Saccharomyces cerevisiae BUF Protein Binds to Sequences Participating in DNA Replication in Addition to Those Mediating Transcriptional Repression (URS1) and Activation

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The heteromeric BUF protein was originally shown to bind to URS1 elements which are situated upstream of many genes in Saccharomyces cerevisiae and mediate negative control of their transcription. Among the genes regulated through the URS1 site and the proteins interacting with it are those participating in carbon, nitrogen, and inositol metabolism; electron transport; meiosis; sporulation; and mating-type switching. We show here that pure BUF protein, in addition to binding to the negatively acting URS1 site, also binds to CAR1 sequences supporting transcriptional activation (upstream activation sequences). To determine the BUF protein structure, we cloned and sequenced the BUF1 and BUF2 genes and found them to be identical to the RF-A (RP-A) genes whose products participate in yeast DNA replication as single-stranded DNA binding proteins. These data argue that BUF protein-binding sites serve multiple roles in transcription and replication.

Regulated expression of the arginase (CARI) gene in Saccharomyces cerevisiae is accomplished through the coordinated action of multiple positively and negatively acting transcription factors and the availability of arginine (23, 34, 37, 49–51). The binding sites for some of these factors have been arbitrarily divided into four functional units in order to facilitate their study. There are three upstream activation sequences (UASs), designated UASC1, UASC2, and UAS5 (23), and one upstream repression sequence, URS1 (31, 50). The first two UAS elements mediate inducer-independent transcriptional activation and consist of multiple ABFI and RAP1 binding sites (24, 25). ABFI and RAP1 are general transcription factors found upstream of many yeast genes (4, 9, 26). Inhibition of transcription supported by these inducer-independent UASs and the proteins that bind to them is mediated by a stronger negatively acting site (URS1) and the protein(s) that binds to it (23, 29, 31). Mutation or deletion of the URS1 site eliminates this inhibition and results in CARI expression supported by the inducer-independent UASs (49, 50). Therefore, in wild-type cells grown without arginine, the balance between activation and repression of CARI transcription is tipped toward reduced expression (23, 29, 31). On the other hand, when arginine is present, the third UAS, UAS1, whose operation is completely inducer dependent, joins the two inducer-independent UASs, and the combined activation capability of the three UASs tips the balance in the direction of increased transcription (60).

The CARI URS1 site was originally identified by a single point mutation (CARI-0*) which rendered the inducible CARI gene constitutive (49). Saturation mutagenesis of the CARI URS1 site revealed it to be the nonanucleotide 5'-AGCCGCGCA-3' (31). Sites homologous to URS1 have recently been identified upstream of many yeast genes, including those associated with carbon (6, 8) and nitrogen metabolism (12, 57) and respiratory apparatus components (7, 17, 64) and those associated with meiosis (11, 55), sporulation (33), and mating-type switching (47). In an increasing number of cases, deletion or mutation of the URS1-homologous sequences upstream of these genes has led to markedly increased expression, suggesting that they function in a manner similar to that proposed for CARI (6–8, 31, 33, 47, 55, 57, 64). The protein that binds to the CARI URS1 site was purified to homogeneity and shown to be a heteromer composed of 37.5- and 73.5-kDa monomers designated BUF1 and BUF2, respectively (29). The protein exists in its heteromeric form both in the presence and in the absence of DNA (29).

Transcriptional repression mediated by the URS1 site, however, involves more than the BUF protein alone. At least one other protein, encoded by the CAR80 (UME6) gene, is also required (40, 63). Like mutation or deletion of the CAR1 URS1 site, disruption of CAR80 results in inducer-independent expression of CARI (48, 63). Formation of the BUF-URS1 DNA complex does not appear to require the CAR80 (UME6) product because it can be demonstrated with extracts from a car80 (ume6) disruption strain (40). There is a lower-molecular-weight DNA-protein complex formed with the wild-type extract that disappears when the car80 (ume6) disruption mutant extract is used, but its physiological significance has not yet been demonstrated (40). This observation does raise the possibility, however, that the CAR80 (UME6) product also binds to DNA.

Studies of the biochemical mechanisms through which the BUF and CAR80 products function will require structural information about these proteins. In addition, at least for BUF protein which binds to a defined cis-acting element, the proposed mechanisms must also explain how the protein contributes to accomplishing the biological functions of the cis-acting elements to which it binds. In this work, we define new biological functions in which BUF protein-binding sites participate and hence must be accounted for by future mechanistic studies; we demonstrate that BUF protein binds to DNA sequences that mediate transcriptional activation as well as repression. In addition, we have used monoclonal antibodies generated against the purified BUF proteins as probes to identify λ-gt11 phage expressing BUF1 and BUF2.
antigenic determinants. These phages permitted us to clone the BUF1 and BUF2 genes and to deduce the amino acid sequences of the proteins which they encode. This in turn permitted identification of yet a third biological process in which BUF protein is a participant.

(A preliminary report of this work has already appeared [30].)

MATERIALS AND METHODS

Screening the λ-gt11 library. The λ-gt11 genomic library for protein expression was prepared from yeast strain EJ101 (65). Twenty 150-mm-diameter petri dishes containing LB-Am- plex medium (5 g of yeast extract, 5 g of NaCl, 10 g of Bacto Tryptone, 1 ml of 1 M NaOH, 2 mM MgCl2, 15 g of agar, 50 mg of ampicillin per liter) were freshly prepared. A 0.2-ml aliquot of an exponentially growing culture of Escherichia coli Y1090 was infected with 2.7 × 10^10 phage. After 10 min of incubation at 37°C, the infected cells along with 8 ml of top agar (5 g of yeast extract, 5 g of NaCl, 10 g of Bacto Tryptone, 1 ml of 1 M NaOH, 2 mM MgCl2, 7.5 g of agar, 50 mg of ampicillin per liter) were added to each plate. The plates were incubated at 42°C for 3.5 h. Nitrocellulose filters were presoaked in 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the moist filters were used to cover the plates containing phage. Overnight incubation at 37°C allowed expressed protein to bind to the filters. Cell debris was removed from the filters by washing in TBST (50 mM Tris [pH 7.0], 150 mM NaCl, 0.05% Tween) for 5 min, and the filters were then air dried. The dry filters were treated with TBST containing 20% fetal calf serum for 1 h and then washed in TBST. Primary-antibody incubations were performed with a 20-fold dilution of a monoclonal antibody mixture of antibody (Ab) 36 (Ab 36 recognizes BUF2 protein) and antibody 62 (Ab 62 recognizes BUF1 protein) in TBST for 90 min. The filters were then washed three times in TBST for 5 min each. Secondary-antibody incubations in TBST were performed with Bio-Rad goat anti-mouse Immunoglobulin G (H + L) horseradish peroxidase conjugate (no. 170-6516; Bio-Rad) at a 1/3,000 dilution for 60 min. Subsequent washes, once in TBST and twice in TBS (TBST without Tween) for 5 min each, preceded incubation in horseradish peroxidase conjugate substrate (no. 170-6431; Bio-Rad) for 30 min. Twenty-four hours later, single plaque purified three times. Subsequent screening also included antibody 270 (Ab 270 also recognizes the large protein, BUF2).

Characterization of the lambda clone insert. All 24 primary phage isolates were grown to near confluency on 90-mm-diameter petri dishes containing Luria-Bertani agar with ampicillin (50 mg/liter) at 37°C. A 5-ml aliquot of SM buffer (100 mM NaCl, 0.2% MgSO4, 50 mM Tris [pH 7.5], 0.1% gelatin) was added to each plate, and the plates were incubated at 4°C under constant agitation for 12 h. The resulting plate lysates were added to 2-ml DEAE columns (18). Phage was eluted with 0.6 ml of elution buffer (10 mM Tris [pH 8.0], 50 mM magnesium acetate) and digested with 1 μg of proteinase K–0.3% sodium dodecyl sulfate (SDS) at 25°C for 5 min. For protein precipitation, 0.35 M potassium acetate was added, and the mixture was heated to 88°C for 20 min and then transferred to ice. After centrifugation at 12,000 × g for 10 min, 20 μg of muscle glycogen and 1 volume of isopropanol were added to the supernatant to precipitate the phage DNA. Following 30 min of incubation on ice, the DNA was pelleted at 12,000 × g and washed with 100% ethanol. The DNA precipitate was dried and resuspended in 25 μl of Tris-EDTA (pH 8.0). A 12.5-μl sample of the DNA was used for restriction site analysis.

Lambda lysogen preparation. Preparations of the above phages were lysogenized in E. coli Y1089. Heat-sensitive lysogens were chosen and superinfected with lambda CR. The lambda CR-resistant isolates were isolated and grown in 250 ml of LB-Amp broth to a density of 5 × 10^8/ml. The temperature and the volume of the growing cultures were abruptly raised by the addition of an equal volume of 60°C Luria-Bertani medium, and the cells were grown at 42°C for 30 min. Host cells were finally incubated at 37°C for 3 to 4 h to allow for phage amplification. Cells were harvested after incubation on ice for several minutes and were pelleted at 20,000 × g for 10 min. The pellets were then resuspended in 25 ml of SM buffer containing 6 μg of DNase (pancreatic; Sigma) per ml and lysed with 5 to 10 drops of chloroform. The lysates were incubated at 4°C overnight and afterwards centrifuged at 12,000 × g for 30 min. The phages were collected by centrifugation (30,000 × g for 2 h), and the pellets were resuspended in 1.5 ml of SM buffer to stabilize the recovered phage. A 25-μl volume of 20% SDS was added to each sample, and the samples were incubated at 60°C for 5 min. The samples were cooled on ice, phenol extracted, and precipitated with 1/10 of a volume of 5 M sodium acetate and 2 volumes of ethanol. The dried DNA pellets were resuspended in 500 μl of Tris-EDTA.

Plasmid constructions. The polymerase chain reaction (PCR) was employed to construct genomic deletions of the BUF genes (strategies used in Fig. 1) as well as to clone them downstream of the T7 promoter in plasmid pT7-7 (strategies used in Fig. 2) for expression in E. coli (53). PCR primers (Fig. 3) were synthesized to contain a 24- to 27-bp match to the template at their respective 3′ termini. The 5′ termini each contained a restriction enzyme recognition sequence to enable the resulting PCR product to be ligated into plasmid pBR322 or pT7-7. For each PCR mixture, 0.2 pmol of template DNA was used. The primer-to-template ratio was 100:1. Reaction mixtures also contained 10 μl of 10× reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM MgCl2, 0.1% gelatin), 1.25 mM concentrations of each of the four deoxynucleotide triphosphates, 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and water for a final volume of 100 μl. Reactions were performed with a Perkin-Elmer Cetus DNA Thermal Cycler with an amplification cycle of 94°C for 3 min. Thirty cycles were performed according to the standard protocol of the manufacturer. Following amplification, the PCR product was phenol extracted, precipitated, and resuspended for restriction enzyme digestion. The resulting double-stranded DNA fragment was gel purified and ligated into the respective vector. The nucleotide sequence of all PCR-derived DNA was verified prior to further utilization.

E. coli extract preparation. A 100-ml Luria-Bertani culture of exponentially growing E. coli BL21 (DE3) containing a T7 expression plasmid was induced by the addition of IPTG to a final concentration of 1.0 mM. After a 3-h induction, the cells were harvested by centrifugation at 10,000 rpm for 10 min. In some cases, the cell pellets were frozen overnight at −80°C. The pellets were resuspended in 5 ml of sonication buffer (20 mM Tris [pH 7.4], 100 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Cell suspensions were sonicated on ice with a microtip set at 30% power. Sonication cycles of 10 s on and 10 s off were repeated 15 times. Sonicates were centrifuged at 10,000 rpm for 30 min to remove cell debris and were transferred to...
prechilled tubes containing 1.0 ml of glycerol. After being mixed, the extract was aliquoted and stored at −80°C.

**Protein sequencing.** Amino acid sequence determinations of purified BUF protein preparations were accomplished with the Applied Biosystems model 477A Peptide Microsequencing System.

**Miscellaneous procedures.** We used the method of Tabor and Richardson for DNA sequence analysis (54). SDS-polyacrylamide gel electrophoresis (PAGE) analysis was performed as described by Laemmli (27). Western blot (immunoblot) analysis was accomplished by the procedures of Towbin et al. (56). BUF protein purification was performed by the procedures of Luche et al. (29). β-Galactosidase assays and electrophoretic mobility shift assays (EMSAs) were performed as described previously (31). Expression of BUF protein in *E. coli* was by the procedure of Tabor (53).

**RESULTS**

**BUF protein binding to UAS elements upstream of CAR1.** BUF protein has previously been repeatedly shown to bind to DNA fragments containing negatively acting sites with homology to UR SI (29–31, 49–51). However, during our original genetic analysis of the CAR1 UR SI site, we recognized that a transversion (C to G) at position −153 resulted not only in the loss of UR SI-mediated repression of CAR1 UAS activity but also in a 10-fold increase in that activity (Fig. 3) (31). This mutation is identical to the CAR1-0− mutation whose structure we originally identified (49) from a mutant isolated by Wiam (63). To assess whether this increased reporter gene expression derived from the generation of a UAS element, we cloned all of the transversion mutations of the parent UR SI fragment into a heterologous expression vector and measured their abilities to support reporter gene expression. As shown in Fig. 4, only the DNA fragment containing the transversion mutation at position −153 supported transcriptional activation.

We assayed the ability of a DNA fragment containing the UR SI site transversion mutation at position −153 (Fig. 3, fragment RL193/194) to bind BUF protein by an EMSA. The DNA fragment formed a complex whose mobility was identical to that previously documented (29) to be generated by the BUF protein and was successfully inhibited by an excess of the wild-type UR SI fragment RL153/154 (data not shown). As shown in Fig. 5, lane R, the mutant fragment also bound to pure BUF protein as previously documented for the wild-type UR SI fragment (29). We further tested this
The observation by assessing the ability of the URSI position -153 transversion mutant fragment (RL193/194) to serve as a competitor of wild-type URSI (DNA fragment RL153/154) binding to pure BUF protein. The mutant fragment (RL193/194 [Fig. 5, lanes A to G]) was as effective a competitor of a DNA fragment containing a wild-type CARI URSI element for protein binding as was the wild-type element itself (RL153/154 [Fig. 2, lanes A to G, in reference 29]).

A search of the CARI upstream region identified a sequence with close homology to the one on the position -153 transversion mutant DNA fragment. This sequence was contained on a 30-bp DNA fragment from the UAS element promoter region of CARI (CARI positions -280 to -251) previously shown to support transcriptional activation in a heterologous expression vector assay system (plasmid plK78 [Fig. 5 in reference 23]). As shown in Fig. 5, lanes N to Q, a DNA fragment (RL195/196) containing CARI sequences -303 to -248 bound to both crude and pure BUF protein. DNA fragment RL195/196 was used as a competitor of the wild-type CARI URSI fragment for binding to pure BUF protein in an EMSA. DNA fragment RL195/196 from CARI UAS element was just as effective a competitor as the position -153 transversion mutant fragment (RL193/194 [Fig. 5, lanes G to M]). In contrast, when the 6 nucleotides of this DNA fragment homologous to CARI URSI were mutated (DNA fragment BS52/53), its ability to serve as a competitor was lost (Fig. 6).

To ascertain the functional capabilities of the wild-type and mutated DNA fragments assayed as shown in Fig. 5 and 6, they were cloned into expression vector plasmid pNG15 (40). As shown in Fig. 7, a plasmid containing the wild-type DNA fragment (pS1-1) supported high-level reporter gene expression. The mutated DNA fragment, on the other hand (contained in plasmid pS2-1), supported reporter gene expression that was no higher than that observed with the vector alone (Fig. 7, plasmid pNG15). Together, these data suggest that the 6 nucleotides required for the 56-bp DNA fragment to support transcriptional activation are also required for binding to BUF protein.

**BUF protein binding to the SDP1 site.** Finally, during
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FIG. 4. Capability of various URS1 element transversion mutants to support transcriptional activation in a heterologous expression vector assay system. Oligonucleotides containing the mutant URS1 elements were cloned into plasmid pMG17 (63). The mutant plasmids were then used as the source of DNA to transform yeast strain RH218. Transformants were assayed for their abilities to trans-activate β-galactosidase production. The activity of the parent plasmid vector alone was 84 U.

data indicated that more than a single protein was likely involved and that none of these proteins was SIN3 (UME4) (62). The observation that the ume4 and umes6 mutants share some phenotypic characteristics (40, 59) further argued that we might expect to see some relationship between the SDP1 binding site and BUF protein. Therefore, we tested the ability of crude BUF protein to bind to the SDP1 site in a competition assay using the CARI URS1 fragment as a probe. As shown in Fig. 8A, the DNA fragment containing the SDP1 site was an effective competitor of the CARI URS1 fragment binding to BUF protein. When the converse experiment was performed with a DNA fragment containing the SDP1 site as the radioactive probe, a more complex result was observed. The strong BUF-DNA complex was observed regardless of whether the radioactive probe contained the CARI URS1 site (Fig. 8A) or the SDP1 site (Fig. 8B). Moreover, the DNA fragment containing CARI URS1 was a successful competitor in both cases. However, when the SDP1 site-containing fragment was used as the probe, a pair of higher-molecular-weight complexes were detected. Increasing concentrations of competitor DNA containing the SDP1 site but not the CARI URS1 site eliminated these higher-molecular-weight complexes.

Isolation of the BUF genes. The above experiments document markedly expanded transcriptional functions mediated by the URS1 site and therefore possibly by the protein(s) bound to it, i.e., BUF protein. It was therefore important to obtain information about the structure of the BUF protein as a foundation for studies designed to help us understand how the URS1 site was able to mediate these multiple functions and the role(s) played by BUF protein in them. To that end, a mixture of three monoclonal antibodies generated against purified BUF2 (Ab 36 and Ab 270) and BUF1 (Ab 62) proteins (29) was used to screen a lambda gt11 expression library (5.4 × 107 independent clones) obtained from Alan Clark (38). Twenty-four phages exhibiting positive antibody responses were identified, purified, and preliminarily characterized. These phages were divided into 16 groups on the basis of the particular antibody recognition of the expressed protein and pattern of EcoRI fragments possessed by each phage. Three classes of phages gave positive reactions with antibody Ab 62. We chose two phage clones (phages 2.2 and 6.1) from these groups for further analysis. Phage 2.2 contained three EcoRI fragments 1.5, 0.6, and 0.4 kb long, while phage 6.1 contained fragments 0.8 and 0.3 kb long. The remaining 13 classes contained proteins recognized by antibody Ab 36, and 4 of these were also recognized by antibody Ab 270. We chose one phage from each of two classes that reacted with both antibodies for further analysis, phases 9.2

FIG. 3. Sequences of DNA fragments used in EMSAs, for cloning into heterologous expression vectors, and as PCR primers for construction of the plasmids in Fig. 1 and 2.

studies of BUF protein binding to the upstream regions of several genes not related to nitrogen metabolism, the question of whether BUF protein bound to the SDP1 site situated upstream of the HO gene (62) was raised (46). The SDP1 binding site contained a GC-rich sequence that shared some homology with the CARI URS1 site. In addition, available
and 9.4. Phage 9.2 contained two 1.2-kb EcoRI fragments, while phage 9.4 contained a single 1.7-kb fragment. The EcoRI fragments were subcloned from each phage and designated in descending order of size: pLS1, pLS2, and pLS3 for phage 2.2; pLS4 and pLS5 for phage 6.1; pLS6 for phage 9.2; and pLS7 for phage 9.4. The EcoRI fragments from plasmids pLS1, pLS2, and pLS3 were radioactively labelled by the random priming method and hybridized to a yeast chromosomal blot. DNA from plasmid pLS1 hybridized to chromosome XIV, while plasmids pLS2 and pLS3, derived from the same phage (phage 2.2), hybridized to chromosome XI (data not shown). In a similar experiment, phage 6.1-derived DNA fragments from plasmids pLS4 and pLS5 hybridized to chromosome XIV. Therefore, it appeared that chromosome XIV was the more likely location of the BUF2 gene. DNA fragments from both plasmids pLS6 and pLS7 hybridized to chromosome I, indicating it to be the most likely location of the BUF2 gene. Southern blot analyses using three restriction endonucleases (BamHI, EcoRI, and SalI) demonstrated that DNA fragments on plasmids pLS1, pLS4, and pLS5 were from the same genomic region on chromosome XIV (data not shown). Similar analyses were carried out with inserts of plasmids pLS6 and pLS7, and the data obtained indicated that the fragments were derived from the same region of chromosome I. Together, these data were consistent with the suggestion that plasmids pLS1, pLS4, pLS5, pLS6, and pLS7 likely contained inserts encoding portions of the BUF1 and BUF2 genes.

To obtain complete genomic copies of the putative BUF genes, the inserts of these plasmids (EcoRI fragments) were

**FIG. 5.** (Left panel) Lanes A to M show competition between CARI DNA fragments mediating transcriptional activation (CARI-0" and CARl UAS_{C2}) and one containing a wild-type CARI URSI. In each case, 50 ng of a ^32P-labelled DNA fragment (RL153/154) containing the URSI element was used as probe. Affinity-purified BUF protein (2 μl) was used as the source of protein in this experiment. A 165-fold excess of sonicated and heat-denatured calf thymus DNA was added to each reaction mixture as a nonspecific competitor. Lane G contains no protein, while lanes F and H contain no competitor DNA (−COMP). Lanes E back through A contain increasing amounts (in micrograms) of an unlabelled DNA fragment (RL193/194) containing the CARI-0" sequence. Lanes I through M contain increasing amounts (in micrograms) of an unlabelled DNA fragment (RL195/196) containing CARl UAS_{C2} (Right panel) Lanes N to R show binding of CARI-0" and CARl UAS_{C2} DNA fragments to crude and purified BUF protein. Fifty nanograms of a radioactive DNA fragment (RL195/196) containing the CARl UAS_{C2} sequence was used as probe for lanes N to Q. In lane R, 50 ng of a DNA fragment (RL193/194) containing the CARI-0" sequence was used as the probe. Lane N contains no protein (−EXT.), while lanes O and P contain 0.25 μl of crude yeast extract. Lane P also contains 1 μg of a nonradioactive DNA fragment (RL153/154) containing the CARl URSI element. In lanes Q and R, 2 μl of affinity-purified BUF protein was added as the source of protein.

**FIG. 6.** Requirement of CARI UAS_{C2} sequences mediating transcriptional activation for binding of BUF protein. The experiment was conducted as described in the legend to Fig. 5, except that the competitor DNA fragments were RL195/196 (wild type [WT]) and BS2/33 (mutant). The radioactive probe was the CARI DNA fragment that served as the insert of plasmid pRL58 (31).

**FIG. 7.** Reporter gene expression supported by wild-type (WT) (pS1-1 in Fig. 3) and mutant (pS2-1 in Fig. 3) plasmids containing sequences from the UAS_{C2} region of the CARI promoter. The assay was conducted as described in Materials and Methods. The cells were grown in minimal yeast nitrogen base medium containing 0.1% arginine as the nitrogen source.
FIG. 8. Competition between DNA fragments containing the SDP1 binding site (RL210/211) or the CAR1 URS1 element (RL208/209) for binding to protein derived from a crude extract. In panel A, 50 ng of labelled DNA fragment 208/209 (CAR1 URS1) was used as the probe, while in panel B, 50 ng of labelled DNA fragment RL210/211 (SDP1 site) was the probe. Each reaction mixture contained 0.25 µl of crude yeast extract and a 165-fold excess of sonicated and heat-denatured calf thymus DNA as a nonspecific competitor. In lanes G, extract was omitted (–ext), while in lanes F and H, competitor DNA was omitted (–comp). Lanes E back through A contain increasing amounts (in micrograms) of unlabelled DNA fragment RL208/209 (CAR1 URS1), while lanes I through M contain increasing amounts of unlabelled DNA fragment RL210/211 (SDP1 site).

used as probes to screen an S. cerevisiae library in E. coli (43). Approximately $8 \times 10^4$ transformants were screened with a mixture of the above probes, and 52 candidates were obtained following a secondary screen. These isolates were screened with the inserts of plasmids pLS1 and pLS4 or plasmids pLS6 and pLS7. Fourteen of the candidate clones hybridized to DNA fragments from both plasmids pLS1 and pLS4, while 35 hybridized to the inserts of both plasmids pLS6 and pLS7 (data not shown). Crude restriction maps were prepared for five candidates from each class. Detailed restriction maps of a representative of each class are shown in Fig. 9 along with the regions covered by each of the phage.

FIG. 9. Restriction maps of yeast chromosomal DNA containing BUF1 and BUF2. Plasmid pLS9 contains the ORF of BUF2, while pLS10 contains the ORF of BUF1. ORFs are indicated by solid arrows. The hatched boxes indicate vector sequences. DNA fragments derived from λ-gt11 clones, as well as plasmids containing them, are indicated below the maps.
and plasmid inserts.

**Nucleotide sequence analysis of the BUF genes.** Using the inserts of plasmid pairs pLS1 and pLS4 and pLS6 and pLS7 to indicate the positions of the BUF1 and BUF2 genes, respectively, we completely sequenced both strands of each gene along with their 5' and 3' flanking sequences (Fig. 10 and 11). The small amount of the BUF1 open reading frame (ORF) contained in the insert of plasmid pLS1 raised the possibility that we had cloned the incorrect gene. This doubt was eliminated by comparing a short protein sequence previously determined with purified BUF1 protein with the sequence deduced from the BUF1 DNA sequence (underlined residues in Fig. 10). The sequences matched, indicating that we had cloned the gene encoding the BUF1 protein.

**BUF2** contains an ORF of 1,863 bp putatively encoding a 621-amino-acid protein with a calculated Mr of 70,347 and a pI of 6.13. The deduced amino acid sequence of BUF2 protein contains a C-4X-C-13X-C-2X-C zinc finger motif between residues 486 and 508. **BUF1** contains a discontinuous ORF of 927 bp putatively encoding a 273-amino-acid protein with a calculated Mr of 9,936 and a pI of 4.7. The **BUF1** ORF contained a consensus intron recognition sequence bracketing nucleotide positions +8 to +115 (Fig. 10).

**BUF null mutations are lethal.** Several laboratories, including our own, have attempted to mutate the gene(s) encoding the BUF proteins. Most of the genetic strategies have involved selecting or screening for strains which have lost the negative regulation mediated by the URS1 site situated in the promoter region of a gene with a phenotype able to be selected or scored. Such a lack of success would be expected if the BUF genes were required for cell viability. To test this possibility, deletion mutations of the BUF genes were constructed as described in Materials and Methods. The null gene constructions were used to replace the wild-type alleles by the one-step replacement method of Rothstein with a diploid strain, M1741, as the transformation recipient (44). Stable Ura+ transformants containing each construction were selected, and their genomic DNA was analyzed by Southern blot analysis to determine whether replacement of the wild-type allele with the null allele had occurred. The data obtained demonstrated that the desired null replacement

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**FIG. 10.** Nucleotide sequence of the BUF1 gene. The consensus intron sequence is bracketed and marked. The protein sequence derived from direct N-terminal protein sequence analysis is underlined.

**FIG. 11.** Nucleotide sequence of the BUF2 gene. The zinc finger motif is underlined.
ment had occurred in one of the two diploid homologs in both cases (data not shown). The two heterozygous diploids were sporulated, and the meiotic products were analyzed. For the BUF2 deletion, 17 ascis were analyzed. In all cases, the two viable and two inviable spores were observed. All of the viable spores were ura mutants, indicating that BUF2 was an essential gene. For the BUF1 deletion, 19 ascis were analyzed, with the same results.

**BUF1 and BUF2 expression in E. coli.** Earlier experiments demonstrated that both the BUF1 and BUF2 subunits are found in the protein-URS1 DNA complex (29). To determine which of the BUF proteins bound to the DNA, we individually cloned both BUF genes downstream of the T7 gene 10 promoter contained on plasmid pT7-7, as described in Materials and Methods. The resulting plasmids were designated pLS21 (BUF2) and pLS22 (BUF1). The BUF1 construction (plasmid pLS22) was carried out such that the putative intron was removed; i.e., we spanned the intron with the PCR primer used to make the construction. Plasmid pT7-7, devoid of yeast DNA, and plasmids pLS21 (BUF2) and pLS22 (BUF1) were expressed in E. coli as described in Materials and Methods, and crude extracts from strains carrying each of the three plasmids were resolved on an SDS-polyacrylamide gel. Plasmid pLS22 (BUF1) supported production of a protein that possessed a mobility similar to that of purified yeast BUF1 protein (data not shown). This species was not observed with plasmid pT7-7. When this experiment was repeated with plasmid pT7-7 (vector) and pLS21 (BUF2), no difference between the protein patterns observed on the SDS gel was observed (data not shown). This result suggested that the BUF2 gene may not have been highly expressed in E. coli. Alternatively, BUF2 protein might have been expressed and then degraded. The latter explanation seemed more likely because during the purification of BUF2 protein we observed it to be degraded to discrete lower-molecular-weight species with a common amino terminus demonstrated by determining the N-terminal amino acid sequences of the degradation products (28).

However, to experimentally distinguish the above possibilities, we resolved crude extracts from strains carrying plasmids pT7-7, pLS21, and pLS22 on SDS-polyacrylamide gels and performed a Western blot analysis with monoclonal antibodies against the BUF1 and BUF2 proteins. As shown in Fig. 12, lanes C and F, extract from an E. coli strain carrying plasmid pT7-7 did not possess any proteins that cross-reacted with antibodies against either the BUF1 or BUF2 protein. E. coli carrying plasmid pLS22 produced a single protein that cross-reacted with antibody against BUF1 protein (Fig. 12, lane E). This protein possessed, within the range of experimental error, the same mobility as purified yeast BUF1 protein. In contrast, E. coli carrying plasmid pLS21 contained a ladder of proteins that cross-reacted with BUF2 antibody (Fig. 12, lane B). The largest species observed in Fig. 12, lane B, possessed, within the range of experimental error, a mobility that was the same as that of purified yeast BUF2 protein. This is the pattern of results expected from a protein that is highly susceptible to protein degradation, and these results are similar to the results observed during purification of the yeast BUF2 protein.

Since both crude extracts contained at least some full-length protein, we determined whether either one of them alone was able to bind URS1 DNA in our standard EMSA. Neither preparation gave a positive signal in this assay, even though a variety of protein concentrations were used. The highest concentration used in this experiment was approximately fivefold greater than the one we normally use in these assays and therefore would have been expected to yield a positive signal if binding had occurred.

**BUF protein is required for DNA synthesis.** As part of our characterization of the BUF gene sequences, we searched available protein data bases for homologous sequences. We found that the deduced BUF1 and BUF2 protein sequences were identical to the heteromeric RF-A (RP-A) protein studied by Brill and Stillman and Heyer et al. as a component of the DNA replication apparatus (2, 19). Replication protein or factor A is a trimeric protein consisting of 69-, 36-, and 13-kDa subunits. The principal known function of this replication factor at present is that of a single-stranded DNA binding protein.

The identity of the BUF and RF-A (RP-A) proteins raised an immediate paradox. The main characteristic of RF-A (RP-A), which was the means of its original identification and purification, was that it bound to single-stranded DNA (2). Since we had no evidence of similar single-stranded binding for BUF protein, we determined the relative abilities of single- and double-stranded DNA fragments to compete with a double-stranded DNA fragment containing the wild-type CAR1 URS1 site for binding to pure BUF protein. As shown in Fig. 13A, lanes A to F, a nonradioactive double-stranded DNA fragment was an effective competitor of a double-stranded radioactive probe possessing the same sequence. However, when the single strands from which the double-stranded probe was derived were used as competitors, no competition was observed (Fig. 13, lanes F to K). In fact, the extent of competition observed with the two single-stranded competitors was no better than that found with a DNA fragment containing a totally unrelated protein binding site, the DAL7 UAS NTR site, that we used as a negative control (58) (Fig. 13B, lanes A to F).
A second question generated by the identity of the RF-A (RP-A) and BUF proteins was the whereabouts of the smallest subunit of the RF-A (RP-A) protein in purified BUF preparations. The very small size of the subunit raised the possibility that it escaped our detection by migrating with the salt front or that it may have run off from the bottom of the SDS-polyacrylamide gel when we characterized our most homogeneous preparations of BUF protein. To test these possibilities, we subjected a preparation of pure BUF protein to SDS-PAGE under conditions that would permit the identification of a 14-kDa protein. As shown in Fig. 14, there is a third protein that comigrates with the 14-kDa standard near the front of the polyacrylamide gel.

To ascertain whether the smallest subunit of the RF-A (RP-A) protein (RFA-3) was capable of binding to a DNA fragment containing the CARI URS1 element, we cloned the gene encoding this subunit downstream of the T7 promoter in plasmid pT7-7 (pLS23) as described in Materials and Methods. Large quantities of a protein exhibiting the expected size of RFA-3 appeared when plasmid pLS23 was expressed in E. coli (data not shown). This species was not observed when the vector alone was expressed. When extract from E. coli cells expressing plasmid pLS23 was used as the source of protein in an EMSA along with a DNA fragment containing the CARI URS1 element as the source of DNA, no DNA-protein complex was observed (data not shown).

FIG. 13. Competition between double-stranded (D.S.) DNA fragment (RL153/154) containing the CARI URS1 element and the single DNA strands (RL153 and RL154 [S.S.A. and S.S.B.] [A and B, respectively]) from which it was annealed for binding to affinity-purified BUF protein. EMSA reaction conditions were the same as those described in the legend to Fig. 11. Lanes: F, no protein; E and G, no competitor DNA (comp); E back through A, increasing amounts (picomoles [pm]) of a nonradioactive, double-stranded DNA fragment containing either the CARI URS1 (RL153/154) or DAL7 UAS NTR (JD28) [58] element, respectively; G to K, increasing amounts (picomoles) of either the top (153) (A) or bottom (154) (B) single-stranded DNA fragment; these single-stranded fragments were annealed to produce the CARI URS1 probe (DNA fragment RL153/154).

FIG. 14. SDS-PAGE analysis of affinity-purified BUF protein. A 12% acrylamide minigel was loaded with 20 μl of affinity-purified BUF protein and developed for 50 min at 200 V. The gel was then stained with silver stain. Molecular sizes (in kilodaltons) are on the right.

DISCUSSION

Data presented in this work have identified and characterized BUF1 and BUF2 as two of the three genes encoding a trimeric protein binding to the URS1 site upstream of CARI (29, 31) and a large number of other genes (29, 31). The BUF1 and BUF2 genes are both essential to cell viability, as evidenced by the 2:2 segregation of lethality when either of them was integrated into one homolog of a diploid strain; this homolog was then sporulated, and its meiotic products were analyzed. Analyses of the deduced BUF1 and BUF2 protein sequences revealed that BUF2 contains a zinc finger motif similar to ones that have been previously reported to be possessed by some DNA binding proteins (22). If this motif could be shown to be important for BUF protein binding to
the **URSI** site, it would raise a paradox with data previously reported by Park and Craig (41). These investigators reported that a 40-kDa protein could be cross-linked to a **URSI**-homologous sequence situated upstream of the heat shock gene **HSP70**. The size of the cross-linked protein is far more similar to that of **BUF**1 than that of **BUF**2. Our inability to observe binding of *E. coli*-expressed **BUF** proteins to **URSI**-containing DNA fragments and the results cited above leave the identity of the **BUF** monomer that binds to double-stranded DNA an open question.

This work also demonstrated that **BUF** protein binds to **CARI** DNA fragments that mediate transcriptional activation. One of the small DNA fragments forming a complex with **BUF** protein contained the **CARI-0** mutation of the **CARI** **URSI** site (Fig. 5, lane R). The potential of the **CARI-0** fragment to support transcriptional activation was recognized during genetic analysis of the **URSI** site (31). However, since the experiments reported were not performed with a heterologous expression vector devoid of **UAS** sequences, we could not at that time unambiguously conclude that the fragment indeed contained a **UAS** element. The necessary experiments are presented in this work, and they demonstrate that the C-to-G transversion at position −153 is the only **URSI** transversion mutation capable of generating the **UAS** element. Other changes of the **URSI** element did not generate a sequence capable of supporting reporter gene expression (Fig. 4). The fact that none of the other transversion mutations generated elements that supported **UAS** activity suggests a more stringent sequence specificity for the **UAS** element than that observed for the **URSI** element. Several of the **URSI** transversion mutant fragments retained their abilities to serve as effective upstream repression sequence elements (31). It may legitimately be argued that the **CARI-0** **UAS**, although a functional **UAS** element in yeast cells, is not physiologically significant for **CARI** expression. That argument, however, is not pertinent to the other **CARI** fragments (the insert of plasmids pLK78 and pS1-1 and fragment RL195/196) which were also found to bind **BUF** protein and to contain a GC-rich sequence. This fragment has already been shown to contain a legitimate part of the **CARI** promoter element, **UAS** <sub>2</sub>, and to support inducer-independent transcriptional activation of a reporter gene when cloned into a heterologous expression vector (24; also this work) (Fig. 7). Similarly, while this work was under review, an article by Bowditch and Mitchell which convincingly shows that the **URSI**-homologous sequences upstream of **IME2** are required for transcriptional activation appeared in the literature (1). Our data support the conclusions reached by these authors and extend the information to the level of the purified **BUF** protein.

The findings that **BUF** protein can bind to DNA fragments that contain either **UAS** or upstream repression sequence elements is very reminiscent of the binding characteristics reported for the **ABF**1, **RAP**1, and **MCM**1 general transcription factors. All three of these proteins have been previously shown to specifically bind sequences required for transcriptional activation of the genes whose promoters contain them (1a, 4, 14, 16, 20, 42). They have also been shown to participate in transcriptional repression as well as activation (9, 21, 26, 45, 52). Finally, all three general transcription factors have been shown to be associated in some way with chromosome replication (3, 5, 10, 32). Therefore, it is not surprising that **BUF** protein is identical to the RF-A (RF-A) protein that participates in DNA replication in yeast cells (2, 19).

These analogies notwithstanding, we must distinguish what we do and do not know about **BUF** protein function at this point. We have unambiguously demonstrated that **BUF** protein binds to sequences that mediate both transcriptional repression and activation. We have not, however, demonstrated that **BUF** protein is itself responsible for either the transcriptional repression or activation. In other words, the available data indicate a role for **BUF** protein in transcriptional repression and activation and DNA replication, but they do not identify the biochemical nature of that role.

Our limited knowledge about the precise biochemical function of **BUF** protein is emphasized by recent studies of **CARI** **URSI**-mediated repression which report that **BUF** protein alone is insufficient to repress transcription; at minimum, the **CAR80** (**UME**6) gene product is also required (40). Bowditch and Mitchell report the same requirement for **URSI**-homologous sequences mediating transcriptional activation (1). These results indicate that **BUF** protein operates in a complex with other protein factors that bind either to **BUF** itself or to DNA sequences adjacent to the **URSI** site. Further evidence for this suggestion derives from data presented in Fig. 8. A DNA fragment carrying the SDP1 site studied by Wang and Stillman (62) was an effective competitor for **CARI** **URSI** binding to **BUF** protein; the converse competition experiment also yielded positive results. However, when a DNA fragment containing the SDP1 site was used as the source of probe in an EMSA, two additional, higher-molecular-weight complexes that were not seen when the **CARI** **URSI** fragment was used instead were observed. The complexes were successfully inhibited by the SDP1 fragment but not the one containing **CARI** **URSI**. Further, the **SIN3** (**UME**4) product has been previously shown to be required for regulation mediated by the SDP1-containing DNA fragment (45, 47, 62). Mutation of the **sin3** (**ume4**3) locus, in contrast, does not affect **CARI** **URSI** function (40). These observations suggest that, in addition to the **BUF** binding site, the SDP1 DNA fragment contains a second site at which these additional proteins bind. Consistent with this suggestion is the fact that the SDP1 DNA fragment sequences most homologous to **URSI** are localized on the 5′ end of the fragment (Fig. 3). In sum, **URSI** elements along with distinct sets of proteins appear to mediate the regulation of different genes, the particular set of proteins being gene specific.

The observation that **CAR80** (**UME**6) product is required for the **URSI** element to mediate **CARI** transcriptional repression (40) and perhaps other proteins when other genes such as **HO** are regulated raises the possibility that **BUF** protein may serve only as a docking site for other proteins that participate in the transcriptional repression or activation processes. Alternatively, **BUF** may serve this docking function in association with other protein binding sites situated adjacent to the **URSI** element. The flexibility of sequences previously demonstrated to be capable of serving as **URSI** sites (29, 31) also raises the possibility that docking specificity might be influenced by the conformation adopted by **BUF** protein as it binds to different but related DNA sites. The homeobox-binding proteins in metazoan cells are a documented example of this latter possibility (39).

Two observations made during this work remain unexplained. The first observation is the DNA binding characteristics of **BUF**. We used affinity chromatography as a principal purification method. Double-stranded DNA containing the **CARI** **URSI** site was the affinity ligand (29). In contrast, investigators studying replication used binding to single-stranded DNA as one of their purification methods (2).
These observations suggest that BUF binds to both single- and double-stranded DNA. This is not surprising, since there are examples of proteins possessing this capability (15). However, when we directly tested this possibility, we observed that single-stranded DNA was totally ineffective as a competitor of double-stranded DNA binding to our BUF protein preparations. Even though the ionic strengths of the two binding assays were somewhat different, we expected to see some inhibition of double-stranded DNA binding by the single-stranded DNA. Moreover, there is to date no report of GC-rich sequences being important to DNA repiliation or replication factor binding, although there is a perfect CARI URS1 site situated on the DNA fragment containing ARS121 (61). Second, even though our purification methods are gentle, the yield of BUF protein that we obtain is only a fraction of the yields reported for purification of the single-stranded DNA binding protein. Together, these observations raise the possibility that we may have purified a minor form of BUF protein that possesses DNA binding characteristics quite different from those of the one purified by investigators studying DNA repiliation. It is pertinent to this discussion that the RF-A protein, which is encoded by the same gene as BUF, exists in phosphorylated and unphosphorylated forms. Whether the difference in DNA binding properties between BUF and RF-A is a function of phosphorylation or some other posttranslational modification is not presently known.

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