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Transcriptional Control of the *Saccharomyces cerevisiae PGK* Gene by RAP1

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The promoter of the yeast glycolytic gene encoding phosphoglycerate kinase (*PGK*) contains an upstream activation sequence between bases −538 and −402 upstream of the initiating ATG. The upstream activation sequence contains multiple functional elements, including an essential region called the activator core (AC) sequence and three copies of the pentamer 5′-CTTCC-3′. The AC sequence shows strong homology to the consensus binding sites for the yeast proteins RAP1 (GRF1) and TUF. We have demonstrated that the yeast protein which interacts with the AC sequence is the DNA-binding protein RAP1. Expression of the *PGK* gene is found to be regulated according to the carbon source in the growth medium. *PGK* mRNA levels are high in yeast cells grown in glucose medium but low in yeast cells grown in media containing carbon sources such as pyruvate and acetate. This carbon source regulation of transcription was found to be mediated, in part, via regulation of RAP1 binding to the AC sequence. The promoters of many other yeast glycolytic genes also contain consensus RAP1-binding sites and copies of the CTTCC pentamer. This suggests that RAP1 may be involved in transcriptional control of many other glycolytic genes in addition to the *PGK* gene.

The gene encoding the glycolytic enzyme phosphoglycerate kinase (*PGK*) is one of the most highly expressed genes in the yeast cell, producing up to 5% of the total mRNA and protein (18). We have studied the promoter of the *PGK* gene to determine why it is such an efficient promoter and how control of *PGK* transcription relates to control of the other glycolytic genes.

The *PGK* promoter contains an upstream activation sequence (UAS) located between bases −538 and −402 upstream of the initiating ATG. Deletion of this region causes a dramatic drop in the level of *PGK* mRNA (28). Previous analysis of this UAS, using a combination of fine deletions and gel retardation assays, identified three sequence motifs (9, 32): (i) a region between bases −523 and −496 shown to be the site of a strong DNA-protein interaction in vitro; (ii) a region between −473 and −458, called the activator core (AC) sequence, that is necessary for high-level expression from the promoter and contains a good match to the consensus binding sites for the proteins RAP1 (GRF1) and TUF (5, 6, 19, 30, 35); and (iii) three repeats of the pentamer 5′-CTTCC-3′. Deletion of individual repeats reduces *PGK* expression but not as dramatically as does deletion of the AC sequence. The *PGK* UAS therefore has a complex structure central to which is the AC sequence. The homology between this sequence and the consensus binding sites for the proteins RAP1 (GRF1) and TUF suggested that one of these proteins may interact with the *PGK* UAS.

The protein RAP1 binds to both activator and silencer elements in vitro and has been proposed to play a role in both activating and silencing transcription in vivo (30). The protein GRF1 was isolated independently but is identical in properties to RAP1 (5). Consensus binding sites for RAP1 (GRF1) have been identified in the promoters of several genes, including the glycolytic genes *ADH1*, *PYK1*, and *ENO1* (6, 27). The TUF protein binds to a conserved transcriptional activator sequence (the RPG box) found in the promoters of genes encoding components of the translational machinery (19). Because their consensus binding sites are identical, RAP1 and TUF may be the same factor, although the reported molecular weights of the two proteins are different (20, 30). We have investigated DNA-protein interactions at the *PGK* UAS and have now identified RAP1 as the protein that binds to the AC sequence.

Many of the yeast glycolytic enzymes share the property of being inducible by glucose (25). In the case of *PGK*, this characteristic correlates with a transcriptional effect on the *PGK* gene. The *PGK* gene is highly expressed in yeast cells grown in media containing glucose but less well expressed in yeast cells grown in media containing carbon sources such as pyruvate and acetate (32, 33). We have previously shown that a region of the UAS upstream of −460 is necessary for this carbon source regulation (32). This region includes the RAP1-binding site of the AC sequence and an additional sequence between bases −523 and −496 that is the site of a strong DNA-protein interaction in vitro (32). We have now analyzed deletions that remove different portions of the UAS upstream of −460 in order to identify more precisely the sequences responsible for carbon source regulation and to identify a possible mechanism for this process.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used were *Saccharomyces cerevisiae* DBY745 (α *ade-100 leu2-3 leu2-112 ura3-52*) and MD40/4c (α *ura2 trp1 leu2-3 leu2-112 his3-11 his3-15*). Unless otherwise stated, yeast cells were grown in synthetic complete medium containing 2% glucose as a carbon source (glucose medium) (15). Pyruvate media was synthetic complete medium containing 2% pyruvate.

DNA fragments. The *PGK* Z+ fragment (−473 to −409) was isolated from pSP46-Z+ (9). The Yfp control fragment was a *Bam*HI-*Bgl*II fragment isolated from pSP46-Yfp. This plasmid contains a 28-base-pair oligonucleotide correspond-

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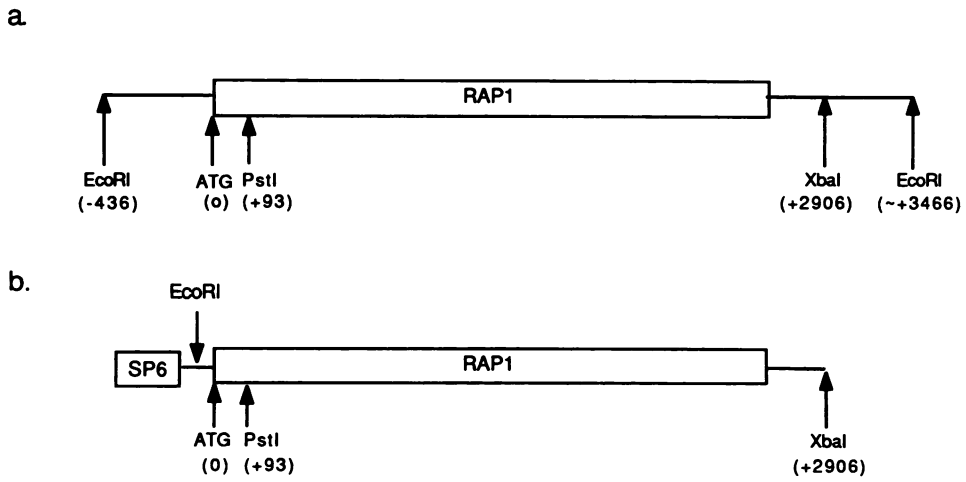

FIG. 1. (a) Structure of λRAP1. The *Eco*RI sites are the ends of the genomic clone isolated from the λ library. Positions of restriction enzyme sites are numbered relative to the ATG (0). (b) Structure of pSP56RT7. SP6 indicates position of the SP6 promoter, immediately adjacent to the ATG of the *RAP1* gene.

ing to the region of the *PGK* UAS between −523 and −496 cloned into the *Hin*cII site of pSP46 (28). *HMR(E)*, *TEF2*, and *RP51* fragments were isolated from pUC13 derivatives containing synthetic oligonucleotides corresponding to the RAP1-binding sites at these loci (30). Fragments were isolated from the pUC13 polylinker by digestion with *Hin*dIII and *Eco*RI. For use in gel retardation assays, these fragments were end labeled by using [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp.) and Klenow polymerase (Bethesda Research Laboratories, Inc.).

Cloning of the *RAP1* gene. A yeast (*S. cerevisiae* X2180) genomic DNA library in λgt11 (Clontech) was screened by hybridization, using a 30-base oligonucleotide made to the sequence between bases 1807 and 1836 in the published *RAP1* sequence (30). Clone λRAP1 was obtained (Fig. 1a). The identity of this clone was confirmed by using restriction enzyme digestion and DNA sequencing. λRAP1 contains the entire *RAP1* coding sequence, 436 base pairs of promoter sequence, and about 560 base pairs of sequence downstream of the *Xba*I site, which is the limit of the published sequence. This clone was tailored for in vitro transcription and translation. An *Eco*RI-*Xba*I fragment containing the *RAP1* gene from λRAP1 was subcloned into pSP56 (28). *RAP1* promoter sequences were removed by digestion of the clone with *Eco*RI and *Pst*I and subsequent regeneration of the 5′ end of the *RAP1* gene by using two oligonucleotides with a total length of 101 base pairs containing the *RAP1* sequence from the ATG to the *Pst*I site at +93. The regenerated 5′ end of the *RAP1* clone was confirmed to be correct by DNA sequencing. This tailored *RAP1* clone (designated pSP56 RT7) therefore contains the *RAP1* gene with the ATG positioned immediately adjacent to the SP6 promoter (Fig. 1b).

Production of RAP1 protein in vitro. pSP56RT7 was linearized with *Xba*I to produce the template for in vitro transcription. Linear DNA (1 μg) was transcribed by using SP6 polymerase under standard conditions (Promega Biotec) in the presence of the cap analog m^{7}G5′ppp5′G (Pharmacia, Inc.). A 1-μg sample of *RAP1* RNA was translated in the presence of [^{35}S]methionine (1,000 Ci/mmol; Amersham) in a rabbit reticulocyte lysate system (Promega Biotec). The total reaction volume was 50 μl. Mock translation reactions were identical but were not primed with any *RAP1* RNA. For the analysis of translation products, 1 to 3 μl of the reaction mixtures was analyzed by electrophoresis through a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel (23). ^{14}C-labeled protein molecular weight markers (Amersham) were run on the gels to allow the approximate sizes of the translation products to be estimated. After electrophoresis, protein gels were fixed in 10% glacial acetic acid, treated with 1 M sodium salicylate, and dried. Labeled proteins were detected by autoradiography at −70°C.

Gel retardation assays. Yeast nuclear protein extracts were prepared as previously described (32). Yeast cells were grown for at least six generations to a cell density of 4×10^6 to 6×10^6/ml before harvesting. The protein concentrations of the nuclear protein extracts were determined by using the Bradford dye-binding assay (4). The gel retardation assay was based on the methods of Fried and Crothers and Garner and Revzin (12, 14). Mixtures for binding reactions using yeast nuclear protein extracts contained 1 μg of nuclear protein extract. This mixture was incubated with the labeled DNA fragment in a buffer containing 20 mM KCl (9). Calf thymus DNA (200 ng) was added to each binding reaction mixture as a nonspecific competitor. After a 30-min incubation at 22°C, DNA-protein complexes were resolved by electrophoresis on a 5% polyacrylamide gel containing 0.5× TBE. The anti-RAP1 and preimmune antisera were prepared in mice against the purified RAP1 protein (30). Each was used at a final dilution of 1:400 in the binding reactions. Binding reaction mixtures were prepared minus the labeled DNA fragment. The antibody was added and allowed to react with the extract for 10 min at 22°C before the labeled fragment was added. Binding reactions using the in vitro produced RAP1 protein were similar to those using the yeast protein extracts. ^{35}S-labeled RAP1 or mock lysate (0.5 to 1 μl) was incubated with the ^{32}P-labeled DNA fragment in the presence of 400 ng of calf thymus DNA. After electrophoresis, the gel was covered with a reversed autoradiography screen and exposed to X-ray film. This procedure allowed the detection of ^{32}P in the labeled DNA fragment without interference from ^{35}S in the translation reaction. In the control experiment in which binding of in vitro-produced RAP1 protein was tested in the pyruvate extract, 0.5 μl of mock or RAP1 lysate was mixed with 1 μg of the pyruvate nuclear protein extract in the presence of 400 ng of calf thymus DNA; the remainder of the assay was carried out by the standard procedure.

Methylation interference analysis. The method used for assaying methylation interference was based on the procedure described by Siebenlist and Gilbert (31). The *PGK* Z+ fragment was end labeled on the noncoding strand at the AC end of the fragment. The labeled fragment was treated with dimethyl sulfate in 50 mM sodium cacodylate–1 mM EDTA (pH 8.0) for 2 min at 22°C to partially methylate the DNA. The fragment was recovered by ethanol precipitation and washed twice in 80% ethanol before use in a standard binding reaction. Fragments present in complex A and in the unbound DNA band were isolated from the gel and phenol extracted before piperidine cleavage. The cleavage products from each fragment were separated on a 20% polyacrylamide sequencing gel and detected by autoradiography.

RNA analysis. Deletions of the *PGK* UAS were produced using BAL 31 exonuclease as previously described (28). Deletion constructs were transformed into yeast cells on a high-copy-number leucine-selectable plasmid by standard procedures (17). Transformants were grown for at least six generations in either glucose or pyruvate medium to a cell density of 4×10^6 to 6×10^6/ml. Cells were harvested and used to prepare both RNA for Northern (RNA) analysis and DNA to check plasmid copy numbers. RNA and DNA were prepared as previously described (9, 32). Northern blots were probed by using a *PGK*-specific probe to detect *PGK* mRNA and a ribosomal-specific probe to measure rRNA as a loading control (both nick translated to a specific activity of 10^8 cpm/μg). Construction of the yeast minimal promoter will be described elsewhere (C. Stanway, A. Chambers, A. J. Kingsman, and S. M. Kingsman, manuscript in preparation). Briefly, the promoter consists of two synthetic oligonucleotides, designated T′ and R, corresponding to sequences around the TATA box and RNA start site in the *PGK* promoter (11). The *PGK* sequences in these oligonucleotides were as follows: T′, −163 to −125; and R, −70 to −35 and −10 to −4. These oligonucleotides were inserted upstream of the human α-interferon-coding sequence in the high-copy-number leucine-selectable plasmid pMA1557 (21). DNA fragments containing regions of the *PGK* UAS were inserted into a *Bam*HI site immediately upstream of the T′ oligonucleotide in pKV560 (see Fig. 8a). Interferon mRNA levels were measured by probing Northern filters with a nick-translated interferon-specific probe. *RAP1* mRNA levels were measured in the untransformed strain DBY745. This strain was grown to a cell density of 4×10^6 to 6×10^6/ml in glucose or pyruvate medium. A 15-μg sample of total RNA was loaded per lane. The filter was probed with a nick-translated internal *Bam*HI fragment from the *RAP1* gene to determine *RAP1* mRNA levels. The filter was then stripped of the *RAP1* probe and reprobed by using *PGK*- and ribosomal-specific probes. Northern blots were quantitated by using an Ambis Beta Scan system.

RESULTS

DNA-protein contacts at the *PGK* AC sequence. A DNA fragment (Z+) (Fig. 2a) from the *PGK* promoter, which contains the AC sequence and the three CTTCC blocks, interacts specifically in vitro with yeast nuclear proteins (9). Because the AC sequence shows strong homology to the consensus binding site for the protein RAP1 and to the RPG box, the binding site of the TUF protein (Fig. 2b), we tested whether one of these proteins interacts with the *PGK* UAS. The end-labeled Z+ fragment was incubated in vitro with a yeast nuclear protein extract as previously described (9). DNA-protein complexes were resolved by the standard band shift assay (12, 14). Three major complexes were observed (see Fig. 4). We have previously shown that complexes A and C are specific and that complex C probably results from partial proteolysis of the protein responsible for complex A (9). Several faint complexes could also be seen, but these were not consistently observed. The faster-migrating complex was nonspecific. DNase I footprinting of complex A had previously localized DNA-protein interactions to the AC sequence, with minor changes in DNase I sensitivity at the CTTCC blocks (9). We have now analyzed the DNA-protein contacts in complex A more closely by using methylation interference footprinting (31). The noncoding strand of the Z+ fragment was end labeled to detect contacts at the G residues opposite the C residues in the AC sequence (Fig. 3); contacts were detected through the AC sequence. The highly conserved G · C base pairs in the RAP1 (GRF1) consensus binding site (6, 30) are all involved in DNA-protein contacts in the complex. Because the residues contacted correspond to these conserved residues, the protein responsible for the complex may be RAP1 (GRF1). Methylation of one residue in the *Bam*HI linker, upstream of the AC sequence, also interfered with complex formation. This contact may not be base specific because this residue is not conserved in other RAP1-binding sites. No DNA-protein contacts were detected at the CTTCC blocks; however, this technique is limited because it detects only contacts in the major groove of the DNA. Further experiments using different footprinting techniques are therefore necessary to determine the extent of DNA-protein interactions at these sites.

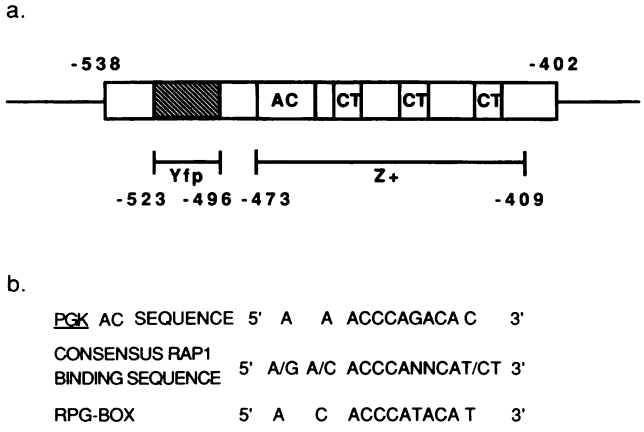

FIG. 2. (a) Functional elements within the *PGK* UAS. AC, Activator core sequence; CT, CTTCC block; Yfp, position of the footprint from the strong in vitro interaction with the Y binding protein; Z+, DNA fragment containing the AC sequence and all three CTTCC blocks. (b) Comparison between the *PGK* AC sequence, the consensus RAP1-binding site, and the RPG box.

Inhibition of complex formation by an anti-RAP1 antibody. An anti-RAP1 antibody (a generous gift from David Shore, Columbia University) was then used to determine whether the protein responsible for the specific complexes was RAP1 (Fig. 4). This mouse antibody was prepared against purified RAP1 protein. It has been shown to react specifically at a concentration of 1:400 with RAP1 protein on Western blots (immunoblots) and was used to screen a λgt11 library at a dilution of 1:250 to isolate RAP1 clones (30). This antibody was added to yeast nuclear protein extracts and allowed to preadsorb the RAP1 protein before the labeled test fragments were added. It was added to standard binding reactions at a final dilution of 1:400. The antibody was allowed to react with the extract for 10 min at room temperature before

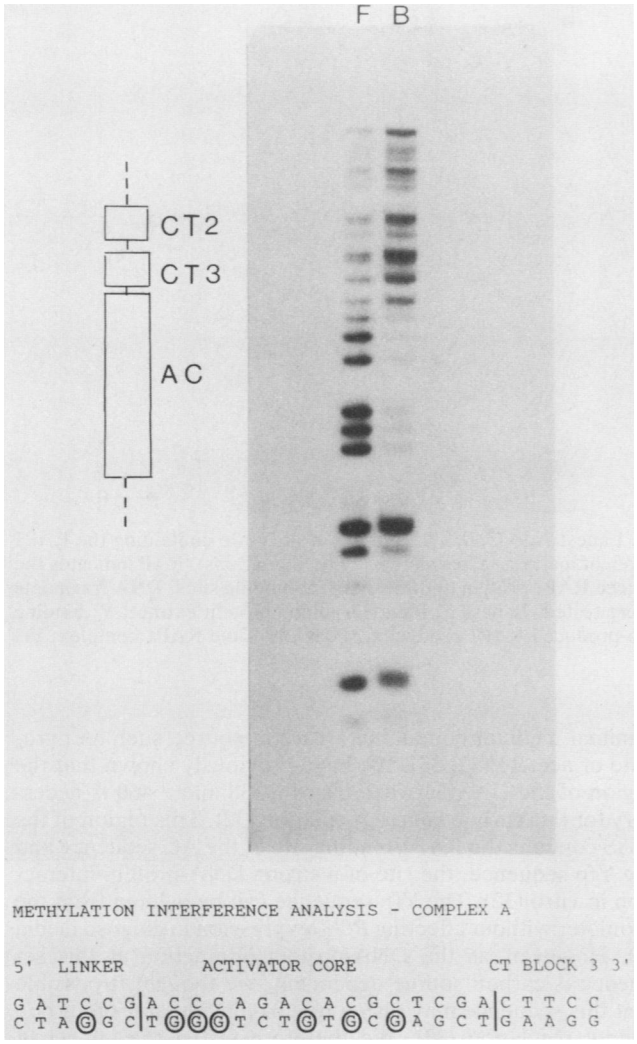

FIG. 3. Methylation interference analysis of Z+ complex A. F, Free fragment; B, fragment bound in complex A. Positions of sequence elements in the *PGK* UAS relative to the autoradiograph are shown on the left. Contacted residues within the Z+ fragment are circled.

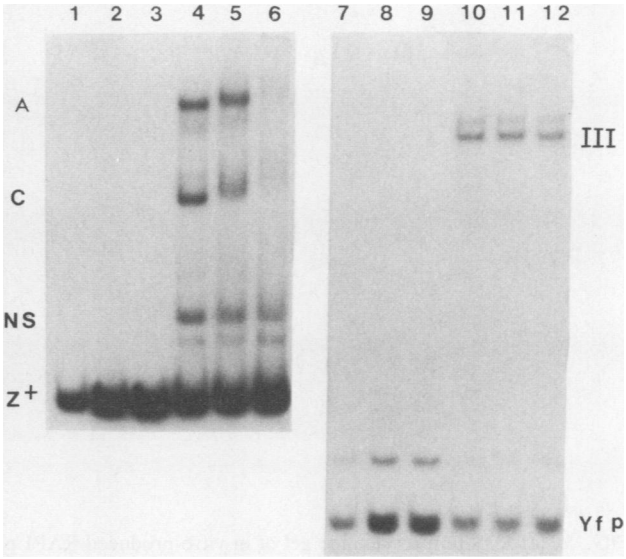

FIG. 4. Inhibition by an anti-RAP1 antibody of formation of Z+-specific complexes. A and C, Z+-specific complexes; N/S, nonspecific complex; III, Yfp complex. Lanes: 1, free fragment; 2, fragment plus preimmune antibody; 3, fragment plus anti-RAP1 antibody; 4, standard binding reaction; 5, binding reaction plus preimmune antibody; 6, binding reaction plus anti-RAP1 antibody; 7 to 12, as 1 to 6 except that the labeled Yfp fragment was used.

the labeled DNA fragment was added; the remainder of the assay was carried out by the standard procedure. Two controls were used. First, a labeled fragment (Yfp) (Fig. 2) containing the region of the UAS between −523 and −496, previously identified as the site of a DNA-protein interaction in vitro (32), was tested in binding reactions with the anti-RAP1 antibody. The protein that binds to this region of the UAS has been shown to be different from the AC-binding protein (9). As a second control, the same dilution of a preimmune serum from the same mouse was tested in binding reactions with the same two DNA fragments. The anti-RAP1 antibody inhibited formation of the Z+-specific complexes A and C but had no effect on the nonspecific complexes (Fig. 2; compare lanes 4 and 6). This antibody had no effect on the complexes formed by the control fragment (compare lanes 10 and 12). In the presence of the same dilution of the preimmune serum, formation of the Z+-specific complexes A and C was not affected (compare lanes 4 and 5). These results show that the RAP1 protein, or a protein that shares with RAP1 a determinant recognized by the antibody, is necessary for formation of the Z+-specific complexes. The anti-RAP1 antibody only identified clones containing the RAP1 gene when it was used to screen a λgt11 library (30). This makes it unlikely that *S. cerevisiae* contains a family of different proteins sharing a protein domain recognized by the antibody.

Binding of in vitro-produced RAP1 protein to the *PGK* UAS. The *RAP1* gene was tailored so as to allow it to be transcribed and translated in vitro (see Materials and Methods). The tailored gene was transcribed by using SP6 RNA polymerase to generate RAP1 mRNA, which was then translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. The major translation product migrated on an SDS-polyacrylamide gel with a mobility consistent with it being the 120-kilodalton full-length RAP1 protein (Fig. 5a, lanes A to C). Identical reactions not primed with the RAP1 RNA produced no detectable labeled proteins (Fig. 5a, lanes D to F). The in vitro-produced RAP1 protein was tested on oligonucleotides containing RAP1-binding sites from the *HMR(E)* silencer region and the UASs from the *TEF2* and *RP51* genes (30) (a generous gift from David Shore, Columbia University) in gel retardation assays (Fig. 5b). In each case, a complex was obtained with the in vitro-produced protein identical in mobility to that obtained with a yeast nuclear protein extract. The in vitro produced-protein was therefore capable of binding to RAP1-binding sites in the same way as the yeast-produced protein. The in vitro-produced protein was then tested for binding to the *PGK* Z+ fragment (Fig. 6). The in vitro-produced protein gave a complex of mobility identical to that of complex A formed in the yeast protein extract. The mock lysate, not primed with RAP1 SP6 RNA, gave no detectable complexes. As a control, the in vitro-produced protein was tested on a DNA fragment that does not contain a consensus RAP1-binding site. The fragment chosen (Yfp) contains an oligonucleotide of the region of the *PGK* UAS between bases −523 and

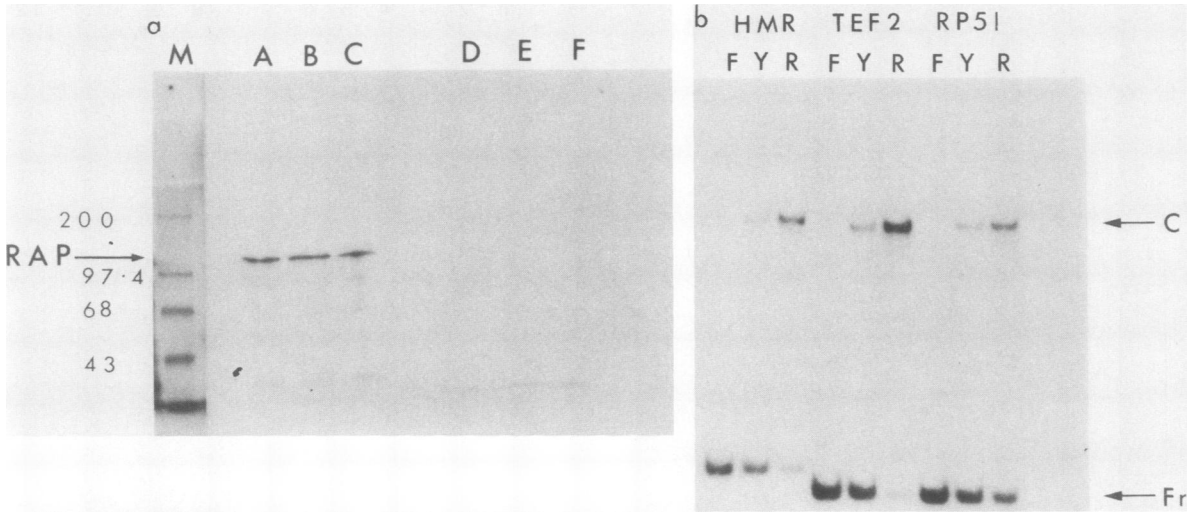

FIG. 5. (a) SDS-polyacrylamide gel of in vitro-produced RAP1 protein. Lanes: A to C, 0.5, 1, and 1.5 μl of lysate containing the RAP1 protein; D to F, identical amounts of a mock lysate; M, protein molecular weight markers (sizes shown are in kilodaltons). RAP indicates the position of the 120-kilodalton RAP1 protein. (b) Binding of the in vitro-produced RAP1 protein to three different binding sites. DNA fragments containing RAP1-binding sites from the *HMR(E)*, *TEF2*, and *RP51* loci were tested. Lanes: F, fragment minus protein extract; Y, binding assay using a yeast nuclear protein extract; R, binding assay using in vitro-produced RAP1 protein. C, Position of the RAP1 complex; Fr, position of the unbound DNA fragment.

−496. The control fragment formed strong complexes with the yeast nuclear protein extract but no complexes with either the in vitro-produced RAP1 or the mock lysate. These results argue strongly that complex A is caused by the RAP1 protein binding to the AC sequence.

Regulated expression requires the RAP1-binding site and flanking sequences. The *PGK* gene is highly expressed in yeast cells grown in minimal medium containing glucose as a carbon source but poorly expressed in yeast cells grown in minimal medium containing a carbon source such as pyruvate or acetate (32, 33). We have previously shown that the region of the UAS between bases −538 and −460 is necessary for this carbon source regulation (32). This region of the UAS contains the RAP1-binding site of the AC sequence and the Yfp sequence, the site of a strong DNA-protein interaction in vitro (32). The Yfp sequence can be deleted from the promoter without affecting *PGK* expression in glucose media (9). However, as the DNA-protein interaction at this sequence is carbon source dependent, we thought it possible that this sequence may play a role as a modulator of carbon source regulation (32). We therefore tested the effects of deletions that removed either all or part of this sequence on carbon source regulation (Fig. 7). When the whole UAS was present (pMA27), the difference in *PGK* mRNA levels between cells grown in glucose and pyruvate media was approximately fivefold. Deletions that removed either the whole Yfp sequence (pMA779 and pMA797) or parts of it (pKV501 and pKV514) showed the same degree of regulation as the construct containing the whole UAS. The loading control shows that there were no differences in the amounts of RNA loaded per gel track. The copy numbers of the test plasmids in yeast cells grown in the different media were also tested (Fig. 7c). For all plasmids except pMA779, the copy number in pyruvate medium was either identical to or slightly higher than that in glucose medium. Therefore, changes in plasmid copy number do not explain the observed decrease in expression from these plasmids in pyruvate-grown cells. pMA779 showed a decrease in copy number in pyruvate-grown cells; this finding does not affect our conclusions because the region deleted in this construct was also absent in pMA797, which did not vary in copy number. The Yfp sequence is therefore not essential for carbon source regulation.

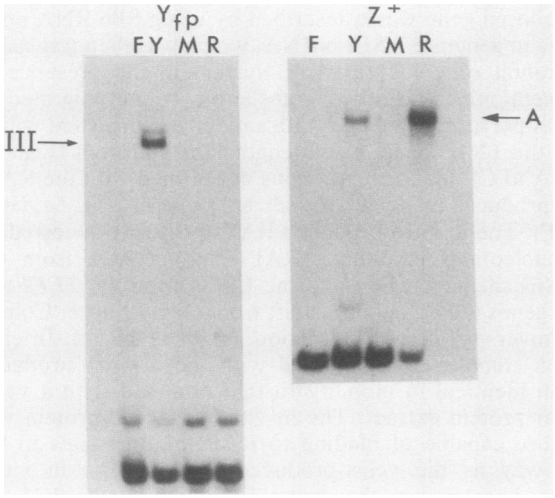

FIG. 6. Binding of in vitro-produced RAP1 protein to the *PGK* Z+ fragment. The Yfp and Z+ fragments were tested in gel retardation assays with the in vitro-produced RAP1 protein. Lanes: F, free fragment; Y, fragment plus yeast nuclear protein extract; M, fragment plus mock lysate; R, fragment plus lysate containing in vitro-produced RAP1. III, Complex III formed on the Yfp fragment in the yeast nuclear protein extract; A, complex A formed on the Z+ fragment in the yeast nuclear protein extract and in the lysate containing RAP1.

Deletions that remove either CTTCC block 1 (−432 to −428) or CTTCC block 2 (−449 to −445) (Fig. 2a) have previously been shown to reduce *PGK* expression in glucose media by 50 and 75%, respectively (9). We tested these

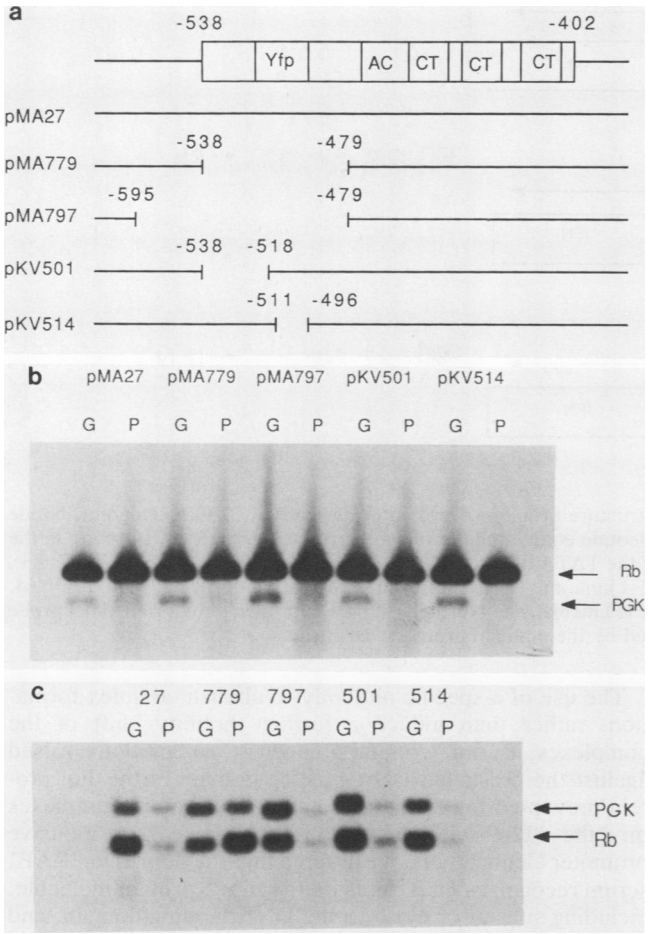

FIG. 7. Demonstration that regions of the UAS upstream of −479 are not essential for carbon source regulation. (a) Structure of the *PGK* UAS showing the extent of the deletions tested. (b) Northern analysis showing carbon source regulation of *PGK* mRNA levels. Lanes: G, RNA isolated from cells grown in glucose medium; P, RNA isolated from cells grown in pyruvate medium. Rb, Position of rRNA; PGK, position of *PGK* mRNA. (c) Southern blot of DNA isolated from the cultures used for RNA analysis. Lanes and abbreviations are as for panel b.

deletions to determine whether either of these CTTCC blocks is necessary for carbon source regulation. When tested, both deletions retained the ability to be carbon source regulated (data not shown). Therefore, although the CTTCC blocks are important for full activation, they are not essential for carbon source regulation.

To investigate further the mechanism of carbon source regulation, we identified the sequences within the UAS required to confer carbon source regulation on a minimal assay promoter. The minimal assay promoter consisted of an oligonucleotide containing a TATA box and an RNA start site, based on the *PGK* sequence, linked to the coding region of human α interferon as a reporter gene (Fig. 8a) (Stanway et al., submitted). DNA fragments were inserted into a *Bam*HI site upstream of the TATA box, and their UAS activities were measured by monitoring the production of interferon mRNA. The parental plasmid produces no interferon mRNA in the absence of UAS sequences upstream of the TATA box (Stanway et al., submitted). When the whole *PGK* UAS was present, interferon mRNA levels were carbon source regulated (Fig. 8b; compare 561G and 561P). This finding confirms the previous observation that the *PGK* UAS contains the sequences through which carbon source regulation is mediated (32). When the *PGK* Z+ fragment was inserted in place of the whole UAS, the level of transcription in glucose-grown yeast cells was less than when the whole UAS was present; however, this transcription was also carbon source regulated (Fig. 8b; compare 564G and 564P). An oligonucleotide containing only the AC sequence and one CTTCC block (*PGK* UAS sequence −473 to −453) was also tested. This oligonucleotide directed no detectable transcription of interferon mRNA (Stanway et al., submitted). These results suggest that flanking sequences, in addition to the RAP1-binding site, are required for both transcriptional activation and carbon source regulation. All constructs tested that were transcriptionally active were also carbon source regulated. We have not been able to identify a separate sequence in the UAS responsible for carbon source regulation.

RAP1 binding to the *PGK* UAS is carbon source regulated. Nuclear protein extracts were made from yeast cells grown in glucose and pyruvate minimal media under the conditions previously used for the RNA analysis. Identical amounts of each protein extract were tested for the ability to form the RAP1 complex (complex A) with the *PGK* Z+ fragment (Fig. 9). Formation of complex A was detected in the glucose extract but not in the pyruvate extract. Formation of the faster-migrating nonspecific complex was not affected by carbon source. Equal samples of the glucose and pyruvate nuclear protein extracts were electrophoresed on an SDS-polyacrylamide gel and visualized by Coomassie staining. Although some proteins were present in the pyruvate extract and absent from the glucose extract, and vice versa, the majority of proteins were equally abundant in both extracts (data not shown). As a control to show that the pyruvate nuclear protein extract did not contain an inhibitor that prevented RAP1 binding to DNA, we also carried out retardation assays, using the pyruvate nuclear protein extract with the addition of the in vitro-produced RAP1 protein. The RAP1 complex (A) was efficiently formed by the in vitro-produced RAP1 protein in the presence of the pyruvate extract, indicating that this extract did not contain an inhibitor of RAP1 binding. These results suggest the hypothesis that regulation of RAP1 binding to the AC sequence may play a role in carbon source regulation of *PGK* transcription in vivo. These results also suggest that in yeast cells grown in pyruvate medium, the RAP1 protein might be less abundant than in cells grown in glucose medium or exist in a modified form that does not bind as efficiently to the *PGK* UAS.

***RAP1* mRNA levels are not regulated by carbon source.** We measured *RAP1* mRNA levels in glucose- and pyruvate-grown cells to determine whether the presence and absence of RAP1-binding activity in the different growth media was due to transcriptional control of the *RAP1* gene. RNA was isolated from untransformed yeast cells grown in glucose and pyruvate media under the conditions used previously. These RNAs were analyzed by agarose gel electrophoresis, followed by Northern blotting (Fig. 10). The Northern filter was initially probed with a *PGK* probe and an rRNA probe as a loading control (Fig. 10a). This assay showed a fivefold difference in chromosomal *PGK* mRNA levels between the two growth media. The same filter was then reprobed with a *RAP1*-specific probe (Fig. 10b). *RAP1* mRNA levels were identical in the two lanes, indicating that *RAP1* mRNA levels are not regulated by carbon source.

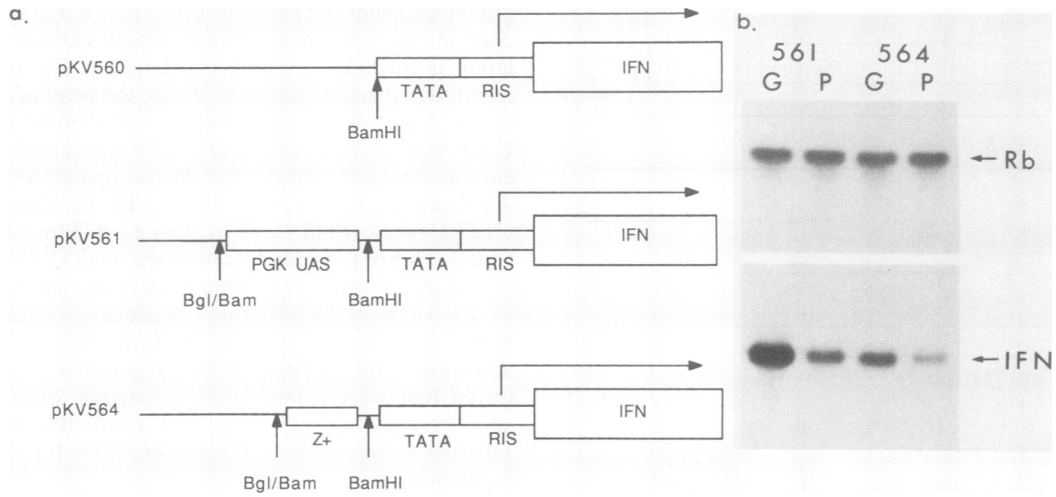

FIG. 8. Carbon source regulation of a minimal assay promoter. (a) Structure of minimal promoter derivatives. TATA, Oligonucleotide containing the TATA box region from the *PGK* promoter; RIS, oligonucleotide containing the region surrounding the RNA start site in the *PGK* promoter; PGK UAS and Z+, DNA fragments inserted upstream of the TATA box in pKV561 and pKV564 (UAS, −538 to −402; Z+, −473 to −409). IFN, α-interferon-coding sequence. Bgl/Bam, *Bgl*II-*Bam*HI junction. (b) Carbon source regulation of pKV561 and pKV564. RNA was isolated from transformants grown on glucose (G) and pyruvate (P) media. The Northern filter was probed with a ribosomal probe as a loading control (Rb) and an interferon probe to detect RNA produced by the minimal promoter constructs (IFN).

DISCUSSION

The AC sequence is essential for high-level expression from the *PGK* promoter (9). Here we have shown that the yeast nuclear protein that binds in vitro to the AC sequence is the protein RAP1. Three different items of data lead to this conclusion: (i) DNA-protein contacts in Z+ complex A map to residues in the AC sequence that are highly conserved between RAP1-binding sites, (ii) an anti-RAP1 antibody inhibited formation of specific complexes on the Z+ fragment, and (iii) RAP1 protein produced in vitro bound strongly and specifically to the Z+ fragment.

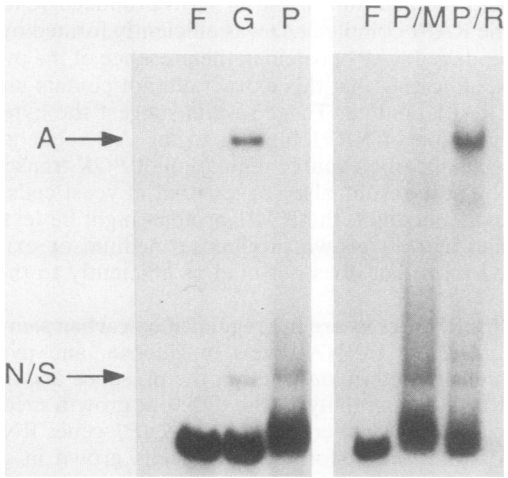

FIG. 9. Demonstration that nuclear protein extracts from pyruvate-grown cells are depleted in RAP1-binding activity. Nuclear protein extract from cells grown in glucose (G) and pyruvate (P) media were tested for binding to the Z+ fragment. As a control experiment, binding of the in vitro-produced RAP1 protein mixed with the pyruvate extract was also tested. F, Free fragment; A, RAP1 complex; N/S, nonspecific complex; P/M, binding reaction containing the pyruvate extract plus a mock translation mix; P/R, binding reaction containing the pyruvate extract plus in vitro-produced RAP1 protein.

The use of a specific antibody to abolish complex formation, rather than induce a further mobility shift of the complexes, is not without precedent: an antibody raised against the presumed DNA-binding domain of the Jun protein prevented the formation of Jun-Fos protein complexes on the 12-*O*-tetradecanoylphorbol-13-acetate-responsive promoter element (29). We assume that the polyclonal RAP1 serum recognizes many sites on the RAP1 protein molecule, including sites at or close to the DNA-binding domain, and that by binding to these sites with high affinity, it prevented RAP1 binding to DNA.

The RAP1 protein produced in vitro in a reticulocyte lysate system bound strongly and specifically to DNA fragments containing RAP1-binding sites, including the *PGK* Z+ fragment. Any posttranslational modifications of the protein necessary for efficient DNA binding must therefore occur in the lysate in the same way as in the yeast cell. The in vitro-produced RAP1 gave a DNA-protein complex with the Z+ fragment of mobility identical to that obtained by using a yeast nuclear protein extract. This finding suggests that the only protein present in complex A, formed in the yeast protein extract, is RAP1. We previously obtained a DNase I footprint of complex A, and we identified minor changes in DNase I sensitivity in the proximity of the CTTCC blocks (9), although DNA-protein contacts at the G · C base pairs within these motifs were not detected in methylation interference footprints. We previously suggested that the changes in DNase I sensitivity might be due to a second protein in the complex that interacts with the CTTCC motifs (9). It now seems more likely that these motifs are involved in secondary interactions with the RAP1 protein.

The interaction between RAP1 and the AC sequence detected in vitro is almost certainly necessary for the UAS to function in vivo. We considered the possibility that a different protein, with a recognition sequence similar to that of RAP1, may actually be important in vivo. The strong binding of the RAP1 protein in vitro could mask the binding of other proteins. This possibility is, however, unlikely because no new DNA-protein complexes were detected when RAP1 binding was inhibited by using the specific antibody.

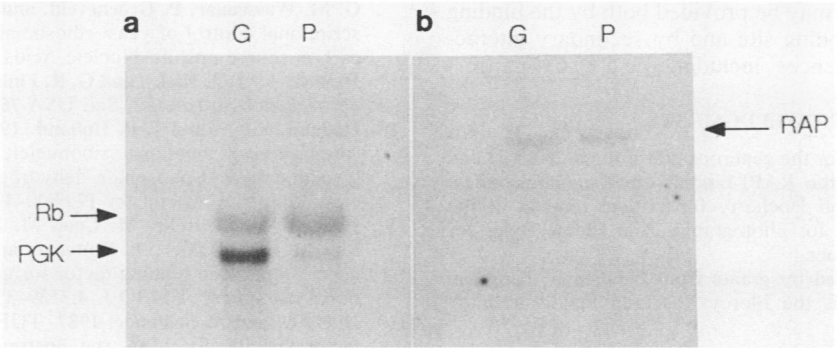

FIG. 10. Demonstration that *RAP1* mRNA levels are not regulated by carbon source. RNA was isolated from glucose (G)- and pyruvate (P)-grown cells. The Northern filter was probed with ribosomal- and *PGK*-specific probes (a) and a *RAP1*-specific probe (b). Rb, rRNA (loading control); PGK, *PGK* mRNA; RAP, *RAP1* mRNA.

We have investigated the mechanism of carbon source control of *PGK* transcription. The previously identified Yfp sequence, the site of a strong DNA-protein interaction in vitro (32), can be deleted from the promoter without affecting carbon source regulation. The role of this sequence within the UAS is unclear, although studies using a minimal assay promoter suggest that it may play a part in creating the correct context for efficient transcriptional activation by RAP1 (Stanway et al., in preparation).

Using the minimal assay promoter, we have confirmed the observation that sequences within the UAS are sufficient to confer carbon source regulation on a heterologous promoter (32). We detected strong binding of RAP1 to the UAS in nuclear protein extracts from glucose-grown cells; however, using protein extracts from pyruvate-grown cells, we detected no specific binding to the UAS. This result argues that carbon source regulation may be mediated in part via the presence or absence of RAP1 binding to the activator core sequence; we saw no evidence for the binding of repressor proteins to the UAS.

Regulation of RAP1-binding activity could occur either by a posttranslational modification of the protein, leading to less efficient binding, or by a reduction in the actual amount of RAP1 protein available to bind. The levels of *RAP1* mRNA do not vary with carbon source; therefore, changes in RAP1-binding activity are not due to transcriptional control of the *RAP1* gene. We have recently shown that dephosphorylation of RAP1 inhibits its ability to bind to DNA (J. Tsang, A. Chambers, A. J. Kingsman, and S. M. Kingsman, submitted for publication). This posttranslational modification could therefore play a role in the differential binding of RAP1 according to carbon source. In addition, we have preliminary evidence that the levels of RAP1 protein are also reduced in pyruvate extracts (unpublished observations).

These data are consistent with a model in which changes in binding of RAP1 to the activator core sequence play an important role in carbon source regulation of *PGK* transcription. This may be an oversimplified view because RAP1-binding sites are found in other promoters and at the *HML* and *HMR* silencers (5, 6, 19, 27, 30, 35). If RAP1-binding activity is regulated by carbon source, binding to these other sites might be affected. Some of these other promoters, such as the promoter of the ribosomal protein gene *L25*, are carbon source regulated (16); however, some of the additional locations might not be expected to be regulated by carbon source. This may be explained by the observation that different RAP1-binding sites within the cell have widely different affinities for the RAP1 protein (6, 30). Clearly, if the level of RAP1-binding activity falls, strong-affinity sites will be less affected than weak-affinity sites. By selecting the correct affinity site for the appropriate location, the yeast cell could have the potential to control many cellular processes by simply changing the level of one protein. Recently, a mechanism for carbon source regulation of the promoter of the glycolytic gene encoding pyruvate kinase (*PYK*) was proposed (27). An upstream repressor sequence located between UAS1 and the TATA box is responsible for repressing *PYK* transcription in nonfermentable carbon sources. UAS1 of the *PYK* promoter contains homologies to the *PGK* promoter, including a RAP1-binding site and CTTCC block (Fig. 11). In the absence of the upstream repressor sequence, expression increased in both glucose and nonfermentable media, but this expression was still carbon source regulated to some extent. This promoter may require an upstream repressor sequence because the RAP1-binding site in the *PYK* promoter is known to be a very high affinity site (6). Therefore, changes in RAP1-binding activity might have less effect on RAP1 binding to the *PYK* UAS than to the *PGK* UAS.

RAP1 consensus binding sites and CTTCC blocks are present in the promoters of the glycolytic genes *PYK1*, *ENO1*, *ADH1*, *PDC1*, and *TPI* in addition to *PGK* (1, 2, 7, 8, 24, 27, 34) (Fig. 11). In each case, where the position of the UAS is known, these motifs are within the UAS. The promoter of the *TDH1* gene (3) also contains a consensus RAP1-binding site, but in this case there is not a CTTCC block in close proximity. Genes encoding enzymes in the first half of the glycolytic pathway, such as the *HXK1* and *HXK2* genes encoding hexokinase isoenzymes, appear not to contain obvious matches to the consensus RAP1-binding site within their promoters (13, 22). We propose that the consensus RAP1-binding site and the CTTCC motif are part of a common system of transcriptional control of many glycolytic genes. The specificity of action of the RAP1 protein

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TPI                          -420 5'  AACCCAT-CAGG~35bpCTTCC 3'
TDH1                         -565 5'  AACCCGTACAT            3'
PGK                          -476 5'AAAACCCAGACACGCTCGACTTCC 3'
ENO1   -483 5' CTTCC------ACTAGGATAGCACCCAAACACC 3'
PYK                          -656 5'TACACCCAGACA--TCGGGCTTCC 3'
PDC                          -635 5'   ACCCATAC - - - -CTTCC 3'
ADH1   -683 5' CTTCCTCTTTTCTGGCAACCAAACCCATACATCG 3'
```

FIG. 11. Conserved sequences in glycolytic promoters.

on glycolytic promoters may be provided both by the binding affinity of the RAP1-binding site and by secondary interactions with flanking sequences, including the CTTCC motif.

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LITERATURE CITED

1. **Alber, T., and G. Kawasaki.** 1982. Nucleotide sequence of the triose phosphate isomerase gene of *Saccharomyces cerevisiae*. J. Mol. Appl. Genetics **1**:419–434.
2. **Bennetzen, J. L., and B. D. Hall.** 1982. The primary structure of the *Saccharomyces cerevisiae* gene for alcohol dehydrogenase I. J. Biol. Chem. **257**:3018–3025.
3. **Bitter, G. A., and K. Egan.** 1984. Expression of heterologous genes in *Saccharomyces cerevisiae* from vectors utilising the glygeraldehyde-3-phosphate dehydrogenase gene promoter. Gene **32**:263–274.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Buchman, A., W. J. Kimmerley, J. Rine, and R. 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. **Burke, R. L., P. Tekamp-Olson, and R. Najarian.** 1983. The isolation, characterisation and sequence of the pyruvate kinase gene of *Saccharomyces cerevisiae*. J. Biol. Chem. **258**:2193–2201.
8. **Butler, G., and D. J. McConnell.** 1988. Identification of an upstream activation site in the pyruvate decarboxylase structural gene (PDC1) of *Saccharomyces cerevisiae*. Curr. Genet. **14**:405–412.
9. **Chambers, A., C. Stanway, A. J. Kingsman, and S. M. Kingsman.** 1988. The UAS of the yeast *PGK* gene is composed of multiple functional elements. Nucleic Acids Res. **16**:8245–8260.
10. **de Blas, A. L., and H. M. Cherwinski.** 1983. Detection of antibodies on nitrocellulose paper: immunoblots with monoclonal antobodies. Anal. Biochem. **113**:214–219.
11. **Dobson, M. J., M. F. Tuite, N. Roberts, A. J. Kingsman, S. M. Kingsman, R. E. Perkins, S. C. Conroy, B. Dunbar, and L. A. Fothergill.** 1982. Conservation of high efficiency promoter sequences in *Saccharomyces cerevisiae*. Nucleic Acids Res. **10**: 2625–2637.
12. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
13. **Frohlich, K. U., K. D. Entian, and D. Menke.** 1985. The primary structure of the yeast hexokinase PII gene (*HXK2*) which is responsible for glucose repression. Gene **36**:105–111.
14. **Garner, M. M., and A. Revzin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. Nucleic Acids Res. **9**:3037–3060.
15. **Hawthorne, D. C., and R. K. Mortimer.** 1960. Chromosome mapping in *Saccharomyces cerevisiae*: centromere linked genes. Genetics **45**:1085–1110.
16. **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.
17. **Hinnen, A., J. B. Hicks, and G. R. Fink.** 1978. Transformation of yeast. Proc. Natl. Acad. Sci. USA **75**:1929–1933.
18. **Holland, M. J., and J. P. Holland.** 1978. Isolation and identification of yeast messenger ribonucleic acids coding for enolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase. Biochemistry **17**:4900–4907.
19. **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 machinery. EMBO J. **4**:3539–3547.
20. **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.
21. **Kim, S., J. Mellor, A. J. Kingsman, and S. M. Kingsman.** 1986. Multiple control elements in the *TRP1* promoter of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:4251–4258.
22. **Kopetzki, E., K. D. Entian, and D. Mecke.** 1985. The complete nucleotide sequence of the hexokinase PI gene (*HXK1*) of *Saccharomyces cerevisiae*. Gene **39**:95–102.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:68–85.
24. **Machida, M., H. Uemura, Y. Jigami, and H. Tanaka.** 1988. The protein factor which binds to the upstream activating sequence of the *Saccharomyces cerevisiae ENO1* gene. Nucleic Acids Res. **16**:1407–1422.
25. **Maitra, P. K., and Z. Lobo.** 1971. A kinetic study of glycolytic enzyme synthesis in yeast. J. Biol. Chem. **246**:475–488.
26. **Mellor, J., M. J. Dobson, N. A. Roberts, M. F. Tuite, J. S. Emtage, S. White, P. A. Lowe, T. Patel, A. J. Kingsman, and S. M. Kingsman.** 1983. Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. Gene **24**: 1–14.
27. **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.
28. **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 activating sequence but does not require TATA sequences. Mol. Cell. Biol. **6**:4335–4343.
29. **Sassone-Corsi, P., L. J. Ransone, W. W. Lamph, and I. M. Verma.** 1988. Direct interaction between fos and jun nuclear oncoproteins: role of the leucine zipper domain. Nature (London) **336**:692–695.
30. **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.
31. **Siebenlist, U., and W. Gilbert.** 1980. Contacts between *E. coli* RNA polymerase and an early promoter of phage T7. Proc. Natl. Acad. Sci. USA **77**:122–126.
32. **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.
33. **Tuite, M. F., M. J. Dobson, N. A. Roberts, R. M. King, D. C. Burke, S. M. Kingsman, and A. J. Kingsman.** 1982. Regulated high efficiency expression of human interferon-alpha in *Saccharomyces cerevisiae*. EMBO J. **1**:603–608.
34. **Uemura, H., T. Shiba, M. Patterson, Y. Jigami, and H. Tanaka.** 1986. Identification of a sequence containing the positive regulatory region of *Saccharomyces cerevisiae* gene *ENO1*. Gene **45**:67–75.
35. **Vignais, M. L., L. P. Woudt, G. M. Wassenaar, W. H. Mager, A. Sentenac, and R. J. Planta.** 1987. Specific binding of TUF factor to upstream activation sites of yeast ribosomal protein genes. EMBO J. **6**:1451–1457.