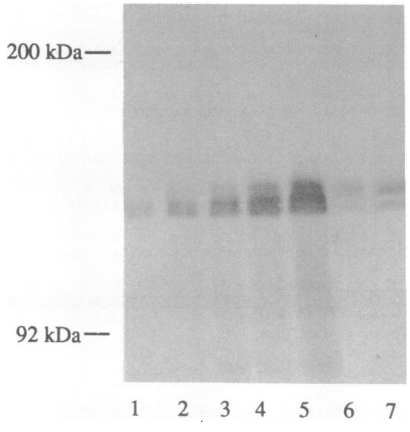

FIG. 3. OBF1 overexpression in yeast. Crude extracts, fraction I, were prepared as described (15). Extracts were prepared from cells harboring pMHOBF1 that were induced by galactose, uninduced cells, and cells lacking pMHOBF1. Aliquots of extracts were applied to a SDS/6% polyacrylamide gel as before; after electrophoresis the gel was analyzed by immunoblotting as described (7). Lanes: 1–5, 1, 5, 10, 50, and 100 μg of protein from the induced cells, respectively; 6 and 7, 200 μg of protein extracts from uninduced cells and from cells lacking pMHOBF1, respectively. Protein size markers (Rainbow prestained from Amersham), myosin (200 kDa), and phosphorylase *b* (92 kDa) were also included. Proteins were determined by the Bio-Rad protein assay.

tion was identified as phosphothreonine. Identical results were obtained when electrophoresis was done in two different buffer systems.

DISCUSSION

We have presented evidence indicating that (*i*) OBF1 is identical to the ABF-I and BAF1 proteins and (*ii*) OBF1 is multiply phosphorylated at serine and threonine residues.

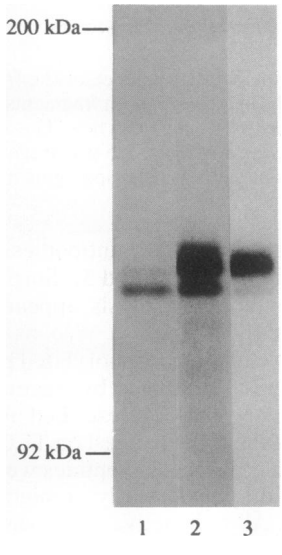

FIG. 4. All OBF1 polypeptides are phosphorylated. OBF1 was ^{32}P-labeled *in vivo* and purified. Radioactively labeled OBF1 was subjected to electrophoresis on SDS/5% polyacrylamide gel, as for Fig. 3. Proteins were transferred to nitrocellulose and exposed 24–48 hr at −70°C to an x-ray film with intensifying screens; then the nitrocellulose filter was treated with anti-OBF1 antiserum to visualize OBF1 protein. Lanes: 1, as a standard, 12 μg of the originally purified OBF1 (15) visualized by the antibody reaction as in Fig. 3; 2, purified OBF1, labeled *in vivo* with ^{32}P and visualized by the antibody reaction; and 3, OBF1 in lane 2 as identified by autoradiography. Protein size markers are the same as in Fig. 3.

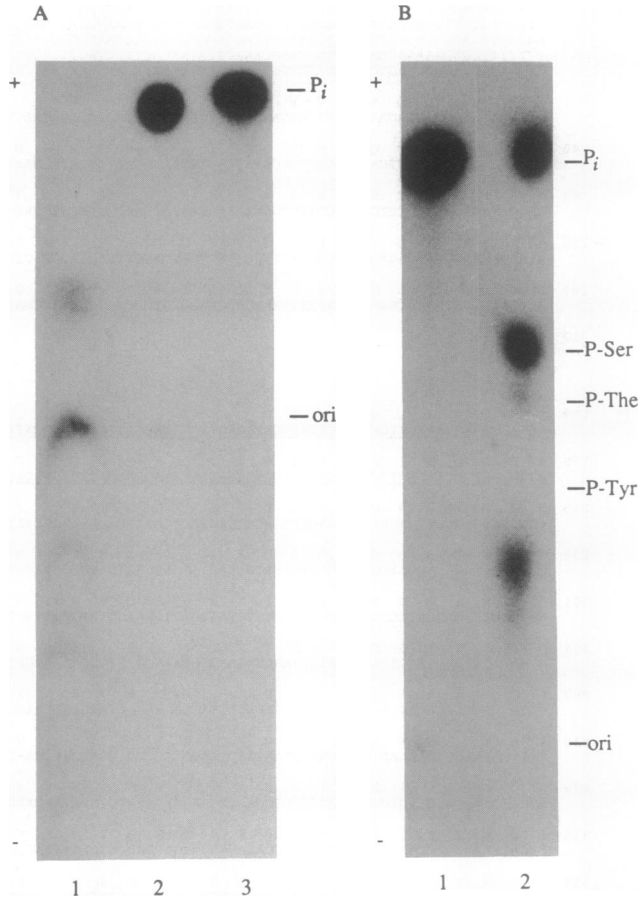

FIG. 5. ^{32}P-labeled OBF1 tryptic peptides and phosphoamino acids analysis by electrophoresis on TLC-cellulose plates. ^{32}P-labeled OBF1 was purified and applied to a SDS/polyacrylamide gel, as for Fig. 4. After electrophoresis, the OBF1 band was identified by autoradiography. The radioactive band was then excised and treated with trypsin as described. (*A*) Autoradiogram of tryptic peptides separated upon electrophoresis on a TLC-cellulose plate. Lanes: 1, ^{32}P-labeled tryptic peptides; 2, tryptic peptides after treatment with calf intestinal phosphatase; and 3, migration of ^{32}P-labeled inorganic phosphate. (*B*) Migration of ^{32}P-labeled material after acid hydrolysis, as described. Lanes: 1, free inorganic phosphate; 2, aliquot of acid hydrolysate mixed with *O*-phosphoserine, *O*-phosphothreonine, and *O*-phosphotyrosine (0.5 μg of each) (Sigma). After autoradiography for 5 days at −70°C, staining by ninhydrin (0.2% in acetone) revealed location of the phosphoamino acid. ori, Position at which samples were applied. The bottom radioactive spot probably represents partially hydrolysed peptides. *P*-Ser, *P*-The, and *P*-Tyr, positions of migration of phosphoserine, phosphothreonine, and phosphotyrosine, respectively, as identified by ninhydrin staining.

These results suggest that phosphorylation may modulate OBF1 functions *in vivo*.

We have suggested that OBF1 is involved in activating yeast origins of replication primarily because of our demonstration that (*i*) OBF1 DNA-binding sites are in a broad spectrum of origins (7) and (*ii*) the OBF1 DNA-binding sites in ARS*121*, a single copy ARS that functions as an origin of replication in its natural chromosomal location (S. S. Walker and S.E., unpublished work), has a role as an enhancer of DNA replication (8, 9). This enhancer works in an orientation-independent manner and can function even when situated at 1.2 kb from the ARS core sequence essential for origin activation. Furthermore, linker substitution mutations within the binding site obliterated the enhancer activity *in vivo* and the binding of OBF1 to the enhancer *in vitro* (11), suggesting that OBF1 is the enhancer protein. Other studies with the ABF-I and BAF1 proteins suggested that OBF1 may have a role in transcriptional regulation as well. OBF1 DNA-binding

sites were identified upstream of several genes, including *DED1* (10), the intergenic region of *YPT1/TUB2* (14) and others (see Introduction). Recently, Buchman and Kornberg (17) suggested that the ABF1 protein acts synergistically in conjunction with other weak activating elements to activate *DED1*, an essential gene of unknown function. Studies with BAF1 implicated the protein in the activation of two divergently transcribed *YPT1* and *TUB2* genes (14). In addition, evidence was reported suggesting a function for ABF-I in transcriptional repression. The B and E elements, ABF-I and RAP1 DNA-binding sites, respectively, were important components of the transcription silencer in the mating-type locus (26, 27); silencer activity was significantly reduced only in a double mutant in which both B and E sites were mutated (27).

Thus, OBF1 is a multifunctional regulatory protein, having a role in replication and transcription. Proteins with such dual functions have also been identified in mammalian cells (28). The question how the diverse activities of OBF1 are regulated in the cell is intriguing. From the findings described here it is tempting to postulate that OBF1 functions are modulated by phosphorylation. The protein kinase that phosphorylates OBF1 is not known, although the Cdc28 and Cdc7 proteins, both serine/threonine kinases (29, 30), are potential candidates. The fact that several OBF1 tryptic peptides associate covalently with phosphate implies that a number of OBF1 domains become phosphorylated. Therefore, phosphorylation/dephosphorylation of specific domains, as occurs in the simian virus 40 large tumor antigen (31–33), could determine whether OBF1 is destined for replication or transcription.

How OBF1 works in replication is not clear. Because OBF1 enhancer works when present at long distances from the essential element of the ARS, to stimulate the origin of replication, OBF1 bound to the enhancer probably has to interact with constituents of the essential element of the ARS. This result could be accomplished by DNA looping, which would place the OBF1 in proximity to a target site, enabling direct interaction of the regulatory protein with a component, presumably a protein, of the replication apparatus. For OBF1 to fulfil a role in transcription, interactions with other proteins, constituents of the transcriptional machinery, may be necessary. Thus, phosphorylation of specific OBF1 domains may modulate these potentially diverse protein–protein interactions. In addition, the phosphorylation may impact the affinity of OBF1 binding to DNA, which may account for the range of binding affinities observed for OBF1 in several ARSs (7).

This paper is dedicated to the memory of Martha Ethlyn Francesconi. We thank Scott S. Walker and William A. Whalen for the careful reading of the manuscript and their useful comments. This work was supported by a National Science Foundation Grant DMB-8916358.

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