MOLECULAR AND CELLULAR BIOLOGY, Sept. 1990, p. 4872–4885 Vol. 10, No. 9
0270-7306/90/094872-14$02.00/0
Copyright © 1990, American Society for Microbiology

Multiple Factors Bind the Upstream Activation Sites of the Yeast Enolase Genes *ENO1* and *ENO2*: ABFI Protein, like Repressor Activator Protein RAP1, Binds *cis*-Acting Sequences Which Modulate Repression or Activation of Transcription

PAUL K. BRINDLE,[1] JANICE P. HOLLAND,[1] CATHERINE E. WILLETT,[1] MICHAEL A. INNIS,[2] AND MICHAEL J. HOLLAND[1]*

Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616,[1] and Cetus Corporation, 1400 Fifty-third Street, Emeryville, California 94608[2]

Received 4 April 1990/Accepted 21 June 1990

Binding sites for three distinct proteins were mapped within the upstream activation sites (UAS) of the yeast enolase genes *ENO1* and *ENO2*. Sequences that overlapped the UAS_1 elements of both enolase genes bound a protein which was identified as the product of the *RAP1* regulatory gene. Sequences within the UAS_2 element of the *ENO2* gene bound a second protein which corresponded to the ABFI (autonomously replicating sequence-binding factor) protein. A protein designated EBF1 (enolase-binding factor) bound to sequences which overlapped the UAS_2 element in *ENO1*. There was a good correlation among all of the factor-binding sites and the location of sequences required for UAS activity identified by deletion mapping analysis. This observation suggested that the three factors play a role in transcriptional activation of the enolase genes. UAS elements which bound the RAP1 protein or the ABFI protein modulated glucose-dependent induction of *ENO1* and *ENO2* expression. The ABFI-binding site in *ENO2* overlapped sequences required for UAS_2 activity in wild-type strains and for repression of *ENO2* expression in strains carrying a null mutation in the positive regulatory gene *GCR1*. These latter results showed that the ABFI protein, like the RAP1 protein, bound sequences required for positive as well as negative regulation of gene expression. These observations strongly suggest that the biological functions of the RAP1 and ABFI proteins are similar.

Expression of genes encoding glycolytic enzymes in the yeast *Saccharomyces cerevisiae* is regulated in response to heat shock (34), entry into stationary phase (2, 24, 30; unpublished data), carbon source (9, 28, 31, 37), and, in addition, by the *trans*-acting regulatory gene *GCR1* (7, 8). As a model for studying glycolytic gene regulation, *cis*-acting regulatory elements were mapped within the 5′-flanking sequences of the enolase genes *ENO1* (10, 40–42) and *ENO2* (9). Each gene contains a complex regulatory region composed of multiple upstream activation sites (UAS). *ENO1* and *ENO2* contain at least two adjacent UAS elements, each of which is independently capable of activating transcription (9, 10). The *ENO1* gene also contains an upstream repression site (URS) located between the TATAAA box and UAS elements. The URS element represses *ENO1* expression in vegetative cells grown on a medium containing glucose as the carbon source (10).

We showed that glucose-dependent induction of *ENO1* (10) and *ENO2* (9) is mediated by UAS elements within the 5′-flanking regions of the respective genes. *GCR1*-dependent regulation of *ENO2* expression is also modulated by one of the UAS elements (19). The *GCR1* gene appears to play a central role in coordinate regulation of glycolytic gene expression, since null mutations in this gene cause substantial reductions in the specific activities of many glycolytic enzymes (1, 7, 8). The *GCR1* gene product was shown to be a *trans*-acting positive regulator of transcription of the two enolase genes (20), all three glyceraldehyde-3-phosphate dehydrogenase genes (20), and probably the other affected glycolytic genes (7).

* Corresponding author.

We report here the identification of three distinct DNA-binding proteins that interact specifically with sequences within the 5′-flanking regions of the *ENO1* and *ENO2* genes. The binding sites for these factors were mapped and found to correlate with essential regions of UAS elements identified from deletion mapping analysis (9, 10). The role of these specific *trans*-acting factors in regulation of enolase gene expression in response to carbon source and the *GCR1* gene product is discussed.

MATERIALS AND METHODS

Materials. Oligonucleotides for DNA-binding assays were provided by the DNA Synthesis Group, Cetus Corp., Emeryville, Calif., and by J. Bruenn, State University of New York at Buffalo.

Strains and growth conditions. *S. cerevisiae* S173-6B (**a** *leu2-3 leu2-112 his3-1 trp1-289 ura3-52*) was provided by F. Sherman, University of Rochester, Rochester, N.Y. *S. cerevisiae* S173-G is identical to strain S173-6B except that it carries a deletion of 90% of the coding sequences of the *GCR1* gene and an insertion of a functional *URA3* gene at the site of the deletion mutation (20). Yeast strains were grown at 30°C and harvested in early log phase (A_{660} of 1). Cells were grown in YP medium (1% yeast extract, 2% peptone; Difco Laboratories, Detroit, Mich.) supplemented with either 2% glucose or 2% glycerol plus 2% lactate.

DNA probes. DNase I-dependent footprinting and methylation interference analysis of the UAS_1 regulatory element within the 5′-flanking region of the *ENO1* gene were performed with a probe extending from an *Ava*II site at position −320 (^{32}P-labeled with polynucleotide kinase) to an *Mbo*II site at position −492. For DNase I footprinting analysis of

the UAS_2 regulatory element in *ENO1*, a segment of 5'-flanking sequence extending from a *Sal*I site at position −415 to an *Xba*I site at position −768 was subcloned into the polylinker region of pUC18. DNA probes were generated by 5' labeling at the *Sal*I site with polynucleotide kinase or 3' labeling at the *Pst*I site with terminal transferase, followed by cleavage of the labeled DNAs with *Xba*I. DNA probes were gel purified before use.

Double-stranded oligonucleotides corresponding to those portions of the UAS_1 and UAS_2 elements from the *ENO1* gene that were protected from DNase I digestion were synthesized and designated *ENO1*/UAS_1 (5'-***TCGA***CTAG GATAGCACCCAAACACCTGCATATTTG-3', where nonenolase sequences added to create a *Sal*I cohesive 5' terminus are indicated in boldface italics) and *ENO1*/UAS_2 (5'-AAAAATACCGCTTCTAGGCGGGTTATCTGCTAATCC GAG-3'), respectively. A double-stranded oligonucleotide corresponding to the RAP1 protein-binding site within element E of the silent mating type locus *HMR* (35) was synthesized and designated HMRE (5'-CTTGCAAAAAC CCATCAACCTTGAAAAAAAGTAGAC-3'). Similarly, a double-stranded oligonucleotide corresponding to the ABFI protein-binding site within element B of the silent mating type locus *HMR* (4, 5) was synthesized and designated HMRB (5'-CAATACATCATAAAATACGAACGA-3'). A double-stranded oligonucleotide corresponding to the EBF2-binding site within the 5'-flanking sequences of the *ENO2* gene was made as described previously (19) and designated *ENO2* (5'-***TCGAC***GAACGCGGCGTTATGTCACTAACGA CGTGCACCATTTTTG-3', where nonenolase sequences added to create a *Sal*I cohesive 5' terminus are indicated in boldface italics).

Single-stranded oligonucleotides were 5'-end labeled with polynucleotide kinase and then annealed in the presence of an excess (1.2-fold) of the unlabeled complementary oligonucleotide to generate ^{32}P-labeled double-stranded oligonucleotide probes.

DNA-binding assays. Gel mobility shift DNA-binding assays were performed as described previously (19) with the double-stranded oligonucleotide probes described above. Competition DNA-binding assays were performed in the absence of poly(dI-dC). The indicated molar excesses of unlabeled competitor double-stranded oligonucleotides were added to the binding reaction mix with the labeled probe prior to the protein extracts. Lambda DNA (restriction endonuclease digested) served as a nonspecific competitor DNA.

DNase I-dependent footprinting and methylation interference analyses were performed as described previously (19). A portion of each respective DNA probe was cleaved by the method of Maxam and Gilbert (29) and used as molecular size markers.

Mono S chromatography of S100 extracts. Whole-cell S100 extracts were prepared by ammonium sulfate precipitation as described previously (19, 38). Portions of S100 extracts containing 1 to 2 mg of protein, as determined by the method of Warburg and Christian (26), were mixed with buffer A (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.4], 0.2 mM EDTA, 2 mM dithiothreitol, 17% glycerol) and buffer B (buffer A with 1 M KCl) to yield a 1-ml solution that was 50 mM KCl. The protein solutions were then chromatographed at room temperature on a Mono S HR5/5 fast protein liquid chromatography (FPLC) column (Pharmacia LKB) equilibrated with buffer A containing 50 mM KCl. The columns were washed with 5 ml of buffer A containing 50 mM KCl, and then DNA-binding proteins were eluted with a 15-ml linear KCl gradient in buffer A (50 to 300 mM KCl). The columns were then step eluted with 2 ml of buffer B. Fractions (1 ml) were collected and stored at −80°C. DNA-binding activities were localized by gel mobility shift assays with specific DNA probes. Samples (2 μl) of each fraction were used in the standard 5-μl binding reaction mixes containing poly(dI-dC) at a final concentration of 25 μg/ml (19).

Synthesis of RAP1 protein in *E. coli*. A DNA sequence encoding the entire yeast RAP1 protein (35) was synthesized from genomic DNA isolated from *S. cerevisiae* S173 6B by the polymerase chain reaction (PCR). Novel sequences were incorporated at the 5' termini of the PCR primers to facilitate subcloning and subsequent expression of RAP1 protein with an *Escherichia coli* expression vector. The 5' PCR primer (5'-GGT***ATCGAT***AAGCAATATGTCTAGTCCAGATTTTG-3') contained the first 19 nucleotides of the translated portion of the *RAP1* gene (underlined) linked to 5' sequences that correspond to the *E. coli trp* promoter ribosome-binding site, which includes a *Cla*I restriction endonuclease cleavage site (boldface italics). The 3' PCR primer (5'-CC***GATATCGAAT TCC***TCATAACAGGTCCTTCTC-3') corresponded to codons 824 to 828 and the TGA termination codon of the *RAP1* gene (underlined) linked to cleavage sites for *Eco*RI and *Eco*RV (boldface italics). The PCR reaction mix (50 μl) contained 20 mM Tris hydrochloride (pH 8.5), 25 mM KCl, 1.5 mM $MgCl_2$, 50 mM each dATP, dGTP, dCTP, and dTTP, 0.05% Tween 20, 20 pmol of the 5' and 3' PCR primers, 2 U of *Thermus aquaticus* DNA polymerase (Perkin Elmer-Cetus), and 100 ng of yeast genomic DNA. Thirty cycles of the PCR reaction were performed with a Perkin Elmer-Cetus thermal cycler as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. A final expression reaction was performed at 72°C for 5 min. These PCR reaction conditions minimize potential incorporation errors caused by mispriming or misextension during the reaction (23). The PCR reaction product was extracted once with chloroform-isoamyl alcohol (48:1) and precipitated from 2 M ammonium acetate–50% isopropyl alcohol. A unique PCR product of the expected size was observed after agarose gel electrophoresis that contained appropriate restriction endonuclease cleavage sites predicted from the nucleotide sequence of the *RAP1* gene (35).

Ten percent of the *RAP1* PCR product was digested with *Cla*I and *Eco*RI and subcloned into the polylinker of the Bluescript M13+ vector (Stratagene) to create an in-frame fusion of the *RAP1* coding sequences and the *lacZ* gene. Synthesis of a fusion protein containing 33 N-terminal residues encoded by the *lacZ* gene linked to the RAP1 polypeptide was induced by addition of IPTG (isopropylthiogalactopyranoside) to log-phase *E. coli* cultures carrying the recombinant Bluescript vector. To test the activity of the PCR product, extracts prepared from four independent subclones were analyzed by gel mobility shift assay with the HMRE double-stranded oligonucleotide. Specific gel mobility shift complexes of the expected mobilities were observed with all four extracts. The *Cla*I-*Eco*RI fragment containing the RAP1 coding sequences was isolated from one of the recombinant Bluescript vectors and subcloned into a high-level *E. coli* expression vector derived from pHCW701 (44), which contains the *E. coli trp* promoter and the *Bacillus thuringiensis cry* terminator in order to produce authentic RAP1 protein without the N-terminal sequences from the *lacZ* gene. A subclone carrying the pHCW701/RAP1 plasmid was grown in a minimal salts medium without tryptophan containing 0.5% glucose. Cells were harvested in log-phase growth and disrupted with an Eaton press as described

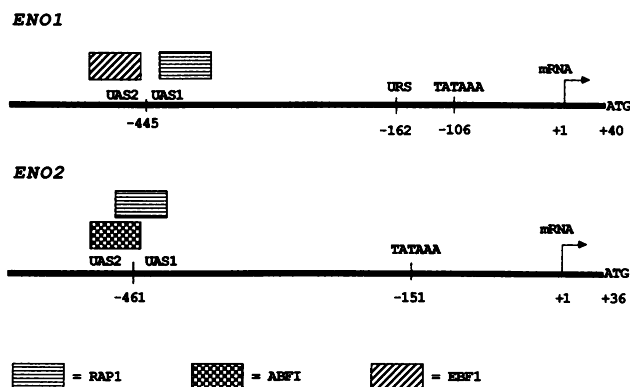


FIG. 1. *cis*-Acting regulatory sequences within the 5′-flanking regions of the enolase genes *ENO1* and *ENO2*. The locations of sequences involved in regulation of transcription of the yeast enolase genes were previously defined by deletion mapping analysis (9, 10). The transcriptional initiation sites (+1) and TATAAA boxes are indicated for both genes. UAS are located immediately upstream and downstream from positions −445 and −461 in *ENO1* and *ENO2*, respectively. A URS is located at position −162 in *ENO1*. The locations of binding sites for RAP1, ABFI, and EBF1 within the 5′-flanking regions of *ENO1* and *ENO2* are indicated by shaded boxes. Binding of ABFI and RAP1 to overlapping sites in *ENO2* is depicted by overlapping boxes.

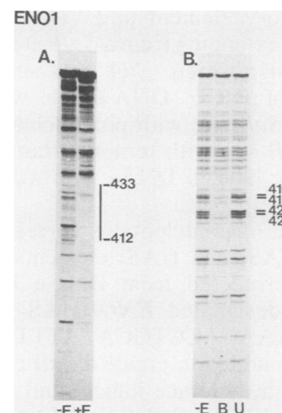


FIG. 2. Mapping of a RAP1 protein-binding site within the UAS_1 element of *ENO1*. (A) DNase I protection analysis with a fragment of DNA corresponding to *ENO1* 5′-flanking sequences extending from positions −320 to −492. The ^{32}P-labeled DNA fragment was incubated with (+E) or without (−E) S100 extract isolated from *S. cerevisiae* S173-6B grown in a medium containing glucose as the carbon source. DNase I digestion and polyacrylamide gel electrophoresis were performed as described in Materials and Methods. The location of a DNase I footprint extending from positions −412 to −433 is indicated. (B) Methylation interference analysis was performed with the same DNA fragment described above except that the opposite strand of the DNA was ^{32}P-labeled. Gel mobility shift analysis and chemical cleavage of the isolated DNA were performed as described in Materials and Methods. A polyacrylamide gel of chemically cleaved DNA is shown for ^{32}P-labeled DNA without incubation with S100 extract (−E) and for bound (B) and unbound (U) DNA isolated after gel mobility shift analysis. Methylation of G residues at positions −416, −417, −423, and −424 interfered with protein-DNA complex formation.

above for yeast cells. Preparation of an S100 extract and Mono S ion-exchange chromatography were performed as described above for yeast strains.

RESULTS

RAP1 protein binds to UAS in the *ENO1* and *ENO2* genes. Multiple UAS were previously mapped within the 5′-flanking regions of the yeast enolase genes *ENO1* and *ENO2* (9, 10). As illustrated in Fig. 1, UAS elements are located immediately upstream and downstream from sites located 445 base pairs and 461 base pairs upstream from the transcriptional initiation sites in *ENO1* and *ENO2*, respectively. To a first approximation, the UAS regions from the two enolase genes appear to be functionally similar, since they can be exchanged between the two genes with no significant effect on gene expression (10). Machida et al. (27) showed previously that a protein factor binds between positions −413 and −433 within the 5′-flanking region of *ENO1*. This binding site overlaps the UAS_1 element in *ENO1* (Fig. 1). Buchman et al. (6) showed that a double-stranded oligonucleotide corresponding to the UAS_1 element from *ENO1* binds specifically to a protein factor which they designated GRF1. The consensus binding site for GRFI is identical to the binding site for the TUF factor first described by Huet and Sentenac (21, 22). The TUF factor and GRFI appear to be identical to a factor described by Shore and Nasmyth (35), which is encoded by the *RAP1* gene.

We have confirmed that a factor binds to a region of *ENO1* 5′-flanking sequence extending from positions −412 to −432 by DNase I-dependent footprinting analysis with a yeast whole-cell extract (S100) (Fig. 2A). Methylation interference studies performed with the same *ENO1* probe implicated four G residues on one of the DNA strands as important contact sites for binding of this factor (Fig. 2B). All four G contact sites were within the region protected from DNase I digestion (Fig. 2A). Based on these observations and previous studies from other laboratories (6, 21, 22, 27, 35), we concluded that the factor which binds the *ENO1* UAS_1 element was the product of the *RAP1* gene. A confirmation of this conclusion is described in a subsequent section of this report. A double-stranded oligonucleotide corresponding to *ENO1* sequences extending from positions −407 to −435 was synthesized and used for subsequent characterization of the *ENO1* UAS_1 binding activity.

We showed in the preceding report (19) that sequences overlapping the UAS_2 element in *ENO2* bound specifically to a factor, designated EBF2, present in partially purified yeast cell extracts. A double-stranded oligonucleotide corresponding to *ENO2* sequences between positions −448 and −486 bound EBF2 specifically in S100 yeast extracts. Although the *ENO2* and *ENO1* UAS elements have little nucleotide sequence similarity, we showed previously that they function similarly when exchanged between the two enolase genes (10).

To test the possibility that EBF2 was RAP1 protein, competition DNA-binding studies were performed with a yeast S100 extract and the *ENO1*/UAS_1 and *ENO2* double-stranded oligonucleotides described above. As illustrated in Fig. 3, a probe corresponding to the *ENO2* oligonucleotide formed a discrete complex with a factor in the yeast extract. This complex was not competed with by unlabeled *ENO1*/UAS_1 oligonucleotide at concentrations up to a 100-fold molar excess, suggesting that EBF2 was not RAP1 protein. Surprisingly, the complex formed with the *ENO1*/UAS_1 probe was competed with by the unlabeled *ENO2* oligonucleotide. These latter results suggested that the *ENO2* oligonucleotide also contained a RAP1 protein-binding site. The failure to observe RAP1 protein binding to the *ENO2* probe with the S100 extract suggested that EBF2 binds preferen-

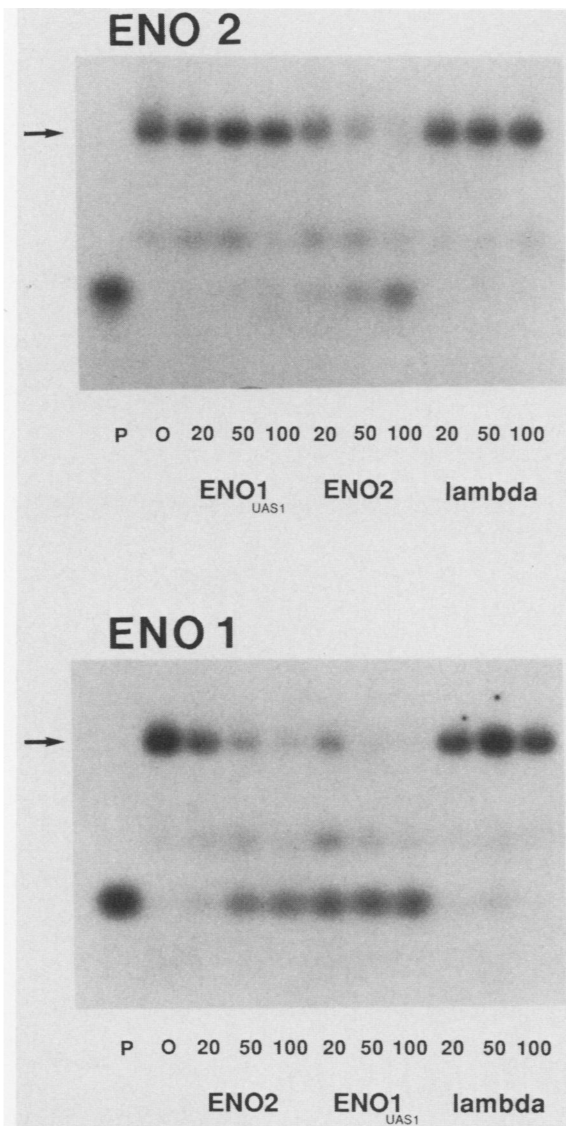


FIG. 3. Competition DNA-protein binding analysis with double-stranded oligonucleotides corresponding to the *ENO1* UAS_1 element and the *ENO2* UAS elements. Gel mobility shift assays were performed with a ^{32}P-labeled *ENO2* oligonucleotide (top panel) and a ^{32}P-labeled *ENO1*/UAS_1 oligonucleotide (bottom panel) in the presence of increasing amounts of competitor DNA. Unlabeled *ENO2* and *ENO1*/UAS_1 oligonucleotides and lambda DNA were used as competitors. DNA-protein binding reactions were performed as described in Materials and Methods in the presence or absence (P) of an S100 extract isolated from *S. cerevisiae* S173-6B grown in a medium containing glucose as the carbon source. Binding reaction mixes contained either no competitor DNA (O) or a 20- to 100-fold molar excess of the indicated competitor DNA. The arrows indicate the locations of specific complexes formed with each of the ^{32}P-labeled oligonucleotides. The intermediate-size complex observed in the agarose gels is due to the interaction of a small amount of single-stranded ^{32}P-labeled oligonucleotide with a single-stranded DNA-binding protein present in the S100 extract.

tially to this oligonucleotide and that EBF2 and RAP1 protein binding to the *ENO2* oligonucleotide may be mutually exclusive.

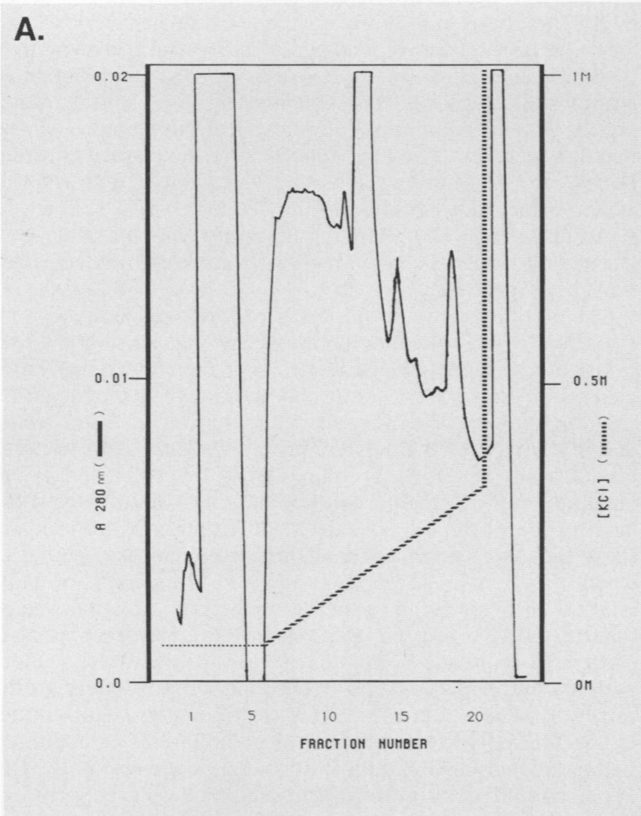
Fractionation of DNA-binding activities that interact with sequences within the *ENO1* and *ENO2* UAS. To further characterize EBF2, RAP1, and other factors capable of interacting specifically with the UAS elements from *ENO1* and *ENO2*, DNA-binding activities from whole-cell yeast S100 extracts were partially purified. Crude cellular extracts were prepared from a wild-type yeast strain grown on a medium containing either glucose or glycerol plus lactate as carbon source and from a strain carrying a *gcr1* null mutation grown on a medium containing glycerol plus lactate. These extracts were subjected to Mono S cation-exchange chromatography. DNA-binding activities were eluted with a 50 to 300 mM linear KCl gradient, followed by a 1.0 M KCl wash. Fractionation of DNA-binding activities was monitored by gel mobility shift assays with specific double-stranded oligonucleotide probes.

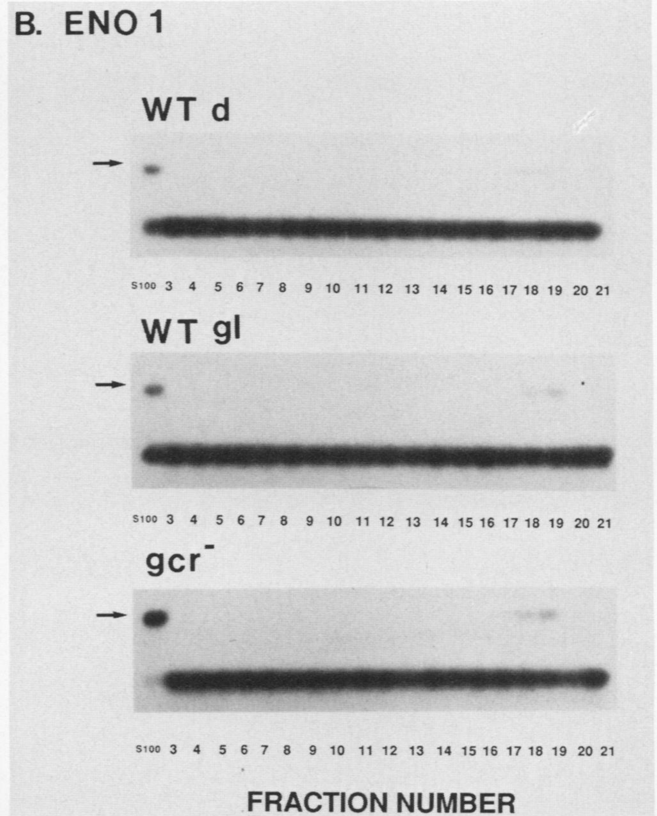
The elution profile of the RAP1 protein was assayed with the *ENO1*/UAS_1 oligonucleotide probe and an oligonucleotide probe, designated HMRE, that corresponds to the RAP1 protein-binding site within the silent mating type locus *HMR* (35). As shown in Fig. 4B and D, the majority of the binding activity observed with each probe with an S100 extract eluted from the Mono S column in fractions 18 and 19. A small amount of residual binding activity was observed for each probe in the 1.0 M KCl wash (fraction 21). Based on these data, we concluded that the binding activity eluted in fractions 18 and 19 corresponded to the RAP1 protein. Additional evidence that this latter binding activity was in fact the RAP1 protein came from Southwestern (DNA-protein blot) analysis. Samples of the putative RAP1-binding activity, purified by Mono S chromatography, were transferred to nitrocellulose membranes following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with a labeled HMRE double-stranded oligonucleotide. The apparent molecular mass of the major binding activity observed in this analysis was approximately 100 kilodaltons (kDa) (data not shown), which is close to the 120 kDa to 116 kDa reported for purified RAP1 protein and GRFI protein (6, 35).

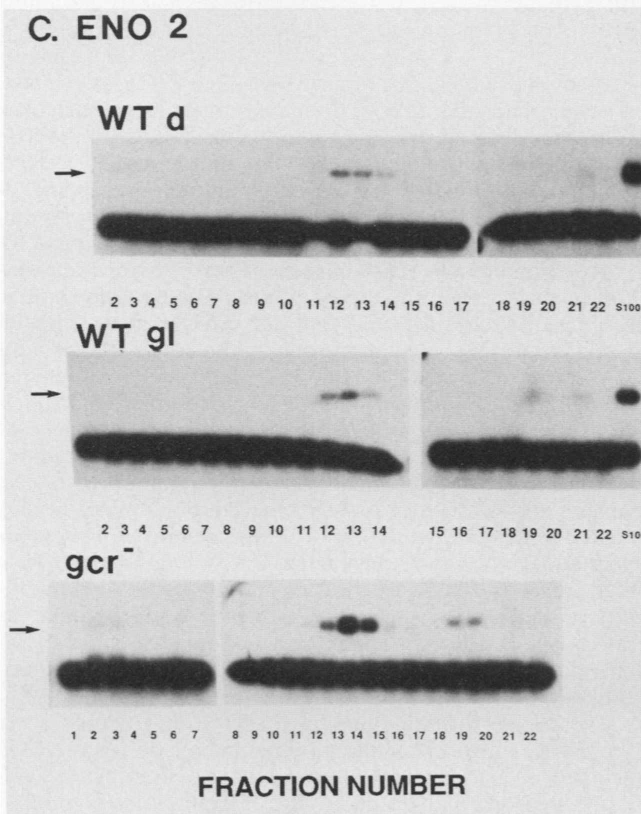
Fractions eluted from the Mono S columns were also assayed with the *ENO2* double-stranded oligonucleotide (Fig. 4C). The major binding activity observed with the S100 extract eluted in fractions 12 to 14 at approximately 150 mM KCl. A minor binding activity eluted in fractions 18 and 19. From this analysis we concluded that the major binding activity corresponded to EBF2 and the minor binding activity corresponded to RAP1 protein. These assignments were consistent with the previously discussed competition studies (Fig. 3), which suggested that the *ENO2* oligonucleotide binds the RAP1 protein more weakly than the EBF2 protein.

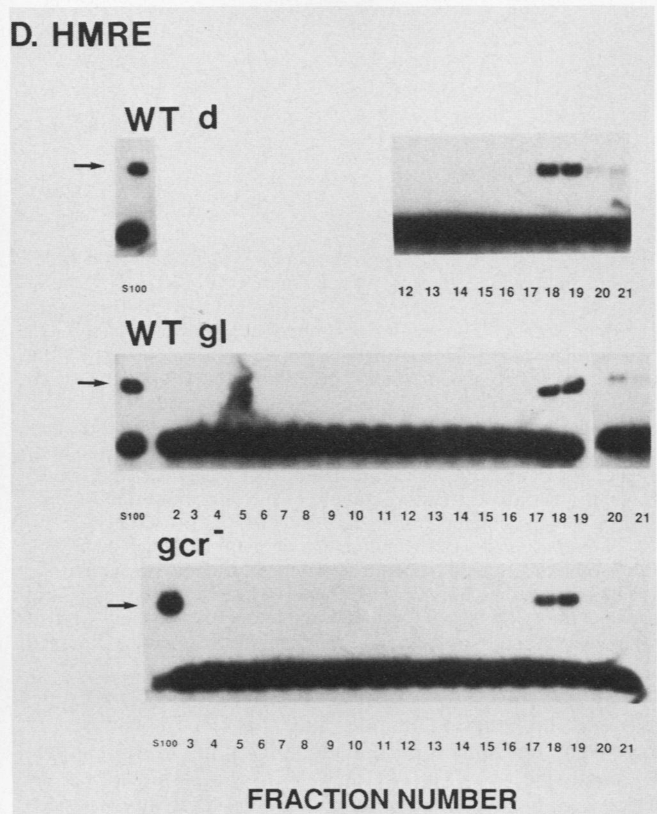
We showed previously that the complex UAS elements from *ENO1* and *ENO2* mediate carbon source-dependent control of transcription of each respective enolase gene (9, 10). We also showed that the product of the *GCR1* regulatory gene is required for maximal transcription of the two enolase genes (20). While it is possible that carbon source- and/or *GCR1*-dependent regulation of transcription involves modification of a *trans*-acting factor which binds to the UAS elements of the enolase genes, no chromatographic (Mono S) differences were observed for EBF2 or RAP1 protein isolated from a wild-type strain grown in glucose versus glycerol plus lactate or a strain carrying a *gcr1* null mutation (Fig. 4B to D).

Two distinct activities bind the *ENO2* UAS. To confirm that the *ENO2* oligonucleotide probe binds EBF2 and RAP1 protein, a series of competition binding experiments were performed. As illustrated in Fig. 5A, the *ENO2* double-stranded oligonucleotide probe formed the same complex with an S100 extract and with the factor which eluted in


A.
0.02
0.01
0.0
A 280 nm
1M
0.5M
0M
[KCl]
1
5
10
15
20
FRACTION NUMBER


B. ENO 1
WT d
S100 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
WT gl
S100 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
gcr⁻
S100 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
FRACTION NUMBER


C. ENO 2
WT d
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 S100
WT gl
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 S100
gcr⁻
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
FRACTION NUMBER


D. HMRE
WT d
S100
12 13 14 15 16 17 18 19 20 21
WT gl
S100 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
gcr⁻
S100 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
FRACTION NUMBER

fraction 13 (EBF2) following Mono S chromatography. Consistent with the data in Fig. 3, formation of this latter complex was competed with efficiently by unlabeled *ENO2* oligonucleotide but was not competed with by the *ENO1*/UAS_1 or HMRE oligonucleotide at concentrations up to a 20-fold molar excess. The *ENO2* oligonucleotide probe also formed a discrete complex with the activity which eluted in fraction 19 (RAP1) following Mono S chromatography (Fig. 5A). As expected, formation of this latter complex was competed with efficiently by the *ENO1*/UAS_1 and HMRE oligonucleotides, which bind the RAP1 protein. A second set of competition binding experiments was performed with the HMRE probe. Formation of a discrete complex with the HMRE probe and either S100 extract or the factor eluted in fraction 19 (RAP1) after Mono S chromatography was competed with by the *ENO1*/UAS_1 and *ENO2* oligonucleotides, although the *ENO2* oligonucleotide was a poorer competitor (Fig. 5B). These data confirm that the *ENO2* oligonucleotide bound specifically to EBF2 and RAP1 proteins and that EBF2 bound more tightly to this probe than did RAP1 protein.

To confirm that the activity eluted in fractions 18 and 19 following Mono S chromatography of yeast extracts was in fact the RAP1 protein, the chromatographic and DNA-binding properties of this latter factor were compared with those of RAP1 protein synthesized in *E. coli*. An *E. coli* expression vector carrying the yeast *RAP1* gene under the control of the tryptophan promoter was constructed as described in Materials and Methods. Extracts were prepared from *E. coli* cells with and without the expression vector carrying the yeast *RAP1* structural gene. These extracts were subjected to Mono S chromatography under the conditions described in the preceding section for yeast S100 extracts. RAP1 protein-binding activity was monitored by gel mobility shift assay with the *ENO1*/UAS_1 oligonucleotide probe.

The chromatographic properties of the major *ENO1*/UAS_1 oligonucleotide-binding factor isolated from *E. coli* carrying the *RAP1* gene were similar to those observed for factor isolated from yeast cells (Fig. 6a and b). The RAP1-binding activity synthesized in *E. coli*, however, eluted one fraction earlier than did RAP1 activity from *S. cerevisiae*. Faster-migrating discrete gel mobility shift complexes were observed in fractions 19 and 21 eluted after Mono S chromatography of the *E. coli* extract. These latter complexes probably represent proteolyzed forms of the RAP1 protein, since no binding activity was observed after chromatography of extracts prepared from *E. coli* which lacked the *RAP1* structural gene (Fig. 6c).

The DNA-binding properties of RAP1 protein synthesized in *E. coli* were examined by gel mobility shift assays. As illustrated in Fig. 7, *E. coli* RAP1 protein, purified by Mono S chromatography, formed a complex with the *ENO1*/UAS_1 oligonucleotide with mobility very similar to that of the complex formed with fraction 19 eluted after Mono S chromatography of a yeast S100 extract. The *ENO2* oligonucleotide also formed a discrete complex of the expected mobility, and the apparent affinity of the *E. coli*-derived RAP1 protein for the *ENO2* probe was lower than that of the *ENO1*/UAS_1 probe. *E. coli* RAP1 protein did not form a complex with the HMRB probe (described below), which does not contain a RAP1 protein-binding site, nor did any of the probes form a complex with a fraction eluted after Mono S chromatography of an extract derived from *E. coli* which lacked the RAP1 protein expression vector (Fig. 7). Based on these comparative data, we conclude that the binding activity eluted in fractions 18 and 19 after Mono S chromatography of yeast extracts is the product of the *RAP1* structural gene.

EBF2- and RAP1-binding sites within the UAS region of the *ENO2* gene overlap. To further define the relationship between the EBF2- and RAP1-binding sites within the *ENO2* gene, methylation interference studies were performed with the *ENO2* oligonucleotide. As shown in Fig. 8, methylation of G residues at positions −470, −468, and −461 within the oligonucleotide interfered with formation of the complexes observed with yeast S100 extract and fraction 13 eluted after Mono S chromatography of the S100 extract. These G contact sites were identical to those observed previously with yeast extracts purified after phosphocellulose chromatography (19). In contrast, methylated G residues at positions −461, −457, −455, and −454 interfered with formation of the complex observed with fraction 19 (RAP1) eluted after Mono S chromatography of a yeast extract. These data showed that the binding sites for EBF2 and RAP1 overlap. It is likely, therefore, that binding of these two factors to the *ENO2* gene is mutually exclusive, as suggested by the competition binding data described earlier (Fig. 3).

For comparative purposes, methylation interference studies were performed with the HMRE oligonucleotide. Methylation of two specific G residues interfered with formation of the complexes observed with yeast S100 extract and fraction 19 (RAP1) eluted after Mono S chromatography of the yeast extract (Fig. 9). The methylation interference data for RAP1 protein binding to *ENO1* (Fig. 2), *ENO2* (Fig. 8), and HMRE (Fig. 9) were aligned to maximize common G residue contact sites (Fig. 10). Although only one G residue contact site was shared by all three RAP1 protein-binding sites, the alignment clearly defined similar nucleotide sequences. Comparison of the three binding sites with a reported (6) GRFI/RAP1 consensus binding site (5′-RMAC CCANNCAYY-3′, where R is A or G, M is A or C, and Y is T or C) revealed a 13 of 13 match for *ENO1*/UAS_1, a 12 of 13 match for HMRE, and a 9 of 13 match for *ENO2*. The poor match between the RAP1 protein-binding site in *ENO2* and the consensus sequence may explain why RAP1 bound more weakly to the *ENO2* site in vitro than *ENO1*/UAS_1 and HMRE.

EBF2 factor is identical to the ABFI factor. Having established that EBF2 and RAP1 are distinct DNA-binding fac-

FIG. 4. Mono S chromatography of *ENO1* and *ENO2* UAS DNA-binding activities. Mono S chromatography was performed with S100 extracts isolated from *S. cerevisiae* S173-6B grown in medium containing glucose (WT d) or glycerol plus lactate (WT gl) as the carbon source and *S. cerevisiae* S173-G, which carries a *gcr1* null mutation (gcr^-). DNA-binding activities were eluted from Mono S columns with a linear KCl gradient (50 to 300 mM), followed by step elution with 1 M KCl (fraction 21). DNA-binding activities were monitored by gel mobility shift assay with *ENO1*/UAS_1, *ENO2*, and HMRE double-stranded oligonucleotide probes as described in Materials and Methods. (A) A typical Mono S chromatogram for the glucose-grown yeast S100 extract. (B, C, and D) Mono S chromatograms of the and S100 extracts assayed with the *ENO1*/UAS_1, *ENO2*, and HMRE oligonucleotide probes, respectively. Control gel mobility shift assays were performed with each S100 extract and each oligonucleotide probe (S100). The arrows indicate the locations of major DNA-protein complexes formed with each of the oligonucleotide probes. Gel mobility shift assays for fractions 2 to 11 (panel D, glucose-grown cells [WT d]) were electrophoresed on polyacrylamide gels and contained no detectable HMRE-binding activity (data not shown).

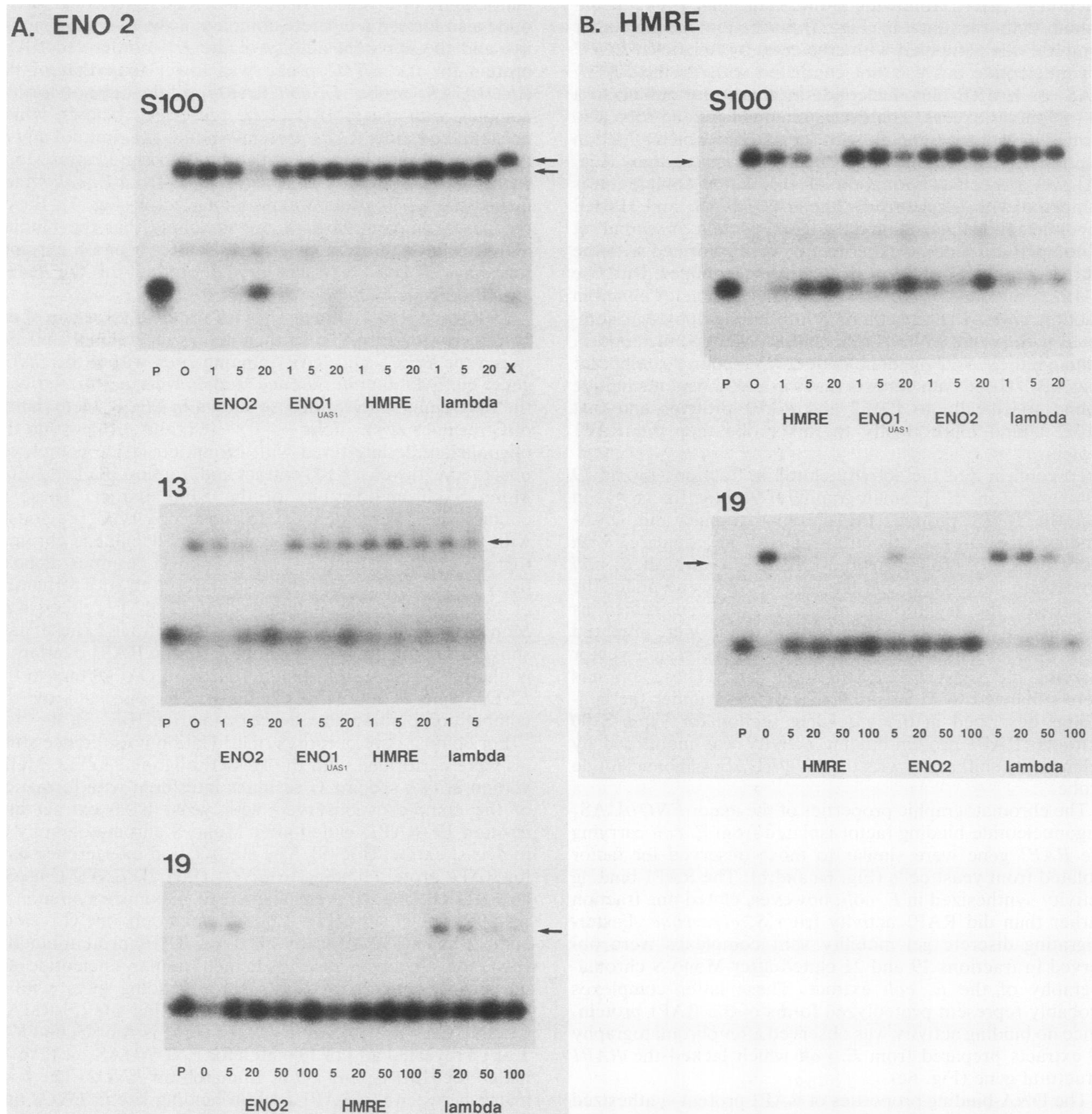


FIG. 5. Competition DNA-protein binding analysis with double-stranded oligonucleotides corresponding to the *ENO2* UAS and the RAP1-binding site within the E element of the yeast silent mating type locus *HMR*. Gel mobility shift assays were performed with a ^{32}P-labeled *ENO2* oligonucleotide (A) and a ^{32}P-labeled HMRE oligonucleotide (B) in the presence of increasing amounts of competitor DNA. Unlabeled *ENO2*, HMRE, and *ENO1*/UAS_1 oligonucleotides and lambda DNA were used as competitors. DNA-protein binding reactions were performed as described in Materials and Methods in the presence or absence (P) of an S100 extract isolated from *S. cerevisiae* S173-6B grown in a medium containing glucose or with fractions 13 and 19 obtained after Mono S chromatography of the S100 extract. Binding reaction mixes contained either no competitor DNA (O) or a 1- to 20-fold molar excess of the indicated competitor DNA (a 5- to 100-fold molar excess of competitor DNA was used for assays with fraction 19). The arrows indicate the locations of major DNA-protein complexes formed with each oligonucleotide probe. A gel mobility shift assay for the *ENO2* probe incubated with fraction 19 in the absence of competitor DNA was included on the same gel with the S100-*ENO2* competitions (X). Note that the DNA-binding activity present in fraction 19 makes a slower-migrating complex with the *ENO2* probe than observed with the S100 extract.

tors, we wished to know whether the EBF2-binding factor was related to any previously reported DNA-binding factor. The core EBF2-binding site in *ENO2* (5'-GTCACTAAC GACGTG-3') that included all the G contact sites determined from methylation interference analysis (19) was compared with consensus sequences for other known DNA-binding factors. A promising match was made with the consensus binding site for a factor, designated GFI, which

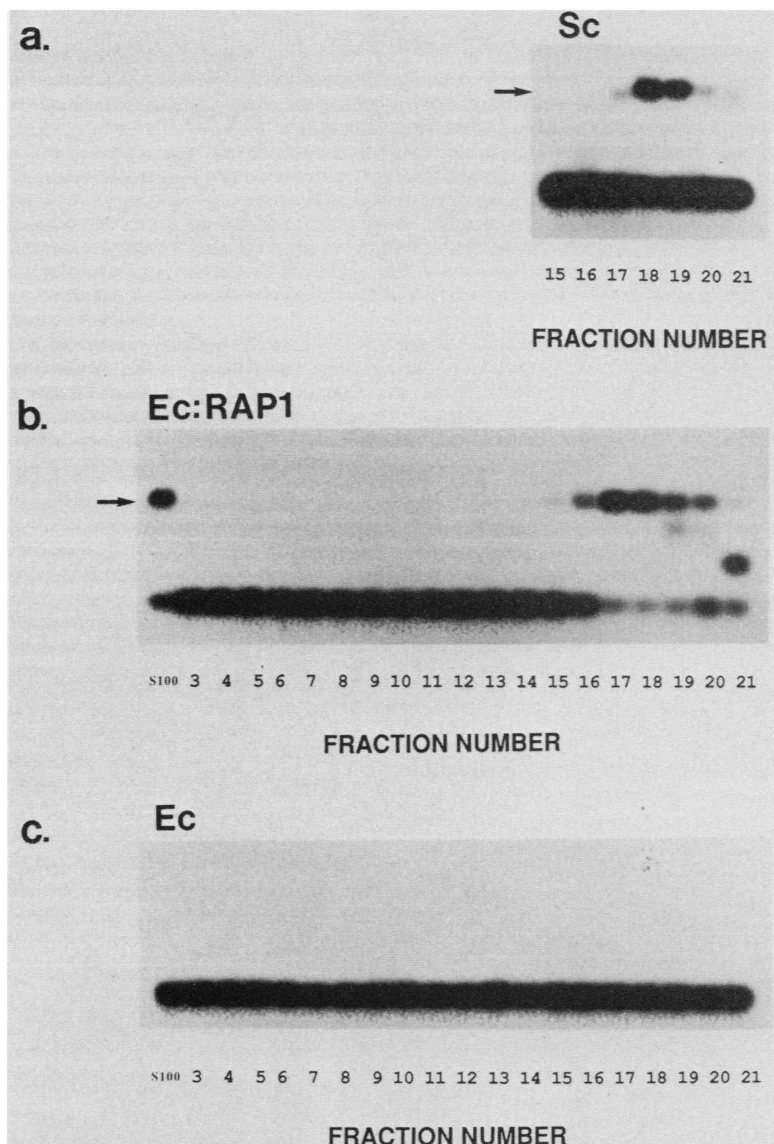


FIG. 6. Mono S chromatography of RAP1 protein expressed in *E. coli*. Preparation of S100 extracts and Mono S FPLC chromatography were as described in Materials and Methods. RAP1 protein was eluted from Mono S columns with a linear KCl gradient (50 to 300 mM), followed by step elution with 1 M KCl (fraction 21). RAP1-binding activity was monitored by gel mobility shift assay with an *ENO1*/UAS$_1$ double-stranded oligonucleotide probe. (a) Mono S chromatogram of S100 extract isolated from *S. cerevisiae* S173-6B grown in a medium containing glucose as the carbon source (Sc). (b) Mono S chromatogram of S100 extract isolated from *E. coli* cells carrying a yeast *RAP1* gene expression vector (Ec:RAP1). (c) Mono S chromatogram of S100 extract isolated from *E. coli* cells without the *RAP1* expression vector (Ec). A gel mobility shift assay was performed with the initial S100 extracts (S100) and served as a control. The arrows indicate the locations of the major specific complexes formed with the *ENO1*/UAS$_1$ probe.

has been implicated in regulation of expression of yeast genes involved in mitochondrial biogenesis (13). The reported GFI consensus binding site is 5'-RTCRNNNNN NACGNR-3', where R is A or G and N is any nucleotide. This consensus sequence, while quite degenerate, matches exactly the *ENO2* EBF2-binding site. Additionally, all but one of the five G contact sites deduced from methylation interference studies on *ENO2* corresponded to conserved or semiconserved residues in the GFI consensus binding site. Dorsman et al. (13) reported that GFI was in fact identical or closely related to another factor, designated ABFI (autonomously replicating sequence [ARS]-binding factor). Binding sites for the ABFI factor have been identified near ARS elements as well as a diverse array of genetic elements, including the silencer of the silent mating locus *HMR*, where ABFI binds within 30 base pairs of a RAP1-binding site (4, 5).

To test the hypothesis that EBF2 was ABFI, a double-stranded oligonucleotide containing the ABFI-binding site found within element B of the *HMR* silencer (HMRB) (4, 5) was synthesized. This oligonucleotide was used in gel mobility shift assays to identify ABFI activity. Figure 11a shows that the chromatographic profiles of ABFI and EBF2 activities were identical after Mono S fractionation of a yeast S100 extract. Competition DNA-binding assays demonstrated that formation of a discrete complex between the HMRB probe and fraction 13 eluted after Mono S chromatography of a yeast extract was effectively competed with by

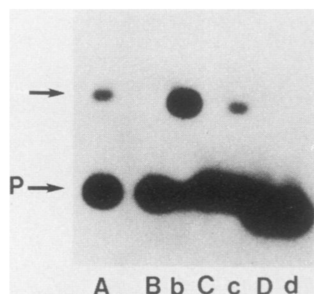


FIG. 7. DNA-binding properties of RAP1 protein isolated from *E. coli* cells carrying a yeast *RAP1* gene expression vector. Gel mobility shift assays and extract preparations were done as described in Materials and Methods. Lane A, RAP1 protein, partially purified by Mono S chromatography (fraction 18) of an S100 extract isolated from *S. cerevisiae* S173-6B grown in a medium containing glucose as the carbon source, incubated with the *ENO1*/UAS_1 oligonucleotide probe. Lanes B, C, and D, Fraction 18 obtained after Mono S chromatography of an S100 extract isolated from *E. coli* cells without the *RAP1* expression vector incubated with *ENO1*/UAS_1, *ENO2*, and HMRB oligonucleotide probes, respectively. Lanes b, c, and d, Partially purified RAP1 (fraction 18) obtained after Mono S chromatography of an S100 extract isolated from *E. coli* cells carrying a yeast *RAP1* gene expression vector incubated with *ENO1*/UAS_1, *ENO2*, and HMRB oligonucleotide probes, respectively. The locations of the major DNA-protein complexes (arrow) and the unbound oligonucleotide probes (arrow P) are indicated.

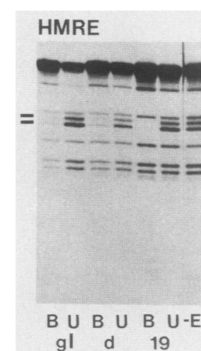


FIG. 9. Mapping the RAP1-binding site in the HMRE oligonucleotide. Methylation interference analysis, gel mobility shift assays, and chemical cleavage of methylated DNA were performed as described in Materials and Methods. HMRE oligonucleotide (^{32}P labeled) was partially methylated and incubated with and without (−E) S100 extracts isolated from *S. cerevisiae* S173-6B grown in medium containing either glucose (d) or glycerol plus lactate (gl) as the carbon source and partially purified RAP1 (fraction 19 eluted after Mono S chromatography of a yeast S100 extract). A polyacrylamide gel of chemical cleavage products is shown for bound (B) and unbound (U) DNA isolated after gel mobility shift assays with each of the binding reaction mixes. Methylation of two G residues (bars) interfered with DNA-protein complex formation with all of the extracts tested.

unlabeled *ENO2* oligonucleotide (Fig. 11b). Conversely, formation of a discrete complex between the *ENO2* probe and fraction 13 from the Mono S column was competed with by unlabeled HMRB oligonucleotide (Fig. 11b). Methylation interference analysis was performed for both strands of the HMRB oligonucleotide with the factor that was partially purified by Mono S chromatography (data not shown). When the ABFI G contact sites in HMRB were aligned with the G contact sites for EBF2 binding of the *ENO2* oligonucleotide (Fig. 10), a very similar sequence was defined. All three of the G contacts found for ABFI binding to HMRB corresponded to G contacts for EBF2 binding to *ENO2*. Taken together, these data strongly suggest that EBF2 and ABFI are identical.

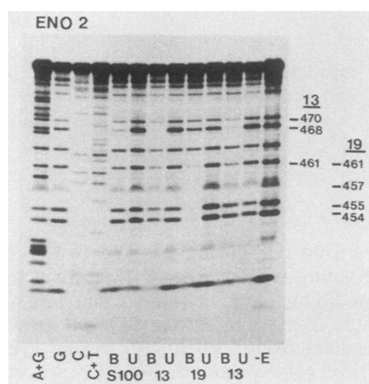


FIG. 8. Mapping of EBF2 and RAP1 protein-binding sites within the *ENO2* oligonucleotide. Methylation interference analysis, gel mobility shift assays, and chemical cleavage of methylated DNA were performed as described in Materials and Methods. *ENO2* oligonucleotide (^{32}P labeled) was partially methylated and incubated with and without (−E) a yeast S100 extract, partially purified EBF2 (fraction 13 eluted after Mono S chromatography of a yeast S100 extract), and partially purified RAP1 (fraction 19 eluted after Mono S chromatography of a yeast S100 extract). A polyacrylamide gel of chemical cleavage products is shown for bound (B) and unbound (U) DNA isolated after gel mobility shift assays with each of the binding reactions. DNA sequencing reaction mixes for the *ENO2* oligonucleotide were electrophoresed in parallel lanes on the polyacrylamide gel. Methylation of G residues at positions −461, −468, and −470 interfered with DNA-protein complex formation with the S100 extract and partially purified EBF2 (13). Methylation of G residues at positions −454, −455, −457, and −461 interfered with DNA-protein complex formation with partially purified RAP1 (19).

EBF1 binds to the UAS_2 element in the *ENO1* gene. As discussed earlier, complex transcriptional regulatory regions

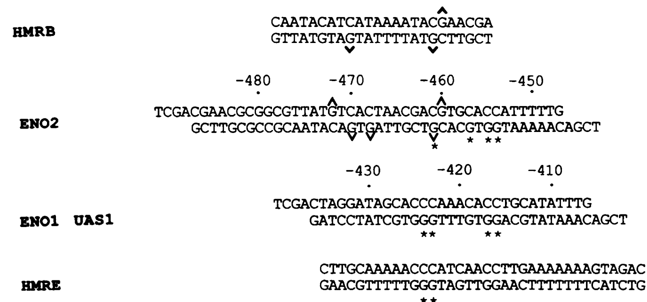


FIG. 10. Summary of the ABFI- and RAP1-binding sites in *ENO1* and *ENO2*. G residues implicated by methylation interference studies as being important for ABFI binding to the *ENO2* and HMRB oligonucleotides are indicated by carets. G residues implicated by methylation interference analysis as being important for RAP1 binding to the *ENO2*, *ENO1*/UAS_1, and HMRE oligonucleotides are indicated by asterisks. The numbers above the *ENO2* and *ENO1*/UAS_1 oligonucleotide sequences indicate the position of the nucleotides relative to the transcriptional initiation sites in *ENO2* and *ENO1*, respectively. All of the sequences have been aligned to maximize common G residue contact sites and matches to the consensus binding site sequences for ABFI (RtCrYyNNNNACg; see Table 1) and RAP1 (RMACCCANNCAYY [6]).

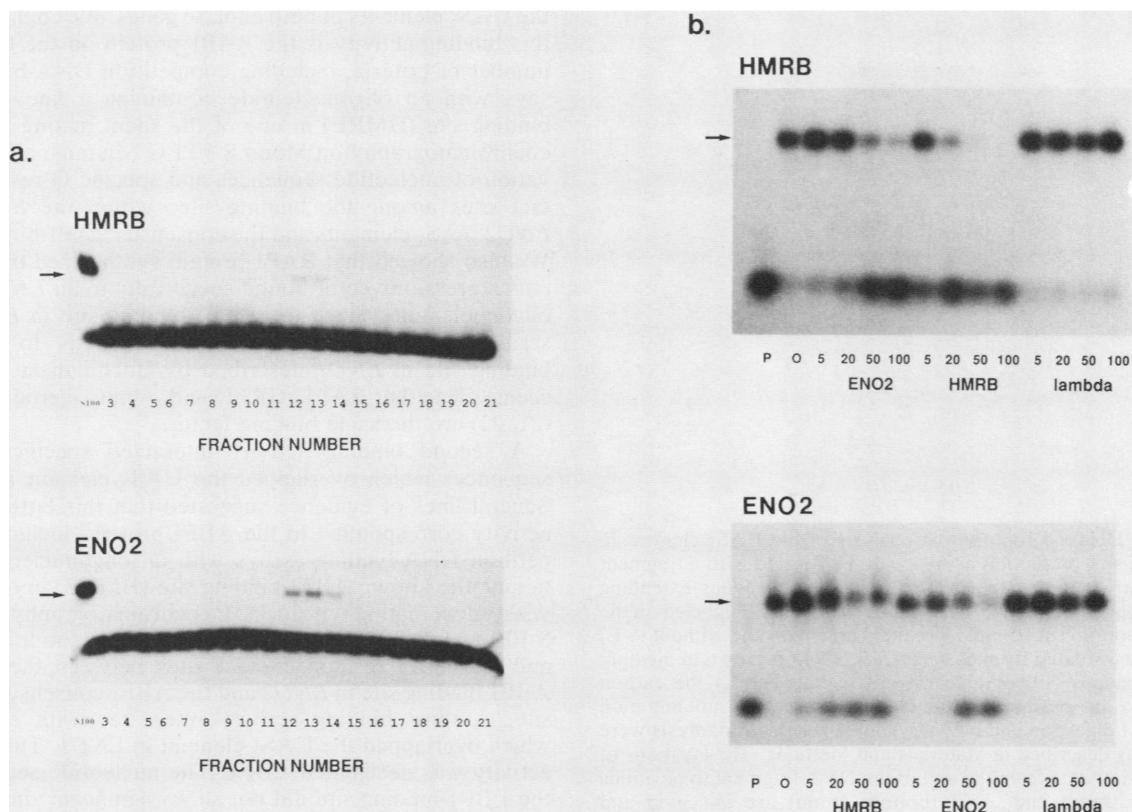


FIG. 11. Comparison of the ABFI and EBF2 DNA-binding proteins. (a) Mono S chromatograms of an S100 extract isolated from *S. cerevisiae* S173-6B grown in a medium containing glucose as the carbon source. Mono S chromatography was performed as described in Materials and Methods. ABFI- and EBF2-binding activities were monitored by gel mobility shift assay with the HMRB (top panel) and *ENO2* (bottom panel) oligonucleotide probes, respectively. Control gel mobility shift assays were performed with the S100 extract and each of the probes (S100). The arrows indicate the positions of the major DNA-protein complexes formed with each oligonucleotide probe. (b) Competition DNA-protein binding analysis with the HMRB and *ENO2* oligonucleotide probes. Gel mobility shift assays were performed with ^{32}P-labeled HMRB oligonucleotide (top panel) and ^{32}P-labeled *ENO2* oligonucleotide (bottom panel) in the presence of increasing amounts of competitor DNA. Unlabeled HMRB and *ENO2* oligonucleotides and lambda DNA were used as competitors. DNA-protein binding reactions were performed in the presence or absence (P) of an S100 extract isolated from *S. cerevisiae* S173-6B grown in a medium containing glucose as the carbon source. Binding reaction mixes contained either no competitor DNA (O) or a 5- to 100-fold molar excess of the indicated competitor DNA. The arrows indicate the locations of the major DNA-protein complexes formed with each oligonucleotide probe.

consisting of at least two adjacent UAS elements are located within the 5'-flanking regions of the *ENO1* and *ENO2* genes (Fig. 1) (9, 10). The regulatory regions from both genes are capable of mediating glucose-dependent induction of gene expression (9, 10), and maximal transcription of both genes requires the *GCR1* gene product (20). Given these functional similarities and the discovery that there is a RAP1 protein-binding site within the UAS_1 elements of both genes, the UAS_2 element in *ENO1* (Fig. 1) was investigated to determine whether, by analogy to *ENO2*, it contained an ABFI-binding site.

To test this possibility, DNase I footprinting analysis was performed with a yeast S100 extract and an *ENO1* probe extending from positions −415 to −767. As illustrated in Fig. 12, two distinct footprints were observed with this probe. The first extended from the end of the probe (−415) to position −433 and undoubtedly corresponded to the binding site for RAP1 protein. The second footprint extended from positions −448 to −474. This latter footprint overlapped UAS_2 (10).

The footprinting analysis demonstrated that RAP1 binding to the UAS_1 element in *ENO1* occurred commensurate with binding of a factor to the UAS_2 element. This latter factor was designated EBF1 (enolase-binding factor). To determine whether EBF1 was identical to ABFI, a double-stranded oligonucleotide, designated *ENO1*/UAS_2, was synthesized which corresponded to the EBF1-binding site defined by the footprinting analysis. This oligonucleotide was used in a gel mobility shift assay to monitor EBF1 activity. EBF1 activity was localized in the 1.0 M KCl was fraction following Mono S chromatography of a yeast S100 extract (Fig. 13). No EBF1 activity was detected in fractions which contained either ABFI or RAP1 protein-binding activity. These data, together with the observation that the nucleotide sequence of the EBF1-binding site appears to be unrelated to the ABFI and RAP1 consensus binding sites, suggested that EBF1 was not ABFI or RAP1. The binding sites for the RAP1, ABFI, and EBF1 factors within the UAS elements of the *ENO1* and *ENO2* genes are summarized in Fig. 1.

DISCUSSION

Three distinct DNA-binding activities interacted specifically with the upstream regulatory regions of *ENO1* and *ENO2*. One of these binding activities corresponded to the RAP1 protein (35) and bound to sequences which overlapped

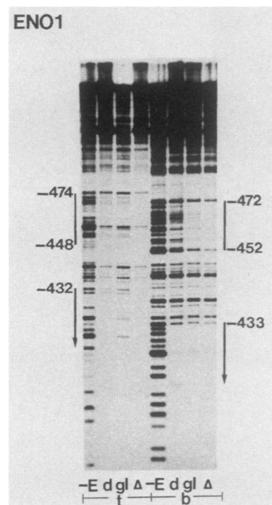


FIG. 12. DNase I footprinting analysis of the UAS_2 element in *ENO1*. DNase I protection assays were performed with a fragment of DNA corresponding to *ENO1* 5'-flanking sequences extending from positions −415 to −767. The DNA fragment, ^{32}P labeled on the top (t) and bottom (b) strands, was incubated with and without (−E) S100 extracts isolated from *S. cerevisiae* S173-6B grown in medium containing glucose (d) or glycerol plus lactate (gl) as the carbon source and *S. cerevisiae* S173-G, which carries a *gcr1* null mutation (Δ). DNase I digestion and polyacrylamide gel electrophoresis were performed as described in Materials and Methods. The locations of footprints extending from the end of the DNA fragment to positions −432 (top strand) and −433 (bottom strain) are indicated and correspond to RAP1 binding to the UAS_1 element of *ENO1*. The locations of footprints extending from positions −448 to −474 (top strand) and positions −452 to −472 (bottom strand) are also indicated and correspond to EBF1 binding to the UAS_2 element of *ENO1*.

the UAS_1 elements of both enolase genes. We confirmed that this binding activity is the RAP1 protein on the basis of a number of criteria, including competition DNA-binding assays with an oligonucleotide containing a known RAP1-binding site (HMRE) in one of the silent mating type loci, cochromatography on Mono S FPLC columns, and conservation of nucleotide sequences and specific G residue contact sites among the binding sites within the *ENO1* and *ENO2* UAS_1 elements and the consensus RAP1-binding site. We also showed that RAP1 protein synthesized from an *E. coli* expression vector bound specifically to an *ENO1* UAS_1 oligonucleotide. Since the RAP1-binding site in *ENO1* described in this report corresponded exactly to a GRFI-binding site in *ENO1* described by Buchman et al. (6), it seems clear that RAP1, GRFI, and, almost certainly, TUF (21, 22) are the same binding factor.

A second binding activity interacted specifically with sequences which overlapped the UAS_2 element in *ENO2*. Several lines of evidence suggested that this latter binding activity corresponded to the ABFI protein, including competition DNA-binding assays with an oligonucleotide containing the known ABFI-binding site (HMRB) in one of the yeast silent mating type loci (5), cochromatography on Mono S FPLC columns, and conservation of nucleotide sequence and critical G residue contact sites between the putative ABFI-binding site in *ENO2* and the ABFI consensus binding site. A third binding activity interacted with sequences which overlapped the UAS_2 element in *ENO1*. This binding activity was designated EBF1. The nucleotide sequence of the EBF1-binding site did not show significant similarity to binding sites for other known yeast DNA-binding proteins.

The binding sites for the three factors overlapped sequences within the 5'-flanking regions of the enolase genes that were previously shown to be required for UAS activity

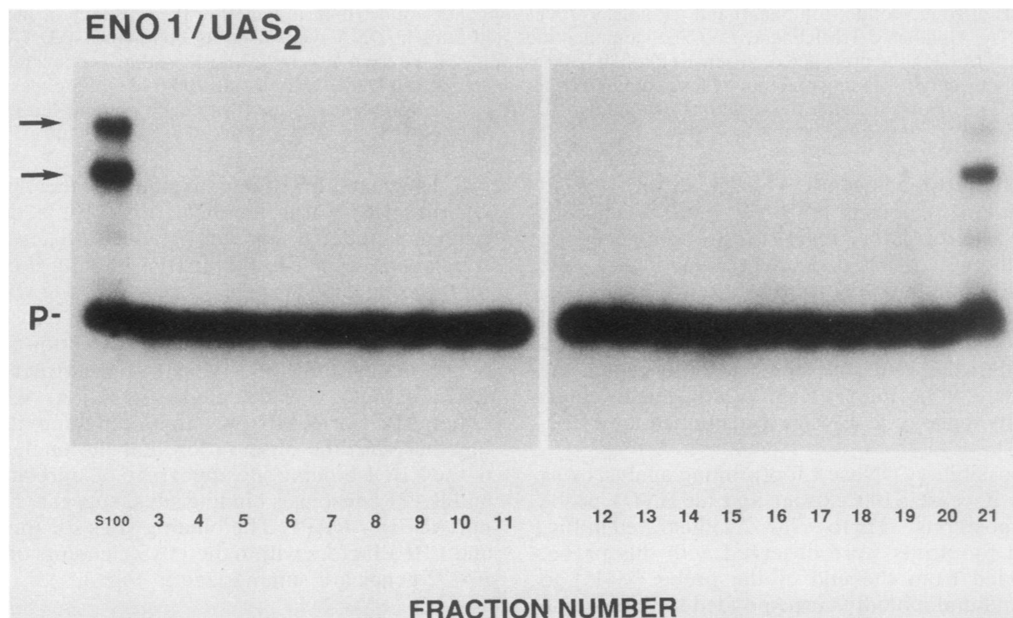


FIG. 13. Mono S chromatography of EBF1. Mono S FPLC chromatography was performed with an S100 extract isolated from *S. cerevisiae* S173-6B grown in a medium containing glucose as the carbon source. EBF1 protein was eluted from the Mono S column with a linear KCl gradient (50 to 300 mM), followed by step elution with 1 M KCl. EBF1-binding activity was monitored by gel mobility shift assay with an *ENO1*/UAS_2 oligonucleotide probe which corresponded to *ENO1* sequences which were protected from DNase I digestion after EBF1 binding. A gel mobility shift assay was performed with the initial S100 extract (S100) and served as a control. The locations of the unbound probe (P) and two major DNA-protein complexes formed with the *ENO1*/UAS_2 oligonucleotide (arrows) are indicated.

(9, 10). In the case of the RAP1 protein, there is clear evidence that a synthetic oligonucleotide corresponding to the RAP1/GRFI-binding site within the *ENO1* UAS_1 element functions as a UAS when cloned into UAS-less test genes (6). The relative UAS activities of oligonucleotides corresponding to RAP1/GRFI-binding sites in different genes are, however, highly variable (6). RAP1-binding sites in glycolytic genes are often found adjacent to one or more sequence elements with the general structure GCTTCCA (6). The presence of this latter element has been correlated with efficient transcriptional activation of some glycolytic genes (6, 10, 32). Specific mutations within GCTTCCA elements or deletion of these elements caused 5- to 17-fold reductions in the UAS activity of oligonucleotides that also contained a RAP1-binding site (6). These results are consistent with our earlier studies showing that *ENO1* deletion mutations that removed the UAS_2 element and the terminal G of the GCTTCCA sequence (centered at position −440) located upstream from the RAP1-binding site caused a pronounced loss of *ENO1* gene expression not observed for deletions which only removed the UAS_2 element (10). RAP1 protein binding appears, therefore, to be insufficient to account for the full activity of these UAS elements. In the case of the *ENO2* gene, the RAP1 protein-binding site determined from methylation interference analysis and the UAS_1 element identified by deletion mapping analysis are located immediately downstream from position −461. The 5′ boundary of the *ENO2* UAS_1 element is located between positions −461 and −433 (9). These data suggest an important role for the RAP1 protein in transcriptional activation of the enolase genes; however, the mechanism of RAP1-dependent transcriptional regulation is unclear.

ABFI bound to sequences that overlap essential sequences within the UAS_2 element of *ENO2*, suggesting that ABFI also plays a role in transcriptional activation. The 3′ boundary of the *ENO2* UAS_2 element is located between positions −479 and −461 (9). Like RAP1, ABFI-binding sites are found associated with diverse genetic elements, including ARS elements, silencers, and the 5′, coding, and 3′ regions of many genes (5, 13). Oligonucleotides containing ABFI-binding sites function less efficiently as UAS elements in vivo than do oligonucleotides containing RAP1- or GAL4-binding sites (4, 5). Nevertheless, the UAS_2 element identified by deletion mapping analysis (9) overlaps the ABFI-binding site in *ENO2*.

We compared the nucleotide sequences of specific protein-binding sites within the 5′-flanking regions of a number of yeast genes with a consensus binding site for ABFI and found some intriguing matches. Stanway et al. (37) described a factor which bound within the upstream regulatory site of the phosphoglycerate kinase (*PGK*) gene at a site designated the modulator domain (M domain). The *PGK* M domain is important for carbon source control of transcription but is not itself sufficient to activate transcription. As illustrated in Table 1, the *PGK* M domain-binding site closely matches the ABFI consensus binding site. Nucleotide sequences located immediately downstream from the M domain are sufficient to activate transcription of the *PGK* gene. Interestingly, this latter activation domain of the *PGK* gene includes a RAP1 consensus binding site and the 5′-GCTTCCA-3′ sequence motif which appears to modulate the efficiency of RAP1-dependent UAS elements (6). The UAS element from the ribosomal protein gene *TCM1* binds a factor designated TAF (16). TAF binds to a sequence with a 13 of 13 match to the ABFI consensus binding site (Table 1). Competition DNA-binding analysis showed that TAF and RAP1 are different

TABLE 1. Comparison of nucleotide sequences of known and putative ABFI-binding sites[a]

Binding site[b]	Sequence	Reference
HMRE B	ATCATAAAATACG	36
ARS1	AGCATTTTTGACG	36
*HMR*I	ATCGCCATATACG	5
*HML*I	ATCATTGCAAACG	5
2μm	ATCTTTGTTAACG	5
DED1′	ATCATTCTATACG	5
DED1″	GTCATTCTGAACG	5
CIII subunit II	ATCATTCCCAACG	13
CIII-Subunit VIII	GTCACGTGGAACG	13
CYC1	GTCGTCTCACACG	13
PHO5	ATCGTTAATGACG	13
ARS 120	ATCATTATGCACG	14
ARS 121 I	ATCACTAAATACG	43
ARS 121 II	GTCATTATATACC	43
ENO2	GTCACTAACGACG	19
Consensus	RtcrYyNNNNACg	
TCM1	ATCGTTTTGTACG	16
PGK	ATCACGAGCGACG	37

[a] The top section of the table shows an alignment of 15 known or probable ABFI-binding sites. Each of the 15 sequences shown lies within a protein-binding site defined by DNase I footprinting analysis, and DNA-protein complex formation with each of these sequences was shown to be competed with by oligonucleotides or DNA fragments containing known ABFI-binding sites (5, 13, 14, 36, 43). The most conserved nucleotides among the binding sites are shown in boldface. The consensus ABFI-binding site, RtCrYyNNN NACg (where R is A or G, Y is C or T, and N is any nucleotide; uppercase letters indicate that the position is invariant in the 15 sequences used to compile the consensus sequence, and lowercase letters indicate that there is some sequence variability at that position), derived from this sequence comparison is indicated. The lower section of the table shows sequences from within DNase I footprints formed with a UAS from the *TCM1* gene (16) and the M domain of the *PGK1* gene (37). These latter sequences correspond closely to the ABFI consensus binding site.

[b] CIII, Yeast cytochrome *c* oxidoreductase.

factors (16). Although the relationship between TAF and ABFI has not been tested directly, the similarities between the recognition sites, apparent molecular weights, and chromatographic properties of TAF (16) and ABFI (11, 39) suggest that these two factors may be identical. Halfter et al. (15) recently reported that a factor designated BAF1 that binds to sites between the divergently transcribed *YPT1* and *TUB2* genes is identical to ABFI. Oligonucleotides corresponding to the BAF1/ABFI-binding sites activated transcription of a test gene in an orientation-independent manner (15). It will be of interest to see whether association of ABFI-binding sites with UAS elements is of general significance as more yeast enhancer elements are characterized.

Transcription of the *ENO2* gene is induced approximately 20-fold in cells grown on a medium containing glucose (9, 30). Deletion of either UAS_1 or UAS_2 does not interfere with glucose-dependent induction of *ENO2* expression (9). Deletion mapping experiments showed that the UAS_1 element is required for glucose-dependent induction of *ENO1* expression when the URS element is inactivated (10). Furthermore, a 45-base-pair double-stranded oligonucleotide containing the *ENO2* ABFI- and RAP1-binding sites is sufficient to confer glucose-inducible activation of transcription when cloned into UAS-less *ENO1* and *ENO2* gene cassettes (19; unpublished results). These data suggest a role for the RAP1 protein in glucose-dependent induction of transcription of the yeast enolase genes and possibly other genes containing RAP1-dependent UAS elements, as suggested by Herruer et

al. (17) for yeast ribosomal protein genes. Interestingly, the data suggest a similar role for the ABFI protein.

The EBF1-binding site overlapped essential sequences within the UAS_2 element of the *ENO1* gene identified by deletion mapping analysis (10). The 3' boundary of the *ENO1* UAS_2 element is located between positions −489 and −442 (10). We observed activation of *ENO1* expression by the UAS_2 element only in the absence of a functional URS element; however, transcription was not glucose inducible. The two *ENO1* UAS elements therefore appear to be functionally distinct, in contrast to the case described above for the *ENO2* gene.

In the preceding report (19), we showed that a deletion mutation extending from positions −479 to −461 within the *ENO2* UAS_2 element permitted wild-type levels of *ENO2* transcription in the absence of a functional *GCR1* gene. This latter deletion mutation was entirely within the binding site for a protein initially designated EBF2 and shown in this report to be ABFI. DNA probes containing the −479/−461 deletion mutation failed to bind ABFI but retained their ability to bind RAP1 (data not shown). ABFI could act as a bifunctional repressor/activator protein which is dependent on the activity of the *GCR1* gene product. Such a bifunctional activity has been proposed for RAP1 (repressor/activator protein), which binds to the HMRE site adjacent to the silent mating type locus *HMR*. Alternatively, the ABFI-binding site may overlap the binding site for another protein which acts as a repressor or activator. Dorsman et al. (13) showed that factors that are identical or closely related to ABFI and CP1 (centromere-binding protein) (3) bind within the 5'-flanking region of the yeast cytochrome *c* oxidoreductase subunit VIII gene in a mutually exclusive fashion; however, the binding site does not correspond to the UAS element for this gene. The 45-base-pair double-stranded oligonucleotide (*ENO2*) which includes the ABFI- and RAP1-binding sites from *ENO2* activated transcription when tested in UAS-less *ENO1* and *ENO2* cassettes; however, transcription was not repressed in a *gcr1* genetic background (19). This observation suggests that *GCR1*-dependent regulation of *ENO2* expression is not modulated directly through ABFI or RAP1, since these factors are capable of binding the 45-base-pair oligonucleotide. Furthermore, the RAP1-binding site within the UAS_1 element of the *ENO1* gene does not overlap an ABFI-binding site (unpublished observation), although both enolase genes are regulated by *GCR1*.

Buchman et al. (5) and Kayne et al. (25) have postulated that RAP1 and ABFI may be involved in regulation of chromatin structure, partly because of the ubiquitous nature of their respective binding sites in the yeast genome and partly because deletion mutations within the histone H4 N-terminus affect the activity of the silent mating type loci, where RAP1- and ABFI-binding sites overlap *cis*-acting sequences which are important for silencer function. Hofmann et al. (18) have recently shown that RAP1 is associated with the nuclear scaffold and is required for the formation of DNA loops in vitro within the *HMLα* locus. Pavlović and Hörz (33) reported experiments with the glyceraldehyde-3-phosphate dehydrogenase gene *TDH3* which suggest that the *GCR1* gene product regulates gene expression by modulating chromatin structure within the 5'-flanking regions of *TDH3*. If the *GCR1* gene functions to derepress transcription by controlling chromatin structure within the 5'-flanking region of yeast genes and the RAP1 and ABFI proteins also activate or repress transcription in part by modulating chromatin structure, it is quite possible that specific rearrangements of the UAS elements of *GCR1*-regulated genes, such as the *ENO2* UAS_2 deletion mutations (19), could suppress the requirement for the *GCR1* gene product.

The results described in this article strongly suggest that the ABFI and RAP1 proteins perform similar biological functions. Diffley and Stillman (12) recently reported the nucleotide sequence of the ABFI structural gene. ABFI and RAP1 have extensive amino acid similarity (12), supporting the notion that these two regulatory proteins may perform similar functions in the cell. A puzzling yet common feature of ABFI- and RAP1-binding sites is that they often reside within regions of DNA which do not appear to regulate expression of specific genes. For the RAP1 protein, this paradox is a least partially explained by the fact that the ability of sequences which bind RAP1 protein to activate transcription of specific genes is modulated by the presence or absence of adjacent nucleotide sequences which do not appear to be involved in RAP1 binding (6). Given the observed functional and structural similarity of the RAP1 and ABFI proteins, it is possible that sequences adjacent to ABFI-binding sites play a similar role, although such putative modulator sequences have not yet been identified.

ACKNOWLEDGMENTS

We thank Andrew Carmen and Larry Kirk for supplying protein extracts and Corey Levenson, Dragan Spasic, and Lauri Goda, Cetus Corporation, for providing oligonucleotides.

P.K.B. was a predoctoral trainee on Public Health Service training grant GM07377 from the National Institutes of Health. C.E.W. was a predoctoral trainee on a training grant from the McKnight Foundation. This research was supported by Public Health Service grant GM30307 from the National Institutes of Health and a grant from the March of Dimes Birth Defects Foundation.

LITERATURE CITED

1. **Baker, H. V.** 1986. Glycolytic gene expression in *Saccharomyces cerevisiae*: nucleotide sequence of *GCR1*, null mutants, and evidence for expression. Mol. Cell. Biol. **6**:3774–3784.
2. **Boucherie, H.** 1985. Protein synthesis during transition and stationary phase under glucose limitation in *Saccharomyces cerevisiae*. J. Bacteriol. **161**:385–392.
3. **Bram, R. J., and R. D. Kornberg.** 1987. Isolation of a *Saccharomyces cerevisiae* centromere DNA-binding protein, its human homolog, and its possible role as a transcription factor. Mol. Cell. Biol. **7**:403–409.
4. **Brand, A. H., G. Micklem, and K. Nasmyth.** 1987. A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell **51**:709–719.
5. **Buchman, A. R., W. J. Kimmerly, J. Rine, and R. D. Kornberg.** 1988. Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:210–225.
6. **Buchman, A. R., N. F. Lue, and R. D. Kornberg.** 1988. Connections between transcriptional activators, silencers, and telomeres as revealed by functional analysis of a yeast DNA-binding protein. Mol Cell. Biol. **8**:5086–5099.
7. **Clifton, D., and D. G. Fraenkel.** 1981. The *gcr* (glycolysis regulation) mutation of *Saccharomyces cerevisiae*. J. Biol. Chem. **256**:13074–13078.
8. **Clifton, D., S. B. Weinstock, and D. G. Fraenkel.** 1978. Glycolysis mutants in *Saccharomyces cerevisiae*. Genetics **88**:1–11.
9. **Cohen, R., J. P. Holland, T. Yokoi, and M. J. Holland.** 1986. Identification of a regulatory region that mediates glucose-dependent induction of the *Saccharomyces cerevisiae* enolase gene *ENO2*. Mol. Cell. Biol. **6**:2287–2297.
10. **Cohen, R., T. Yokoi, J. P. Holland, A. E. Pepper, and M. J. Holland.** 1987. Transcription of the constitutively expressed yeast enolase gene *ENO1* is mediated by positive and negative *cis*-acting regulatory sequences. Mol. Cell. Biol. **7**:2753–2761.

11. **Diffley, J. F. X., and B. Stillman.** 1988. Purification of a yeast protein that binds to origins of DNA replication and a transcriptional silencer. Proc. Natl. Acad. Sci. USA **85:**2120–2124.
12. **Diffley, J. F. X., and B. Stillman.** 1989. Similarity between the transcriptional silencer binding proteins ABFI and RAP1. Science **246:**1034–1038.
13. **Dorsman, J. C., W. C. van Heeswijk, and L. A. Grivell.** 1988. Identification of two factors which bind to the upstream sequences of a number of nuclear genes coding for mitochondrial proteins and to genetic elements important for cell division in yeast. Nucleic Acids Res. **16:**7287–7301.
14. **Eisenberg, S., C. Civalier, and B. K. Tye.** 1988. Specific interactions between a *Saccharomyces cerevisiae* protein and a DNA element associated with certain autonomously replicating sequences. Proc. Natl. Acad. Sci. USA **85:**743–746.
15. **Halfter, H., U. Muller, E.-L. Winnacker, and D. Gallwitz.** 1989. Isolation and DNA-binding characteristics of a protein involved in transcription activation of two divergently transcribed, essential yeast genes. EMBO J. **8:**3029–3037.
16. **Hamil, K. G., H. G. Nam, and H. M. Fried.** 1988. Constitutive transcription of yeast ribosomal protein gene *TCM1* is promoted by uncommon *cis-* and *trans-*acting elements. Mol. Cell. Biol. **8:**4328–4341.
17. **Herruer, M. H., W. H. Mager, L. P. Woudt, R. T. M. Nieuwint, G. M. Wassenaar, P. Groeneveld, and R. J. Planta.** 1987. Transcriptional control of yeast ribosomal protein synthesis during carbon-source upshift. Nucleic Acids Res. **15:**10133–10144.
18. **Hofmann, J. F.-X., T. Laroche, A. H. Brand, and S. M. Gasser.** 1989. RAP-1 factor is necessary for DNA loop formation in vitro at the silent mating type locus *HML*. Cell **57:**725–737.
19. **Holland, J. P., P. K. Brindle, and M. J. Holland.** 1990. Sequences within an upstream activation site in the yeast enolase gene *ENO2* modulate repression of *ENO2* expression in strains carrying a null mutation in the positive regulatory gene *GCR1*. Mol. Cell. Biol. **10:**4863–4871.
20. **Holland, M. J., T. Yokoi, J. P. Holland, K. Myambo, and M. A. Innis.** 1987. The *GCR1* gene encodes a positive transcriptional regulator of the enolase and glyceraldehyde-3-phosphate dehydrogenase gene families in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**813–820.
21. **Huet, J., P. Cottrelle, M. Cool, M.-L. Vignais, D. Thiele, C. Marck, J.-M. Buhler, A. Sentenac, and P. Fromageot.** 1985. A general upstream binding factor for genes of the yeast translational apparatus. EMBO J. **4:**3539–3547.
22. **Huet, J., and A. Sentenac.** 1987. TUF, the yeast DNA-binding factor specific for UAS_{rpg} upstream activating sequences: identification of the protein and its DNA-binding domain. Proc. Natl. Acad. Sci. USA **84:**3648–3652.
23. **Innis, M. A., and D. H. Gelfand.** 1989. Optimization of PCR, p. 3–12. *In* M. Innis, D. Gelfand, J. Sninsky, and T. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., New York.
24. **Jigami, Y., N. Toshimitsu, H. Fujisawa, H. Uemura, H. Tanaka, and S. Nakasato.** 1986. Analysis of expression of yeast enolase 1 gene containing a longer pyrimidine-rich region located between the TATA box and transcription start site. J. Biochem. **99:**1111–1125.
25. **Kayne, P. S., U.-J. Kim, M. Han, J. R. Mullen, F. Yoshizaki, and M. Grunstein.** 1988. Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell **55:**27–39.
26. **Layne, E.** 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol. **3:**447–454.
27. **Machida, M., H. Uemura, Y. Jigami, and H. Tanaka.** 1988. The protein factor which binds to the upstream activating sequence of *Saccharomyces cerevisiae ENO1* gene. Nucleic Acids Res. **16:**1407–1422.
28. **Maitra, P. K., and Z. Lobo.** 1971. A kinetic study of glycolytic enzyme synthesis in yeast. J. Biol. Chem. **246:**475–488.
29. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74:**560–564.
30. **McAlister, L., and M. J. Holland.** 1982. Targeted deletion of a yeast enolase structural gene. J. Biol. Chem. **257:**7181–7188.
31. **Nishizawa, M., R. Araki, and Y. Teranishi.** 1989. Identification of an upstream activating sequence and an upstream repressible sequence of the pyruvate kinase gene of the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**442–451.
32. **Ogden, J. E., C. Stanway, S. Kim, J. Mellor, A. J. Kingsman, and S. M. Kingsman.** 1986. Efficient expression of the *Saccharomyces cerevisiae PGK* gene depends on an upstream activation sequence but does not require TATA sequences. Mol. Cell. Biol. **6:**4335–4343.
33. **Pavlovic´, B., and W. Hörz.** 1988. The chromatin structure at the promoter of a glyceraldehyde phosphate dehydrogenase gene from *Saccharomyces cerevisiae* reflects its functional state. Mol. Cell. Biol. **8:**5513–5520.
34. **Piper, P. W., B. Curran, M. W. Davies, K. Hirst, A. Lockheart, J. E. Ogden, C. A. Stanway, A. J. Kingsman, and S. M. Kingsman.** 1988. A heat shock element in the phosphoglycerate kinase gene promoter of yeast. Nucleic Acids Res. **16:**1333–1348.
35. **Shore, D., and K. Nasmyth.** 1987. Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell **51:**721–732.
36. **Shore, D., D. J. Stillman, A. H. Brand, and K. A. Nasmyth.** 1987. Identification of silencer binding proteins from yeast: possible roles in *SIR* control and DNA replication. EMBO J. **6:**461–467.
37. **Stanway, C., J. Mellor, J. E. Ogden, A. J. Kingsman, and S. M. Kingsman.** 1987. The UAS of the yeast *PGK* gene contains functionally distinct domains. Nucleic Acids Res. **15:**6855–6873.
38. **Swanson, M. E., and M. J. Holland.** 1983. RNA polymerase I-dependent selective transcription of yeast ribosomal DNA. J. Biol. Chem. **258:**3242–3250.
39. **Sweder, K. S., P. R. Rhode, and J. L. Campbell.** 1988. Purification and characterization of proteins that bind to yeast ARSs. J. Biol. Chem. **263:**17270–17277.
40. **Uemura, H., Y. Jigami, H. Tanaka, N. Toshimitsu, M. Paterson, and S. Nakasato.** 1985. Nucleotide sequence of the 5′ flanking region responsible for the enhancement of the expression of yeast enolase 1 gene. J. Biochem. **98:**859–862.
41. **Uemura, H., T. Shiba, M. Machida, I. Matsui, Y. Jigami, and H. Tanaka.** 1987. A positive regulatory sequence of the *Saccharomyces cerevisiae ENO1* gene. J. Biochem. **102:**181–189.
42. **Uemura, H., T. Shiba, M. Paterson, Y. Jigami, and H. Tanaka.** 1986. Identification of a sequence containing the positive regulatory region of *Saccharomyces cerevisiae* gene *ENO1*. Gene **45:**67–75.
43. **Walker, S. S., S. C. Francesconi, B. K. Tye, and S. Eisenberg.** 1989. The OBF1 protein and its DNA-binding site are important for the function of an autonomously replicating sequence in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**2914–2921.
44. **Wong, H. C., and S. Chang.** 1986. Identification of a positive retroregulator that stabilizes mRNAs in bacteria. Proc. Natl. Acad. Sci. USA **83:**3233–3237.