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Identification of an Upstream Activating Sequence and an Upstream Repressible Sequence of the Pyruvate Kinase Gene of the Yeast *Saccharomyces cerevisiae*

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To clarify carbon source-dependent control of the glycolytic pathway in the yeast *Saccharomyces cerevisiae*, we have initiated a study of transcriptional regulation of the pyruvate kinase gene (*PYK*). By deletion analysis of the 5′-noncoding region of the *PYK* gene, we have identified an upstream activating sequence ($UAS_{PYK}1$) located between 634 and 653 nucleotides upstream of the initiating ATG codon. The promoter activity of the *PYK* 5′-noncoding region was abolished when the sequence containing the $UAS_{PYK}1$ was deleted from the region. Synthetic $UAS_{PYK}1$ (26mer), in either orientation, was able to restore the transcriptional activity of UAS-depleted mutants when placed upstream of the TATA sequence located at −199 (ATG as +1). While the $UAS_{PYK}1$ was required for basal to intermediate levels of transcriptional activation, a sequence between −714 and −811 was found to be necessary for full activation. On the other hand, a sequence between −344 and −468 was found to be responsible for transcriptional repression of the *PYK* gene when yeast cells were grown on nonfermentable carbon sources. This upstream repressible sequence also repressed transcription, although to a lesser extent, when glucose was present in the medium. The possible mechanism for carbon source-dependent regulation of *PYK* expression through these *cis*-acting regulatory elements is discussed.

The glycolytic pathway is a major metabolic flux in the cell cycle of the yeast *Saccharomyces cerevisiae*. Yeast cells undergo glycolytic growth when a fermentable carbon source such as glucose is present in the medium and enter into a "transition" phase as the amount of fermentable sugar decreases to a certain level or that of a nonfermentable carbon source increases (14, 23). In the transition phase, or gluconeogenic growth, activity of the glycolytic enzyme drops, glycogen concentration increases (14, 23), and gluconeogenic enzymes are derepressed (11). Glycolytic enzyme levels and their respective mRNA levels are induced by glucose (25); hence, the shift in growth mode of yeast cells can be correlated with the change in glycolytic enzyme levels. However, overall control of glycolytic enzyme genes is not clarified yet.

The pyruvate kinase gene (*PYK*) is regarded as a highly expressed gene among glycolytic enzyme genes of *S. cerevisiae* (13), and its product is induced by glucose (25) and is thought to play a key role in regulation of glycolysis (13). Thus, clarification of the regulation mechanism of *PYK* expression will lead to an understanding of control of the glycolytic pathway. In higher eucaryotes, transcription of a PYK isozyme is induced by glucose through insulin action (30). In yeasts, cyclic AMP is suggested to be the mediator of regulation of carbohydrate metabolism (14, 28), and the *GCR1* gene is known to act as a positive regulator of many glycolytic enzymes including PYK (1, 7). *GCR1* regulates the transcription of, at least, enolase and glyceraldehyde 3-phosphate dehydrogenase genes (16). *PYK* also could be regulated at the transcription level, but the mechanism by which these factors, in response to a carbon source shift, exert their effects on *PYK* transcription is not known yet. On the other hand, *cis*-acting positive elements, or upstream activating sequences (UAS), have been identified in several glycolytic enzyme genes including *ENO1* (9), *ENO2* (8), *PGK* (31), and *ADH2* (2). UAS appear to be the binding sites of transactivation factors (40) and play a crucial role in transcriptional activation of genes downstream from them.

To understand how these *trans*- and *cis*-acting factors and sequences function in regulating the transcription of *PYK*, we have initiated analysis of the 5′-noncoding region of the *PYK* gene to identify *cis*-acting regulatory elements required for its expression. Our results demonstrate that the *PYK* promoter contains a UAS (UAS1) consisting of, at most, 20 base pairs (bp) that can function in either orientation and direct transcription in a heterologous system. We also show that another sequence (UAS2) upstream of UAS1 is necessary for full activation of transcription and that a transcription repression sequence (upstream repressible sequence [URS]) resides between UAS1 and the TATA sequence. The possible mechanism for the carbon source-dependent regulation of *PYK* expression by UAS and URS is discussed.

MATERIALS AND METHODS

Strains and growth conditions. *S. cerevisiae* X2180-1B (α *SUC2 mal mel gal2 CUP1*), 20B-12 (α *trp1 pep4-3*), and pyk1-5 (**a** *pyk1-5 ade1 leu1 met14 ura3*) were obtained from the Yeast Genetic Stock Center. Yeasts were grown in YP medium containing 2% peptone and 1% yeast extract or in a defined medium containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.) supplemented with appropriate nutrients. The carbon source was 2% glucose or 2% glycerol plus 2% ethanol.

Materials. All enzymes we used were purchased from commercial sources and used as instructed by the suppliers. An oligonucleotide probe for screening the *PYK* clone and primers for sequencing deletion endpoints were synthesized by a model 381A DNA synthesizer (Applied Biosystems). The *xylE* gene clone (Transgene) was obtained from J.-P.

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Lecocq (44). Pyrocathecol was from Nakarai Chemicals, Kyoto, Japan.

DNA and RNA isolation. Plasmids were constructed by using gel-purified DNA fragments (27) and were isolated from *Escherichia coli* according to the method of Birnboim and Doly (4). Total yeast DNA was prepared as described by Davis et al. (10). Total RNA was isolated from yeast cells grown to a density of 10^7/ml as described by Struhl and Davis (41), except that 50 U of zymolyase 100,000 was used instead of lyticase.

Transformation of *E. coli* and *S. cerevisiae*. *E. coli* was transformed by the $CaCl_2$ method (26). *S. cerevisiae* was transformed by the lithium acetate method as described by Ito et al. (19).

Cloning of *PYK* gene. Chromosomal DNA was isolated from *S. cerevisiae* X2180-1B grown to a density of 10^8 cells per ml; 30 μg of the DNA was digested partially with *Sau*3A, and the digested fragments of >5 kilobase pairs (kb) were fractionated and collected through a 5 to 20% sucrose density gradient. The fragments were ligated to pBR322 previously linearized with *Bam*HI. Transformation of *E. coli* HB101 with the ligation mixture produced an *S. cerevisiae* gene bank consisting of approximately 2×10^4 clones. An oligonucleotide, 5'-CTAATCTAGACATTGTGATG-3', which was complementary to the sequence from −7 to +13 of the yeast *PYK* gene (A of the initiating ATG of *PYK* was referred to +1 [6]), was used to screen the *S. cerevisiae* gene bank. Two positive clones were obtained and the plasmids were isolated from them. After digestion with *Hin*dIII followed by Southern analysis (38), one plasmid was found to contain a ca. 8.8-kb fragment including the *PYK* coding region. Isolation of the *PYK* gene was confirmed by the agreement of the restriction map of the fragment with that reported previously (Fig. 1a) (6) and by complementation of the *pyk1-5* mutation of *S. cerevisiae*.

Construction of the expression vector pKY54. An 8.8-kb *Hin*dIII fragment containing the *PYK* gene was digested with *Eco*RI and *Hae*III, and a *Hae*III-*Eco*RI fragment (ca. 1.0 kb) containing the 5'-noncoding region and a *Hae*III-*Hae*III fragment (ca. 500 bp) containing the stop codon and the 3'-noncoding sequence were isolated (Fig. 1b). The *Hae*III terminal of the *Hae*III-*Eco*RI fragment was converted into *Hin*dIII by cloning the fragment into the polylinker site of pUC8. The *Hae*III-*Hae*III fragment was also cloned into pUC8 to convert the stop codon-proximal terminus into a *Bam*HI site and the distal terminus into an *Eco*RI site. The 8.8-kb fragment was also cleaved with *Eco*RI and *Xba*I to isolate an *Eco*RI-*Xba*I fragment (ca. 500 bp; Fig. 1b). The initiation codon of *PYK* was removed from this fragment by digestion with *Bal*31 exonuclease, and a *Bam*HI linker was ligated to the ends with T4 ligase. The fragment was then cleaved with *Ava*II and *Bam*HI, and the 5'-noncoding fragment containing the transcription start point (6) was isolated. The fragment having an appropriate deletion endpoint (at −3) was selected. The *Eco*RI-*Xba*I fragment (ca. 500 bp) in the 5'-noncoding region was also cleaved with *Ava*II, and the fragment upstream of the *Ava*II site was isolated. Three fragments, *Hin*dIII-*Eco*RI, *Eco*RI-*Ava*II, and *Ava*II-*Bam*HI, were combined and ligated to generate a ca. 1.4-kb fragment containing the entire 5'-noncoding region of *PYK* but lacking the last two nucleotides (Fig. 1b). The *Hin*dIII-*Bam*HI fragment containing the 5'-noncoding sequence and the *Bam*HI-*Eco*RI fragment containing the 3'-noncoding region were combined and ligated in the presence of a 2μm-*TRP1* *Eco*RI-*Hin*dIII fragment and pBR322, to generate expression vector pKY54 (Fig. 1c).

Construction of *PYK* promoter deletions. (i) Construction of pKY56. A *Bam*HI-*Bgl*II fragment containing the entire coding sequence of the *xylE* gene of *Pseudomonas putida* (18, 44) was ligated to pKY54 previously linearized by *Bam*HI cleavage to generate plasmid pKY56 (Fig. 2). To construct deletions in the *PYK* promoter region, the *Hin*dIII site of pKY56 at the 5' boundary of the upstream region of *PYK* was converted into either an *Xho*I (pKY56X) or a *Bgl*II (pKY56Bg) site by partial digestion of pKY56 with *Hin*dIII followed by filling in with Klenow enzyme and ligation in the presence of the respective linkers. Plasmid pKY56 was referred to as the wild-type plasmid; hence, the activity of the *xylE* gene product, catechol 2,3-dioxygenase ($CatO_2$ase), in the yeast transformants harboring pKY56 was the same as the wild-type level of enzyme activity.

(ii) Deletions in the 5'→3' direction of the *PYK* promoter region. Digestion of pKY56X with *Xho*I followed by treatment with *Bal*31 exonuclease generated a series of DNA fragments retaining between 904 and 173 bp of the 5'-noncoding sequences. The termini of the deletion were filled in with Klenow enzyme and ligated with an *Xho*I linker, and the resulting fragments were cut with *Bam*HI and *Xho*I. The *Xho*I-*Bam*HI fragment from a pKY56X series deletion was ligated to the *Bam*HI-*Xho*I fragment of pKY56X which was lacking the *PYK* 5'-noncoding sequence. The resulting molecules have deletions in the 5'→3' direction in the *PYK* 5' region. They were termed "X" series (Fig. 2).

(iii) Internal deletions in the *PYK* promoter region. A second series of deletions was generated by removing sequences in the 3'→5' direction with *Bal*31 from pKY56Bg previously cut with *Bam*HI. This produced a set of deletions with endpoints between −260 and −845. The ends of the deletion molecules were converted to *Xho*I sites as described above. The resulting fragments were digested with *Bgl*II and *Xho*I. The *Bgl*II-*Xho*I fragment containing the *PYK* 5' region was combined with an *Xho*I-*Bam*HI fragment containing the *PYK* 5' region from an X-series deletion to generate molecules having internal deletions in the *PYK* 5' region. The 5' endpoint of each deletion was provided from a pKY56Bg series deletion, and the 3' endpoint was from an X-series deletion. This internal deletion set was named "Y" or "W" series (Fig. 2).

All deletion endpoints were sequenced by the dideoxy sequencing method (35), using synthetic oligonucleotide primers. The coordinate of each endpoint refers to the position of the last remaining base and is used to designate individual plasmid number; for example, X217 plasmid had the deletion endpoint at position −217. Since the 3' endpoint of the deletions in the Y series was fixed at −217, numerals following Y indicated the 5' endpoints of the internal deletions. With the W series, the positions of both endpoints were used to designate particular plasmid number (Fig. 3).

Assay methods. $CatO_2$ase was assayed with either whole cells (plate assay) or crude extracts. The plate assay was performed by spraying the plates with 0.5 M pyrocatechol in water and observing the development of yellow color on yeast colonies (44). Crude extracts were prepared as follows. Yeast transformants were inoculated into 3.5 ml of defined medium and incubated at 30°C for 18 h. A 100-μl portion of the culture was inoculated into 50 ml of fresh defined medium, which was incubated at 30°C until the A_{600} of the culture reached 1.0. Yeast cells were harvested from 2.5 ml of the culture, washed once with 0.01 M phosphate buffer, pH 7.0, and suspended in 1 ml of 0.1 M phosphate buffer, pH 7.0, containing 10% acetone (34). An equal volume of glass beads (0.45 mm in diameter) was added to the tube, and the

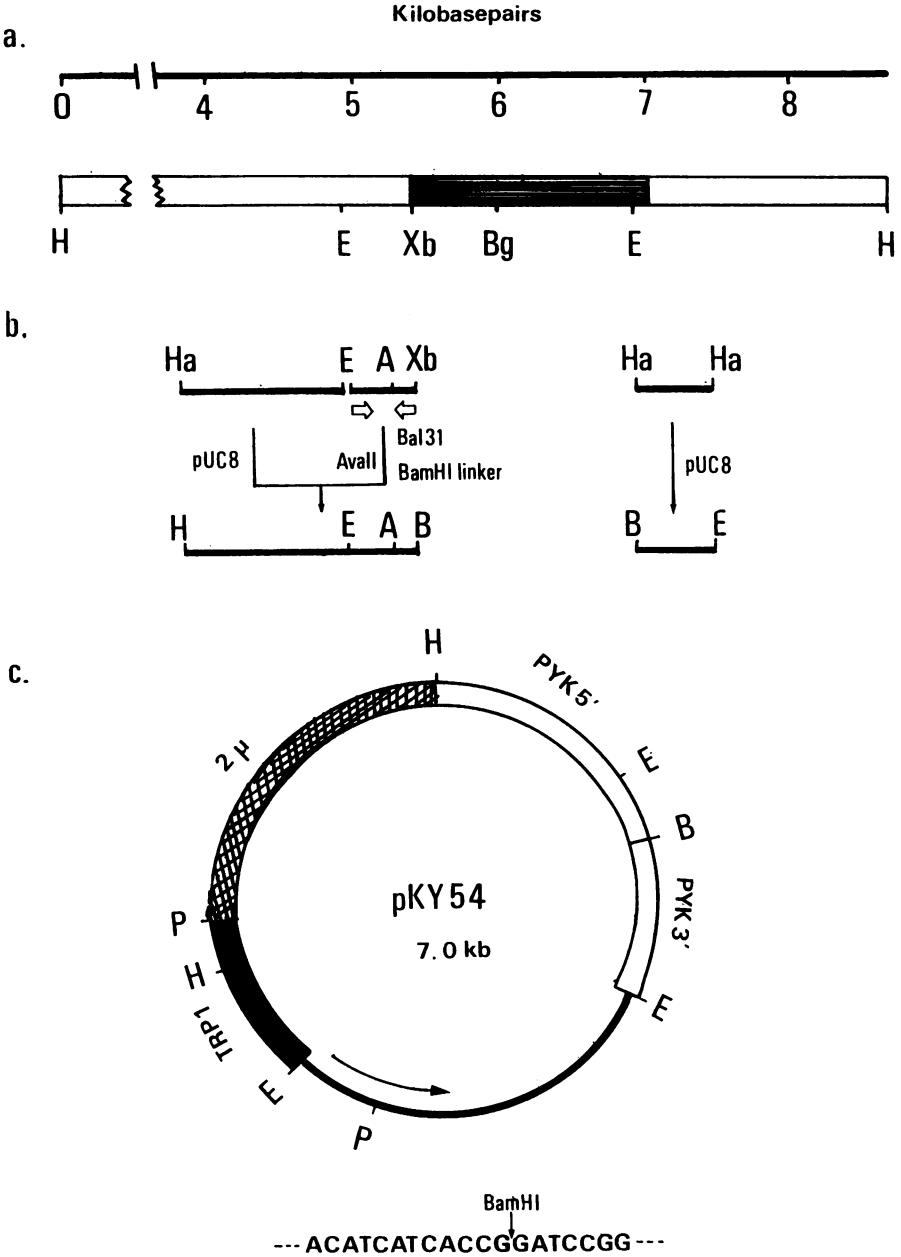

FIG. 1. Cloning of the *PYK* gene and construction of pKY54. (a) The open box represents the cloned 8.8-kb *Hin*dIII fragment, and the closed box designates the protein-coding region (6). (b) The solid bars designate the DNA fragments used to construct pKY54. The open arrows indicate the direction of digestion with *Bal*31 exonuclease. The construction procedure is described in detail in the text. (c) pKY54 carries 1.4- and 0.5-kb DNA fragments derived from 5′- and 3′-noncoding regions of *PYK*, the yeast 2μm origin (*Hin*dIII-*Pst*I fragment of B form), *TRP1* marker, and the pBR322 sequence containing origin of replication and the ampicillin resistance gene (indicated by the arrow inside the circle). The partial nucleotide sequence containing the *Bam*HI cloning site is shown at the bottom. Restriction enzymes are as follows: A, *Ava*II; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae*III; P, *Pst*I; Xb, *Xba*I.

cells were disrupted by vortexing the tube at 4°C. Cell debris and glass beads were removed by centrifugation at 1,600 × *g* for 5 min, and the supernatant was used as the enzyme source.

A 600-μl amount of the crude extract was added to the tube containing 2.4 ml of 0.1 M phosphate buffer, pH 7.0, and the reaction was started by adding 20 μl of 10 mM pyrocatechol in water at 30°C. The increase in A_{375} was recorded with a Shimadzu UV-160 spectrophotometer. One unit of $CatO_2$ase was defined as the amount causing the oxidation of 1 nmol of catechol per min at 30°C, using a molar extinction value of 3.3×10^4 of the oxidation product (22).

Protein was assayed by the modified Lowry method (32). The activity of β-galactosidase was assayed as described by Miller (29).

Estimation of plasmid copy number. Total DNA from yeast transformants was digested with *Eco*RI, electrophoresed on a 0.7% agarose gel, and transferred to Biodyne transfer membrane (Paul Corp., Glen Cove, N.Y.) by the method described by Southern (38). Southern filters were probed with the *TRP1*-specific fragment which was 5′ end labeled

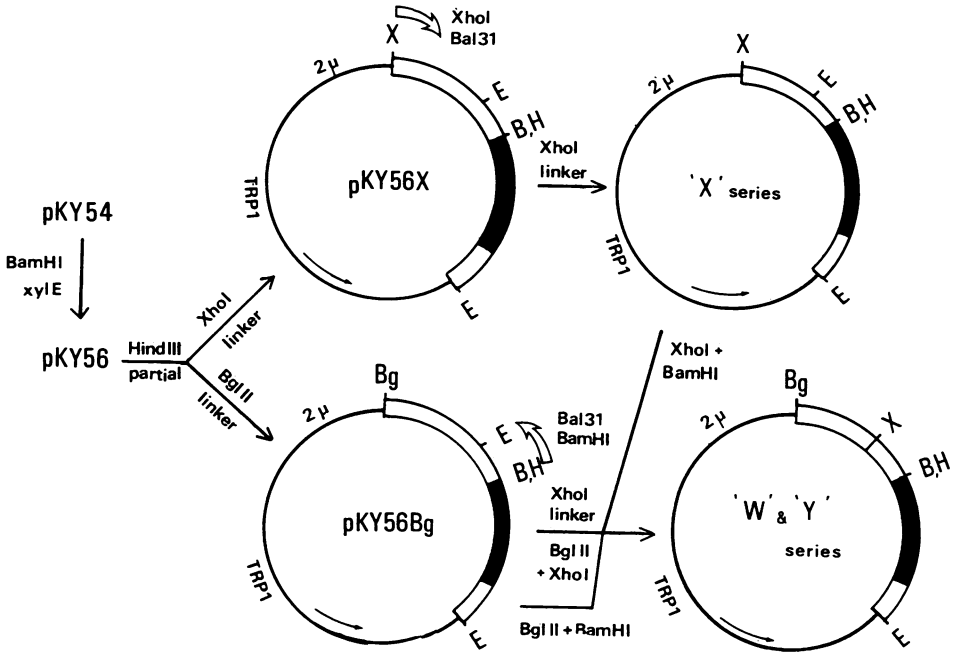

FIG. 2. Construction of deletions in the 5'-noncoding region. Construction of pKY56 is described in Materials and Methods. Since pKY56 had three *Hin*dIII sites (two from pKY54 and one from the polylinker sequence preceding the *xylE* coding sequence), the molecule which had been cut uniquely at the site located at the 5' end of the *PYK* 5'-noncoding region was selected, and its termini were filled in and ligated in the presence of an *Xho*I or a *Bgl*II linker to generate pKY56X or pKY56Bg. Deletions were generated from the unique *Xho*I site in pKY56X and from the unique *Bam*HI site in pKY56Bg by *Bal*31 exonuclease. Deletion endpoints were filled in and converted to the *Xho*I site by ligation of an *Xho*I linker. For further details of construction of the X, Y, and W series of deletion plasmids, see the text. Each deletion endpoint of the mutant is signified by an *Xho*I site. Since an *Eco*RI site is located at −477 in the 5'-noncoding region, the *Xho*I-*Bam*HI fragment derived from the X-series deletion plasmid does not always possess an *Eco*RI site. Symbols: Open line, *PYK* 5'- and 3'-noncoding regions; solid line, *xylE* coding region; thin line, pBR322, *TRP1*, and 2μm portion of pKY54. X is *Xho*I; other restriction enzymes are as given in the legend to Fig. 1.

with [γ-^{32}P]ATP, (5,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) at 42°C for 18 h, rinsed, and exposed to Kodak X-Omat film.

Transcription start site mapping. The mRNA start site of selected deletion mutants was determined by the S1 mapping method (27). A *PYK* probe was prepared as follows: plasmid X217 was cut with *Ava*I, dephosphorylated with calf intestine alkaline phosphatase, and digested with *Xho*I to generate a 645-bp *Xho*I-*Ava*I fragment. This fragment was 5' end labeled on the strand complementary to the mRNA, using [γ-^{32}P]ATP and T4 polynucleotide kinase. Total yeast RNA (200 μg) was hybridized with the probe (ca. 7.7 × 10^5 cpm/15 ng) at 80°C for 15 min and then at 37°C for 3 h, and the hybrids were digested with 20 U of S1 nuclease at 30°C for 30 min. The digests were electrophoresed on 6% polyacrylamide–urea sequence gel to resolve protected fragments.

RESULTS

Cloning of the *PYK* gene and construction of pKY54. Cloning of the *PYK* gene of *S. cerevisiae* was reported by Kawasaki and Fraenkel (21) and Burke et al. (6). We synthesized an oligonucleotide probe according to their published sequence and have cloned the *PYK* gene by screening a yeast gene bank with the probe. Restriction mapping analysis (Fig. 1) and the ability of the cloned DNA fragment to complement the *pyk1-5* mutation (data not shown) confirmed the identity of the isolated *PYK* gene. By using ca. 1.4 kb of the 5'-noncoding and 0.5 kb of the 3'-noncoding regions of the *PYK* gene, the expression vector pKY54 was constructed (Fig. 1). pKY54 was able to direct expression of the *xylE* gene of *P. putida* (18, 44) in yeasts, indicating that the 5'-noncoding region of *PYK* on pKY54 has a promoter activity and that $CatO_2$ase of *P. putida* was active in yeasts as well as in *E. coli* and *Bacillus subtilis* (44). This enabled us to use the *xylE* system to monitor the activity of the *PYK* promoter.

Mapping of *cis*-acting positive regulatory elements within 5'-noncoding region. To identify the *cis*-acting positive elements of *PYK* transcription, we made a series of deletions in the 5'-noncoding region of the *PYK* gene and analyzed their effect on expression of the *xylE* gene when yeast cells were grown on glucose (Fig. 3).

In X-series deletion mutants, expression of $CatO_2$ase activity was obtained if the 5'-noncoding region retained 653 nucleotides but became undetectable on plates when deletions were extended to position −641 and beyond to position −217. These results suggest that the apparent 5' endpoint of a positive regulatory element maps between positions −653 and −641. When $CatO_2$ase was assayed in crude extracts prepared from selected mutants, the results coincided with those of the plate assay: the enzyme activity dramatically decreased to almost zero when the deletion was extended to −642, while the X653 mutant plasmid resulted in only a 20% loss in activity. Plate-negative clones tested gave no detectable activity in crude extracts.

Y- and W-series deletion mutants have internal deletions. Mutant plasmids having deletions covering the region between −652 and −603 failed to express $CatO_2$ase activity with a few exceptions (W652-311, Y652, and Y714 in Fig. 3).

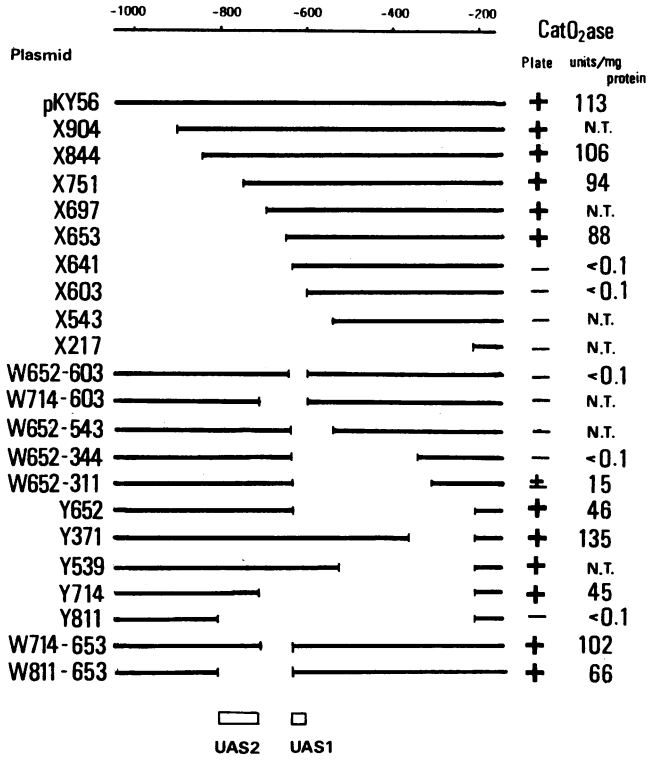

FIG. 3. Diagrammatic representation of the *PYK* 5′-noncoding region showing the internal deletions and their effects on *xylE* expression. Coordinates of deletion endpoints are given relative to the ATG of the *PYK* coding region and are shown as plasmid numbers. The ATG of *xylE* is located 22 bp downstream from that of *PYK* in all deletion plasmids because of intervention between the *PYK* 5′-noncoding sequence and the *xylE* coding sequence of a *Bam*HI linker and the polylinker sequence of M13tg127 to which the gene had been cloned. $CatO_2ase$ activities are as follows: +, development of yellow color within a few minutes; ±, pale yellow color; −, no color change observed 1 h after treatment; NT, not tested. Enzyme activities given are the average of three separate experiments. Standard deviations are between 3.08 and 16.5. Two UAS regions identified are shown as open boxes at the bottom.

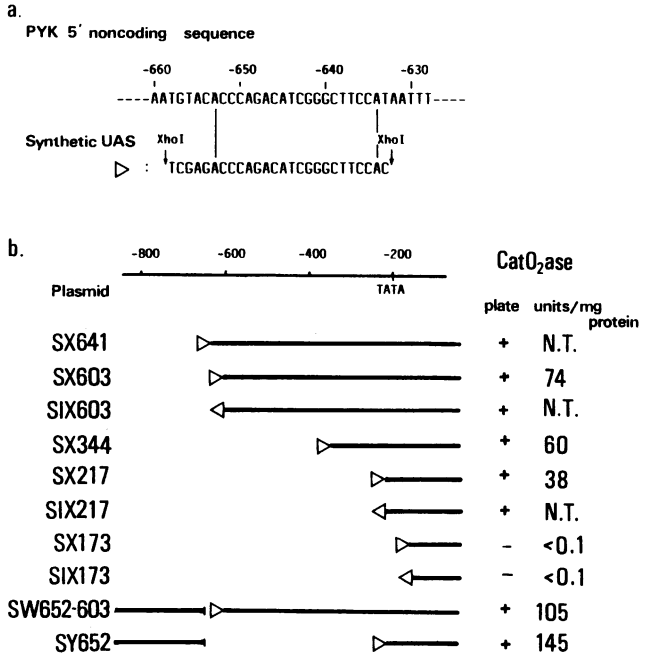

FIG. 4. Nucleotide sequences of the $UAS_{PYK}1$ region and the synthetic UAS (a) and recovery of expression of $CatO_2ase$ activity by insertion of the synthetic $UAS_{PYK}1$ (SU) into deletion plasmids (b). (b) SU was inserted at the 3′ endpoint of each deletion, and its orientation is designated by the direction of an open arrowhead. The resulting plasmids were named by adding S or SI, according to the orientation of SU, before the names of the plasmids into which the oligonucleotide was inserted. For $CatO_2ase$ activities, see the legend to Fig. 3.

(These exceptional cases will be mentioned below.) On the other hand, all plasmids retaining this region were able to direct the $CatO_2ase$ expression regardless of the position of the deletion (Fig. 3). These results suggest that the sequence between −653 and −603 contains at least part of the essential *cis*-acting positive regulatory element whose 5′ endpoint is between −653 and −641.

The plasmids lacking the sequence between −653 and −603 but positive by the plate assay (W652-311, Y652, and Y714 in Fig. 3) appeared to express much lower activities than the wild type. The enzyme activity became detectable only when the 3′ endpoint of the internal deletion was extended to −311, and it reached 40% of the wild-type level when the 3′ endpoint was at −217. The expression level was not changed by the extension of the 5′ endpoint of deletion from −652 to −714 (Y652 and Y714 in Fig. 3), but was reduced to an undetectable level when the 5′ endpoint was at −811 (Y811). These results indicate that the sequence between −811 and −714 functions as another *cis*-acting positive regulatory element depending on the distance from the TATA sequence. The data with plasmids W714-653 and W811-653 (Fig. 3) suggest another possible role of this element: plasmid W714-653 containing both positive regulatory elements could direct approximately the wild-type level of $CatO_2ase$ expression, whereas plasmid W811-653 lacking the "upstream" element (the sequence between −811 and −714) showed a 40% loss of activity. This indicates that the upstream element may be required for full activation of the *PYK* promoter while the "downstream" element (the sequence downstream of −653) is essentially required for the promoter activity.

Synthetic UAS_{PYK} can restore promoter activity. To confirm that the downstream element is an essential one, we synthesized an oligonucleotide corresponding to the sequence from −653 to −634 and having an *Xho*I site at both ends (Fig. 4a). The synthetic DNA (termed SU) was inserted at the 3′ endpoint of the deletions, and the resulting plasmids (named by adding S before the names of the plasmids into which SU was inserted) were analyzed for ability to direct $CatO_2ase$ expression (Fig. 4b). All but two plasmids gave $CatO_2ase$-positive transformants, indicating that SU could recover the promoter activity of the *PYK* 5′-noncoding region of the deletion plasmids. Since plasmids SW652-603 and SY652 could direct the wild-type level of $CatO_2ase$ expression, we conclude that, at most, 20 bp corresponding to the sequence from −653 to −634 is essential for the promoter function of the *PYK* 5′-noncoding region, and we call this sequence $UAS_{PYK}1$. Figure 4 also shows that $UAS_{PYK}1$ was functional in either orientation.

SW652-603 and SY652 provided wild-type levels of $CatO_2ase$ activity (Fig. 4). Since they have the upstream element in the 5′-noncoding region, it is likely that the upstream element is required for full activation of the *PYK* promoter. Therefore, we termed it $UAS_{PYK}2$.

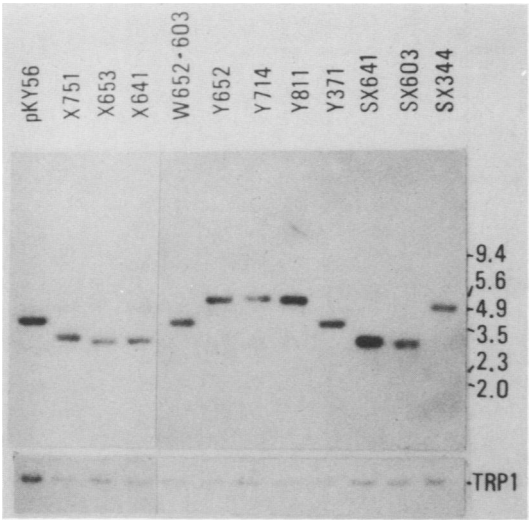

FIG. 5. Copy numbers of deletion plasmids by Southern analysis of *Eco*RI-digested total DNA from transformants. Southern filters were probed with a *TRP1*-specific fragment labeled with ^{32}P. The relative intensities of plasmid *TRP1* to chromosomal *TRP1* bands (inserted at the bottom) gave a rough estimate of plasmid copy number. Plasmid *TRP1* bands were developed after overnight exposure, and chromosomal *TRP1* bands were developed after 1 week of exposure. The positions of *Eco*RI and *Hin*dIII digests of lambda DNA are also shown (in kilobase pairs).

Plasmids SX173 and SIX173, which failed to restore $CatO_2$ase expression, had 3′ endpoints of the deletion at −173. In these cases, one of the two possible TATA sequences (at −199 and −148 [6]) was eliminated. Thus, it is possible that the TATA sequence at −199 is required for transcriptional activation of the *PYK* promoter.

$CatO_2$ase activity correlates with the level of *xylE* mRNA. Since we assayed the promoter activity of the *PYK* 5′-noncoding region as the $CatO_2$ase activity on a multicopy plasmid, it was necessary to confirm that the level of the enzyme activity really represented that of the transcriptional activity of the promoter. For this purpose, we used Southern analysis (38) to estimate plasmid copy number and determined the level and start site of the mRNA by the S1 mapping method (27).

Figure 5 shows the result of Southern analysis of DNA isolated from selected mutants, using a ^{32}P-labeled *TRP1* fragment as a probe. Because an *Eco*RI site is located at −477 in the *PYK* 5′-noncoding region (Fig. 1 and 2), the size of an *Eco*RI fragment containing the *TRP1* sequence is different in individual plasmids. Using the signal intensity provided by the chromosomal *TRP1* sequence as reference, we can conclude that there was no significant difference in the copy number of the plasmids tested; W652-603 and other mutant plasmids which conferred no detectable activities of $CatO_2$ase (Fig. 3) were present in copy numbers equivalent to that of the pKY56 wild-type plasmid (Fig. 5). This indicates that loss of $CatO_2$ase activity was not caused by a concomitant loss of plasmids from the cell.

Next, we analyzed the *xylE* mRNA by S1 mapping techniques. All protected fragments migrated similarly (Fig. 6), indicating that transcription started at the same or very close to the original site (position −33 [6]) in deletion mutants and that there was no readthrough transcription from the vector in tested mutants. The signal intensity of the protected fragments in Fig. 6 shows good correlation with the $CatO_2$ase activity conferred by individual plasmids (Fig. 3 and 4): the intensity decreased in parallel with enzyme activity, and no protected fragments were observed in the absence of activity (X641, Y811, and W652-603 in Fig. 6). Insertion of SU into the deletion plasmids restored transcription and the wild-type levels of the mRNA (S series in Fig. 6), confirming that $UAS_{PYK}1$ is an essential positive element. $UAS_{PYK}2$ could also direct the initiation of transcription from or close to the original site (Fig. 6, Y652 and Y714), indicating that this element is able to function as a transcriptional activating sequence in particular constructions. From these results, we conclude that the $CatO_2$ase assay system used in this study reflects the transcriptional activity of the *PYK* promoter region.

$UAS_{PYK}1$ functions in *CYC1-lacZ* system. To determine whether $UAS_{PYK}1$ is active in a heterologous system, a *CYC1-lacZ* hybrid (15) was used. Plasmid pAKI004 was constructed from pLGΔ-312 (15) by removing the two UAS of the *CYC1* gene while leaving the TATA sequence intact (17), resulting in a loss of expression of the *lacZ* gene. When $UAS_{PYK}1$ was placed upstream of the TATA sequence by inserting SU (S15-1 plasmid), expression of β-galactosidase activity was restored (Fig. 7). This clearly shows that $UAS_{PYK}1$ functions as a transcription activator in a heterologous system.

Mapping of *cis*-acting negative regulatory element within the 5′-noncoding region. Since glucose is known to induce the expression of glycolytic enzymes including *PYK* (25), we studied whether a sequence responsible for this induction resides within the 5′-noncoding region of *PYK* by cultivating yeast cells harboring pKY56 in a medium containing either glucose or glycerol plus ethanol as the carbon source and by assaying the $CatO_2$ase level. Under gluconeogenic conditions, a 70% reduction in transcription was observed with pKY56 (Fig. 8), suggesting that the *PYK* expression was carbon source dependent and that the 5′-noncoding region mediated the regulation. Therefore, we analyzed internal deletion mutants to map the sequence responsible for the carbon source dependency. SY652 provided ca. 2.5-fold more activity than the wild-type plasmid under glycerol-plus-ethanol conditions (Fig. 8), indicating the presence of a transcription repression sequence between −652 and −217. We observed a 2.7-fold increase when the 5′ end of the deletion was at −468 and the 3′ end was at −344, and the activity decreased to the wild-type level when the 5′ end of the deletion was at −371 or the 3′ end of the deletion was brought to −445 (Y371 and W580-445, Fig. 8). These results suggest that the region between −468 and −344 contains a repressible sequence responsible for low transcriptional activity of the *PYK* promoter when yeast cells utilize glycerol plus ethanol as the carbon source. Plasmid X653, which does not have the $UAS_{PYK}2$ sequence, and plasmid W580-538, which has a 43-bp deletion between the UAS and TATA, were subject to repression of transcription similar to that of the wild type under gluconeogenic conditions, indicating that $UAS_{PYK}2$ does not play a major role in repression of transcription and that the activation observed in internal deletion mutants is not caused by a mere change in spacing between the UAS and TATA. Therefore, we termed the sequence between −468 and −344 an upstream repressible sequence (URS_{PYK}).

Removal of the URS_{PYK} also activated transcription under glucose conditions (Fig. 8). With plasmid Y468, which has an internal deletion between −468 and −217, the $CatO_2$ase activity increased by 1.6-fold over the wild-type level. This suggests that URS_{PYK} represses transcription

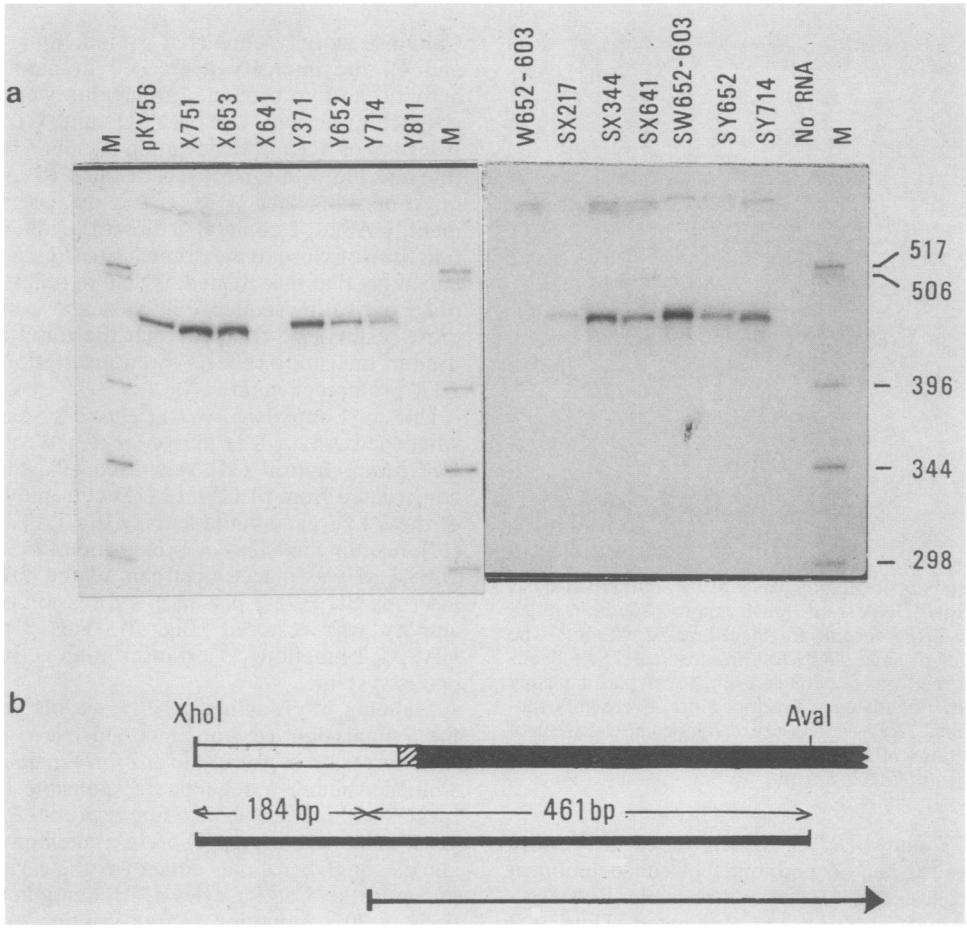

FIG. 6. Mapping of the 5′ end of the *xylE* mRNA transcribed from wild-type and mutant plasmids. (a) Total RNA was isolated from transformants harboring an individual plasmid (indicated at the top of the gel), hybridized with the labeled DNA probe, digested with S1 nuclease, and analyzed as described in Materials and Methods. A control reaction containing no RNA was carried out and analyzed simultaneously. *Hin*fI digests of pBR322 were used as marker DNA, and their sizes (in base pairs) are given on the right. The faint uppermost band on the gel is undigested DNA probe. (b) Diagram of the structure of *PYK-xylE* hybrid. Symbols: Open box, *PYK* 5′-noncoding sequence; hatched box, linker sequence; solid box, *xylE* coding sequence. The solid bar indicates the fragment used as DNA probe, and the arrow designates the expected direction of transcription.

under both fermentable and nonfermentable carbon sources, but the repression is more drastic under gluconeogenic conditions.

To confirm the presence of a URS between −468 and −344, we isolated a 198-bp *Eco*RI-*Rsa*I fragment corresponding to the DNA sequence between −472 and −275, inserted it into the *XhoI* sites of plasmids Y468 and Y652, and assayed the $CatO_2$ase level. Y468A showed a reduction in transcription to the wild-type level and Y652A abolished transcription. Surprisingly, when this fragment was inserted in an inverted direction, transcription was repressed almost completely in both cases (Y481IA and Y652IA, Fig. 8). We also inserted a 112-bp chromosomal DNA fragment at the *XhoI* site of plasmid Y468, resulting in the same level of activation of transcription as URS deletions (data not shown). These results indicate that URS_{PYK} actually resides within the *Eco*RI-*Rsa*I fragment, that the repression is much more potent when the URS is placed in an inverted orientation between the UAS and the TATA sequence, and that the distance effect observed in the transcriptional activation by $UAS_{PYK}2$ (Fig. 3) can be explained by the presence of the URS.

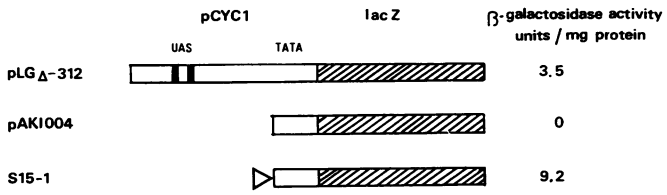

FIG. 7. Diagram of *CYC1-lacZ* hybrid and insertion of the synthetic $UAS_{PYK}1$. Only regions of interest on each plasmid are shown. Symbols: Open box, *CYC1* 5′-noncoding region; solid box, two UAS of *CYC1*; hatched box, *lacZ* coding sequence. Open arrowhead indicates the synthetic $UAS_{PYK}1$.

DISCUSSION

We have initiated an analysis of *cis*-acting regulatory elements in the 5′-noncoding region of the yeast *PYK* gene to clarify regulation of *PYK* expression by the carbon source. In the work we describe, we have identified two UAS and a URS of the *PYK* gene, $UAS_{PYK}1$, $UAS_{PYK}2$, and URS_{PYK}, between positions at −653 and −634, −811 and −714, and −468 and −344, respectively. We conclude that $UAS_{PYK}1$ is an essential *cis*-acting positive regulatory element of the

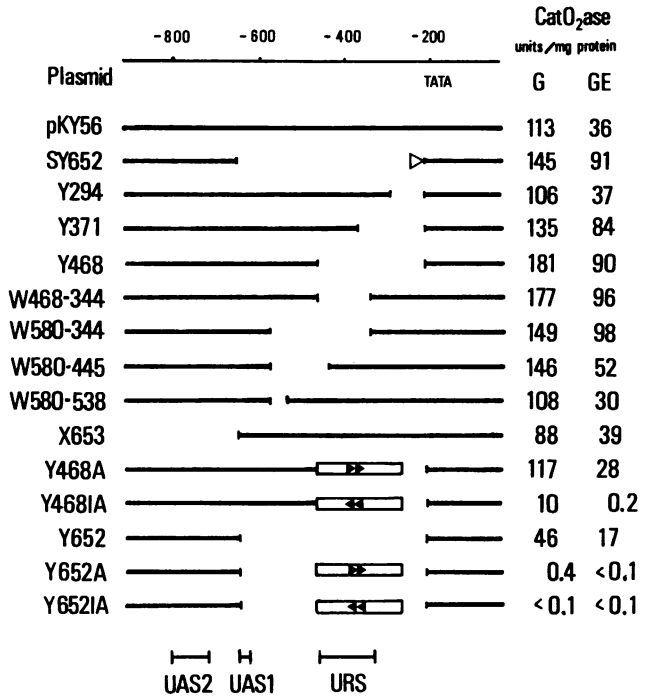

FIG. 8. Mapping of a URS in the *PYK* 5'-noncoding region. Coordinates of deletion endpoints are as in the legend to Fig. 3. All values of enzyme activities are averages of three separate experiments, using extracts from glucose-grown (G) or glycerol-plus-ethanol-grown (GE) cells. The standard deviations are between 3.08 and 16.5. An open arrowhead indicates SU, and open boxes indicate a 198-bp *Eco*RI-*Rsa*I fragment. Closed arrowheads designate the orientation of the fragment.

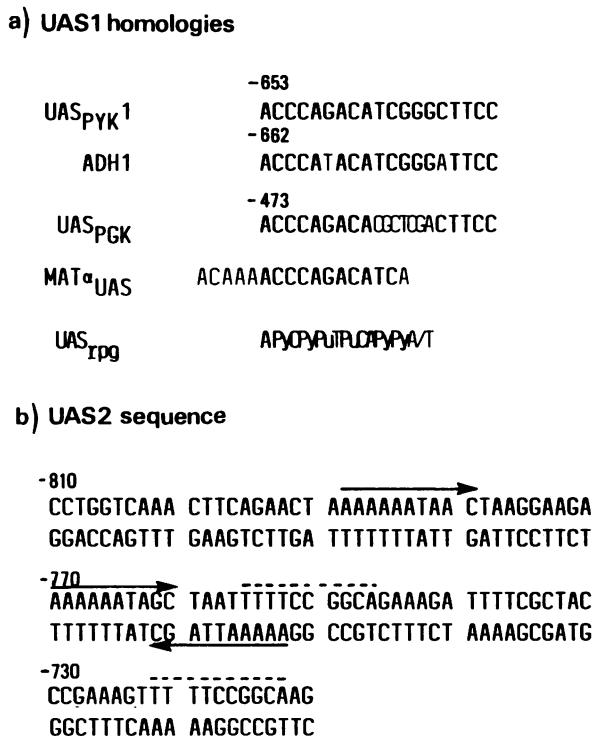

FIG. 9. Homologous sequences with $UAS_{PYK}1$ and the nucleotide sequence of the $UAS_{PYK}2$ region. (a) $UAS_{PYK}1$ homolog found in 5'-noncoding regions of *ADH1*, *PGK*, *MATα*, and the consensus sequence of UAS_{rpg}. Matching nucleotides are in boldface (b) Nucleotide sequence of the $UAS_{PYK}2$ region. Arrows indicate a possible direct repeat and an inverted repeat. A dashed line above the sequence shows another direct repeat.

PYK promoter. Like other yeast UAS, $UAS_{PYK}1$ is functional in either orientation and does not show strict position dependency as long as it is placed upstream of the TATA at −199. $UAS_{PYK}1$ does not contain dyad symmetry as observed in UAS_{GAL} (42) or repeated sequences as in UAS_{PGK} (31).

When we looked for homologies with $UAS_{PYK}1$ in other glycolytic enzyme genes of *S. cerevisiae* by a computer search with an IDEAS program (20) and by eye, a strikingly homologous sequence was found in the 5'-noncoding region of *ADH1*: 19 bp starting from position −662 (ATG as +1 [3]) coincided exactly, with the exception of two nucleotides (Fig. 9a). We do not know whether this sequence in the *ADH1* upstream region is a UAS of *ADH1*. Another sequence with less homology (14 of 21 nucleotides) was found in the UAS of the *PGK* gene (Fig. 9a) (31). These homologies suggest the possibility of a common regulation of these genes. Apart from glycolytic enzyme genes, the *MATα1/α2* UAS (37) has a homologous sequence (11 of 17 nucleotides; Fig. 9a), and the matched nucleotides show a coincidence with the consensus sequence of the UAS of ribosomal protein genes (43).

$UAS_{PYK}2$ is defined as an element required for full transcriptional activation of the *PYK* promoter and is able to activate transcription if the sequence containing a URS has been removed. A *cis*-acting positive element required for full activation is also reported in the long terminal repeat of human immunodeficiency virus type 2 (12). The $UAS_{PYK}2$ sequence contains direct repeats and an inverted repeat (Fig. 9b), suggesting that this region could provide protein-binding sites to activate transcription.

The result obtained with plasmid SY652 (Fig. 4) indicates that the two UAS and the TATA sequence are the only essential elements for transcriptional activation in the 5'-noncoding region and that their spacings are flexible (W714-653, Fig. 3).

The URS functions as a transcription repression sequence in cells grown on either a fermentable or a nonfermentable carbon source, although the strength of repression depends on the carbon sources. Cohen et al. reported the presence of a URS in the 5'-noncoding region of *ENO1*, which represses the glucose-dependent induction of *ENO1* expression and thus maintains its constitutive expression (9). URS_{PYK} also represses transcription to some extent in glucose-grown cells, but its major role seems to be that of repressing transcription when cells are grown on gluconeogenic carbon source. Glucose is known to induce yeast pyruvate kinase (25). Our finding of a URS in the 5'-noncoding region of *PYK* suggests the possibility of derepression of transcription by glucose. We speculate that the URS plays a key role in the regulation of *PYK* expression in response to the carbon source in the medium. When a nonfermentable carbon source becomes prevalent in the medium, or the amount of fermentable carbon source decreases as yeast cells grow, the URS of *PYK* could shut off *PYK* expression in response to the carbon source shift to enable cells to make a change in the metabolism flux from glycolysis to gluconeogenesis.

Recently, Purvis et al. reported the existence of the *cis*-acting positive regulatory sequence in the *PYK* coding region (downstream activation site [33]). Our data show that two UAS and the TATA are required for activation, and a

URS is required for repression, of transcription of a gene placed downstream of the *PYK* 5′-noncoding region. The downstream activation site might be involved in the control mechanism of *PYK* transcription together with the UAS and the URS.

Although we have identified three regulatory sites by deletion analysis, the use of a multicopy plasmid raises a problem that a fluctuation in copy number might result in a variation of the expression level. We cannot exclude this possibility in the case of $UAS_{PYK}2$ since UAS2 contributes only a 20 to 40% change of the expression level in its requirement for full activation. Identification of a UAS2 binding factor will be required to solve this problem.

cis-acting positive and negative sites are often the binding sites of *trans*-acting factors, which interact directly or indirectly with RNA polymerase to regulate initiation of transcription (40). Two UAS and a URS of *PYK* may represent such binding sites, and the carbon source-dependent regulation of *PYK* expression could be realized through formation or modulation or both of activities of UAS and URS complexes. An observation supporting this notion comes from URS deletion mutants: the level of transcriptional activity in a transformant grown on glucose suggests that the UAS complex is more active under glucose conditions than gluconeogenic conditions (Fig. 8). On the other hand, the observation that repression of transcription is more severe under gluconeogenic conditions implies that a more potent URS complex is formed under gluconeogenic conditions to secure the repression of *PYK* expression. Thus, we hypothesize that both UAS and URS complexes are modulated in response to the carbon source in the medium: the amount and/or activity of the UAS complexes increases and those of the URS complex decrease when cells grow on glucose, and vice versa when cells grow on a gluconeogenic carbon source.

A silencer binding protein, RAP1 (36) or GRF1 (5, 24), is reported to bind to the *MATα* UAS, and is likely to be a transactivation factor that binds to $UAS_{PYK}1$. Requirement of $UAS_{PYK}2$ for full activation of transcription suggests the possibility that a UAS2-protein complex modifies the activity of a UAS1-RAP1/GRF1 complex by interacting with it. A URS-protein complex may interfere with transcriptional activation by both UAS complexes. Thus, a combination of a general factor (RAP1/GRF1) and a specific factor(s) may control expression of the *PYK* gene. Stanway et al. argued that a modulator domain in the UAS_{PGK} is responsible for the carbon source-dependent regulation of the *PGK* gene (39). However, we found no significant homology between the modulator sequence and $UAS_{PYK}2$ or URS_{PYK} except for a "GAAAG" motif found in these sequences (at −746 and −728 in $UAS_{PYK}2$, at −416 and −409 on a complementary strand in the URS, and at −527 in *PGK*). This motif is also found in the UAS of the *ENO2* gene (at −444 [8]). No significant homologies were observed in the *ENO1* URS either.

At present, we do not know the nature of a UAS2 factor or a URS factor, but we have detected those binding factors by gel mobility-shift analysis (M. Nishizawa, unpublished observation). The *GCR1* product functions as a positive regulator of many glycolytic enzyme genes including *PYK* (1, 7; Nishizawa, unpublished observation). Thus, it may affect some step in the transcriptional activation process. Currently, we are investigating the nature of UAS2 and URS factors and the effect of a *gcr1* mutation on formation of UAS and URS complexes. Those studies will lead to further clarification of the regulation of *PYK* expression and overall control of the glycolytic pathway in *S. cerevisiae* by the carbon source.

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