# *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 (*CAR1*) gene in *Saccharomyces cerevisiae* is accomplished through the co-ordinated 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 $UAS_{C1}$, $UAS_{C2}$, and $UAS_{I}$ (23), and one upstream repression sequence, *URS1* (31, 50). The first two UAS elements mediate inducer-independent transcriptional activation and consist of multiple ABF1 and RAP1 binding sites (24, 25). ABF1 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 *CAR1* expression supported by the inducer-independent UASs (49, 50). Therefore, in wild-type cells grown without arginine, the balance between activation and repression of *CAR1* transcription is tipped toward reduced expression (23, 29, 31). On the other hand, when arginine is present, the third UAS, $UAS_{I}$, 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 *CAR1 URS1* site was originally identified by a single point mutation (*CAR1-0⁻*) which rendered the inducible *CAR1* gene constitutive (49). Saturation mutagenesis of the *CAR1 URS1* site revealed it to be the nonanucleotide 5'-AGCCGCCGA-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 *CAR1* (6–8, 31, 33, 47, 55, 57, 64). The protein that binds to the *CAR1 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 *CAR1* (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

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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-Amp medium (5 g of yeast extract, 5 g of NaCl, 10 g of Bacto Tryptone, 1 ml of 1 M NaOH, 2 mM $MgCl_2$, 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 $2.7 \times 10^4$ 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 $MgCl_2$, 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-thiogalacto-pyranoside), 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 time. 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 primary isolates were 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% $MgSO_4$, 50 mM Tris [pH 7.5], 0.01% 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 resus-

pended 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$_{vir}$. The lambda$_{vir}$-resistant survivors were isolated and grown in 250 ml of LB-Amp broth to a density of $5 \times 10^8$/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 3 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 $MgCl_2$, 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* BL 21(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 $MgCl_2$, 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
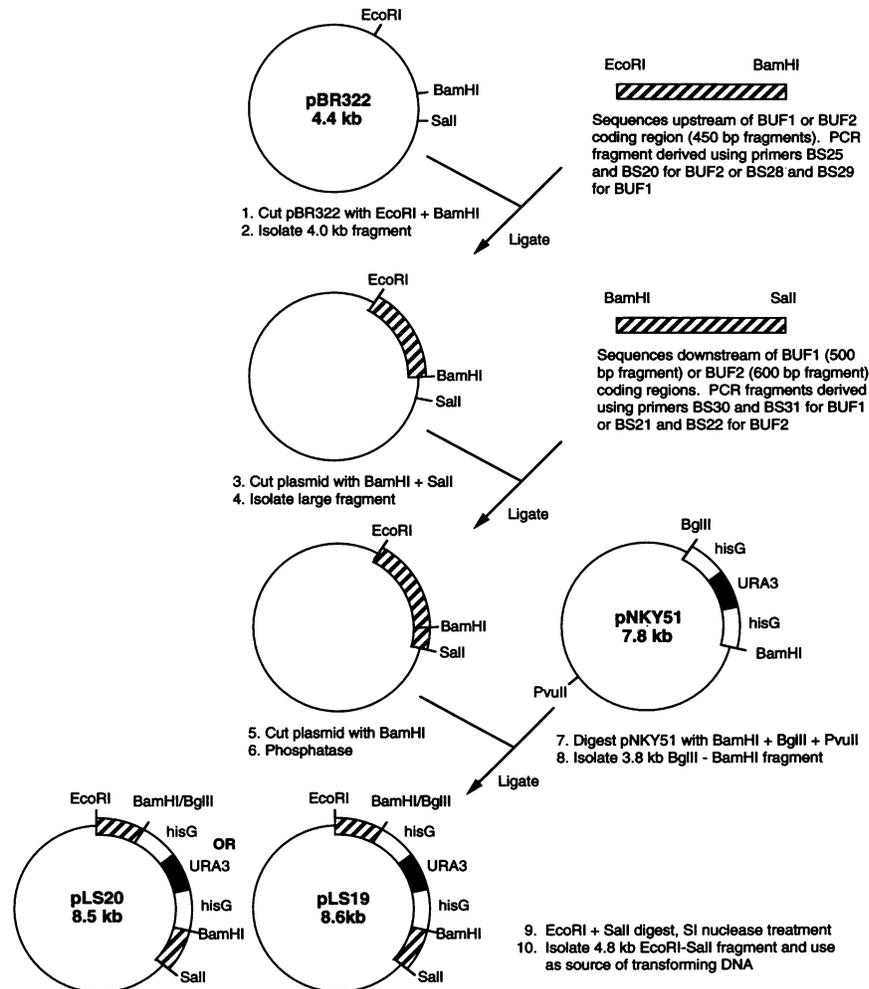
FIG. 1. Strategy used to construct plasmids containing deletions of the *BUF1* (pLS20) or *BUF2* (pLS19) genes.

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 *URS1* (29–31, 49–51). However, during our

original genetic analysis of the *CAR1 URS1* site, we recognized that a transversion (C to G) at position −153 resulted not only in the loss of *URS1*-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 Wiame (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 *URS1* 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 *URS1* 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 *URS1* 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 *URS1* fragment (29). We further tested this

1. Digest pT7-7 with:
   a. NdeI and HindIII for BUF1
   b. NdeI and BamHI for RPA-3

2. Isolate large fragment

pLS22 (BUF1)     pLS23 (RPA-3)

Ligate     pT7     pT7

HincII     bla

BglII     pT7-7
2469 bp

ClaI
HindIII
PstI
SalI
XbaI
BamHI
SmaI
EcoRI

Δ10     XbaI

rbs  ATG  NdeI

T7 promoter

CGTTCGAACTTCTGATAGACTTCGAAATTAATACGACTCACTATAGGGAGACCACAAC
AsuII        AsuII

Met ala arg ile
GGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG GCT AGA ATT
XbaI                              rbs        NdeI        EcoRI

arg ala arg gly ser ser arg val asp leu gln pro lys seu ile asp ...
CGC GCC CGG GGA TCC TCT AGA GTC GAC CTG CAG CCC AAG CTT ATC GAT ...
Smal          XbaI  SalI  PstI        HindIII  ClaI
BamHI

Ndel          Hindlll
BUF1

Ndel          BamHI
RPA-3

Generate PCR fragments using primers:
a. BS15 and BS23 for BUF1 gene, or
b. BS17 and BS24 for RPA-3 gene

2. Digest PCR derived fragments with:
   a. Ndel and HindIII for BUF1 containing fragment
   b. Ndel and BamHI for RPA-3containing fragment

3. Gel purify fragments

1. Digest pLS9 with AgeI and HindIII
2. Isolate 2.0kb fragment containing BUF2 gene

1. Digest pT7-7 with Ndel and BamHI
2. Isolate large fragment
3. Ligate in synthetic linker BS13/14
4. Digest with AgeI and HindIII
5. Isolate large fragment
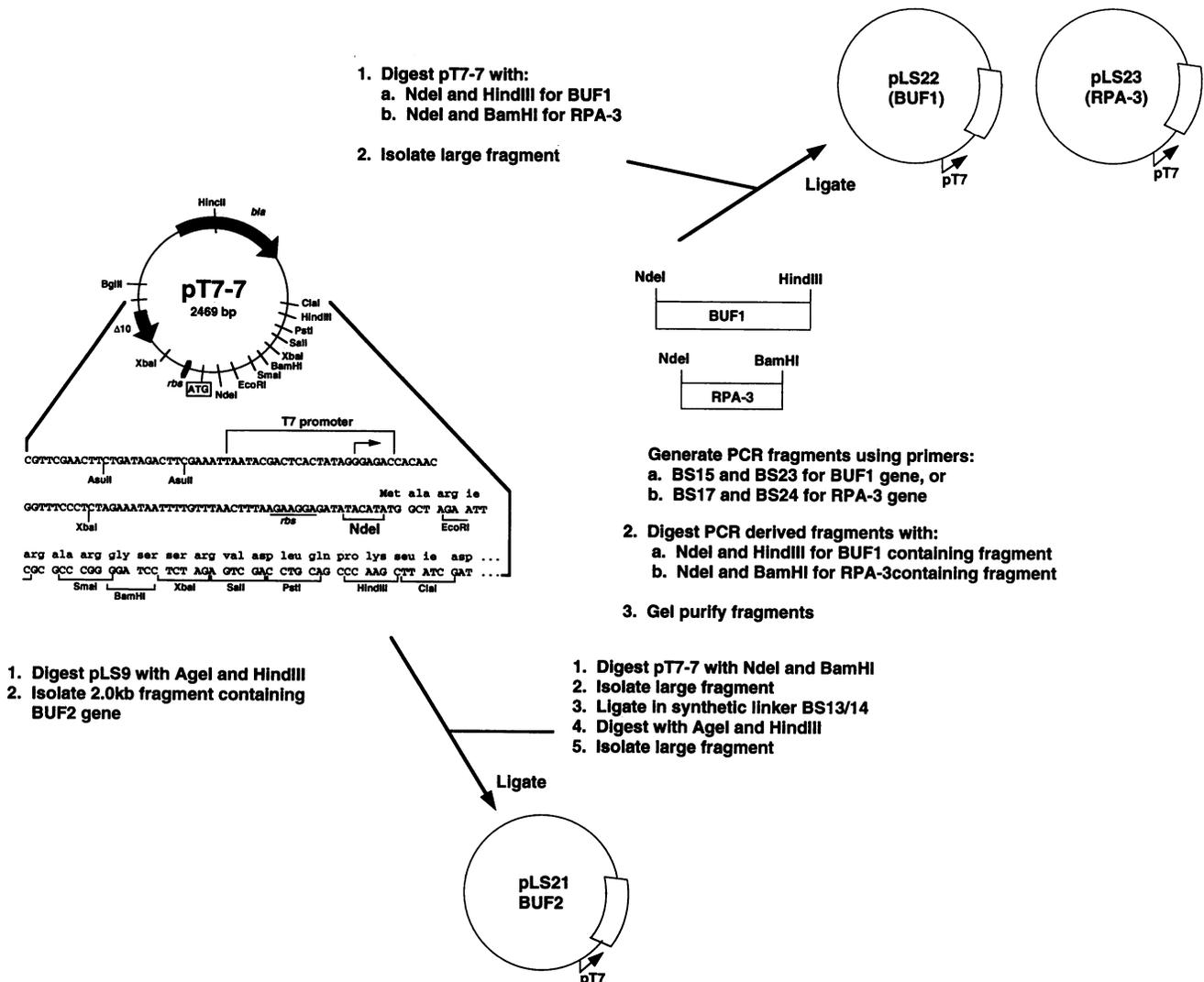
Ligate

pLS21 BUF2

pT7

FIG. 2. Strategy used to construct plasmids containing the *BUF* genes cloned downstream of the T7 promoter. *rbs*, ribosome-binding site.

observation by assessing the ability of the *URS1* position −153 transversion mutant fragment (RL193/194) to serve as a competitor of wild-type *URS1* (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 *CAR1 URS1* 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 *CAR1* 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*$_{C2}$ promoter region of *CAR1* (*CAR1* 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 *CAR1* 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 *CAR1 URS1* fragment for binding to pure

BUF protein in an EMSA. DNA fragment RL195/196 from *CAR1 UAS*$_{C2}$ 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 *CAR1 URS1* 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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                          -161                    -133
RL153 (S.S.A.)  TTCTAGCGCGCTCCTGCCGCACGCGGTAGCCGCCGAGGGGTCTAAAGAGTACTAGC
RL154 (S.S.B.)  AAGATCGCGCGAGGACGGCGTGCGCCATCGGCGGCTCCCCAGATTTCTCATGATCG    CAR1URS1


                  -218              -199    -218             -199
                TCGACTTTGCTTTTCTTATCACATACTCGAGTTTGCTTTTCTTATCACATAC      pJD28
                  GAAACGAAAAGAATAGTGTATGAGCTCAAACGAAAAGAATAGTGTATGCCGG    (DAL7UAS)


                -303                                        -248
RL195           TTTTTTTCTATCCCTTACAAGAAGCCGAGACGCCGCGAAAATATCGGCTAGTGCGA
RL196           AAAAAAAGATAGGGAATGTTCTTCGGCTCTGCGGCGCTTTTATAGCCGATCACGCT    UAS c2


                -303                                        -248
BS52            TTTTTTTCTATCCCTTACAAGAAGCCGAGACtaatatAAAATATCGGCTAGTGCGA
BS53            AAAAAAAGATAGGGAATGTTCTTCGGCTCTGattataTTTTATAGCCGATCACGCT


                -303                                        -248
pS1-1           TCGACTTTTTTTTCTATCCCTTACAAGAAGCCGAGACGCCGCGAAAATATCGGCTAGTGCGAC
                  GAAAAAAAGATAGGGAATGTTCTTCGGCTCTGCGGCGCTTTTATAGCCGATCACGCTGCCGG


                -303                                        -248
pS2-1           TCGACTTTTTTTTCTATCCCTTACAAGAAGCCGAGACtaatatAAAATATCGGCTAGTGCGAC
                  GAAAAAAAGATAGGGAATGTTCTTCGGCTCTGattataTTTTATAGCCGATCACGCTGCCGG


                          -161                    -133
RL193           TTCTAGCGCGCTCCTGCCGCACGCGGTAGCgGCCGAGGGGTCTAAAGAGTACTAGC
RL194           AAGATCGCGCGAGGACGGCGTGCGCCATCGcCGGCTCCCCAGATTTCTCATGATCG    CAR1O-


                -165              -141
RL208           CTTAGCGGTAGCCGCCGAGGGGTCT
RL209           GAATCGCCATCGGCGGCTCCCCAGA    CAR1URS1


RL210           CTGCCGGTGCCTGCGATGAGATACG    SDP1 site
RL211           GACGGCCACGGACGCTACTCTATGC


                EcoRI
BS25            TCGCGGAATTCTGTGCTGCAGTAGGCAAAGTTCCA  }  PCR primers for BUF2
                                                        upstream region
BS20            CCGCGGATCCTCTTGTAAGTATAATCTGGTCTT
                BamHI


                BamHI
BS21            CCGCGGATCCTTCTATTTCGTCAAGTTTTTGAA  }  PCR primers for BUF2
                                                      downstream region
BS22            ATCGCGGTCGACTATTTGGAGAAGGAAGATGAA
                     SalI


                EcoRI
BS28            TCGCGGAATTCGAGCAGCAATAACACTAGATGTGG  }  PCR primers for BUF1
                                                        upstream region
BS29            CCGCGGATCCGCCTGTTCTAGATAGTCGCTATCG
                BamHI


                BamHI
BS30            TCGCGGATCCTGGAACCCGTTCCAAGAAGGGGT  }  PCR primers for BUF1
                                                      downstream region
BS31            TCGCGTCGACGAGCCCTGAATCAGACATTCACCAC
                     SalI


                NdeI
BS13            TATGAGCAGTGTTCAACTTTCGAGGGGCGATTTTCATAGCATCTTCACCAATAAGCAAAG
BS14            ACTCGTCACAAGTTGAAAGCTCCCCGCTAAAAGTATCGTAGAAGTGGTTATTCGTTTC

                              AgeI  BamHI
BS13 (cont)     GTACGATAATCCCACCGGTG      Linker for insertion of BUF2 gene
BS14 (cont)     CATGCTATTAGGGTGGCCACCTAG  into NdeI/BamHI digested pT7-7


                NdeI
BS15            GCCGCATATGGCAACCTATCAACCATATAACGAATATT  }  PCR primers for
                                                            BUF1 gene
BS23            ATGCCGAAGCTTTTCATAGGGCAAAGAAGTTATTGTC
                     HindIII


                NdeI
BS17            GCCGCATATGGCCAGCGAAACACCAAGAGTT  }  PCR primers for
                                                    RPA-3 gene
BS24            GCCGGATCCTTTAGCTAGTATATTTCTGGGTATTTCTT
                BamHI
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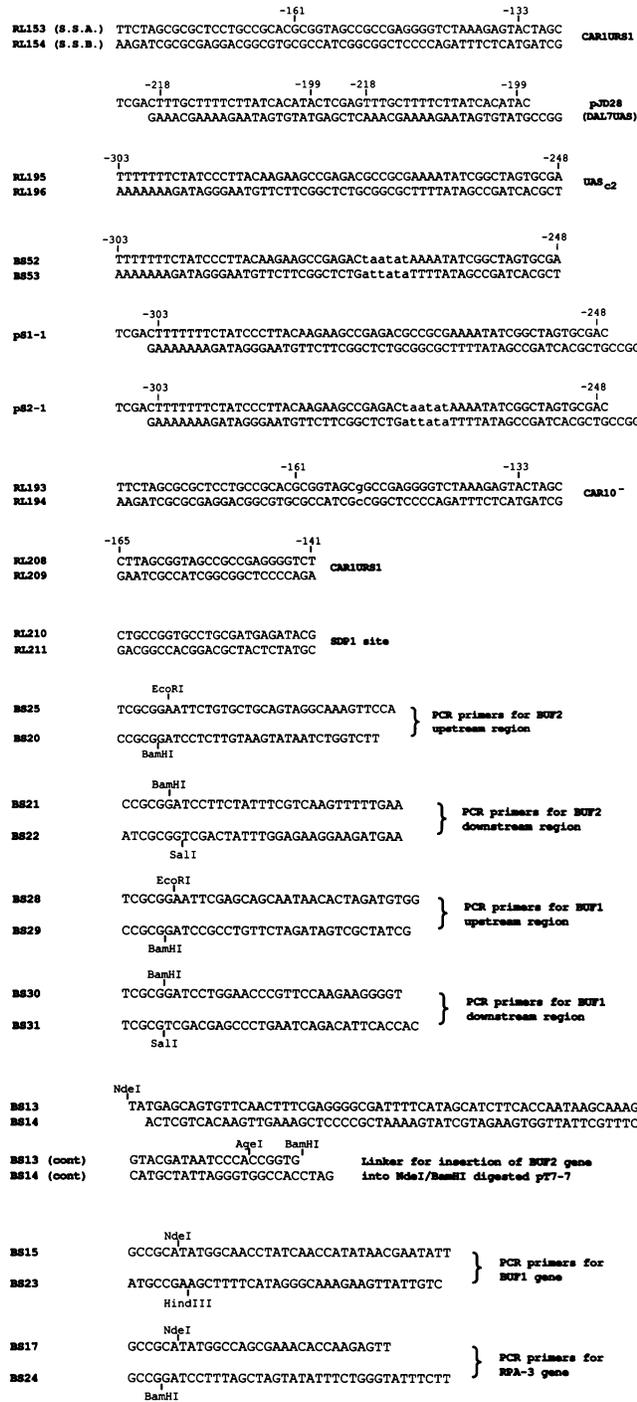
FIG. 3. Sequences of DNA fragments used in EMSAs, for cloning into heterologous expression vectors, and as PCR primers for construction of the plasmids in Fig. 1 and 2.

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      -158                                                -146
       |                                                   |
5'— G   T   A   G   C   C   G   C   C   G   A   G   G — 3'

       ↓   ↓   ↓   ↓   ↓   ↓   ↓   ↓   ↓   ↓   ↓   ↓   ↓

    ↓                                                   ↓

       C   A   T   C   G   G   C   G   G   C   T   C   C
      14  30  16  38  50 1189 87  66  22  14  30  23  14
```
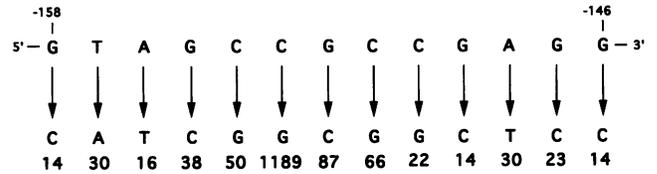
FIG. 4. Capability of various *URS1* element transversion mutants to support transcriptional activation in a heterologous expression vector assay system. Oligonucleotides containing the mutant *URS1* elements were cloned into plasmid pNG17 (63). The mutant plasmids were then used as the source of DNA 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 one of these proteins was SIN3 (UME4) (62). The observation that the *ume4* and *ume6* 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 *CAR1 URS1* fragment as a probe. As shown in Fig. 8A, the DNA fragment containing the SDP1 site was an effective competitor of the *CAR1 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 *CAR1 URS1* site (Fig. 8A) or the SDP1 site (Fig. 8B). Moreover, the DNA fragment containing *CAR1 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 *CAR1 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 × 10⁵ 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 *Eco*RI 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 *Eco*RI 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, phages 9.2
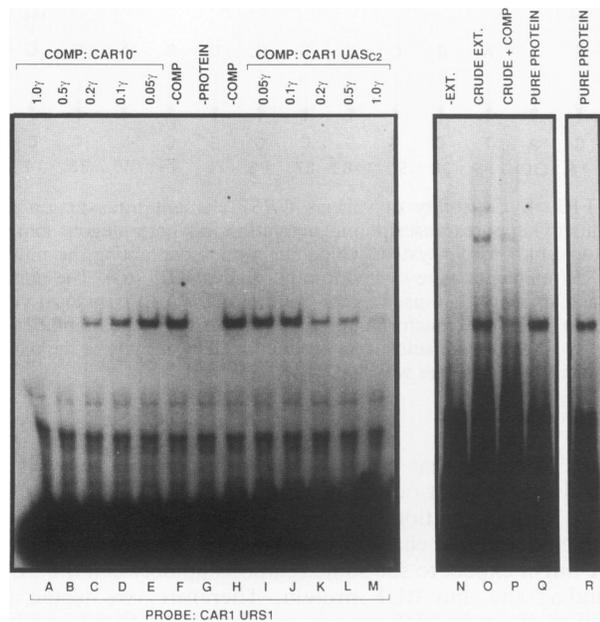
studies of BUF protein binding to the upstream regions of several genes not related to nitrogen metabolism, the question of whether BUF protein bound to the SDP1 site situated upstream of the *HO* gene (62) was raised (46). The SDP1 binding site contained a GC-rich sequence that shared some homology with the *CAR1 URS1* site. In addition, available

FIG. 5. (Left panel) Lanes A to M show competition between *CARI* DNA fragments mediating transcriptional activation (*CARI-0⁻* and *CARI UAS*<sub>C2</sub>) and one containing a wild-type *CARI URS1*. In each case, 50 ng of a $^{32}$P-labelled DNA fragment (RL153/154) containing the *URS1* 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 *CARI UAS*<sub>C2</sub>. (Right panel) Lanes N to R show binding of *CARI-0⁻* and *CARI UAS*<sub>C2</sub> DNA fragments to crude and purified BUF protein. Fifty nanograms of a radioactive DNA fragment (RL195/196) containing the *CARI UAS*<sub>C2</sub> 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 *CARI URS1* 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*<sub>C2</sub> 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 BS52/53 (mutant). The radioactive probe was the *CARI* DNA fragment that served as the insert of plasmid pRL58 (31).

and 9.4. Phage 9.2 contained two 1.2-kb *Eco*RI fragments, while phage 9.4 contained a single 1.7-kb fragment. The *Eco*RI 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 *Eco*RI 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 *BUF1* 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 (*Bam*HI, *Eco*RI, and *Sal*I) 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 (*Eco*RI fragments) were



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*<sub>C2</sub> 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.
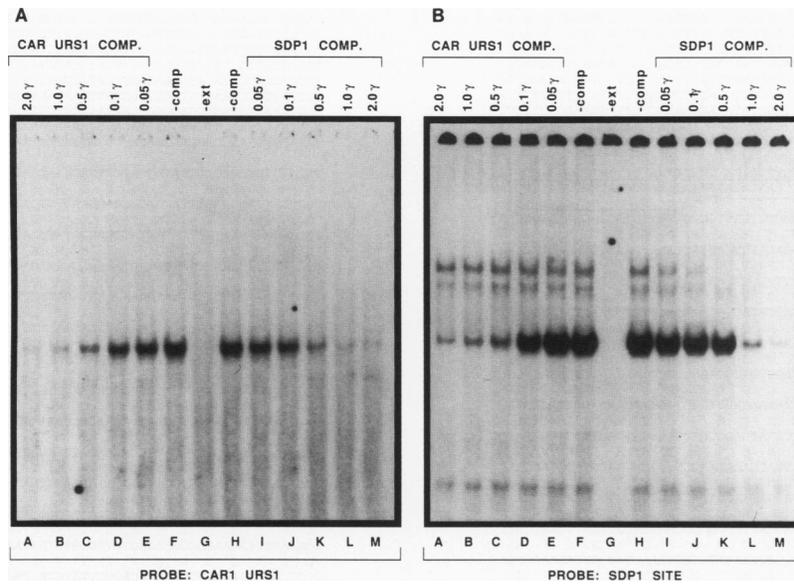
FIG. 8. Competition between DNA fragments containing the SDP1 binding site (RL210/211) or the *CAR1 URS1* element (RL208/209) for binding to protein derived from a crude extract. In panel A, 50 ng of labelled DNA fragment 208/209 (*CAR1 URS1*) was used as the probe, while in panel B, 50 ng of labelled 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 × 10⁴ 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
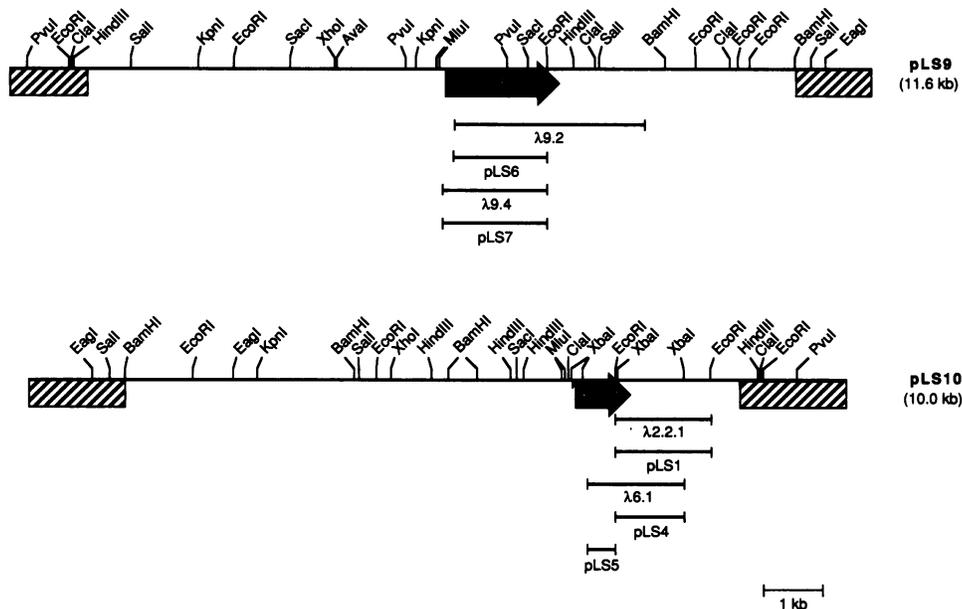


FIG. 9. Restriction maps of yeast chromosomal DNA containing *BUF1* and *BUF2*. Plasmid pLS9 contains the ORF of *BUF2*, while pLS10 contains the ORF of *BUF1*. ORFs are indicated by solid arrows. The hatched boxes indicate vector sequences. DNA fragments derived from λ-gt11 clones, as well as plasmids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

TCATTTTCTTGGCCCATTTTGAAAACCTCAACGTTAAGAAAGTCATTTAGAGCAGCAATA
ACACTAGATGTGGATATTTCTCCCGAAATACTAATTTTTCCCTTAAATTTGTCCACAAAT
TGCTGTACTAGCTGGTATGATTCACCACTGATGATATGTTCAGCCTTTTTCACATATGTC
TGGTCGAAAGAACCACTTTTCAGAGATTCTACTTCTGCAGACAGCAATAATTTTGCATGC
AATAACGGTCTCCATAATTTTGCAACCACTAGTTTACTTTTTGCCTGGTCTGATAGCTTC
TTTTTAGCAATTCCTTTGGCCTCGATGAGCTTCCATTTTCAATTTTCATCTTTTTTTTCA
CGATGCGAAGTTTCTTAGATAATTATCGCCATATACGAAACGCGTTAGGAAACGCGTTCT
TTCCTTTATAATAAGCAATATTATATCAACTTAAGGTGGAAGTAATGTGGCAAAATCGAT
AGCGACTATCTAGAACAGGCTAGTTTAAGCATATACATAATGGCAAGTATGTATTAGTGC
                                        M  A  T├──── Intron ────
TAGGAATTGGAGGATTTGCTCTTTTCTGCATCATTTTTTTTATTTTTATTGGCGAATGTT
AAAATTACTAACAATGGATGGGATTCCAATACAGCCTATCAACCATATAACGAATATTCA
                          Y  Q  P  Y  N  E  Y  S
TCAGTAACGGGCGGTGGCTTTGAGAACTCTGAGTCCCGCCCAGGTAGTGGGGAGTCGGAA
S  V  T  G  G  F  E  N  S  E  S  R  P  G  S  G  E  S  E
ACTAACACTAGAGTTAACACCTTGACACCTGTGACGATCAAACAAATTCTAGAGTCCAAA
T  N  T  R  V  N  T  L  T  P  V  T  I  K  Q  I  L  E  S  K
CAGGATATTCAGGACGGCCCCTTCGTTTCGCATAACCAAGAACTTCATCACGTTTGTTTT
Q  D  I  Q  D  G  P  F  V  S  H  N  Q  E  L  H  H  V  C  F
GTAGGTGTGGTGAGAAACATTACAGACCATACTGCAAATATTTTTTTAACTATTGAGGAT
V  G  V  V  R  N  I  T  D  H  T  A  N  I  F  L  T  I  E  D
GGAACTGGTCAAATAGAAGTGAGAAAATGGAGCGAAGATGCAAATGACTTGGCTGCCGGT
G  T  G  Q  I  E  V  R  K  W  S  E  D  A  N  D  L  A  A  G
AACGATGACTCTTCTGGTAAAGGTTATGGTTCGCAAGTCGCCCAACAATTTGAAATTGGC
N  D  D  S  S  G  K  G  Y  G  S  Q  V  A  Q  Q  F  E  I  G
GGTTACGTAAAAGTTTTTGGTGCTTTGAAAGAGTTTGGTGGTAAGAAAAATATACAGTAT
G  Y  V  K  V  F  G  A  L  K  E  F  G  G  K  K  N  I  Q  Y
GCGGTGATTAAGCCCATAGATTCATTCAATGAAGTGTTGACGCATCACTTGGAAGTCATC
A  V  I  K  P  I  D  S  F  N  E  V  L  T  H  H  L  E  V  I
AAATGTCATTCCATAGCCAGTGGAATGATGAAACAACCTTTGGAGAGTGCATCCAACAAC
K  C  H  S  I  A  S  G  M  M  K  Q  P  L  E  S  A  S  N  N
AATGGGCAATCATTATTTGTCAAGGATGATAACGATACATCTTCCGGCTCCAGTCCGTTA
N  G  Q  S  L  F  V  K  D  D  N  D  T  S  S  G  S  S  P  L
CAAAGAATTCTAGAATTTTGTAAGAAGCAATGTGAGGGCAAAGACGCTAATTCATTCGCT
Q  R  I  L  E  F  C  K  K  Q  C  E  G  K  D  A  N  S  F  A
GTTCCCATTCCATTGATCTCGCAATCCTTGAATTTGGATGAAACTACCGTCAGAAACTGC
V  P  I  P  L  I  S  Q  S  L  N  L  D  E  T  T  V  R  N  C
TGTACGACCTTGACTGACCAGGGTTTTATCTACCCAACTTTTGATGACAATAACTTCTTT
C  T  T  L  T  D  Q  G  F  I  Y  P  T  F  D  D  N  N  F  F
GCCCTATGAAAATATATAGATTATTTTTTTAGCATTTCTTAGTATTCGTTTGTGTATTTC
A  L  *
TACTAGGTAAGTACCAAAATTTGCCGGAAATAGAGTGGAAAATGGAACCCGTTCCAAGAA
GGGGTAAGATGCATGAATATTAAGTTGGTTGCATAAATATGCTATATAAAGTCCACTACA
AAAAGTCATTTATTTTTGCCCGTAAGAATAAAGAACGAATCCGTCCTTATCTCTGACTTC
AATGGGAACATTTACACGCAGTACGGGCTTCAATAATAGTTCCCAGTCGTATCTGGTTCT
AGGTGTACATAATGAAAAATGTCTCAGCATTCTTATTTTGACAGCTTTGAATTTTGACAA
ATTTCTCAGGAAACACCAGAGGTTAATATTTGGAATATCTTTAAAATTCTCTGGATCTTG
GAAATCATTGAAAAAATTTTCCAATGTCACATTTTGTAAGAACTCCTCCACATCTAAATC
TCTATATTCGATGGTTCGGTTCTTCAATTTGATTAAACTATCTCTTAGGCAACGCCTTAC
AGAGTTGTTCCAATAGTAAGTGTGGTGAATGTCTGATTCAGGGCTCATTACTGTACTTAA
ATTTGGAACTGCATTAGCATTTGGTATGTTA

**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.

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 $M_r$ 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 $M_r$ of 29,936 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

CACATCGGCAAGATTGTTCAAATGCAGCCACAGCACATCTTCTAACATAAGCCTCAAAGG
TACCGACAAAAATACATTTTGCTGTGCTGCAGTAGGCAAAGTTCCAGATAACACACCACA
AATACCCCATTTCCTTAGAAGTTTGATGTGATCTATGTCAAATACTAGCGGTGGCATAAA
AACCTCCTCGCCTTTTGCCGTTCTTTTTGCATATATGTTAATAGCTATGCGCGTACCACC
TTCTTCAGCCATATACGAAAGGATCTATGCTAATTGGGTGGATTTCTTATCGCATCACTT
TCTTATTCTCTAACTCTTCTTCGCTGCTCAATAAAATATCTCTGCTTGGAAAAATGTCAC
GCGTAAAAATTAGACGAGGCGACAAAAGACGCGTGAACTAACATAAAGGTTTACTTGAAC
AAATGTTATCACTTTAAGTTCTACACGTAAAACAAAAACTATTCACTCTTCTTATTACGG
TAAAGGCGAAACCAGCAAGAAGACCAGATTATACTTTACAAGAGATGAGCAGTGTTCAACT
                              M  S  S  V  Q  L
TTCGAGGGGCGATTTTCATAGCATCTTCACCAATAAGCAAAGGTACGATAATCCCACCGG
S  R  G  D  F  H  S  I  F  T  N  K  Q  R  Y  D  N  P  T  G
TGGCGTTTATCAAGTTTATAACACCAGGAAATCTGATGGGGCTAACAGCAACAGAAAGAA
G  V  Y  Q  V  Y  N  T  R  K  S  D  G  A  N  S  N  R  K  N
TTTGATCATGATTTCCGATGGTATTTACCATATGAAGGCTCTGTTGAGAAACCAAGCTGC
L  I  M  I  S  D  G  I  Y  H  M  K  A  L  L  R  N  Q  A  A
ATCCAAGTTCCAGTCAATGGAACTACAAAGGGGTGATATCATTCGCGTGATAATTGCAGA
S  K  F  Q  S  M  E  L  Q  R  G  D  I  I  R  V  I  I  A  E
ACCTGCTATTGTCAGGGAAAGAAAGAAATACGTTCTTTTAGTAGATGACTTTGAGTTGGT
P  A  I  V  R  E  R  K  K  Y  V  L  L  V  D  D  F  E  L  V
CCAGTCGCGTGCTGATATGGTCAACCAAACTAGTACTTTTTTGGATAACTATTTCTCAGA
Q  S  R  A  D  M  V  N  Q  T  S  T  F  L  D  N  Y  F  S  E
GCATCCAAATGAAACCTTAAAAGACGAAGATATAACTGACAGTGGTAATGTTGCCAATCA
H  P  N  E  T  L  K  D  E  D  I  T  D  S  G  N  V  A  N  Q
AACAAACGCCAGCAATGCTGGTGTCCCTGATATGCTGCATTCAAACTCAAACTTGAATGC
T  N  A  S  N  A  G  V  P  D  M  L  H  S  N  S  N  L  N  A
AAATGAGAGAAAATTCGCCAATGAAAACCCTAATTCGCAAAAAACCAGACCAATTTTTGC
N  E  R  K  F  A  N  E  N  P  N  S  Q  K  T  R  P  I  F  A
CATCGAACAACTGTCTCCATACCAAAACGTTTGGACTATCAAAGCAAGAGTTTCCTACAA
I  E  Q  L  S  P  Y  Q  N  V  W  T  I  K  A  R  V  S  Y  K
GGGAGAAATTAAAACGTGGCACAATCAAAGAGGTGATGGTAAACTATTCAATGTCAACTT
G  E  I  K  T  W  H  N  Q  R  G  D  G  K  L  F  N  V  N  F
CTTGGATACCTCTGGAGAAATCCGAGCCACGGCGTTTAATGATTTTGCTACAAAATTTAA
L  D  T  S  G  E  I  R  A  T  A  F  N  D  F  A  T  K  F  N
CGAAATTTTACAAGAAGGCAAAGTATACTATGTATCAAAGGCAAAACTCCAACCAGCTAA
E  I  L  Q  E  G  K  V  Y  Y  V  S  K  A  K  L  Q  P  A  K
GCCCCAATTTACTAATCTAACACACCCTTATGAACTGAATTTGGATAGAGACACTGTTAT
P  Q  F  T  N  L  T  H  P  Y  E  L  N  L  D  R  D  T  V  I
AGAAGAATGTTTCGATGAAAGTAATGTTCCGAAAACCCATTTCAATTTCATCAAACTAGA
E  E  C  F  D  E  S  N  V  P  K  T  H  F  N  F  I  K  L  D
TGCTATTCAGAACCAGGAAGTAAATTCCAACGTAGACGTCCTCGGTATTATCCAAACTAT
A  I  Q  N  Q  E  V  N  S  N  V  D  V  L  G  I  I  Q  T  I
AAACCCACATTTTGAGCTAACTTCAAGGGCTGGGAAGAAATTCGATCGTCGTGACATCAC
N  P  H  F  E  L  T  S  R  A  G  K  K  F  D  R  R  D  I  T
AATTGTTGACGACTCTGGGTTTTCTATCTCTGTTGGCCTATGGAATCAGCAAGCCCTTGA
I  V  D  D  S  G  F  S  I  S  V  G  L  W  N  Q  Q  A  L  D
TTTCAACCTTCCTGAAAGTTCTGTTGCTGCCATTAAAGGTGTTCGTGTGACGGATTTTGG
F  N  L  P  E  G  S  V  A  A  I  K  G  V  R  V  T  D  F  G
TGGCAAAATCTTTGTCTATGGGATTTTCTAGTACCCTGATTCCGAATCCAGAAATTCCTGA
G  K  S  L  S  M  G  F  S  S  T  L  I  P  N  P  E  I  P  E
GGCATATGCCTTAAAGGGTTGGTATGATTCCAAGGGCCGCAACGCAAACTTCATCACTTT
A  Y  A  L  K  G  W  Y  D  S  K  G  R  N  A  N  F  I  T  L
AAAGCAAGAACCCGGTATGGGTGGTCAATCGGCTGCTAGCTTAACAAAATTCATTGCTCA
K  Q  E  P  G  M  G  G  Q  S  A  A  S  L  T  K  F  I  A  Q
GCGTATTACTATTGCTAGAGCTCAAGCTGAAAATCTAGGAAGAAGCGAGAAAGGTGACTT
R  I  T  I  A  R  A  Q  A  E  N  L  G  R  S  K  E  G  D  F
TTTTAGTGTTAAAGCTGCTATAAGTTTCTTAAAAGTTGATAATTTTGCATATCCTGCCTG
F  S  V  K  A  A  I  S  F  L  K  V  D  N  F  A  Y  P  A  C
TTCTAATGAGAATTGTAATAAGAAAGTTCTGGAACAGCCTGATGGTACTTGGAGATGTGA
S  N  E  N  C  N  K  K  V  L  E  Q  P  D  G  T  W  R  C  E
GAAGTGCGACACCAATAATGCAAGGCCAAATTGGAGATACATCTTGACAATATCAATTAT
K  C  D  T  N  N  A  R  P  N  W  R  Y  I  L  T  I  S  I  I
TGACGAAACCAATCAACTATGGCTCACTTTATTTGACGACCAAGCTAAACAATTATTGGG
D  E  T  N  Q  L  W  L  T  L  F  D  D  Q  A  K  Q  L  L  G
TGTTGATGCTAATACATTAATGTCTTTGAAGGAAGAAGACCCCAACGAATTCACAAAAAT
V  D  A  N  T  L  M  S  L  K  E  E  D  P  N  E  F  T  K  I
TACTCAAAGTATCCAAATGAACGAATATGACTTTAGGATTAGAGCGCGTGAGGATACATA
T  Q  S  I  Q  M  N  E  Y  D  F  R  I  R  A  R  E  D  T  Y
CAATGATCAAAGCAGAATTAGATATACCGTTGCTAACCTACACAGCTTGAATTACAGGGC
N  D  Q  S  R  I  R  Y  T  V  A  N  L  H  S  L  N  Y  R  A
TGAAGCCGACTATCTTGCCGATGAGTTATCCAAGGCTTTGTTAGCTTAATGTATCAAATA
E  A  D  Y  L  A  D  E  L  S  K  A  L  L  A  *
ATCAAGTACTATTTAATCTATGTAACATATGAGAAATGTAAAAAAAAAACTTCTATTTCG
TCAAGTTTTTGAAGGGCGGTTCAGCATCTCTTGATGACTGGATATGAAACTACACGTTCA
GTCATATGAATTTGAACCCATAATGCACAAATGAATAAAAATGCTAACAAATTCTAATTA
ATGGCATGGTACTAACACCCAACAATTTACCGCCCTTTAGGTGTACAGCTTCAAATAACA
AAGCTTTACAAATTCACCTTCAACATTTACGTTCAGTAGTATGAAATTACCATCATGACG
TGCTCCTATCAAAGTGAGATTATTGCACTTCGTATTATTAGGAAATTACCAAAAAATGCA
AAATTTTTCAATTCTGTGTCAAAGAAATCGCGGTAGGCATGACTATACCTCTTCAGTGTC
AGTTGCCTATTTAAACTTTCTCCTTTTGAAGAGAAACCTATGATGACTGCATCTTAATTA
TCCTCGTATAATCTGTAGGGATTGTGAACCGTTCTACAAGCAAGTTATTACCTCTAACAT
CATATTGCTCTCTCGTCCGAAGATCGATAGCTATTTCTTCCTCTTTGTTCTAGTCCTTGCT
GCTGTTCTGCCTCATCGACCTCATCAAATTCATCTTCCTTCTCCAAATAGTC

**FIG. 11.** Nucleotide sequence of the *BUF2* gene. The zinc finger motif is underlined.

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. These 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 replace-

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 asci 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 asci 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 plasmids 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 the 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 proteolysis, and these results are similar to the results observed during purification of the yeast BUF 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
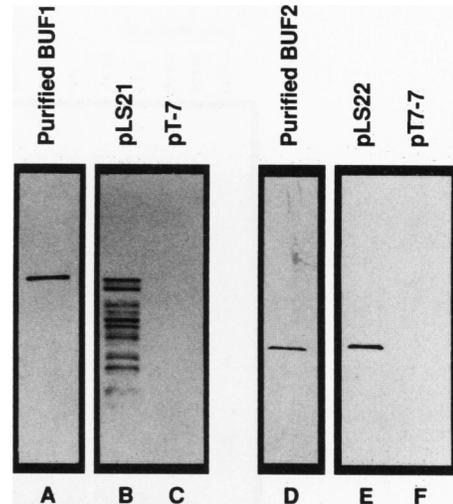


FIG. 12. Western blot analysis of purified BUF protein from *S. cerevisiae* and BUF protein expressed in *E. coli*. Lanes: A and D, 2 µl of affinity-purified BUF protein; B, approximately 1 µg of *E. coli* crude extract derived from cells expressing plasmid pLS21 (*BUF2*); C and F, approximately 1 µg of *E. coli* extract derived from cells expressing vector plasmid pT7-7; E, extract derived from cells expressing plasmid pLS22 (*BUF1*). Lanes A to C were probed with Ab 36, specific for the BUF2 protein. Lanes D to F were probed with Ab 62, which is specific for BUF1 protein. All of these data were derived from a single SDS-polyacrylamide gel. One empty lane was left between lanes A and B, C and D, and D and E to permit us to cut the blot into pieces for optimizing development of the chromophore reaction.

assays and therefore would have been expected a priori 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).
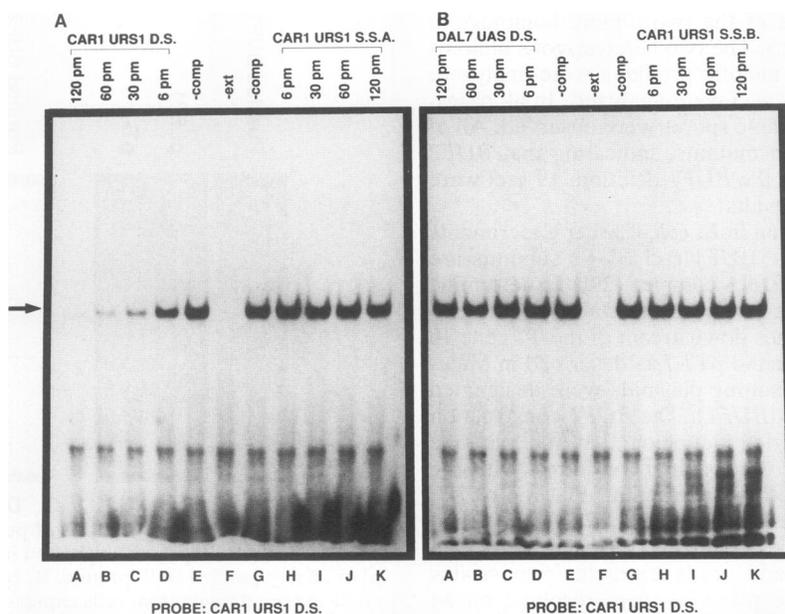
FIG. 13. Competition between double-stranded (D.S.) DNA fragment (RL153/154) containing the *CAR1 URS1* element and the single DNA strands (RL153 and RL154 [S.S.A. and S.S.B.] [A and B, respectively]) from which it was annealed for binding to affinity-purified BUF protein. EMSA reaction conditions were 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 *CAR1 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 *CAR1 URS1* probe (DNA fragment RL153/154).

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 pro-
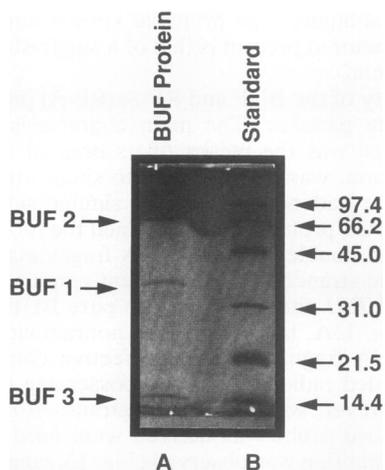


FIG. 14. SDS-PAGE analysis of affinity-purified BUF protein. A 12% acrylamide minigel was loaded with 20 μl of affinity-purified BUF protein and developed for 50 min at 200 V. The gel was then stained with silver stain. Molecular sizes (in kilodaltons) are on the right.

tein 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 *CAR1 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 *CAR1 URS1* element as the source of DNA, no DNA-protein complex was observed (data not shown).

## 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 *CAR1* (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 *URS1* 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 *URS1*-homologous sequence situated upstream of the heat shock gene *HSP70*. The size of the cross-linked protein is far more similar to that of BUF1 than that of BUF2. Our inability to observe binding of *E. coli*-expressed BUF proteins to *URS1*-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 *CAR1* DNA fragments that mediate transcriptional activation. One of the small DNA fragments forming a complex with BUF protein contained the *CAR1-0⁻* mutation of the *CAR1 URS1* site (Fig. 5, lane R). The potential of the *CAR1-0⁻* fragment to support transcriptional activation was recognized during genetic analysis of the *URS1* 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 *URS1* transversion mutation capable of generating the UAS element. Other changes of the *URS1* 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 *URS1* element. Several of the *URS1* transversion mutant fragments retained their abilities to serve as effective upstream repression sequence elements (31). It may legitimately be argued that the *CAR1-0⁻* UAS, although a functional UAS element in yeast cells, is not physiologically significant for *CAR1* expression. That argument, however, is not pertinent to the other *CAR1* 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 *CAR1* promoter element, $UAS_{C2}$, 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 Bowdish and Mitchell which convincingly shows that the *URS1*-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 ABF1, RAP1, and MCM1 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 (RP-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 *CAR1 URS1*-mediated repression which report that BUF protein alone is insufficient to repress transcription; at minimum, the *CAR80* (*UME6*) gene product is also required (40). Bowdish and Mitchell report the same requirement for *URS1*-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 *URS1* 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 *CAR1 URS1* 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 *CAR1 URS1* fragment was used instead were observed. The complexes were successfully inhibited by the SDP1 fragment but not the one containing *CAR1 URS1*. Further, the *SIN3* (*UME4*) 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*) locus, in contrast, does not affect *CAR1 URS1* 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 *URS1* are localized on the 5′ end of the fragment (Fig. 3). In sum, *URS1* 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* (*UME6*) product is required for the *URS1* element to mediate *CAR1* 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 *URS1* element. The flexibility of sequences previously demonstrated to be capable of serving as *URS1* 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 *CAR1 URS1* 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 replication or replication factor binding, although there is a perfect *CAR1 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 replication. 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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