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Concerted Action of the Transcriptional Activators REB1, RAP1, and GCR1 in the High-Level Expression of the Glycolytic Gene *TPI*

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In *Saccharomyces cerevisiae*, the *TPI* gene product, triosephosphate isomerase, makes up about 2% of the soluble cellular protein. Using in vitro and in vivo footprinting techniques, we have identified four binding sites for three factors in the 5′ noncoding region of *TPI*: a REB1-binding site located at positions −401 to −392, two GCR1-binding sites located at positions −381 to −366 and −341 to −326, and a RAP1-binding site located at positions −358 to −346. We tested the effects of mutations at each of these binding sites on the expression of a *TPI*::*lacZ* gene fusion which carried 853 bp of the *TPI* 5′ noncoding region integrated at the *URA3* locus. The REB1-binding site is dispensable when material 5′ to it is deleted; however, if the sequence 5′ to the REB1-binding site is from the *TPI* locus, expression is reduced fivefold when the site is mutated. Because REB1 blocks nucleosome formation, the most likely function of its binding site in the *TPI* controlling region is to prevent the formation of nucleosomes over the *TPI* upstream activation sequence. Mutations in the RAP1-binding site resulted in a 10-fold reduction in expression of the reporter gene. Mutating either GCR1-binding site alone had a modest effect on expression of the fusion. However, mutating both GCR1-binding sites resulted in a 68-fold reduction in the level of expression of the reporter gene. A LexA-GCR1 fusion protein containing the DNA-binding domain of LexA fused to the amino terminus of GCR1 was able to activate expression of a *lex* operator::*GAL1*::*lacZ* reporter gene 116-fold over background levels. From this experiment, we conclude that GCR1 is able to activate gene expression in the absence of REB1 or RAP1 bound at adjacent binding sites. On the basis of these results, we suggest that GCR1 binding is required for activation of *TPI* and other GCR1-dependent genes and that the primary role of other factors which bind adjacent to GCR1-binding sites is to facilitate or modulate GCR1 binding in vivo.

The primary metabolic flux in many cells is sugar metabolism through the glycolytic pathway. In *Saccharomyces cerevisiae*, the enzymes of glycolysis constitute a major fraction (30 to 60%) of the soluble protein (18, 23). Holland and Holland (25) have provided evidence that the most abundant mRNA species in yeast cells code for glycolytic enzymes. Thus, the genes encoding glycolytic enzymes are among the most highly expressed in *S. cerevisiae*.

A number of *trans*-acting factors have been shown to be involved in the high-level expression of genes encoding glycolytic enzymes; these include the DNA-binding proteins GCR1 (2, 14, 15, 26, 28), RAP1 (5, 9, 10, 12, 30, 31, 34, 40, 41), REB1 (13), and ABF1 (9, 11). In general, the upstream activation sequence (UAS) elements of glycolytic genes tend to be complex in nature, most having multiple binding sites for several different *trans*-acting factors. For example, all known GCR1-binding sites are located adjacent to RAP1-binding sites (28). In addition, several of the genes have binding sites for ABF1 (9, 11), REB1 (13), and as yet unidentified factors (9) in the vicinity of their GCR1- and RAP1-binding sites. The respective roles of the factors bound at adjacent sites in the UAS elements of glycolytic genes and how they act together to bring about high-level expression of the cognate genes remains to be elucidated.

In addition, *GAL11*/*SPT13* (35), *GCR2* (42), and *GCR3* (44) have been shown to be necessary for full expression of one or more of the glycolytic genes. Recently, Uemura and Jigami provided genetic evidence for a direct physical interaction between GCR1 and GCR2 (43). These workers suggested that GCR1 and GCR2 function together as a transcriptional activation complex.

In this study, we extend our analysis of the regulatory region of the gene, *TPI*, encoding the glycolytic enzyme triosephosphate isomerase. In wild-type cells, *TPI* is constitutively expressed, and triosephosphate isomerase makes up about 2% of the soluble protein (36). Mutations in *GCR1* results in a 17-fold reduction in triosephosphate isomerase activity (14) which is mirrored by a reduction in the steady-state level of the *TPI* transcript (40). Mutations in *GCR2* also result in severe reductions (20-fold) in triosephosphate isomerase activity (42). The level of the *TPI* transcript has not been measured in a *gcr2* mutant strain; however, *gcr2* lesions are known to reduce severely the levels of other glycolytic transcripts (42). Mutations in *GCR3* result in a twofold reduction in triosephosphate isomerase activity in strains growing on fermentable carbon sources, whereas no reduction in triosephosphate isomerase enzymatic activity is observed in cells growing on nonfermentable carbon sources (44). In addition to the strong requirements for GCR1 and GCR2, RAP1 is required for the expression of *TPI* (40). In vivo mapping studies revealed four binding sites for proteins in the *TPI* UAS (UAS_{TPI}) region (28). Three of the binding sites, two GCR1-binding sites surrounding a RAP1-binding site, had been previously identified by mutational analysis (40) or by DNase I footprinting studies (28). The fourth site,

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TABLE 1. Strains used

Strain	Genotype	Source or reference
S. cerevisiae		
S150-2B	*MATa leu2-3,112 his3Δ trp1-289 ura3-52*	A. Lewin
HBY4	*MATa gcr1Δ::HIS3 leu2-3,112 his3 trp1-289 ura3-52*	40
HBY10	*MATa leu2-3,112 his3Δ trp1-289 ura3-52*::pJH199(*lex$_{op}$::GAL1::lacZ URA3*)	This study
DFY641	*MATa gcr2Δ::URA3 leu2-3,112 ura3-52*	D. Fraenkel (42)
DFY642	*MATa leu2-3,112 ura3-52*	D. Fraenkel (42)
JF1052	*MATa leu2 ura3-52 his4-917 lys2-128δ spt13-201::LEU2*	J. Fassler
E. coli		
C600/pPET11a	F$^-$ *thi-1 leuB6 tonA21 lacY1 supE44*/pPET11a	J. Warner
C600/pPET11a-REB1	F$^-$ *thi-1 leuB6 tonA21 lacY1 supE44*/pPET11a-REB1	J. Warner

which mapped 5′ to the other sites, lay over a near-consensus REB1-binding site; however, the ability of REB1 to interact with the site was not determined. In this report, we extend our analysis of the *TPI* controlling region and suggest a model by which the factors that bind at adjacent sites in the UAS$_{TPI}$ element act in concert to mediate high-level expression of *TPI*.

MATERIALS AND METHODS

Strains and growth conditions. Strains used for this study are described in Table 1. Yeast cultures were grown in yeast-peptone medium (38) supplemented with 2% glucose or 2% lactate and 2% glycerol. *Escherichia coli* cultures were grown in LB medium (32) supplemented with appropriate antibiotics.

Preparation of yeast and *E. coli* extracts. Unless otherwise stated, cell extracts were prepared by passage through a French pressure cell as described previously (15).

Nucleic acid manipulations. Techniques used throughout this study are described in the standard reference manuals (1, 38, 39). *S. cerevisiae* DNA was prepared by the method of Rose et al. (38) except that no incubations were carried out at temperatures above 37°C.

Plasmid constructions. Plasmid YIpES90 was used as the starting material for a series of internal deletions in the 5′ noncoding region of *TPI*. This plasmid contains a *TPI::lacZ* fusion with 853 bp of DNA 5′ to the translational start of *TPI* derived from plasmid pES35-2 (40) cloned into the *Hin*dIII-*Kpn*I sites of YIp56 (Fig. 1). The unique *Sph*I site of plasmid YIpES90 located at position −220 with respect to the translational start site served as the origin of a set of *Bal* 31-induced deletions which extended in the 5′ direction toward the *Hin*dIII site that lies within the vector sequence adjacent to position −853 in the 5′ noncoding region of *TPI* (Fig. 1). Plasmid YIpES90 was linearized with *Sph*I and treated for various times with exonuclease *Bal* 31. The large fragment of DNA polymerase I was used to fill in the ends, and *Sph*I linkers were added. The resulting material was then digested with *Hin*dIII and *Sph*I and gel purified. The desired bands were excised from the gel and subcloned into the vector portion of plasmid YIpES90 that had been digested with *Hin*dIII and *Sph*I. The ligation mixture was then used to transform *E. coli* to ampicillin resistance. Plasmid DNA was purified from several of the transformants, and the precise deletion endpoints were determined by double-stranded DNA sequencing using a primer that allowed sequencing through the *Sph*I site at −220.

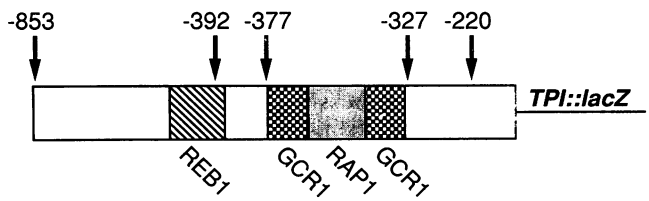

FIG. 1. Schematic representation of the *TPI::lacZ* reporter gene and 5′ noncoding region of *TPI* carried on plasmid YIpES90. Rectangle, 5′ noncoding region of *TPI* with relative positions of REB1-, GCR1-, and RAP1-binding sites indicated (not drawn to scale); solid line, *TPI::lacZ* structural gene.

Plasmid pLexA (1–87)-GCR1(1–844) is a derivative of plasmid pLexA(1–87)+pl (kindly provided by M. Ptashne) in which the entire *GCR1* structural gene was cloned in frame behind *lexA*(1–87). Plasmid pLexA(1–87)-GCR1(1–844) gives rise to a hybrid polypeptide consisting of the first 87 amino acid residues of LexA followed by a 9-amino-acid residue linker region with the sequence GFELGTRGS followed by amino acid residues 1 to 844 of GCR1.

Transformations and integrations. *E. coli* strains were transformed with plasmid DNA by the method of Enea et al. (16). The method of Ito et al. (29) was used to transform *S. cerevisiae*. The *TPI::lacZ* fusion constructs were integrated at the *URA3* locus as described previously (40). The *lex* operator (*lex$_{op}$*)::*GAL1::lacZ* reporter gene carried on plasmid pJH199 (provided by M. Ptashne) was integrated at the *URA3* locus after the plasmid was linearized by digestion with *Stu*I. Yeast transformation experiments were carried out on yeast nitrogen base minimal medium with the appropriate supplements.

β-Galactosidase assays. β-Galactosidase activities of glass bead extracts of yeast cultures were determined by the method of Miller (32) and are expressed as millimicromoles of *o*-nitrophenol per minute per milligram of protein. Reported values represent averages from at least five different cultures. Variation between individual cultures was less than 20%. Glass bead extracts of yeast cultures were prepared essentially by the method of Himmelfarb et al. (24). Protein concentrations of the extracts were determined by the method of Bradford (6).

In vitro synthesis of RAP1. RAP1 was synthesized in vitro by using rabbit reticulocyte lysates as described previously (3).

DNA band shift assays. DNA band shift assays, based on the procedures of Fried and Crothers (19) and Garner and Revzin (20), were carried out as described previously (40).

DNase I protection studies. DNase I footprints were carried out be a modification of the method of Pfeifer et al. (37) as described previously (28).

TABLE 2. Effects of deletions in the 5′ noncoding region of *TPI* on expression of *TPI*::*lacZ*

Construct	Activity[a]	% Activity
S150-2B/YIpES90	3,215	100
S150-2B/YIpES90Δ −300 to −220	3,733	116
S150-2B/YIpES90Δ −336 to −220	1,904	59
S150-2B/YIpES90Δ −347 to −220	167	5
S150-2B/YIpES90Δ −367 to −220	123	4

[a] Expressed as defined in Materials and Methods.

In vivo DNA methylation protection studies. The method of carrying out genomic footprints of the native *TPI* promoter has been described in detail elsewhere (28).

RESULTS

***TPI* gene expression is controlled by a single complex UAS element.** Previously, using a series of *Bal* 31-induced deletions, we showed that sequences sufficient for high-level expression of a *TPI*::*lacZ* gene fusion integrated at *URA3* resided between the fusion joint and 392 nucleotides preceding the start of the structural gene (40). More recently, through a series of in vivo methylation protection studies of the native *TPI* promoter, we identified an area of protection 5′ to position −392 in the vicinity of UAS_{TPI} (28). Inspection of the protected sequence revealed a near-consensus binding site for the protein REB1 (13, 33). The presence of protected bases 5′ to position −392 suggested that there might be additional UAS elements upstream of the UAS element that we previously mapped to between positions −392 and −327. Thus, we prepared a set of *Bal* 31-induced deletions originating at position −220 and extending in the 5′ direction towards the known UAS_{TPI} element. The extents of the deletions were determined by DNA sequence analysis. The deletion constructs were integrated into the genome of strain S150-2B at the *URA3* locus. Transformants were screened by Southern blot analysis to ensure that unit-copy integrants were used for subsequent expression studies (data not shown). Table 2 shows the effects of deletions in the 5′ noncoding region of *TPI* on the expression of the reporter gene. A deletion that removed material from positions −220 to −300 had no significant effect on expression of the *TPI*::*lacZ* gene fusion, whereas a deletion that extended to position −367, which removed a portion of the known UAS element, resulted in a 26-fold reduction in expression of the reporter gene. From this analysis, we conclude that there are no sequences 5′ of position −367 capable of independently directing expression of the reporter gene.

Identification and characterization of a REB1-binding site in the *TPI* controlling region. To determine whether REB1 was capable of interacting with the near-consensus REB1-binding site located at positions −401 to −392, DNA band shift assays were performed with a radiolabeled DNA fragment extending from positions −487 to −348 and with extracts prepared from *E. coli* expressing a polypeptide carrying the carboxy-terminal two-thirds of REB1, which includes the functional REB1 DNA-binding domain (kindly provided by B. Morrow and J. Warner). Figure 2 shows the appearance of shifted bands when the fragment was incubated with extract prepared from yeast strain S150-2B and with extract prepared from *E. coli* expressing the REB1 DNA-binding moiety but not with extract from an *E. coli* strain carrying the parental plasmid, pPET11a. The migration differences of the shifted bands observed with *E. coli* and yeast extracts

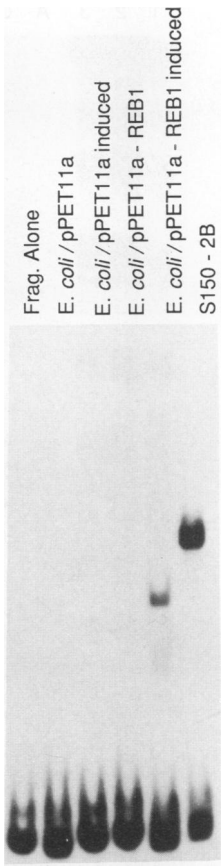

FIG. 2. Binding of REB1 to DNA from the *TPI* controlling region. DNA band shift assays were performed with a radiolabeled 120-bp *Hin*dIII-*Alu*I restriction fragment from pES36 (40) which carried the putative REB1-binding site located at positions −401 to −392. The radiolabeled DNA was incubated in binding buffer with the protein extract indicated above each lane. Nucleoprotein complexes were resolved from free DNA by nondenaturing polyacrylamide gel electrophoresis and were revealed by autoradiography. Lanes: Frag. Alone, radiolabeled DNA fragment; *E. coli*/pPET11a, protein extract from a culture of *E. coli* C600 harboring plasmid pPET11a; *E. coli*/pPET11a induced, protein extract from a culture of *E. coli* C600 harboring plasmid pPET11a which had been induced with isopropylthiogalactopyranoside (IPTG) 3 h prior to harvest; *E. coli*/pPET11a-REB1, protein extract from a culture of *E. coli* C600 harboring plasmid pPET11a-REB1; *E. coli*/pPET11a-REB1 induced, protein extract from a culture of *E. coli* C600 harboring plasmid pPET11a-REB1 which had been induced with IPTG 3 h prior to harvest; S150-2B, protein extract from yeast strain S150-2B.

are attributable to differences in the REB1 polypeptide produced by the strains. The *E. coli* strain produced a truncated version of REB1 (apparent molecular weight of 80,000), whereas, the yeast strain produced native REB1 (apparent molecular weight of 125,000).

DNase I protection studies were then carried out to map the site of REB1 binding in vitro. Both extracts from an *E. coli* strain expressing the functional DNA-binding domain of REB1 and yeast strain S150-2B were used as a source of REB1 in the DNase I protection assays shown in Fig. 3. The degree of protection observed was greater with protein extracts from the *E. coli* strain overexpressing the truncated REB1 polypeptide than with protein extracts from yeast strain S150-2B. In addition to the protected region, an area

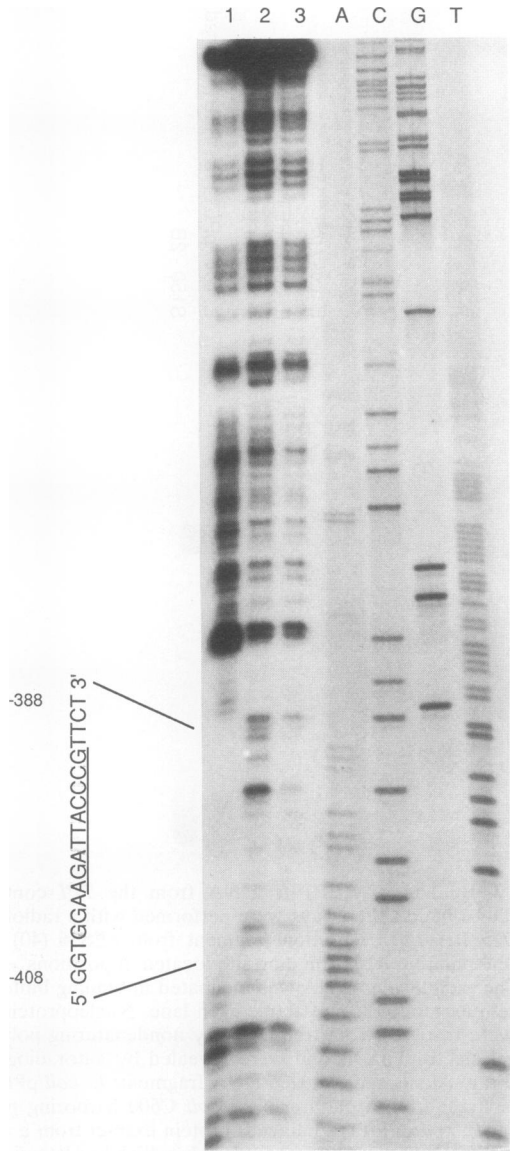

FIG. 3. In vitro DNase I footprint of the REB1-binding site in the *TPI* controlling region. Analysis of the REB1-binding site in UAS_{TPI} was carried out with an extract of *E. coli* C600/pPET11a-REB1 expressing the functional DNA-binding domain of REB1, an extract of yeast strain S150-2B, and a radiolabeled fragment of DNA carrying sequence from positions −448 to −220 of the *TPI* 5′ noncoding region. Lanes: 1, sample in which a nucleoprotein complex was allowed to formed between the *E. coli* extract containing the functional DNA-binding domain of REB1 and the DNA fragment prior to treatment with 1.0 U of DNase I; 2, DNA fragment treated with DNase I in the absence of other proteins; 3, sample in which a nucleoprotein complex was allowed to form between the S150-2B yeast extract and the DNA fragment prior to treatment with 1.0 U of DNase I; A, C, G, and T, products of dideoxy sequencing reactions of M13es2-2, which served as molecular weight standards. The sequence protected is indicated on the left.

of hypersensitivity was observed adjacent to the REB1-binding site with the *E. coli* extract containing REB1. The difference in the degree of protection observed is probably attributable to a higher concentration of the functional DNA-binding domain of REB1 in the *E. coli* extracts. In both cases, however the near-consensus REB1-binding site located at positions −401 to −392 was protected from DNase I digestion. Therefore, we conclude that the sequence from positions −401 to −392 is, in fact, a binding site for REB1.

In vivo methylation protection studies of UAS_{TPI}. The UAS elements of genes encoding glycolytic enzymes tend to be complex in nature; among the UAS elements of these genes there are binding sites for GCR1 (28), RAP1 (5, 9, 10, 12, 30, 31, 34, 40, 41), REB1 (13), ABF1 (9, 11), and as yet unidentified factors (9). There are reports in the literature that the DNA-binding ability of one of these factors, RAP1, may be affected by the nutritional composition of the medium (12). In addition, *GAL11/SPT13* and *GCR2* have been implicated in the expression of one or more of the glycolytic genes (35, 42). In an effort to determine the effects of mutations in *GAL11/SPT13* and *GCR2* on the ability of the other proteins, namely, REB1, GCR1, and RAP1, to bind at UAS_{TPI} and to correlate expression with factor-binding site occupancy, we carried out a series of in vivo guanosine methylation protection experiments of the *TPI* controlling region in several genetic backgrounds and in the wild-type strain under both gluconeogenic and glycolytic growth conditions. Figure 4 shows the results of a series of in vivo guanosine methylation protection footprints of the native *TPI* promoter in wild-type, *gcr1*, *gcr2*, and *gal11/spt13* mutant strains. Four areas of protection were observed over the UAS_{TPI} element with DNA isolated from wild-type, *gcr2*, and *gal11/spt13* mutant strains, whereas only two areas of protection were observed with DNA isolated from the *gcr1*-deletion mutant strain. In wild-type, *gcr2*, and *gal11/spt13* mutant strains, protection was observed over the REB1-binding site, the GCR1-binding sites, and the RAP1-binding site. Protection was not observed over the GCR1-binding sites in the *gcr1*-deletion mutant strain, although the REB1- and RAP1-binding sites were protected. Identical patterns of factor binding at UAS_{TPI} were observed with DNA isolated from wild-type cultures exposed to dimethyl sulfate under glycolytic and gluconeogenic growth conditions. However, it should be noted that in the experiments presented, dimethyl sulfate treatment was carried out in washed cells; therefore, we may have missed nutritionally specific interactions that occur only in actively growing cells. In addition, we may have missed interactions occurring at binding sites that do not involve guanosine residues.

Site-directed mutagenesis of factor-binding sites in the *TPI* controlling region. We have identified four binding sites for transcriptional regulatory proteins in the 5′ noncoding region of *TPI* from positions −401 to −324. Site-directed mutagenesis of each of the four binding sites and the TATA element was carried out to assess the role of each of these sites in the high-level expression of *TPI*. In the case of the RAP1-binding site, we also introduced mutations adjacent to the site (see below) to eliminate the possibility of RAP1 binding at an adjacent partially overlapping sequence (ACAC CCCTTTTC) which resembles a RAP1-binding site. In addition, we prepared a construct in which both GCR1-binding sites were mutated. The mutations were introduced into plasmid YIpES90, which carries the *TPI*::*lacZ* reporter gene in addition to 853 bp of the *TPI* 5′ noncoding region. After the constructs were integrated at the *URA3* locus in unit copy, we tested the effect of the factor-binding site mutations on the level of expression of the *TPI*::*lacZ* reporter gene (Table 3). A mutation in the REB1-binding site (AG ATTACCCG to AGATTgaaCG) resulted in a fivefold reduction in expression. Mutations in either GCR1-binding site by

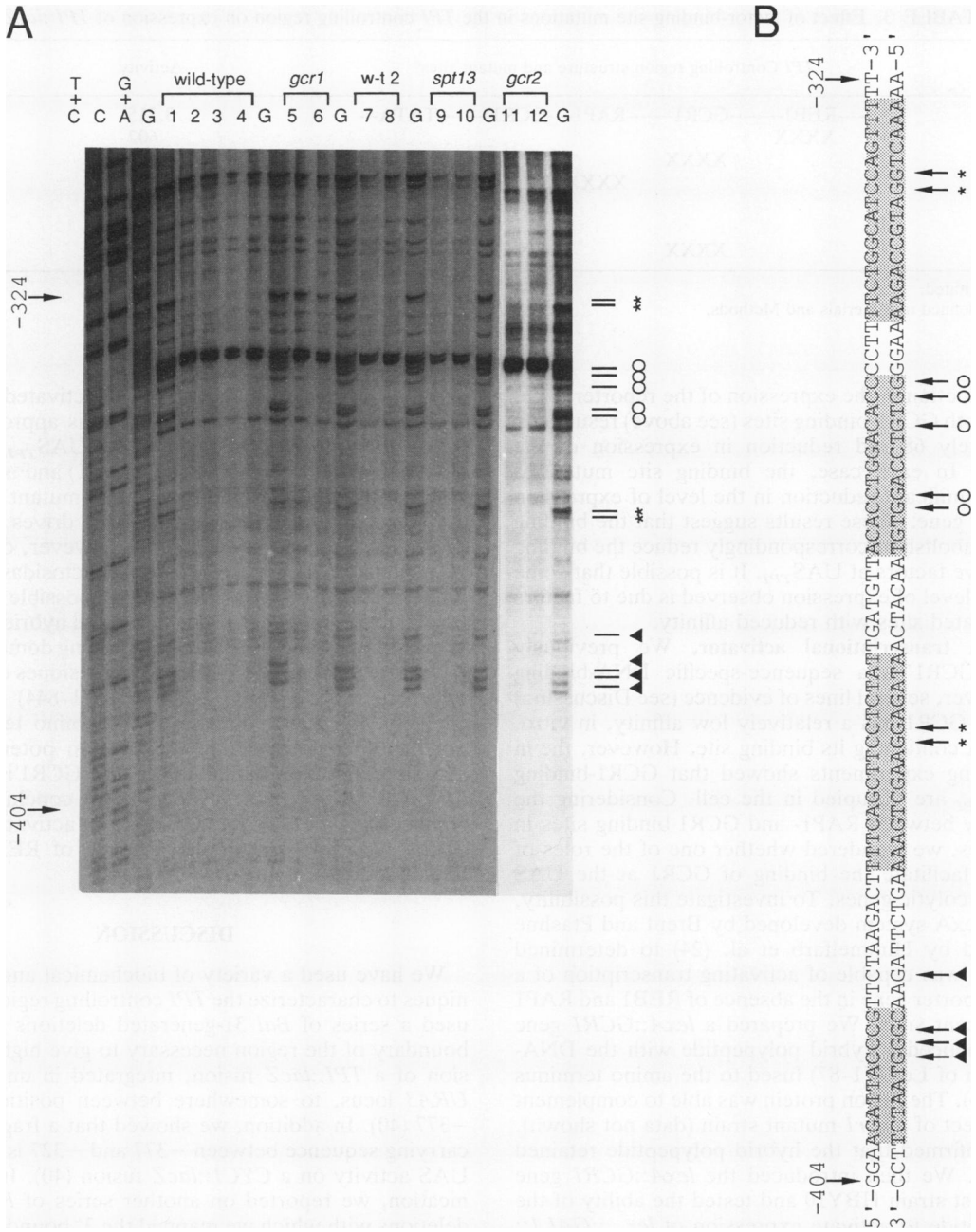

FIG. 4. Genomic footprint of the *TPI* controlling region. (A) Lanes: T+C, C, G+A, and G, genomic sequencing reactions of UAS_{TPI} (the strand sequenced corresponds to the bottom strand shown in panel B); 1 and 2, in vivo guanosine methylation of wild-type strain S150-2B grown in yeast extract-peptone medium supplemented with 2% glucose (YPD); 3 and 4, in vivo guanosine methylation of wild-type strain S150-2B grown in yeast extract-peptone medium supplemented with 1.5% glycerol and 2% lactic acid (YPGly+Lact); 5 and 6, in vivo guanosine methylation of *gcr1* mutant strain HBY4 grown in YPGly+Lact; 7 and 8, in vivo guanosine methylation of wild-type strain DFY642 grown in YPD; 9 and 10, in vivo guanosine methylation of *spt13/gal11* mutant strain JF1052 grown in YPD; 11 and 12, in vivo guanosine methylation of *gcr2* mutant strain DFY641 grown on YPD (lanes 11 and 12 and the rightmost G lane were run on a different gel from the other lanes in the figure). *, guanosine residues protected by GCR1; ○, guanosine residues protected by RAP1; ◀, guanosine residues protected by REB1. (B) Summary map of the *TPI* controlling region showing the DNA sequence of the *TPI* 5′ noncoding region from positions −404 to −324 with respect to the translational start codon. Stippling denotes binding sites for REB1, GCR1, and RAP1. Other symbols are as indicated for panel A.

itself (TTTCAGCTTCCTCTAT to TTTCAGCaaCCTCTAT or TTCTGGCATCCAGTTT to TTCTGGAaaaCAGTTT) resulted in an approximately threefold reduction in expression. Mutations in the RAP1-binding site and adjacent sequence (ACACCTGGACACCCCTTTTC [RAP1-binding site underlined] to ACACCTGGAgAtatCTgcag) reduced expression 10-fold. A mutation of the TATA sequence element located at positions −175 to −169 (TATAAG to GCGAAG) resulted

TABLE 3. Effect of factor-binding site mutations in the *TPI* controlling region on expression of *TPI::lacZ*

Sequence no.	*TPI* Controlling region structure and mutant sites[a]	Activity	% Activity
1	-----REB1--------GCR1-------RAP1----GCR1--------TATA---	3,215	100
2	XXXX	602	18
3	XXXX	1,241	38
4	XXXX	282	9
5	XXXX	920	28
6	XXXX	169	5
7	XXXX XXXX	47	1

[a] XXXX, site mutated.
[b] Expressed as defined in Materials and Methods.

in a 20-fold reduction in the expression of the reporter gene. Mutations in both GCR1-binding sites (see above) resulted in an approximately 68-fold reduction in expression of the reporter gene. In each case, the binding site mutations resulted in a significant reduction in the level of expression of the reporter gene. These results suggest that the binding site mutations abolish or correspondingly reduce the binding of the respective factors at UAS_{TPI}. It is possible that some of the residual level of expression observed is due to factors binding at mutated sites with reduced affinity.

GCR1 as a transcriptional activator. We previously showed that GCR1 is a sequence-specific DNA-binding protein. However, several lines of evidence (see Discussion) suggested that GCR1 has a relatively low affinity, in vitro, for linear DNA containing its binding site. However, the in vivo footprinting experiments showed that GCR1-binding sites at UAS_{TPI} are occupied in the cell. Considering the close proximity between RAP1- and GCR1-binding sites in glycolytic genes, we wondered whether one of the roles of RAP1 was to facilitate the binding of GCR1 at the UAS elements of glycolytic genes. To investigate this possibility, we used the LexA system developed by Brent and Ptashne (8) as modified by Himmelfarb et al. (24) to determined whether GCR1 was capable of activating transcription of a *GAL1::lacZ* reporter gene in the absence of REB1 and RAP1 bound at adjacent sites. We prepared a *lexA::GCR1* gene fusion that produced a hybrid polypeptide with the DNA-binding domain of LexA(1–87) fused to the amino terminus of GCR1(1–844). The fusion protein was able to complement the growth defect of a *gcr1* mutant strain (data not shown). This result confirmed that the hybrid polypeptide retained GCR1 activity. We then introduced the *lexA::GCR1* gene fusion into yeast strain HBY10 and tested the ability of the hybrid polypeptide to activate expression of lex_{op}*::GAL1::lacZ* reporter gene that had its UAS_{GAL} sequences replaced with two copies of the lex_{op}. As controls, we introduced plasmids pLex(1–87)+pl and pMA411 into strain HBY10. Plasmid pLex(1–87)+pl expresses the DNA-binding domain of LexA and serves as a negative control. Plasmid pMA411 carries a *lexA::GAL4* gene fusion which produces a hybrid polypeptide between LexA(1–87) and GAL4(74–881). As pointed out by Golemis and Brent, this assay system is qualitative in nature and should not be used to compare the relative intrinsic activating activities of different activators (22). The LexA-GCR1 fusion protein was able to activate expression of the reporter gene 116-fold over the level of expression observed when just the DNA-binding domain of LexA is expressed in *trans* to the reporter gene; activities [expressed as described in Materials and Methods) were 6 for LexA(1–87), 694 for LexA(1–87)-GCR1(1–844), and 6,769 for LexA(1–87)-GAL4(74–881). The level of β-galactosidase activity observed with LexA-GCR1-activated expression of the lex_{op}*::GAL1::lacZ* reporter gene is approximately one-fifth of that observed when the native UAS_{TPI} drives expression of *TPI::lacZ* (Table 3, sequence 1) and is over twice as high as the level observed when the mutant UAS_{TPI}, with mutations in the RAP1-binding site, drives expression of *TPI::lacZ* (Table 3, sequence 4). However, one must exercise caution when comparing β-galactosidase levels from different reporter genes because of possible differences in β-galactosidase specific activities of the hybrid polypeptides. We previously mapped the DNA-binding domain of GCR1 to the carboxy-terminal 154 amino acid residues of the polypeptide. The hybrid LexA(1–87)-GCR1(1–844) interacts with lex_{op} via the LexA domain at the amino terminus of the polypeptide. Therefore, the activation potential of GCR1 may be somewhat attenuated because GCR1 is "standing on its head" at lex_{op}. In any event, we conclude from these results that LexA-GCR1 is capable of activating expression of the reporter gene in the absence of REB1 and RAP1 bound at adjacent sites.

DISCUSSION

We have used a variety of biochemical and genetic techniques to characterize the *TPI* controlling region. Initially we used a series of *Bal* 31-generated deletions to map the 5′ boundary of the region necessary to give high-level expression of a *TPI::lacZ* fusion, integrated in unit copy at the *URA3* locus, to somewhere between positions −392 and −377 (40). In addition, we showed that a fragment of DNA carrying sequence between −377 and −327 is able to confer UAS activity on a *CYC1::lacZ* fusion (40). In this communication, we reported on another series of *Bal* 31-induced deletions with which we mapped the 3′ boundary of UAS_{TPI} to somewhere between positions −336 and −300. In addition, the deletions indicated that sequences 5′ to position −347 are not capable of independently acting as a fully functional UAS element.

In the noncoding region of *TPI* from positions −405 to −327, we have identified four binding sites for three proteins (Fig. 4): a REB1-binding site located at positions −401 to −392, two GCR1-binding sites located at −381 to −366 and −341 to −326, and a RAP1-binding site located at −358 to −346. We tested the effects of mutations at each of these sties on the level of expression of a *TPI::lacZ* gene fusion which carried 853 bp of the *TPI* 5′ noncoding region integrated at *URA3*. It is interesting to note that the REB1-binding site is dispensable when material 5′ to it is deleted and the reporter gene is integrated at the *URA3* locus (40); however, when material 5′ to the REB1-binding site is from the *TPI* locus, expression is reduced fivefold as a result of

mutations in the REB1-binding site. This finding suggests that sequence 5′ to the REB1-binding site, at the *TPI* locus, has an inhibitory effect on expression of *TPI* and that the role of REB1 is to antagonize the inhibitory effect of the sequence 5′ to its binding site. Fedor et al., studying Y-factor (also called REB1, GRF2, Q-binding protein), reported that Y-factor binding to its site in the GAL UAS creates a nucleosome-free region of approximately 230 bp (17). Both Fedor et al. (17) and Brandl and Struhl (7) have shown that whereas REB1 has little activating activity by itself, the strong synergistic effect observed between REB1 and transcription factors bound at neighboring sites is strongly distance dependent. These workers suggested that the REB1 effect in transcription is mediated through chromatin structure. Accordingly, we envision that the most likely function of the REB1-binding site in the *TPI* controlling region is to prevent the formation of nucleosomes over UAS_{TPI}, thereby allowing RAP1 and GCR1 access to their binding sites at UAS_{TPI}. From the observation that the REB1-binding site is required for full expression of the *TPI*::*lacZ* reporter gene when material 5′ to its binding site is from the *TPI* locus, we redefine UAS_{TPI} to include the REB1-binding site located at positions −401 to −392. Therefore, we propose that *TPI* expression is controlled by a single complex UAS element extending from positions −401 to −326.

Mutation of the RAP1-binding site resulted in a 10-fold reduction in expression of the reporter gene. In vivo footprinting experiments with *gcr1* mutant strains showed that the RAP1-binding site at the *TPI* locus is occupied, yet expression of triosephosphate isomerase is reduced 17-fold as a result of the *gcr1* mutation. Thus, *RAP1* binding is required for high-level expression of *TPI*; however, its binding is not sufficient for maximal expression of *TPI*.

Mutating either GCR1-binding site alone had a modest (approximately threefold) effect on expression of the fusion. However, mutating both GCR1-binding sites resulted in a 68-fold reduction in the level of expression of the reporter gene. It is interesting to note that mutation of both GCR1-binding sites in UAS_{TPI} had a greater effect on expression of the *TPI*::*lacZ* reporter gene than did a complete deletion of *GCR1*, a 68-fold versus 12-fold (40) reduction in β-galactosidase activity. This discrepancy may be explained in terms of squelching (21). In *gcr1* mutant strains, the residual level of expression of the reporter gene was GCR1 independent. Presumably, in the *GCR1* strain, mutations in GCR1-binding sites render expression of the reporter gene GCR1 independent. In both strains, residual expression of the *TPI*::*lacZ* fusion gene was dependent on the ability of the basal promoter to compete successfully for transcriptional factors. However, the background transcriptional activities of the two strains were very different. In the *gcr1* mutant strain, the transcriptional activity of all of the GCR1-dependent genes, which comprise the most highly expressed genes in *S. cerevisiae*, was reduced to between 2 and 10% of wild-type levels (14); presumably, in this case, transcription factors are freed to transcribe GCR1-independent genes. In $GCR1^+$ strains, in contrast, expression of only the reporter gene is affected by the mutations in the UAS_{TPI} element. In these strains, the glycolytic genes are fully functional and transcriptionally active. Thus, the difference in the level of expression of the reporter gene in the two strain backgrounds is most likely related to the phenomenon of squelching whereby basal transcription factors become limiting in the presence of highly expressed transcriptional activators (21).

In vivo footprinting experiments show that four factor-binding sites in UAS_{TPI} were occupied in wild-type strains grown in both gluconeogenic and glycolytic medium. This finding accords with the observation that *TPI* is constitutively expressed in cultures growing in both media (2, 14). We did not observe any differences in the factor-binding site occupancy state at the RAP1-binding site, nor did we observe any effect on factor binding at UAS_{TPI} as a result of mutations in *GCR2* or *SPT13/GAL11*. This result suggests that the role of *GCR2* and *SPT13/GAL11* is not to facilitate the binding of REB1, RAP1, or GCR1 in the controlling region of *TPI*. *gcr1*-deletion mutations which eliminated factor binding at the GCR1-binding sites did not have any effect on factor binding at the neighboring REB1- and RAP1-binding sites. GCR1 synthesized in vitro by using rabbit reticulocyte lysates binds UAS_{TPI} relatively weakly; 50- to 100-fold more GCR1 than RAP1 is required to achieve the same degree of shifting of UAS_{TPI} in a DNA band shift assay (3, 4). Preliminary experiments with a hybrid MBP-GCR1(690–844) polypeptide which carries the functional GCR1 DNA-binding domain have indicated that the apparent affinity of GCR1 for its site is at least an order of magnitude lower than RAP1 for its site (27). Yet despite these differences in apparent affinity, in vivo the RAP1- and GCR1-binding sites at UAS_{TPI} are occupied to approximately the same extent. These observations suggest that there are factors in the cell which facilitate the binding of GCR1 to its DNA-binding sites.

The ability of the LexA(1–87)-GCR1(1–844) fusion protein to activate expression of the $lexA_{op}$::*GAL1*::*lacZ* reporter gene suggests that REB1 and RAP1 binding at adjacent sites was not required for GCR1-dependent gene expression per se. Therefore, the role of these factors may be to facilitate binding of GCR1 at UAS_{TPI}. We propose that REB1 plays an indirect role by preventing the formation of nucleosomes over UAS_{TPI}, thereby allowing the binding of RAP1 and GCR1. We envision that RAP1 plays a more direct role in the binding of GCR1 at UAS_{TPI}. Considering the proximity of RAP1- and GCR1-binding sites at UAS_{TPI} and in the UAS elements of other glycolytic genes (28), we think it likely that RAP1 binding is required at sites adjacent to GCR1-binding sites for GCR1 binding in vivo. RAP1 may facilitate the binding of GCR1 at UAS_{TPI} by cooperative protein-protein interactions between the two factors bound at neighboring sites, or RAP1-induced bending (45) may alter the topology of adjacent GCR1-binding sites, resulting in the formation of high-affinity GCR1-binding sites.

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