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Purification of the heteromeric protein binding to the URS1 transcriptional repression site in *Saccharomyces cerevisiae*

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ABSTRACT **The protein that binds to the URS1 site situated upstream of many genes in *Saccharomyces cerevisiae* is a central element responsible for global negative control of transcription in this organism. Among the genes whose expression is regulated by this protein are those that participate in nitrogen metabolism, carbon metabolism, electron transport, inositol metabolism, heat shock response, meiosis, and sporulation. This factor, binding URS1 factor (BUF), has been purified and shown to be a heteromeric protein composed of 37.5- and 73.5-kDa monomers. The heteromeric form of BUF is stably maintained both in solution and bound to its DNA target site.**

The control of gene expression in eukaryotes has been shown to involve one or more of several basic mechanisms. Basal level transcription may involve the TATA sequence and subsequent core transcriptional apparatus but no additional upstream elements. More commonly, tissue- or function-specific genes also contain from one to as many as a dozen upstream cis-acting elements, which serve as binding sites for transcriptional regulators: activation and repression factors and accessory proteins that are neither activators nor repressors but are required for their operation (1). Transcriptional control is then accomplished by the response of these activators, repressors, or accessory proteins to environmental signals. These signals may result in the onset or termination of production of these proteins, binding to their target DNA sites upstream of the regulated genes, their operation once bound to these targets, or their interaction with one another.

Expression of the arginase (*CAR1*) gene in *Saccharomyces cerevisiae* provides a system for studying the interactions of multiple positively and negatively acting transcription factors. *CAR1* transcription occurs only when arginine is available within the cell for degradation (2) and is mediated by three major upstream activation sequences (UASs). Two of these UAS elements, UAS_{C1} and UAS_{C2}, mediate transcription that is completely independent of the inducer, arginine. UAS_{C1} consists of two binding sites for ABF1 and a RAP1 site (3). UAS_{C2} consists of three RAP1 sites, a site with homology to the GCR1 site, and a site for an unidentified transcriptional activation factor (L. Kovari and T.G.C., unpublished data). The structure and identification of protein binding sites in the third and weakest UAS, UAS_I, have not yet been resolved, but the operation of UAS_I is absolutely dependent on the presence of arginine. In addition to these sites, a negatively acting upstream repression sequence (*URS1*) has been identified and characterized genetically (4). Induced *CAR1* expression has been suggested to be accomplished as follows (5, 6). In the absence of inducer, only UAS_{C1}, UAS_{C2}, and their associated transcriptional activation factors can operate. However, the operation of these activator proteins is prevented by the transcriptional repression mediated by *URS1* and the proteins that bind to it. When inducer is provided, the UAS_I and its associated proteins operate as well. The combined strength of the three UASs functioning together overcomes the negative effects of transcriptional repression mediated by the *URS1* site, and *CAR1* expression occurs.

The *URS1* site was originally thought to be an arginine pathway-specific element (7). However, more specific delineation of the site by saturation mutagenesis (8) permitted identification of *URS1* homologous sequences upstream of many genes, ranging from those associated with carbon (9, 10) and nitrogen metabolism (11–14) to respiratory chain components (15–17) and those associated with meiosis (18), sporulation, and mating-type switching (19). In a number of cases, deletion of the *URS1* homologous sequence has resulted in marked increases in expression of the gene containing it, leading to the suggestion that it was functional (17, 20, 21).

Mechanistic studies of the *URS1* site and transcriptional repression of the many genes containing it have been impeded by a lack of knowledge about the protein(s) associated with *URS1*. Others have reported chemical crosslinking experiments with a partially purified protein preparation and concluded that a 40-kDa protein bound to *URS1* (22). Further purification of the protein was not, however, reported. In view of the importance of this protein to the regulation of gene expression in *S. cerevisiae* and the deficiency of information about it, we decided to purify it. The following work describes purification of the protein binding to *URS1* (BUF; binding URS1 factor) and demonstration that it exists both in solution and bound to the URS1 site as a stable heteromer. The monomers of BUF exhibit molecular masses of 37.5 and 73.5 kDa.

MATERIALS AND METHODS

Yeast (1–2 kg) growing in yeast extract/peptone/dextrose medium was harvested, washed in an equal volume of cold distilled water, and resuspended in 1/10th vol of refrigerated extraction buffer [200 mM Tris·HCl, pH 8.0/400 mM $(NH_4)_2SO_4$/10 mM $MgCl_2$/1 mM EDTA/10% (vol/vol) glycerol/1 mM phenylmethylsulfonyl fluoride (PMSF)/1 mM dithiothreitol (DTT)]. An equal volume of acid-cleaned glass beads (diameter, 0.45–0.50 mm), cooled to −20°C, was added to the suspended cells, which were then broken in a Waring blender operated at top speed until the mixture reached 15°C. The mixture was then placed at −80°C until the temperature cooled to 2°C. This procedure was repeated four times. Debris was removed by a 1-hr centrifugation (16,000 × *g*). Ammonium sulfate was added to the supernatant (313 g/liter) to achieve 50% saturation. After 30 min of incubation at 0°C, the precipitated protein was recovered by centrifugation and dissolved in 160 ml of dialysis buffer (20 mM Hepes/2 mM EDTA/10% glycerol/0.05 M KCl/1 mM PMSF/1 mM DTT). This extract (final vol, 400–500 ml) was dialyzed two or more

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Abbreviations: UAS, upstream activation sequence; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay.

times against a 40-fold excess of the buffer described above until the final salt concentration was at or below 0.1 M, as determined with a conductivity meter.

The dialyzed extract was loaded onto four DEAE-cellulose (Whatman DE52) columns (5.0 × 20.0 cm), equilibrated in M buffer (20 mM Tris·HCl, pH 7.9/20% glycerol/0.2 mM EDTA/0.05 M KCl/1 mM PMSF/1 mM DTT) (23). After all unbound protein was eluted, M buffer containing 0.3 M KCl was used to recover bound protein. The protein-containing fractions were pooled and dialyzed against a 40-fold excess of dialysis buffer.

The dialyzed preparation was loaded onto two 300-ml columns of Whatman phosphocellulose (P11) and equilibrated with M buffer; unbound protein was eluted. Bound protein was eluted with M buffer containing 0.3 M KCl.

Chemically synthesized oligonucleotides RL-173 (5′-GATCTAGCGGTAGCCGCCGAGGG-3′) and RL-174 (5′-GATCCCCTCGGCGGCTACCGCTA-3′) contain the URS1 site and were used to produce a double-stranded DNA fragment, which was annealed and ligated into a 400- to 800-base-pair polymer. URS1 oligonucleotide concatamers were phenol extracted, precipitated, and resuspended in 10 mM potassium phosphate buffer (pH 8.2). CNBr-activated Sepharose 4B affinity matrix (Pharmacia no. 17-0430-01) was swollen, washed in 1 mM HCl, and activated with 10 mM potassium phosphate. The oligonucleotide polymer was added (500 μg of DNA per g of Sepharose), the mixture was allowed to incubate overnight at 4°C, and coupling groups were deactivated in 0.1 M Tris·HCl (pH 8.0) for 4–6 hr. The affinity matrix was washed once in 0.1 M potassium phosphate (pH 8.0) and three times in 10 mM Tris·HCl (pH 8.0) containing 1 mM EDTA and 0.5 M NaCl, and two 30-ml columns (2.5 × 10 cm) were prepared and equilibrated with M buffer containing 0.3 M KCl. The phosphocellulose eluent was loaded onto these affinity columns and unbound protein was eluted with M buffer containing 0.3 M KCl. The column was then developed with a 0.3–1.5 M KCl linear gradient in M buffer. Fractions were assayed by electrophoretic mobility-shift assay (EMSA) (8). Fractions that assayed positively were pooled, dialyzed, and again subjected to affinity chromatography. Active fractions were pooled, concentrated, and stored at −80°C.

Competition EMSAs were performed as described (8). Synthetic oligonucleotides used in these assays are shown in Fig. 1.

Native gel electrophoresis was performed as described by Laemmli (24) except that SDS was omitted from all solutions. Protein was electroeluted from this gel at 15°C into running buffer supplemented with 1 mM DTT.

Western blot analysis was performed according to Towbin *et al.* (25) using the Trans-Blot apparatus (Bio-Rad no. 170-3930) and nitrocellulose as a blotting medium. The air-dried nitrocellulose membrane was analyzed by probing the filters with primary murine monoclonal antibodies. Secondary goat antibodies were obtained from Bio-Rad [no. 170-6516 blotting grade affinity-purified goat anti-mouse IgG (H+L) human IgG adsorbed horseradish peroxidase conjugate]. Antibody exposure and the horseradish peroxidase color development procedure were followed according to the manufacturer's instructions. Monoclonal antibody preparations were produced by standard procedures. The nearly homogeneous protein was used in the ELISAs that identified the desired clones.

RESULTS

Specificity of BUF for DNA Binding. Since our initial identification of the URS1 site upstream of genes encoding enzymes associated with arginine metabolism, *URS1* homologous sequences have been observed in the promoters of

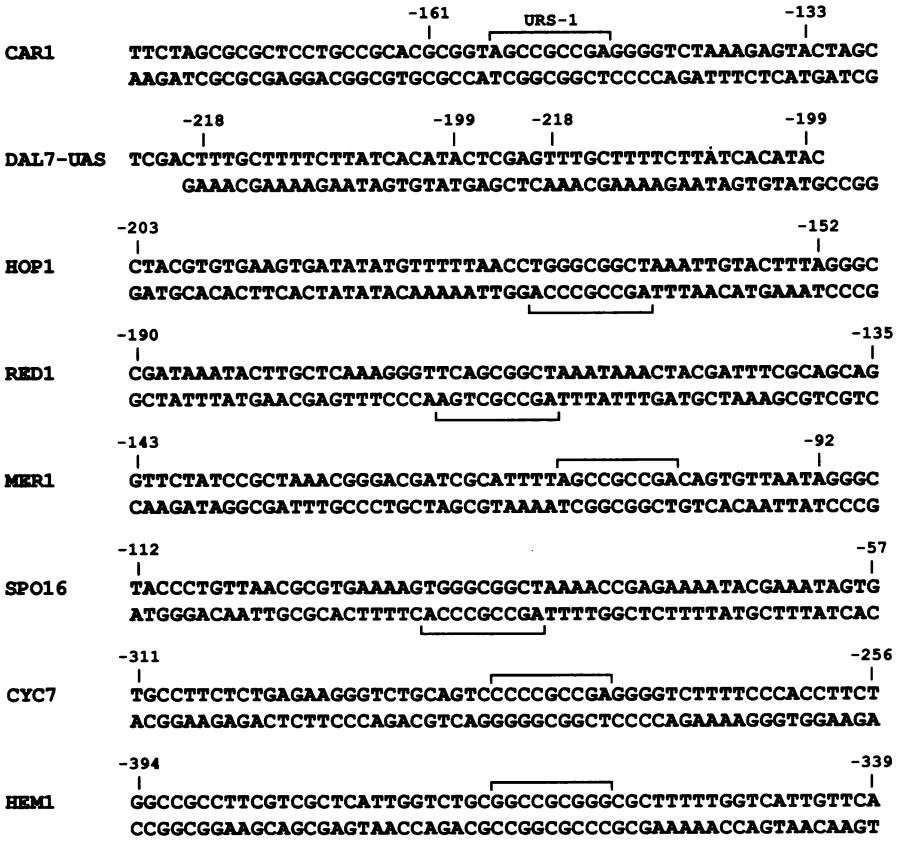

FIG. 1. Synthetic oligonucleotides used in this work.

meiosis and sporulation-specific genes among others. If DNA fragments from those promoters could be shown to be effective competitors of the *CAR1 URS1* site for protein binding, it would indicate that the bound proteins were not specific to the arginine degradative genes but were more likely factors globally mediating negative transcriptional regulation. As shown in Fig. 2, DNA fragments derived from the upstream regions of a representative sample of these genes, *HOP1, RED1, CYC7, HEM1**, *MER1* (26), and *SPO16* (18), were all effective competitors of *CAR1 URS1*–protein complex formation. In contrast, a DNA fragment containing the *DAL7 UAS* element (27) was not, as expected, an effective competitor, thus serving as a control for specificity of the EMSA.

Purification of BUF. As a first step toward understanding how the *CAR1 URS1* element mediated negative transcriptional control, we purified the protein(s) that bound to it. The purification procedure described in *Materials and Methods* was used to purify BUF to near homogeneity. It was not possible to determine protein concentrations in later steps of the purification scheme because of the very small amounts present. This difficulty made construction of a meaningful purification table impossible. However, DEAE-cellulose chromatography resulted in loss of ≈80% of the soluble protein without significant loss of DNA binding activity. As shown in Fig. 3*A*, DNA binding protein was thereafter eluted from a phosphocellulose column behind the main protein peak that was eluted with the 0.1 M salt wash. The activity elution profile from the first affinity column developed with a linear salt gradient is shown in Fig. 3*B*. Protein eluted from the second affinity column was found to be nearly homogeneous, as evaluated by gel electrophoresis (see below). SDS/PAGE analysis of our purest preparations yielded two polypeptide species estimated to be 73.5 and 37.5 kDa, respectively (Fig. 4). This result suggested that BUF might be a heteromeric protein. Additional experiments demonstrated that both polypeptides are sensitive to proteolysis, with the larger species being the more sensitive. The smaller species, however, is not derived from the larger (data not shown).

BUF Is a Heteromeric Protein. Finding two polypeptides in our most purified preparations generated two possibilities: either a single protein bound to the DNA and was contaminated with a second one, or, alternatively, the two polypeptides were members of a heteromeric protein that bound to the DNA. Our first attempt to resolve this issue was to ascertain the behavior of the proteins when subjected to native gel electrophoresis. An entire preparation of purified protein was electrophoresed through a 7.5% native gel, which was set up with seven identical, heavily loaded lanes. After electrophoresis, we cut off the two outside lanes and stained them. Fig. 5 (lane A) depicts one of these stained lanes. The stained lanes were then precisely reassembled, with the center portion of the gel containing the five unstained lanes. Using a straight edge aligned with the stained lanes as a guide, we cut out the lower half of the single band that occurred on the gel (cut lines are indicated by the positions of the arrows in lane A). A very light band appears above the overloaded band, representing the vast majority of the protein loaded onto the gel. The identity of this minor contaminant is unknown. The protein contained in the excised portion of the gel was electroeluted into buffer as described in *Materials and Methods*, and the resulting protein solution was divided into two portions. The first portion was subjected to SDS/PAGE (lane B), while increasing amounts of the second portion were used as the source of protein in an EMSA (lanes D–F). Two polypeptide species of 73.5 and 37.5 kDa were observed in the stained SDS gel (lane B) just as they were when the purified preparation was directly analyzed by SDS/PAGE (Fig. 4). As shown in Fig. 5 (lanes D–F), the electroeluted protein formed a single complex with *URS1*-containing DNA, which migrated in a manner identical to that observed when a sample of crude extract was used in its place (lane C).

The experiment described above indicated that the single protein-containing band obtained from native gel electrophoresis contained two polypeptides and also formed a complex with a DNA fragment containing the *URS1* site. These observations did not, however, directly demonstrate that both polypeptides were contained in the DNA–protein complex that appeared in the EMSA. To more directly address this question, we generated two hybridoma cell lines that produced monoclonal antibodies that were specific to each of the polypeptides in the purified BUF preparation. As shown in Fig. 6 (lanes A–C), each antibody preparation reacted with only a single polypeptide species. Multiple, identical gel-shift assays were performed with purified BUF used as the source of protein for the assay. The gel was then divided into several pieces. One lane of the gel was processed in the standard

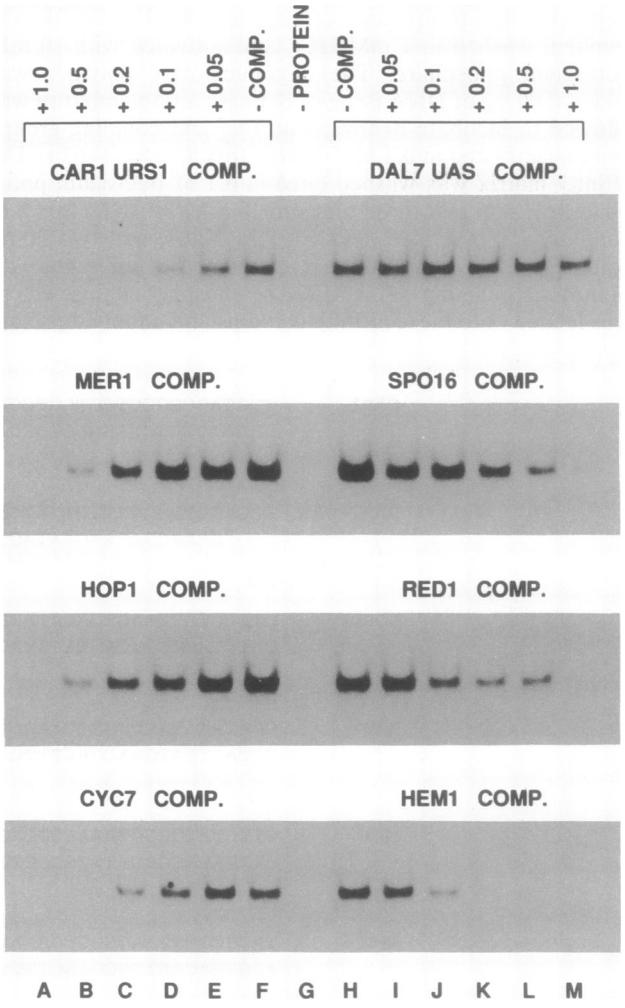

FIG. 2. Competition of DNA fragments derived from the regulatory regions of various yeast genes with a labeled DNA fragment containing the *CAR1 URS1* in an EMSA. In each case, 50 ng of a ^{32}P-labeled *CAR1* oligonucleotide was used as a probe. A 165-fold excess of sonicated calf thymus DNA was added to each reaction mixture as nonspecific competitor. Amounts of competitor DNA (μg) are indicated. One reaction mixture contained no protein. The genes from which the oligonucleotides were derived are indicated, and their sequences are shown in Fig. 1.

*Atcheson, C. L. & Esposito, R. E., Annual Yeast Genetics and Molecular Biology Meeting, May 23–27, 1991, San Francisco, Poster 179B.

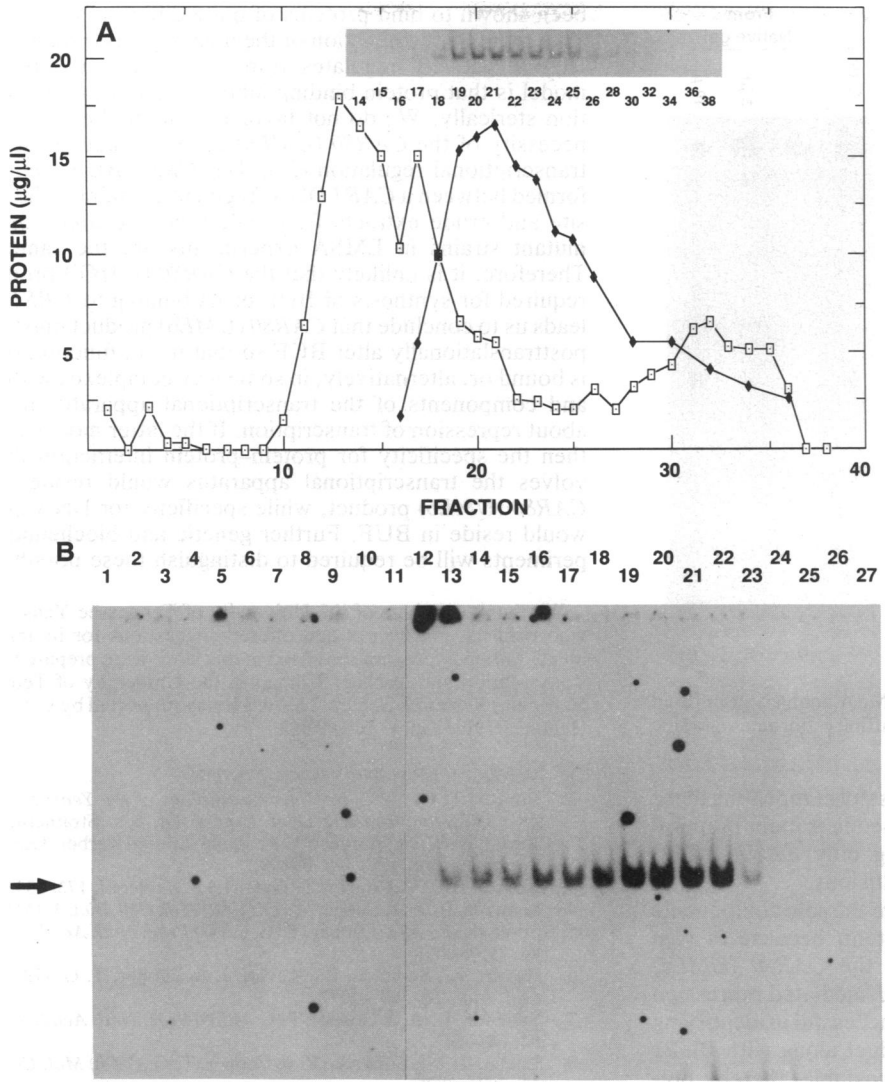

FIG. 3. (*A*) Elution profile of BUF from the phosphocellulose column. The 0.3 M wash was started at fraction 22. □, Protein concentrations observed in the fractions; ◆, complex formation activity as determined by densitometric quantitation of autoradiograms generated by our standard EMSAs. (*Inset*) Primary data from the EMSA. (*B*) Elution profile of the first affinity column-bound fraction as detected by EMSA. Fraction numbers appear at the top. Data are those derived when the column was developed with a 0.2–1.5 M linear KCl gradient. The gradient began and was completed with fractions 1 and 27, respectively. Arrow indicates position of the desired DNA–protein complex determined from other experiments.

manner to visualize the radioactive DNA–protein complex formation (lane D). The contents of two lanes were transferred to a nitrocellulose membrane as described in *Materials and Methods*. The membrane was then cut into two pieces such that one of the two lanes was bisected. The piece of membrane containing the contents from one and a half lanes was treated with antibody specific for the 73.5-kDa polypeptide (lanes E and F), while the other was treated with antibody specific for the 37.5-kDa species (lane F). The contents of two lanes from an identical gel were processed in the same fashion except that the antibody used to treat them was specific for the 37.5-kDa polypeptide (lanes G and H). Antigen–antibody complex was visualized with the peroxidase reaction as described and the pieces of membrane were reassembled. As shown in lanes D–G, both antibody preparations reacted with the same band. This indicated that the 37.5- and 73.5-kDa species were both present in the DNA–protein complex. Note that the purified protein, which was not incubated with DNA, migrated more slowly than the DNA–protein complex (lane H).

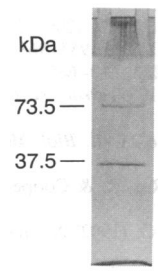

FIG. 4. SDS/PAGE analysis of the preparation that was obtained after the second affinity chromatographic column. Sizes were approximated by using commercial standards (data not shown).

DISCUSSION

Data presented in this and earlier work demonstrate that a heteromeric protein (binding URS1 factor), consisting of 37.5- and 73.5-kDa monomers designated BUF1 and BUF2, respectively, binds to the *CAR1 URS1* site and similar sites upstream of numerous genes in *S. cerevisiae* (8). In several cases, deletion of the *URS1* site has been shown to result in 10- to 20-fold increases in gene expression, leading us to conclude that BUF is the DNA binding protein responsible for repressing transcriptional activation of these genes (5). We also demonstrated that, in solution, BUF1 and BUF2 form a stable BUF complex, since neither of the monomeric polypeptides could be identified alone in any of our analytical electrophoretic experiments.

Our data are consistent with and extend earlier observations (22). These investigators subjected the products of a chemical crosslinking experiment between a DNA fragment containing multiple cis-acting sites, including one with homology to *URS1* and a partially purified protein preparation to molecular sieve chromatography and concluded that the *URS1* binding factor was a 40-kDa protein. A second protein with the characteristics of our 73.5-kDa polypeptide was not observed. If the specificity of cross-linking were sufficiently

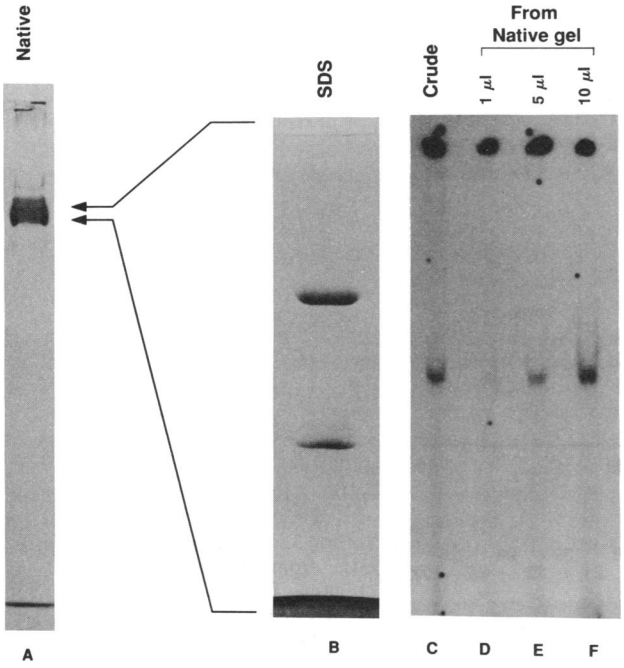

FIG. 5. Native gel, SDS/PAGE, and EMSA analysis of purified BUF protein derived from the second affinity chromatographic column.

directly to the DNA. We were unsuccessful at repeating these experiments with our nearly homogeneous protein preparation and a DNA fragment containing only *CAR1 URS1*. Hence, we cannot confirm their conclusions.

It is unlikely that BUF1 and BUF2 are the sole components of this transcriptional repression system, because *in vivo* experiments have demonstrated that the *CAR80* (*UME6*) gene product is also required for *URS1*-mediated repression of *CAR1* expression (28). We were unsuccessful in identifying a complex containing the *CAR80* product along with BUF1 and BUF2. Such a complex may be unstable, thereby precluding its identification. Alternatively, the *CAR80* (*UME6*) gene product is required in some way for function of BUF, but it does not directly bind to it.

The presence of the BUF binding site in the promoter regions of so many unrelated genes supports the idea that BUF is a global transcriptional factor that negatively regulates expression of these genes. Genes that contain BUF binding sites in their promoter regions have been shown to contain a wide variety of UASs, which, in some cases, have

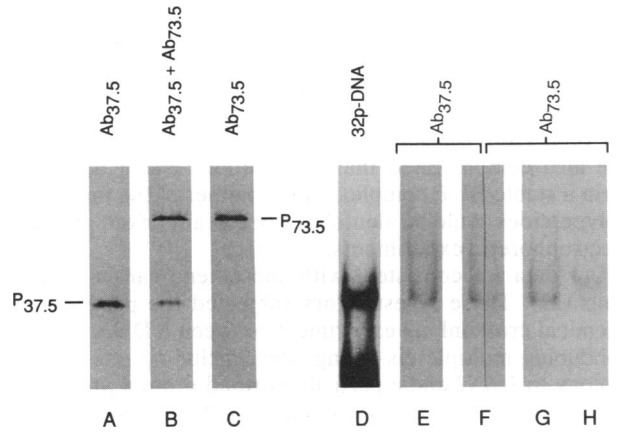

FIG. 6. Western blot analysis of purified BUF protein before and after EMSA.

been shown to bind proteins of quite different structure (1). This prompts the question of the mechanisms through which BUF negatively regulates transcriptional activation. One model is that protein binding inhibits transcriptional activation sterically. We do not favor this model because of the necessity of the *CAR80* (*UME6*) gene product for negative transcriptional regulation (28). The *URS1*–BUF complexes formed between a *CAR1* DNA fragment containing the *URS1* site and crude extracts from wild-type and *ume6* deletion mutant strains in EMSA experiments are the same (28). Therefore, it is unlikely that the *CAR80* (*UME6*) product is required for synthesis of BUF or its binding to *URS1*. This leads us to conclude that *CAR80* (*UME6*) product must either posttranslationally alter BUF so that it can function once it is bound or, alternatively, in some way complexes with BUF and components of the transcriptional apparatus to bring about repression of transcription. If the latter model applies, then the specificity for protein–protein interaction that involves the transcriptional apparatus would reside in the *CAR80* (*UME6*) product, while specificity for DNA binding would reside in BUF. Further genetic and biochemical experiments will be required to distinguish these possibilities.

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