Characterization of *TPI* gene expression in isogenic wild-type and *gcr1*-deletion mutant strains of *Saccharomyces cerevisiae*

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ABSTRACT

In *Saccharomyces cerevisiae* the enzymes of glycolysis constitute 30 – 60 percent of the soluble protein. *GCR1* gene function is required for high level glycolytic gene expression. We have undertaken a biochemical and genetic characterization of *TPI*, a gene affected by *gcr1* lesions. Northern analysis showed that steady-state levels of *TPI* transcripts are severely reduced in *gcr1* mutant strains. However, primer extension experiments revealed that *TPI* transcripts isolated from wild-type and *gcr1* mutant strains have identical 5' ends. To map the 5' boundary of *TPI* controlling region, we employed a *TPI::lacZ* gene fusion carrying 3.5 kb 5' to the translational start of the *TPI* structural gene. Nuclease *Bal31* deletion analysis demonstrated that sequences sufficient for high level expression of *TPI* reside within 392 nucleotides preceding the start of the structural gene. We have identified GRF1/RAP1/TUF-binding site positioned 339 to 349 bp 5' to the translation start of *TPI*. DNA band shift assays were carried out with wild-type and *gcr1* deletion mutant strains, and similar patterns of band shifting were observed.

INTRODUCTION

In *Saccharomyces cerevisiae* the enzymes of glycolysis constitute the major fraction of soluble protein in the cell (1,2). Evidence for coordinate expression of genes encoding glycolytic enzymes came from *gcr1* mutant strains. These strains have severely reduced levels of most glycolytic enzymes, but most other cellular functions are unaffected by *gcr1* lesions (3,4). *GCR1* has been cloned (5) and sequenced (6,7). However, the mechanism by which *GCR1* brings about high level expression of genes encoding glycolytic enzymes remains unknown.

Recently, the regulatory protein known as general regulatory factor 1 or repressor activating protein 1 (GRF1/RAP1) has been implicated in the high level expression of *PGK* (8), *ENO1* (9), and *PYK* (9) which encode the glycolytic enzymes phosphoglycerate kinase, enolase, and pyruvate kinase, respectively. Capieaux et al. (10) have suggested that

GRF1/RAP1 is the same protein as TUF which has been proposed to be a component of a generalized transcriptional mechanism for control of yeast growth (11). Sequences close to the consensus sequence for the GRF1/RAP1/TUF-binding site have been found in front of several other genes encoding glycolytic enzymes (8,10,12). This observation suggests that GRF1/RAP1/TUF may also be involved in their high level expression.

Here we focus our attention on a genetic and biochemical characterization of the controlling region of the gene, *TPI*, which encodes the glycolytic enzyme triose phosphate isomerase (E.C. 5.3.1.1). The expression of *TPI* is affected by *gcr1* mutations (4), and, as pointed out by Chambers et al. (8), in its 5' nontranslated region there is a putative GRF1/RAP1/TUF-binding site. In wild-type yeast triose-phosphate isomerase makes up about 2% of the soluble protein (13). In *gcr1* mutant strains its levels range from 0.18 to 0.48% of the soluble protein depending on the growth medium, with the higher level being observed with extracts from cultures grown in the presence of glucose (3,6). In the present study we characterized the *TPI* transcript, and we used *Bal31* deletion analysis and DNA band shift assays in conjunction with isogenic wild-type and *gcr1*-deletion mutant strains in an effort to identify sequences important for the expression of *TPI* and to further investigate the role *GCR1* plays in the expression of genes encoding glycolytic enzymes.

MATERIALS AND METHODS

Yeast strains

Yeast strains used during the course of this study are shown in Table 1.

Growth conditions and preparation of yeast extracts

Cultures were grown in YP medium (14) supplemented with 2% glucose or 2% lactate and 2% glycerol. In experiments with isogenic wild-type and *gcr1*-deletion mutant strains where the effect of carbon source was tested, cultures were started in YP medium supplemented with 2% lactate and 2% glycerol. Four

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Table 1.

Strain	Genotype	Source, Reference
DFY510	<i>MATa</i> , <i>leu2-3,112</i> , <i>his3-11,15</i> , <i>can1</i>	(6)
DFY520	<i>MATa</i> , <i>gcr1Δ::LEU2</i> , <i>leu2-3,112</i> , <i>his3-11,15</i> , <i>can1</i>	(6)
DFY578	<i>MATa</i> , <i>his3-11,15</i> , <i>can1</i>	This work
S150-2B	<i>MATa</i> , <i>leu2-3,112</i> , <i>his3Δ</i> , <i>trp1-289</i> , <i>ura3-52</i>	(A. Lewin)
HBV4	<i>MATa</i> , <i>gcr1Δ::HIS3</i> , <i>leu2-3,112</i> , <i>his3Δ</i> , <i>trp1-289</i> , <i>ura3-52</i>	This work
YRAP	<i>MAT</i> , <i>ade2-1</i> , <i>ura3-52</i> , <i>leu2</i> , <i>trp1</i> , <i>can1-100</i> , <i>his3</i> , <i>rap1::leu2-pUC-URA3-RAP</i> 894-1584	(D. Shore)

hours prior to harvest, half of each culture was shifted to glycolytic medium by the addition of glucose (final concentration 2%) to the growth medium. The cultures (mid-to late-logarithmic growth phase) were harvested by centrifugation, washed, and suspended in A(200) buffer (15) (3 ml/g of cell pellet), and then the extracts were prepared by passage through a French pressure cell as described previously (4). Cell debris was removed by centrifugation, and the protein concentration was determined by the method of Bradford (16).

Nucleic acid manipulations

Techniques used throughout the course of this study are described in the standard reference manuals (17,18,19). *S. cerevisiae* DNA and RNA was prepared by the methods of Sherman et al. (14) and Struhl and Davis (20), respectively.

Primer extension

Total RNA isolated from yeast strains S150-2B and HBV4 was used to determine the 5' end of the *TPI* transcripts. Total RNA, 10 μ g and 25 μ g from the wild-type and the *gcr1*-deletion mutant strains respectively, was annealed to a radiolabeled primer (CC-ACCGACAAAGAAAGTTCTAGCC) which hybridized near the 5' end of the *TPI* structural gene. The primer was extended with reverse transcriptase, and the extension products were analyzed by polyacrylamide gel electrophoresis. For a molecular weight standard we used DNA sequencing reactions of the 5' noncoding region of *TPI* which had been generated with the aforementioned primer.

Plasmid constructions

The *TPI::lacZ* fusion plasmid, pHB110, was prepared in the following manner. First a 4.4 kb *HindIII* fragment encoding *TPI* and 3.5 kb of DNA 5' to *TPI*'s start codon was cloned from pTPI-C10 (21) into pUC18 to generate plasmid, pHB51. To produce an in frame gene fusion between *TPI* and *lacZ* we chose to utilize the *BglII* site within *TPI* carried on plasmid pHB51. Therefore, we cloned a 3 kb fragment of DNA encoding *lacZ* from plasmid pMC1871 (22) into the aforementioned *BglII* site. The resulting plasmid pHB110 served as a starting point for the isolation of deletions in *TPI*'s 5' noncoding region.

The scheme to introduce *Bal31*-induced deletions in the 5' noncoding region of *TPI* is shown in Figure 1. Plasmid pHB110 was digested with *Tth1111* which cuts at a unique site 854 bp 5' to *TPI*'s translational start. The linearized material then was treated with exonuclease *Bal31* for various times. Following

Figure 1. Scheme to generate 5' deletions in the *TPI* 5' noncoding region. See text for details. Boxes represent the indicated structural genes. The *Tth1111* site occurs 854 bp 5' to the start of the *TPI* structural gene. The *TPI::lacZ* fusion joint occurs at the ninety-ninth codon of *TPI*. Restriction sites are as follows: E, *EcoRI*. S, *SalI*. H, *HindIII*. T, *Tth1111*.

Bal31 treatment Klenow fragment was used to fill in any recessed 3' ends. Then *HindIII*-*SalI*-*HindIII* linkers (CAAGCTTGTCG-ACAAGCTTG) were added. Following linker addition, the material was digested with *SalI* which cuts once in the polycloning site, derived from pUC18, and once in the newly added linker. The effect of this digest was to dropout yeast DNA between the polycloning site and the *TPI* proximal *Bal31*-induced deletion endpoint. The resulting mixture was ligated and used to transform *E. coli*. Plasmid DNA was prepared from the transformants, and the precise deletion endpoints determined by the dideoxy sequencing method using Sequenase (U.S. Biochemical) according to the manufacturer's protocol for sequencing double-stranded DNA. The DNA primer used was similar to the reverse sequencing primer for pUC18.

Once the desired deletion constructs were identified, they were subcloned on *HindIII* fragments into the *HindIII* site of YIp56. The orientation of the fusion with respect to *URA3* was determined by restriction endonuclease analysis.

Transformation

E. coli strains were transformed with plasmid DNA by the method of Enea et al. (23). The method of Ito et al. (24) was used to transform *S. cerevisiae*. The *TPI::lacZ* fusion constructs

were integrated at the *URA3* locus by first digesting the YIp56 derivatives at a unique *Stu1* site, which occurs within *URA3*, of YIp56 prior to transformation. Transplacement experiments were carried out on YNB minimal medium (Difco) with the appropriate supplements.

Gel-transfer hybridization

Southern and Northern analysis were carried out as described previously (6).

β -galactosidase assays

β -galactosidase assays were by the method of Miller (25).

DNA band shift assays

The DNA band shift assays were based on the procedures of Fried and Crothers (26) and Garner and Revzin (27). The binding reactions were incubated for 20 minutes in a 20 μ l volume at ambient temperature. The binding buffer was composed of 12 mM HEPES pH 7.5, 60 mM KCl, 5 mM MgCl₂, 4 mM Tris, 0.6 mM EDTA, 0.6 mM DTT, 10% glycerol, 0.26 μ g/ μ l poly(dI-dC), and 0.3 μ g/ μ l BSA. The yeast extracts, prepared as described above, were diluted to a protein concentration of 1 mg/ml in 1 $\times$ binding buffer immediately prior to their addition to the binding reactions. The typical reaction contained 0.5 to 1 ng of labeled DNA and 1 μ g of protein from a yeast extract. The reaction mix was electrophoresed through a 5% T (82:1) polyacrylamide gel with recirculation of the running buffer (10 mM Tris pH 7.5, 1.0 mM EDTA).

Oligonucleotides used in competition experiments were synthesized on an Applied Biosystems 380B DNA Synthesizer. In experiments where double-stranded (ds) oligonucleotides were used as competitor, complementary oligonucleotides were annealed together. Hybridization of the oligonucleotides was followed spectrophotometrically.

RESULTS

Characterization of the *TPI* transcript in *GCR1* and *gcr1*-deletion mutant backgrounds

Previous studies suggested that *gcr1* lesions bring about a reduction in the levels of mRNAs specifying glycolytic enzymes whose levels are affected by mutations in *GCR1* (3,7). To determine if the steady-state level of *TPI* mRNA was affected by *gcr1* mutations, we prepared probes to detect RNA encoding triose-phosphate isomerase and, as a control, isopropylmalate dehydrogenase which is encoded by *LEU2*. In order to use the *LEU2* transcript as a control it was first necessary to repair the *leu2* mutation in the parent of the *gcr1*-deletion mutant strain DFY520. Therefore, strain DFY578 was prepared by transplacing DFY520's parent, strain DFY510, to leucine prototrophy. RNA then was prepared from strain DFY578 and strain DFY520 grown in the absence and presence of glucose. Then RNA gel transfer hybridization experiments (Northern analysis) were carried out with radiolabeled probe DNA. As shown in Figure 2, the steady-state levels of *TPI* RNA were severely reduced in the *gcr1*-deletion mutant; in addition there appears to be a slight induction by glucose in the amount of *TPI* RNA in both the wild-type and *gcr1*-deletion mutant. On the other hand, the level of RNA encoding isopropylmalate dehydrogenase was unaffected by both the *gcr1* lesion and the presence of glucose.

To determine if the *gcr1* lesion affected the mature 5' end of

Figure 2. Comparison of steady-state *TPI* mRNA levels in wild-type and *gcr1*-deletion mutant strains. RNA isolated from wild-type and *gcr1*-deletion mutant strains, DFY578 and DFY520 respectively, grown in the absence and presence of glucose was electrophoresed through a 0.8% agarose-37% formaldehyde gel and transferred to a nylon filter. Lanes 1 through 4 were loaded, as indicated in the figure, with 10 μ g total RNA. After transfer, the filter was hybridized with a radiolabeled probe complementary to the *TPI* transcript. Following autoradiography the filter was stripped and rehybridized with a radiolabeled probe complementary to the *LEU2* transcript. Arrows indicate the positions of the 1.1 kb *TPI* and 1.5 kb *LEU2* transcripts.

the *TPI* transcript, primer extension studies were carried out with RNA isolated from wild-type and *gcr1*-deletion mutant strains. The primer chosen annealed to the beginning of the *TPI* structural gene. The first base incorporated during the primer extension reactions corresponded to position +2 with respect to the adenine in the initiation codon. The primer extension products were run next to a DNA sequence of the *TPI* nontranslated region which was generated with the same primer used in the primer extension reactions. Figure 3 shows that the mature transcripts had identical 5' ends in wild-type and *gcr1*-deletion mutant strains. The predominant extension products had ends which correspond to a pair of adenines located 29 bases upstream from *TPI*'s translation initiation codon. Thus, mature *TPI* transcripts had a leader of either 29 or 30 nucleotides, depending on which adenine was used, and it appeared that the site of transcription initiation was unaffected by the *gcr1* lesion.

Identification of the 5' boundary of the *TPI* controlling region

A *TPI::lacZ* gene fusion was prepared for use in determining the 5' boundary of the *TPI* controlling region. Since upstream activating sequences may be quite some distance away from a gene's transcriptional start site, our initial studies were carried out with a fusion which contained 3.5 kb of DNA 5' to the translational start site of *TPI*. Use of the fusion in *trans* to *TPI* permitted expression of triose-phosphate isomerase which is required for normal cell growth, while allowing us to follow the expression of the reporter gene product, a hybrid between triose-phosphate isomerase and β -galactosidase which retains β -galactosidase activity.

Subcloning experiments and nuclease *Bal31* deletion analysis

Figure 3. Comparison of the 5' ends of the *TPI* transcripts in wild-type and *gcr1*-deletion mutant strains. (A) Primer extension experiment to determine the 5' end of the *TPI* transcript. Lane 1, extension products obtained after a radiolabeled oligonucleotide complementary to the 5' end of the *TPI* structural gene was hybridized to 10 μ g of total RNA isolated from strain S150-2B and extended with reverse transcriptase. Lane 2, extension products obtained with 25 μ g of total RNA isolated from strain HBY4 treated as above. Lanes 3 through 6, DNA sequencing reactions of the *TPI* 5' nontranslated region generated with the aforementioned primer. Unlabeled arrows indicate the position of the predominant extension products. P, denotes the position of the unextended primer. (B) DNA sequence of the *TPI* 5' nontranslated region. Arrows point to the bases that correspond to the 5' end of the *TPI* transcript.

were used to map the 5' boundary of the *TPI* controlling region. The *Bal31* induced deletions were generated in a pUC18 based vector. After the deletion constructs were isolated and the precise end points of the various deletions determined by DNA sequencing, the constructs were cloned into plasmid YIp56, directly downstream of the *URA3* gene carried on the plasmid. Recombinant plasmids were obtained and the *TPI::lacZ* fusion construct oriented with respect to *URA3*. Recombinant plasmids in which *URA3* and the *TPI::lacZ* fusion were transcribed in the same orientation were chosen for integration into the genome. As a control for interference between transcriptional units, in the case of constructs 92-9 and 35-2 both orientations were chosen for integration.

The YIp56 derivatives were then integrated at the *URA3* locus. Recombinant plasmids were digested with *SnaI* which cuts the plasmids at a unique site in *URA3*, and the linear DNAs were then used to transform strains S150-2B and HBY4 to uracil prototrophy. Since tandem integration events can occur, transformants were screened by Southern blot analysis in order to ensure that transformants used for further study had only one copy of the fusion construct integrated into the genome. DNA was prepared from at least four independent transformants from each transformation mixture and screened in a Southern analysis with radiolabeled YIp56 probe DNA. Of the 124 transformants screened 54 were the result of single integration events and 70 were the result of tandem integration events (data not shown). Except where indicated otherwise, a single integrant of each fusion construct in the genetic background of S150-2B and HBY4 was obtained and used for further study.

Once we obtained the *TPI::lacZ* fusion constructs integrated in unit copy in the genome at *URA3*, we were interested in determining the stability of the fusion since it was flanked by direct repeats of *URA3*. Therefore, we carried out the following experiment with one of the fusion constructs. The S150-2B derivative carrying fusion construct 35-2 was grown to stationary phase in YPD medium diluted and plated on YPD. 100 colonies were then picked and screened on minimal media with and without uracil. In addition, each colony was suspended in a microtiter dish and assayed for β -galactosidase activity. Of the 100 colonies tested, all were uracil prototrophs and all exhibited β -galactosidase activity. Thus the fusion construct was stable; the rate of segregation was less than 1%.

Figure 4 shows the results obtained with the fourteen fusion constructs used in this study when integrated in unit copy and expressed in both wild-type and *gcr1*-deletion mutant strains. First, a comparison of fusion constructs which were integrated in both orientations, 92-9 and 35-2 with 92-9R and 35-2R, showed that there was not an effect of orientation on the levels β -galactosidase activity observed. More importantly, no effect on β -galactosidase activity was observed with deletions that removed DNA 5' to position -392. Thus, all sequences necessary for high level expression of *TPI* reside within 392 base pairs preceding *TPI*'s start codon. The DNA sequence within the fifteen bases between position -392 and -377 was required for full expression of the fusion construct in a wild-type background. Deletion of these bases resulted in a two and one-half to three and one-half-fold reduction in the level of β -galactosidase activity. When an additional eleven bases were deleted leaving 348 bases preceding *TPI*'s start, there was an additional three to four-fold reduction in the level of expression observed. The latter construct, when grown on *YPGL* medium, exhibited twenty-seven units of activity in the wild-type background, one tenth the maximal level of expression. The step-wise reduction in the level of β -galactosidase activity suggests there may be several regulatory sequences in the 44 bp region spanning positions -392 to -348.

The level of expression of *TPI::lacZ* fusion constructs which exhibited maximal levels of expression in a wild-type background was reduced more than ten-fold in a *gcr1* mutant background. Thus, the *gcr1* lesion had the same effect on the fusion as it has on *TPI* itself. It is interesting to note that the residual level of expression of the *TPI::lacZ* fusion in the *gcr1* mutant was not diminished by deletions which removed sequence 5' to position -348, yet such a deletion reduced expression ten-fold in the wild-type background.

Figure 4. Effect of *Bal31*-induced deletions on β -galactosidase levels expressed from a *TPI::lacZ* fusion integrated in unit copy at the *URA3* locus in wild-type and *gcr1*-deletion mutant strains. β -galactosidase activities were determined by the method of Miller (25) from at least two independent cultures (two assays per culture) of strains harboring each deletion construct grown in YP medium supplemented with either 2% glycerol, 2% lactate (YPGL) or 2% glucose (YPD). Except in the case of deletion constructs 92-9R and 35-2R, the deletion constructs were integrated into the genome such that the DNA immediately 5' to the deletion endpoint was sequence which lay 3' to the *URA3* structural gene. Deletion constructed 92-9R and 35-2R were oriented in the reverse orientation. In their case, the DNA sequence immediately 5' to the deletion endpoint is from the vector YIp56. Solid line denotes DNA from the *TPI* region remaining between the deletion endpoint and the *TPI* translational start. Stippled box denotes *TPI::lacZ* gene fusion. SD, standard deviation; ND, not done.

Protein nucleic acid interaction in the *TPI* 5' nontranslated region that correlates with expression

The *Bal31* induced deletion endpoints were marked with a *HindIII* site. Thus, it was possible to isolate DNA fragments which extended from a *SphI* site located at position -220 to any deletion endpoint mapping promoter distal to the *SphI* site. We were interested in determining if we could detect a differential pattern of band shifting with fragments isolated from *TPI::lacZ* fusion constructs which gave different levels of β -galactosidase activity. We were also interested in determining if we could detect any differences in the pattern of band shifting with extracts prepared from wild-type and *gcr1*-deletion mutant strains grown in the absence and presence of glucose. *HindIII-SphI* fragments were prepared from constructs 34-1, 70-1, and 81-3. These constructs gave high, medium, and low levels of expression of the *TPI::lacZ* fusion respectively. As shown in Figure 5, fragments isolated from constructs 34-1 and 70-1 gave rise to a positive band shift reaction with extracts prepared from both *GCR1* and *gcr1*-deletion mutant strains grown in the absence and presence of glucose. However, a band shift was not observed with fragment prepared

from construct 81-3. The inability to detect a positive band shift with fragment isolated from 81-3 suggested that the protein nucleic acid interaction detected with fragments 34-1 and 70-1 was occurring within the 29 bp region between deletion endpoints 70-1 and 83-1, or that the 83-1 deletion end point occurred within the binding site.

Figure 6 shows the results of a competition experiment we carried out to ensure that the band shift we observed was due to specific binding. Two synthetic double-stranded (ds) oligonucleotides were prepared for use as competitors. The first, a 30 mer which contained 17 bp from -422 to -406 of the *TPI* region (see Figure 10), served as a control, and the second, a 66 mer which contained 51 bp from -377 to -327, contained the putative site of protein nucleic acid interaction. The 30 mer was an ineffective competitor in band shift assays with radiolabeled *HindIII-SphI* restriction fragment prepared from deletion construct 34-1. In contrast the 66 mer was an effective competitor in similar assays. 15.6 ng of the 66 mer was more effective as competitor than 1000 ng of the 30 mer. Figure 6 also shows that the 66 mer was only effective as a competitor

Figure 5. DNA band shift analysis of the *TPI* controlling region. *HindIII-SphI* restriction fragments were isolated from deletion constructs which gave rise to high (34-1, lanes 1–5); medium (70-1, lanes 8–12), and low (81-3, lanes 13–17) levels of expression of the *TPI::lacZ* fusion (see Figure 4). Radiolabeled DNA fragments were incubated in binding buffer with yeast extracts containing 1 µg protein prepared from wild-type and *gcr1*-deletion mutant strains grown in the absence and presence of glucose as indicated on the figure. The first lane in each series serves as a control; the respective fragments were incubated in binding buffer without the addition of a yeast extract. Lanes 6 and 7, molecular weight standards (pBR322 digested with *HpaII*). Nucleoprotein complexes were resolved from free DNA by nondenaturing polyacrylamide gel electrophoresis and were revealed by autoradiography. f, free unbound probe; c, specific complex; (H-S), *HindIII-SphI*.

when it was presented as ds DNA. No competition was observed when 1000 ng of either strand of single-stranded (ss) 66 mer was added to the reaction mix.

Next we carried out a series of competition experiments where we used an oligonucleotide, referred to as 31 mer, which contained a consensus GRF1/RAP1/TUF-binding site in the same context as the 30 mer (oligonucleotide a of Figure 6) in addition to the competitors used in the experiment of Figure 6. These oligonucleotides were also radiolabeled and used as probes in the band shift assay. Figure 7 shows that positive band shift reactions were obtained with the *HindIII-SphI* prepared from construct 34-1, the 66 mer, and 31 mer, but not with the 30 mer. Furthermore, the oligonucleotide containing the consensus GRF1/RAP1/TUF-binding site was an effective competitor in the band shift assays which utilized the *HindIII-SphI* fragment prepared from construct 34-1, the 66 mer, and itself as probe.

The fact that the fragment containing the consensus GRF1/RAP1/TUF-binding site was able to compete with the *HindIII-SphI* fragment prepared from construct 34-1, suggested that *RAP1* gene product was responsible for the band shifts we observed with fragments isolated from our deletion constructs. To test this hypothesis we obtained a strain, YRAP, from David Shore's laboratory carrying an altered *RAP1* gene which expresses a functional *RAP* gene product which is 230 amino acids smaller than the wild-type gene product. Figure 8 shows that when protein extract prepared from strain YRAP was used in band shift assays, a faster migrating complex was observed than when the extract was prepared from a wild-type strain. This observation confirms that the *RAP1* gene product binds in the *TPI* controlling region.

The DNA sequence from the *TPI* controlling carried on the 66 mer was scanned for sequences resembling a

Figure 6. Competition of band shift patterns with oligonucleotides which contain sequence from the *TPI* controlling region. Competition experiments were carried out with radiolabeled *HindIII-SphI* restriction fragment isolated from deletion construct 34-1 and protein extract prepared from yeast strain S150-2B grown in the presence of glucose. Increasing amounts of single-stranded (ss) or double-stranded (ds) DNA, as indicated in the figure, were added to the band shift assay reaction mix. In assays where double-stranded DNA was used as competitor, both strands of the competitor were synthesized and annealed together. Nucleoprotein complexes were resolved from free DNA by nondenaturing polyacrylamide gel electrophoresis and were revealed by autoradiography. f, free unbound probe; c, complex; ds, double-stranded; ss, single-stranded; Oligo A, denotes an oligonucleotide with the sequence CAAGCTTGGACCCATC-AGGTTGGCATGCC which contained a sequence spanning the region -406 to -422 (see Figure 10); Oligo B denotes an oligonucleotide with the sequence GGAAGCTTAGCTTCTCTATTGATGTTACACCTGGACACCCCTTTT-CTGGCATCCAGTTGCATGCC which contained the sequence spanning the region -327 to -377 (see Figure 10).

GRF1/RAP1/TUF-binding site. One such sequence (CACCCC-TTTTC) was found extending from -349 to -339 which differs in three positions from the consensus GRF1/RAP1/TUF-binding site proposed by Buchman *et al.* (28). We then prepared an oligonucleotide, named 69RBSM (RAP Binding Site Mutation), in which the above sequence was changed to AACCCATCAGG, the sequence found at position -420 to -410 which Chambers *et al.* (8) had suggested was a GRF1/RAP1/TUF-binding site. Figure 9 shows that fragment 69RBSM shifts weakly if at all.

We next investigated the ability of fragments containing sequences of the *TPI* controlling region to activate expression of a heterologous gene. For this purpose we chose a *CYC::lacZ* fusion construct (29). In a series of subcloning experiments we replaced *CYC*'s upstream activating sequences with the *HindIII-SphI* fragment from deletion construct 34-1, an 81 mer, containing sequence of the *TPI* controlling region from -392, the 3' end point of deletion 34-1, to -327, and a 66 mer containing sequences from the *TPI* controlling region from -377, the 3' end point of deletion 70-1, to -327. The resulting constructs were then integrated into the yeast genome in unit copy at the *URA3* locus. Table 2 shows that each of these three fragments were able to activate expression of the *CYC::lacZ* fusion to the same extent. Thus, these fragments contain an upstream activating sequence from the *TPI* controlling region.

To test the effect of disrupting the GRF1/RAP1/TUF-binding site on expression of the *TPI::lacZ* fusion construct we subcloned the 66 mer and 69RBSM in front of the *TPI::lacZ* fusion construct at position -220. All of the *TPI* controlling region 5' to -220 except those sequences carried on the oligonucleotides (-377

Figure 7. Competition of band shift patterns with an oligonucleotide which contains a consensus GRF1/RAP1/TUF-binding site. Competition experiments were carried out with radiolabeled probe DNA as indicated in the figure. 34-1 H-S denotes a *HindIII-SphI* restriction fragment isolated from deletion construct 34-1; 66 mer, denotes the double-strand form of the oligonucleotide labeled B in Figure 6; 31 mer, denotes the double-strand form of an oligonucleotide with the sequence C-AAGCTTGAACCCATACATGTTGGCATGCC which contains a consensus GRF1/RAP1/TUF-binding site; 30 mer, denotes the double-strand form of the oligonucleotide labeled A in Figure 6, lanes 1, 6, 11 and 16 labeled fragments without protein extract; lanes 2, 7, 12, and 17 radiolabeled DNA fragments incubated with 1 μ g protein prepared from strain S150-2B; lanes 3, 8, 13, and 18 standard band shift reaction mix with the addition of 1 μ g of unlabeled 66 mer added as competitor; lanes 4, 9, 14, and 19 standard band shift reaction mix with the addition of 1 μ g unlabeled 31 mer as competitor; lanes 5, 10, 15, and 20 standard band shift reaction mix with the addition of 1 μ g unlabeled 30 mer as competitor. Nucleoprotein complexes were resolved from free DNA by nondenaturing polyacrylamide gel electrophoresis and were revealed by autoradiography. f, free unbound probe; c, complex.

to -327) were deleted. These constructs were then integrated in the yeast genome at the *URA3* locus in unit copy. Table 2 shows that the 66 mer was able to direct the expression of the *TPI::lacZ* fusion; whereas, the construct with the GRF1/RAP1/TUF-binding site mutation was unable to direct the expression of the fusion.

DISCUSSION

In an effort to investigate the mechanism by which the *GCR1* gene product brings about high level glycolytic gene expression, we carried out a comparative study in which we used isogenic wild-type and *gcr1*-deletion mutant strains to examine the expression of *TPI*, which encodes the glycolytic enzyme triose-phosphate isomerase.

Clifton et al. (4) originally showed that *gcr1* lesions result in a severe reduction in the specific activity of most glycolytic enzymes. In crude extracts of cultures grown on rich medium supplemented with pyruvate, the specific activity of triose-phosphate isomerase was reduced over 17-fold (4). Indirect experiments involving RNA isolation and in vitro cell-free translation suggested that the reduction in the specific activity of the enzymes of glycolysis observed with *gcr1* mutants was due to a corresponding reduction in the level of the mRNAs specifying the affected enzymes (3). Here we used Northern analysis to show that the steady-state level of RNA specifying *TPI* is substantially reduced in *gcr1* mutants. Reductions in steady-state levels of RNA encoding phosphoglucose isomerase,

Figure 8. Band shift analysis of *TPI* controlling region with protein extracts prepared from strains with different alleles of *RAP1*. Band shift experiments were carried out with radiolabeled *HindIII-SphI* restriction fragment isolated from deletion construct 34-1. Yeast S150-2B was used to prepare an extract containing the native *RAP1* gene product, and strain YRAP was used to prepare the extract with the mutant form of the *RAP1* gene product. Wild-type denotes that extract was prepared from strain S150-2B. YRAP denotes that extract was prepared from strain YRAP. Glu, denotes that extracts were prepared from cultures grown in YP medium supplemented with 2% glucose; GlyLac, denotes that extracts were prepared from cultures grown in YP medium supplemented with 2% glycerol and 2% lactate; Pyr, denotes that extracts were prepared from cultures grown in YP medium supplemented with 2% pyruvate. Lane 1, probe alone; lane 2-6 standard band shift reactions using extracts prepared from cultures as indicated on the figure. Nucleoprotein complexes were resolved from free DNA by nondenaturing polyacrylamide gel electrophoresis and were revealed by autoradiography. f, free unbound probe; c, complex.

phosphoglycerate kinase, pyruvate kinase (Baker, unpublished result), and glyceraldehyde-3-phosphate dehydrogenase (7) have also been observed in *gcr1* mutant strains. Thus, it seems likely that the reduction in specific activity of the other enzymes which are affected by *gcr1* lesions is due to a reduction in the steady-state level of the corresponding RNAs.

Primer extension experiments suggested that transcription initiation of *TPI* occurs most frequently at a pair of adenines located 29 to 30 bp 5' to the *TPI* structural gene in both wild-type and *gcr1*-deletion mutant strains. Thus, whereas, the *gcr1* lesion affects the absolute amount of the *TPI* transcript it does not affect the formation of the 5' end of the message.

Deletion analysis showed that sequences sufficient for high level expression of *TPI* reside between the site defined by deletion endpoint 34-1, 392 nucleotide pairs preceding the start of the structural gene and the *TPI::lacZ* fusion joint occurring at codon 99 of *TPI*. Recently, Chambers et al. (8) pointed out that many genes encoding enzymes of the lower half of glycolysis carry a conserved sequence in their promoters which has a high degree of sequence similarity to the GRF1/RAP1/TUF-binding site. In the case of *PGK*, *ENO1*, and *PYK* GRF1/RAP1/TUF-binding has been demonstrated, and the GRF1/RAP1/TUF-binding site has been shown to play a major role in the high level expression of *PGK* (8) and *ENO1* (9). As noted by Chambers et al. (8), *TPI* encodes the sequence ACCCATCA which is very similar

Figure 9. Band shift analysis of an oligonucleotide with a mutant GRF1/RAP1/TUF-binding site. DNA band shift assays were carried out with radiolabeled probe DNA as indicated on the figure. 34-1 H-S denotes a *HindIII-SphI* restriction fragment isolated from deletion construct 34-1; 81 mer denotes the *HindIII-SphI* restriction fragment generated from the double-strand form of an oligonucleotide with the sequence GGAAGCTTGTCTAAGACTTTTCAGCTTCCTCTATTGATGTTACACCTGGACACCCCTTTTCTGGCATCCAGTTGCATGCC; 66 mer denotes the *HindIII-SphI* restriction fragment generated from the double-strand form of the oligonucleotide labeled B in Figure 6; 69 RBSM denotes the *HindIII-SphI* restriction fragment generated from the double-strand form of an oligonucleotide with the sequence GCTAAGCTTAGCTTCCTATTGATGTTACACCTGGAAACCCATCAGGTGGCATCCAGTTGCATGCCAAC; Lanes 1, 4, 7 and 10 labeled fragments without protein extract; lanes 2, 5, 8 and 11 labeled DNA fragments incubated with 1 μ g protein prepared from strain S150-2B; lanes 3, 6, 9, and 12 standard band shift reaction mix with 1 μ g 31 mer as competitor. Nucleoprotein complexes were resolved from free DNA by nondenaturing polyacrylamide gel electrophoresis and were revealed by autoradiography. f, free unbound probe; c, complex.

to the GRF1/RAP1/TUF consensus sequences and the sequence found upstream of other genes encoding glycolytic enzymes. A comparison of deletion constructs 37-2 and 34-1 showed similar levels of β -galactosidase expression from the *TPI::lacZ* fusion. The former construct contains the near consensus GRF1/RAP1/TUF-binding site which has been deleted in the latter (see Figure 10). Furthermore, DNA band shift assays and competition experiments with oligonucleotides containing the sequence ACCCATCA, suggested that this sequence binds RAP1 very weakly if at all. Thus, it is unlikely that this sequence plays a role in the expression of *TPI*. However, we were able to identify a GRF1/RAP1/TUF-binding site (CACCCCTTTTC) in the *TPI* controlling region located at position -349 to -339. Use of extracts prepared from strain YRAP and the use of oligonucleotides with an altered GRF1/RAP1/TUF-binding site demonstrated that the *RAP1* gene product was able to bind to fragments containing the sequence CACCCCTTTTC but was unable to bind to the related sequence AACCCATCAGG. Both of these sequences differ from the consensus GRF1/RAP1/TUF-binding site in three positions (see Figure 10). Furthermore, subcloning experiments showed that an oligonucleotide containing the former sequence was able to direct expression of the *TPI::lacZ* fusion; whereas, an otherwise identical oligonucleotide encoding

Table 2. Identification of a UAS element in the *TPI* controlling region. β -galactosidase assays were performed as described in Figure 4 from strains grown in YPD medium.

Construct	Arrangement	β - Galactosidase Activity	
		Mean	SD
CYC1::lacZ	³⁸⁵ UAS — TATA — CYC1::lacZ	70	9
UAS _{less} CYC1::lacZ	() ²⁴⁴ — TATA — CYC1::lacZ	8	6
34-1 H-S::CYC1::lacZ	(34-1 H-S) ¹⁴³ — TATA — CYC1::lacZ	94	3
81mer::CYC1::lacZ	(81mer) ¹⁴³ — TATA — CYC1::lacZ	101	5
66mer::CYC1::lacZ	(66mer) ¹⁴³ — TATA — CYC1::lacZ	99	7
35-2	⁸⁶³ — TATA — TPI::lacZ	210	11
78-1	²⁷⁸ — TATA — TPI::lacZ	11	10
66mer::TPI::lacZ	^{66mer} (²⁰²) ²⁰⁰ — TATA — TPI::lacZ	129	11
69 RBSM::TPI::lacZ	^{69 RBSM} (²⁰²) ²⁰⁰ — TATA — TPI::lacZ	18	4

Figure 10. Summary map of deletion endpoints in the *TPI* controlling region. The position of the deletion endpoints which occur between -495 and -300 are noted in bold type. Numbers in brackets denote units of β -galactosidase activity observed with the particular deletion construct in a wild-type background from cultures grown in YPD medium. The near consensus GRF1/RAP1/TUF-binding sites are noted by dashed underlining, positions which differ from the consensus are underscored with X. The CTTCC pentamer is overdotted. Sequence contained in the 30 mer and the 66 mer are underlined. The DNA sequence data generated with deletion constructs 37-2, 34-1, and 70-1 revealed a C at position -354, not a T as originally reported by Alber and Kawasaki (21).

the latter sequence in place of the former sequence was unable to direct expression of the fusion. The deletion end point of construct 83-1, the first deletion construct with low level expression of the *TPI::lacZ* fusion, falls within this binding site. Thus, *RAP1* binding in the *TPI* controlling region appears to be important for expression.

Figure 10 shows that the sequence CTTCC is located just upstream of the GRF1/RAP1/TUF-binding site. The sequence CTTCC, in addition to the GRF1/RAP1/TUF-binding site, has been implicated in the high level expression of *PGK(30)*, *ENO1*, and *PYK(9)*, and it is also conserved in the promoter region of

several genes encoding enzymes in the lower half of glycolysis (8). the presence of the CTTCC block in this region suggests that it may play a role in *TPI*'s expression.

Chambers et al. (8) previously reported the inability to detect *RAP1* binding with extracts prepared from cultures grown in medium supplemented with the gluconeogenic carbon source pyruvate. However, using crude whole cell extracts we were able to detect *RAP1* binding with extracts prepared from strains grown in gluconeogenic media. We detected binding with extracts prepared from cultures grown in medium supplemented with glycerol plus lactate and in medium supplemented in pyruvate (Figure 8, lanes 3,4 and 6).

DNA band shift assays utilizing extracts prepared from wild-type and *gcr1*-deletion mutant strains yielded similar patterns of band shifting with a fragment that extended from -392 to -220 (Figure 5, lanes 2-5). The band shift analysis has been extended to include the region from -498 to +32 and no differences in the pattern of band shifting have been observed with extracts from wild-type or *gcr1*-deletion mutant strains (Baker, unpublished result). Thus, under the conditions used in the band shift assays reported here, there is no evidence that *GCR1* encodes a protein which binds to DNA. Since the levels of β -galactosidase activity observed with deletion construct 34-1 are reduced by the *gcr1* lesion, it can be concluded that *GCR1* must exert its effect through sequences that lie 3' to position -392. How *GCR1* acts to bring about high level expression of *TPI* remains unknown. The *GCR1* gene product may act as an adaptor or it may act to modify, in some unknown way, another protein which binds in the controlling region of *TPI* and other genes which are affected by *gcr1* lesions. If the *GCR1* gene product should act to modify another protein, the band shift data would suggest that the modified protein is still able to bind DNA. However, as in the case of *GCR1* itself, one is unable to rule out the possibility of differential DNA binding which is undetected by the DNA binding assay used here.

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