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Characterization of the DNA-Binding Activity of GCR1: In Vivo Evidence for Two GCR1-Binding Sites in the Upstream Activating Sequence of *TPI* of *Saccharomyces cerevisiae*

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***GCR1* gene function is required for high-level glycolytic gene expression in *Saccharomyces cerevisiae*. Recently, we suggested that the CTTCC sequence motif found in front of many genes encoding glycolytic enzymes lay at the core of the GCR1-binding site. Here we mapped the DNA-binding domain of GCR1 to the carboxy-terminal 154 amino acids of the polypeptide. DNase I protection studies showed that a hybrid MBP-GCR1 fusion protein protected a region of the upstream activating sequence of *TPI* (UAS_{TPI}), which harbored the CTTCC sequence motif, and suggested that the fusion protein might also interact with a region of the UAS that contained the related sequence CATCC. A series of in vivo G methylation protection experiments of the native *TPI* promoter were carried out with wild-type and *gcr1* deletion mutant strains. The G doublets that correspond to the C doublets in each site were protected in the wild-type strain but not in the *gcr1* mutant strain. These data demonstrate that the UAS of *TPI* contains two GCR1-binding sites which are occupied in vivo. Furthermore, adjacent RAP1/GRF1/TUF- and REB1/GRF2/QBP/Y-binding sites in UAS_{TPI} were occupied in the backgrounds of both strains. In addition, DNA band-shift assays were used to show that the MBP-GCR1 fusion protein was able to form nucleoprotein complexes with oligonucleotides that contained CTTCC sequence elements found in front of other glycolytic genes, namely, *PGK*, *ENO1*, *PYK*, and *ADH1*, all of which are dependent on *GCR1* gene function for full expression. However, we were unable to detect specific interactions with CTTCC sequence elements found in front of the translational component genes *TEF1*, *TEF2*, and *CRY1*. Taken together, these experiments have allowed us to propose a consensus GCR1-binding site which is 5′-(T/A)N(T/C)N(G/A)NC(T/A)TCC(T/A)N(T/A)(T/A)(T/G)-3′.**

In *Saccharomyces cerevisiae*, expression of glycolytic genes accounts for 30 to 60% of the soluble protein in the cell (31, 39). High-level expression of these genes is dependent on several *trans*-acting factors, namely, GCR1 (4, 22, 23, 41), GCR2 (69), and RAP1/GRF1/TUF (hereafter referred to as RAP1) (12, 42, 66). The involvement of GCR1 and GCR2 in glycolytic gene expression was established when mutations were isolated in the genes that encoded these factors, whereas the role of RAP1 in the expression of these genes was suggested when, through DNA sequence analysis, it was discovered that many glycolytic genes carried sequences in their 5′ noncoding region which matched the consensus RAP1-binding site (16). The role of RAP1 was confirmed by mutational analysis; RAP1-binding sites located in front of glycolytic genes have been shown to be essential features of their upstream activating sequence (UAS) elements (15, 19, 47, 53, 64). In addition, several of the genes have binding sites for ABF1 (12) and REB1 (51)/GRF2 (20)/QBP (10)/Y (30) (hereafter referred to as REB1) in the vicinity of known UAS elements (11, 18, 20, 65); however, the roles played by ABF1 and REB1 in the expression of these genes are unclear.

Recently, we were able to demonstrate that *GCR1* encodes a DNA-binding protein which binds to a fragment of DNA carrying the UAS element for the gene encoding triose-phosphate isomerase, *TPI* (5). Mutational analysis of the DNA fragment suggested that GCR1 was interacting with a CTTCC sequence motif carried on the fragment, but the GCR1-binding site was not determined. Genetic studies had previously implicated CTTCC sequence elements as playing an important role in the expression of genes encoding glycolytic enzymes (13, 17). Bitter et al. (8) recently defined a sequence element [G(A/C)(A/T)TCC(A/T)], related to the CTTCC sequence motif, which they termed GPE for GRF1-site potentiator element. In this study, we used both in vitro and in vivo techniques to further characterize the DNA-binding activity of GCR1 and to define a consensus GCR1-binding site. We propose that *GCR1* encodes the positive *trans*-acting factor that works through the CTTCC/GPE sequence element.

MATERIALS AND METHODS

Nucleic acid manipulations. Techniques used throughout the course of this study are described in the standard reference manuals (3, 60, 61). Oligonucleotides used as radiolabeled probes in this study were initially synthesized on an Applied Biosystems 380B DNA synthesizer and then cloned into plasmid DNA. The oligonucleotides were then purified from the plasmid DNA and used in the band-shift assays as described below.

In vitro synthesis of GCR1. Plasmid pHB66 (5) was used to direct the synthesis of GCR1 RNA. GCR1 transcripts were expressed in vitro under the control of the SP6 promoter. The transcription reactions were carried out in the presence

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TABLE 1. Oligonucleotides used as probes in DNA band-shift assays in this study

Oligonucleotide	Sequence[a]
Linker	**agcttgcatgcctgcaggtcgactctagaggatccccgggtaccgagctcgatt**
UAS_{TPI}	**agcttAGCTTCCTCTATTGATGTTACACCTGGACACCCCTTTTCTGGCATCCAGTTgcat**
TPI_1	**agcttGACTTTTCAGCTTCCTCTATTGATGgcatgcggatccccgggtaccgagctcgatt**
TPI_2	**agcttTTTTTCTGGCATCCAGTTTTTAATgcatgcggatccccgggtaccgagctcgatt**
PGK	**agcttGACTTCCTGTCTTCCTATTGATTGCgcatgcggatccccgggtaccgagctcgatt**
ENO1	**agcttCTAATCCGAGCTTCCACTAGGATAGgcatgcggatccccgggtaccgagctcgatt**
PYK	**agcttAGACATCGGGCTTCCACAATTTTCGgcatgcggatccccgggtaccgagctcgatt**
ADH1	**agcttACAATATGGACTTCCTCTTTTCTGGgcatgcggatccccgggtaccgagctcgatt**
CYC1	**gattcggCCCGGGAGCAAGATCAAGATGTTTTCACCGATCTTTCCGGTCTCTTTGGCCGGGGTTTACGGACGATGA** **CCGAAGACCAAGCGCCAGCTCATTTGGCGAGCGTTGGTTGGTGGATCAAGCCCACGCGTAGGCAATCCTCGA**
$TEF1_1$	**agcttCTTTTTTACTCTTCCAGATTTTCTCgcatgcggatccccgggtaccgagctcgatt**
$TEF1_2$	**agcttTCCCCTCTTTCTTCCTCTAGGGTGTgcatgcggatccccgggtaccgagctcgatt**
TEF2	**agcttAGCGTAGGCGCTTCCCCTGCCGGCTgcatgcggatccccgggtaccgagctcgatt**
CRY1	**agcttTGGTGCAGGGCTTCCTCAGGTAGACgcatgcggatccccgggtaccgagctcgatt**

[a] Uppercase bold lettering denotes sequences from the indicated genes; lowercase lettering denotes sequences from the polylinker of pUC18.

of the cap analog $m^7G(5')ppp(5')G$ with a kit from Promega, according to the manufacturer's specifications. The in vitro-derived transcripts were then translated in a rabbit reticulocyte lysate system in the presence of L-[^{35}S]methionine by using a kit obtained from Promega, according to the manufacturer's specifications. The amount of GCR1 produced in the rabbit reticulocyte lysates was estimated by determining the amount of [^{35}S]methionine incorporated into trichloroacetic acid-precipitable material. Typical in vitro translation reactions yielded approximately 2.4 ng of GCR1 per μl of rabbit reticulocyte lysate. Carboxy-terminal truncations of GCR1 were generated by first linearizing plasmid pHB66 with restriction enzymes which cut within the *GCR1* structural gene prior to the in vitro transcription reactions. The truncated RNAs were then translated in the rabbit reticulocyte lysate system. Translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the radiolabeled proteins were visualized by autoradiography at −70°C.

***malE*::*GCR1* gene fusions.** A series of *malE*::*GCR1* gene fusions which carried various deletions of the 5′ end of the *GCR1* structural gene were prepared. These deletions were generated either by cloning an appropriate restriction fragment carrying a portion of *GCR1* into one of the pMAL vectors available from Bethesda Research Laboratories or by amplifying the desired portion of *GCR1* with appropriate primers (carrying an in-frame *Bam*HI restriction endonuclease site at the 5′ end of the primer) by the polymerase chain reaction. The amplified fragments were then digested with *Bam*HI and *Sal*I (*Sal*I cuts 3′ to the *GCR1* structural gene) and cloned into the *Bam*HI-*Sal*I sites of pMAL-cRI. The in-frame fusions thus constructed direct the synthesis of a hybrid polypeptide between the maltose-binding protein and GCR1 (MBP-GCR1).

Induction of *malE*::*GCR1* gene fusions. *Escherichia coli* strains harboring *malE*::*GCR1* gene fusions were grown at 37°C in LB with ampicillin at 100 μg/ml to an optical density (A_{600}) of 0.5. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added to a final concentration of 2 mM, and the cultures were grown an additional 2 h at 37°C. The cultures were then collected by centrifugation, and the cells were suspended in 3 ml of TEN lysis buffer (50 mM Tris [pH 8.0], 1 mM EDTA, 50 mM NaCl) per g (pellet [wet weight]). Cells were disrupted by passage through a French pressure cell at 20,000 lb/in^2. Cellular debris was removed by centrifugation at 17,000 × *g* for 20 min at 4°C. Cellular extracts prepared in this manner typically resulted with protein concentrations that ranged from 0.068 to 0.2 mg/ml.

Purification of MBP-GCR1 fusion protein. Hybrid MBP-GCR1 fusion protein was purified by virtue of its maltose-binding moiety over an amylose column (59).

DNA band-shift assays. DNA band-shift assays based on the procedures of Fried and Crothers (32) and Garner and Revzin (33) were carried out as described previously. Table 1 shows the sequences of the oligonucleotide probes used in this study. The typical reaction was carried out in a 20-μl volume which contained 0.5 to 1 ng of radiolabeled probe DNA along with 0.2 μg of poly(dI-dC) per μl as nonspecific competitor.

DNase I protection studies. DNase I footprints were carried out by a modification of the method of Pfeifer et al. (58). Nucleoprotein complexes were allowed to form under standard band-shift reaction conditions, and then various amounts of DNase I (0.2 to 0.5 U) were added. The reaction mixture was incubated at ambient temperature for 2 min. The reaction was stopped by the addition of an equal volume of stop solution (50% glycerol, 0.25 M EDTA [pH 8.0]). The resulting mixture was immediately loaded onto a native polyacrylamide gel. Free and complexed DNA were detected by autoradiography of the wet gel. Bound DNA from both the upper and lower complexes was cut out of the gel and eluted from the gel by being soaked overnight at 37°C in 500 mM ammonium acetate–1 mM EDTA. The eluted DNA was then precipitated, washed, suspended in loading buffer, boiled, and loaded onto a denaturing sequencing gel.

In vivo DNA methylation protection studies. Cultures of yeast strains S150-2B and HBY4 were grown in YP medium (60) supplemented with 2% glucose or 2% glycerol and 2% lactic acid, respectively, to an optical density (A_{600}) of 1. The cultures were then harvested, washed three times in 137 mM NaCl–2.7 mM KCl–4.3 mM $NaPO_4$–1.4 mM KPO_4 (pH 7.4) (phosphate-buffered saline [PBS]), and resuspended at a concentration of 10^8 cells per ml in PBS. Aliquots (5 ml) were then treated with 25 μl of dimethyl sulfate (final concentration, 52 mM) for various times (6, 29). The reactions were quenched by the addition of 45 ml of ice-cold PBS, and then the cells were washed two times in 30 ml of PBS. Chromosomal DNA was then isolated from the dimethyl sulfate-treated cells by standard procedures (60), except no incubations were carried out at temperatures above 37°C. The resulting DNA (200 μg) was digested to completion with *Ava*II, after which the DNA was extracted

with phenol, precipitated with ethanol, resuspended in 100 μl of 1 M piperidine, and incubated at 95°C for 30 min. The piperidine-cleaved material was then precipitated two times and lyophilized overnight, following which the DNA was suspended in 105 μl of water and its concentration was determined spectrophotometrically. Aliquots (5 μg) were dried, resuspended in 5 μl of gel loading buffer, and boiled for 5 min prior to being loaded onto a 6% polyacrylamide–8 M urea sequencing gel. In adjacent lanes, chemical cleavage reaction mixtures (48) of yeast genomic DNA were run to identify the areas of protection.

The genomic sequencing reactions were prepared in the following manner: yeast chromosomal DNA was isolated, digested with *Ava*II, and subjected to the standard Maxam and Gilbert (48) sequencing reactions. The resulting DNA was then prepared for electrophoresis as described above. After electrophoresis, the DNA was electroblotted to Hybond N+ (Amersham), and the filter was processed for hybridization (21).

The hybridization probe was prepared by primer extension of an M13 clone of the 5′ noncoding region of *TPI*. A *TPI*-specific primer (5′-GACCTTAATACATTCAG-3′) was designed such that it would anneal with its 5′ end at the *Ava*II site located at −487 with respect to the translational start of *TPI* and would be positioned to prime synthesis towards the start of the *TPI* structural gene (1).

Hybridizations were carried out essentially according to the method of Church and Gilbert (21), except that hybridization volumes were kept to 5 ml, and hybridizations were preformed in a rolling drum incubator (Robbins Scientific, Sunnyvale, Calif.).

Dideoxy DNA sequencing. M13mp18 phage DNA was sequenced by the dideoxy sequencing method with Sequenase (U.S. Biochemical), according to the manufacturer's specifications.

RESULTS

Throughout the course of this study, we used GCR1 synthesized in vitro from rabbit reticulocyte lysates and MBP-GCR1 fusion protein, expressed in *E. coli*, to characterize the DNA-binding activity of GCR1. Both GCR1 synthesized in vitro and the full-length MBP-GCR1 fusion protein were able to form nucleoprotein complexes with DNA carrying the UAS of *TPI* (Fig. 1). The nucleoprotein complexes observed with the fusion protein migrate more slowly than the complexes observed with GCR1 from the rabbit reticulocyte lysate (Fig. 1). This difference is presumably due to the increase in molecular weight of the nucleoprotein complex due to the presence of the maltose-binding moiety of the fusion protein. We routinely observed the appearance of two shifted bands with GCR1 and its derivatives in the band-shift assays described here. The appearance of two shifted bands with GCR1 synthesized in rabbit reticulocyte lysates has been noted previously (5). Figure 1 also shows that no nucleoprotein complexes were observed with untranslated rabbit reticulocyte, *E. coli* extracts prepared from uninduced and induced cultures of strains carrying the parent plasmid, pMAL-cR1, and extracts prepared from uninduced cultures of *E. coli* strains carrying the *malE*::*GCR1* gene fusion.

The DNA-binding domain of GCR1 resides within the carboxy-terminal 154 amino acid residues. In an attempt to map the DNA-binding domain of GCR1, we carried out a series of DNA band-shift experiments with truncated versions of GCR1. Carboxy-terminal truncations of GCR1 were synthesized in vitro. Plasmid pHB66 was linearized with a series of restriction endonucleases that cleaved within the *GCR1* structural gene. The linearized constructs were then used as templates for in vitro RNA synthesis, and runoff

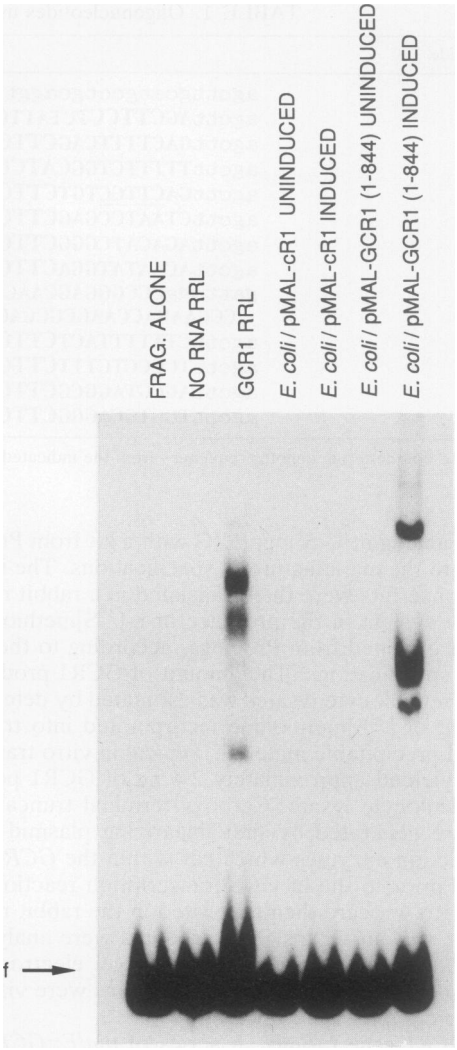

FIG. 1. Comparison of DNA-binding activity of GCR1 and hybrid MBP-GCR1 fusion protein. DNA band-shift assays were carried out with a radiolabeled fragment of DNA carrying UAS_{TPI} (see Table 1 for sequence). The radiolabeled DNA was incubated in binding buffer with the protein extract indicated above each lane. Nucleoprotein complexes were resolved from free DNA by nondenaturing PAGE and were revealed by autoradiography. FRAG. ALONE, radiolabeled DNA fragment; NO RNA RRL, 5 μl of untreated rabbit reticulocyte lysate (RRL); GCR1 RRL, 5 μl of rabbit reticulocyte lysate containing in vitro synthesized GCR1; *E. coli*/pMAL-cR1 UNINDUCED, 1 μl of an extract of an *E. coli* culture harboring plasmid pMAL-cR1; *E. coli*/pMAL-cR1 INDUCED, 1 μl of an extract of an *E. coli* culture harboring plasmid pMAL-cR1 which had been induced with IPTG 2 h prior to harvest; *E. coli*/pMAL-GCR1 (1–844) UNINDUCED, 1 μl of an extract of *E. coli* culture harboring plasmid pMAL-GCR1 (1–844) (numbers in parentheses denote amino acid residues of GCR1 present in the expressed polypeptide); *E. coli*/pMAL-GCR1 (1–844) INDUCED, 1 μl of an extract of an *E. coli* culture harboring plasmid pMAL-GCR1 (1 to 844) which had been induced with IPTG 2 h prior to harvest; f, free unbound probe.

transcripts were translated in vitro by using rabbit reticulocyte lysates. Production of polypeptides of the desired molecular mass was confirmed by SDS-PAGE and autoradiography (data not shown). The rabbit reticulocyte lysates were then used in a series of band-shift experiments to determine which, if any, of the truncated proteins had DNA-binding activity. Figure 2A shows that only full-length GCR1 had binding activity.

Next we were interested in carrying out a similar set of experiments with an amino-terminal deletion series. Thus, we constructed a set of *malE::GCR1* gene fusions. Various subgenic portions of *GCR1* were fused in frame to *malE* in the fusion vector pMAL-cR1. *E. coli* lysates prepared from strains harboring the *malE::GCR1* gene fusions were prepared. Production of MBP-GCR1 hybrid polypeptide of the desired molecular mass was confirmed by SDS-PAGE, and the fusion protein was visualized by Coomassie blue staining (data not shown). In general, there was an inverse correlation between the size of the fusion polypeptide and the amount of material observed. *E. coli* lysates containing the hybrid MBP-GCR1 polypeptide were then tested by DNA band-shift assays (Fig. 2B). All constructs that carried the carboxy-terminal 154 amino acids of GCR1 were able to bind to the UAS of *TPI*. Lysates from strains expressing fusion protein carrying the carboxy-terminal 138 amino acids of GCR1 were unable to bind the DNA fragment used in the study. A summary of the mapping data is shown in Fig. 2C. From these results, we concluded that the DNA-binding domain of GCR1 resides somewhere within the carboxy-terminal 154 amino acids of GCR1.

DNase I footprint of GCR1-binding site. We recently reported results which suggested that GCR1 interacts with the CTTCC sequence motif found in front of many genes encoding glycolytic enzymes (5). Therefore, we were interested in determining the sequence that GCR1 protected from DNase I digestion. For these experiments, we used purified preparations of the smallest MBP-GCR1 (690 to 844) fusion protein which retained DNA-binding activity in the band-shift assay. Figure 3 shows the results of the DNase I protection studies using purified MBP-GCR1 (690 to 844) fusion protein. Two areas of protection were observed: one clear area of protection centered over the CTTCC motif and another area of partial protection centered over the related sequence CATCC. We were somewhat surprised to see an area of protection over the CATCC sequence element, since we previously showed that changing the CTTCC sequence element to CAACC on a fragment that carried both elements abolished the ability of the fragment to bind GCR1 (5). At least two possibilities exist to explain the discrepancy: the concentration of GCR1 in the rabbit reticulocyte lysate was not high enough to detect binding at the CATCC sequence element, or the concentration of MBP-GCR1 was so high that artifactual binding at secondary sites was detected in the experiments presented here. To determine whether either of these alternatives was correct, we sought to carry out in vivo G methylation protection studies using the *TPI* promoter.

Genomic footprint of the native *TPI* promoter. To determine whether the sequences protected in the in vitro footprinting studies were occupied in vivo, we carried out G methylation protection experiments with the wild-type strain, S150-2B (*MAT***a** *leu2-3,112 his3*Δ *trp1-289 ura3-52*) and the *gcr1*-deletion mutant strain, HBY4 (*MAT***a** *gcr1*Δ*::HIS3 leu2-3,112 his3*Δ *trp1-289 ura3-52*). We used a hybridization probe that allowed us to detect protection in the region of the *TPI* UAS element. Figure 4 shows four areas of protection with DNA isolated from the wild-type strain, whereas only two areas of protection were observed for DNA isolated from the isogenic *gcr1*-deletion mutant strain. In the wild-type strain, protection was observed for a known REB1-binding site (65), for the G doublet that corresponds to the C doublet in the CTTCC pentamer, for a known RAP1-binding site (64), and for the G doublet that corresponds to the C doublet in the CATCC pentamer. Identical patterns of protection were observed for G methylation protection experiments carried out with S150-2B grown in YP medium supplemented with glycerol plus lactic acid (data not shown). In the *gcr1* mutant strain, protection was observed only for the REB1- and RAP1-binding sites. No areas of protection were observed for the CTTCC or CATCC sequence elements in the *gcr1* mutant strain. Thus, these results showed that there are two in vivo GCR1-binding sites in UAS_{TPI}.

GCR1 interacts with promoters of other glycolytic genes. To date, our studies concerning the DNA-binding characteristics of GCR1 have utilized DNA carrying sequences from the *TPI* promoter. In an effort to define a consensus GCR1-binding site, we synthesized DNA with putative GCR1-binding sites found in front of a number of other genes encoding glycolytic enzymes, namely, *PGK*, *ENO1*, *PYK*, and *ADH1*. These sequences were picked for the following reasons. Deletion analysis showed that three CTTCC sequence elements are important for expression of *PGK* (17). We arbitrarily chose one of these elements for study. The CTTCC elements from *ENO1* and *PYK* were chosen because genetic evidence shows that they are important determinants of their respective UAS elements (13). We predicted that the CTTCC element found adjacent to the RAP1-binding site in the *ADH1* promoter would likely bind GCR1; therefore, we decided to test it for its ability to bind GCR1. In addition, we decided to test the two GCR1-binding sites in the *TPI* UAS individually. The oligonucleotides which carried 25 nucleotides from the genes of interest with the CTTCC motif under study located at the center of each oligonucleotide were synthesized. Because of the relative positions of the elements in UAS *PGK*, the oligonucleotide carrying the *PGK* sequence contained two CTTCC sequence elements. The oligonucleotides were then tested for their ability to interact with the DNA-binding domain of GCR1 in a series of DNA band-shift assays. As negative controls, we included the polylinker from pUC18 and a fragment of DNA containing both UAS elements of *CYC1* (35), a gene not known to be dependent on GCR1 for expression, in the band-shift assays. Table 1 shows the sequences of the oligonucleotides used as probes in this study. The results of the band-shift assays are shown in Fig. 5. The appearance of shifted bands was not detected when the polylinker from pUC18 or when the *CYC1* UAS elements were used as probes. On the other hand, each of the putative GCR1-binding sites gave rise to the appearance of shifted bands, thus providing evidence that GCR1 is capable of interacting with these sequences.

Santangelo and Tornow (62) have reported that the expression of the genes encoding elongation factor EF-1α (*TEF1* and *TEF2*) and ribosomal protein 59 (*CRY1*) is reduced in *gcr1* mutant strains. We were interested in determining whether these genes contained sequences that would act as potential GCR1-binding sites in their 5′ noncoding region. The RAP1 site of *TEF1* is bracketed by two CTTCC sequence elements (TTTACTCTTCCAGATT and CTCTTT CTTCCTCTAG) (26, 52). A site (TAGGCGCTTCCCCTGC) (52) of potential GCR1 interaction was located upstream of the RAP1-binding site in *TEF2*. Likewise, *CRY1* also har-

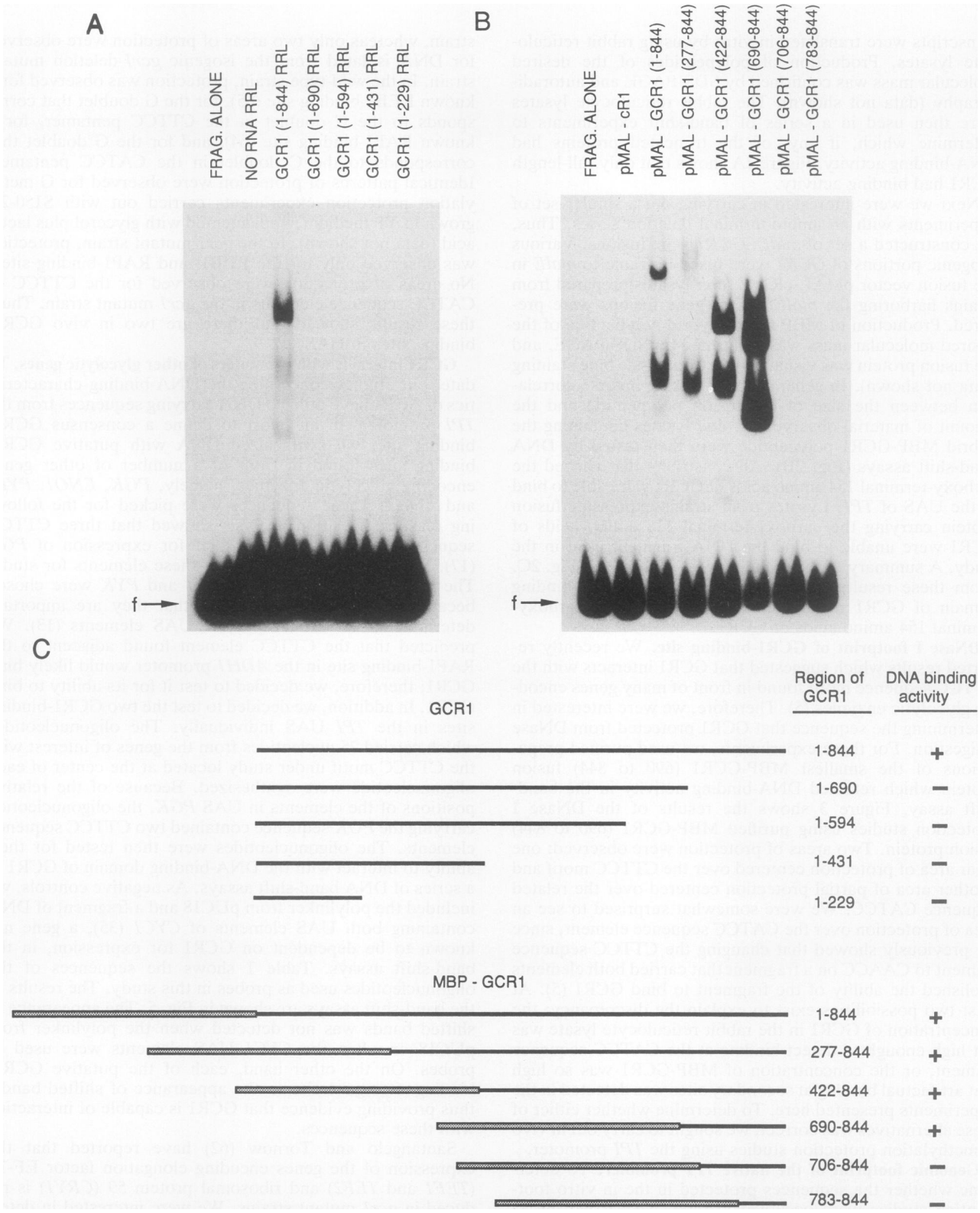

FIG. 2. Carboxy-terminal 154 amino acid residues of GCR1 confer DNA-binding activity. DNA band-shift assays were carried out as described in the legend to Fig. 1. (A) DNA band-shift assays with in vitro synthesized GCR1 carboxy-terminal deletion series. The numbers in parentheses denote the amino acid residues of GCR1 expressed in the rabbit reticulocyte lysates (RRL). Five microliters of each rabbit reticulocyte lysate was used in the assays. (B) DNA band-shift assays with GCR1 amino-terminal deletion series hybrid MBP-GCR1 fusion protein. One microliter of an extract of the induced *E. coli* culture indicated above each lane was added to the standard band-shift reaction mixture, as described in the legend to Fig. 1. Numbers in parenthesis denote the amino acid residues of GCR1 contained in the hybrid polypeptide. (C) Schematic representation of the GCR1 polypeptides used in the mapping study and summary of results obtained. Solid lines represent regions of GCR1 carried in the polypeptide. The stippled lines indicate maltose-binding protein moiety carried in the fusion protein.

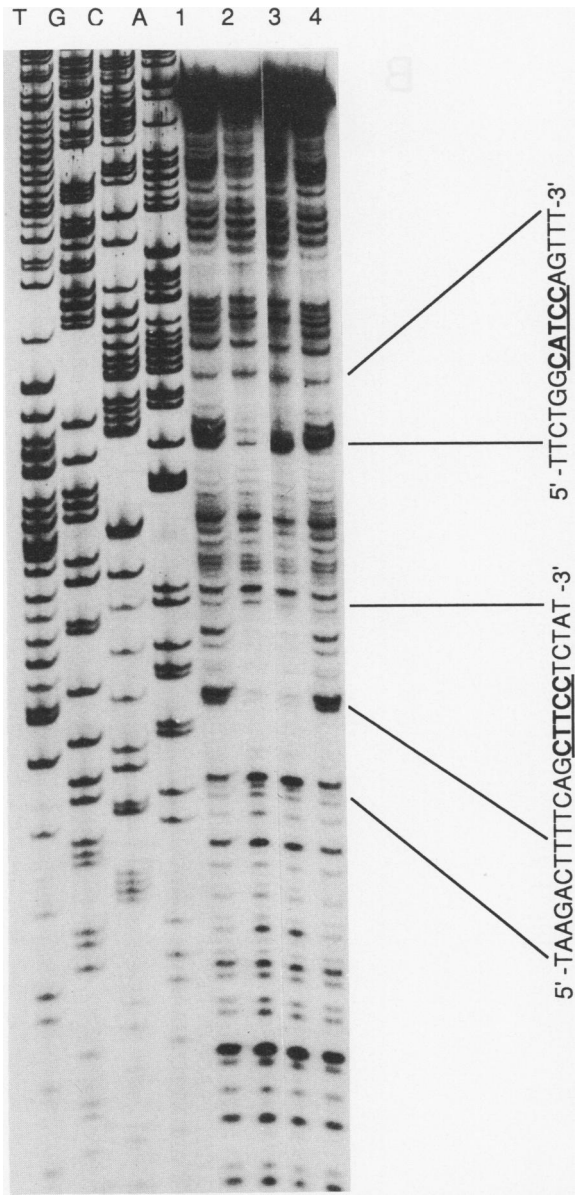

FIG. 3. GCR1 DNA-binding domain protects the CTTCC sequence motif and a related sequence element, CATCC, in UAS_{TPI} from DNase I cleavage. Analysis of UAS_{TPI} was carried out with purified MBP-GCR1 (690 to 844) and a radiolabeled 234-bp fragment carrying the UAS of *TPI*. Lanes T, G, C, and A, are the products of the dideoxy sequencing reactions of M13mp18 and serve as molecular weight standards; lanes 1 and 4, free fragment treated with DNase I; lane 2, nucleoprotein complex treated with 0.2 U of DNase I, as described in Materials and Methods; lane 3, nucleoprotein complex treated with 0.5 U of DNase I. The sequences of protected areas are denoted on the right. The exact extent of the area protected over the CTTCC sequence element could not be determined because of the lack of bands in the control lanes (lanes 1 and 4); therefore, two 5′ boundaries are indicated on the figure.

bored a potential GCR1-binding site (GCAGGGCTTCC TCAGG) (46) downstream of its RAP1-binding site. Thus, in each case, we were able to locate CTTCC sequence elements in the vicinity of a known RAP1-binding site. We were interested in determining whether these sequences were able to bind GCR1; therefore, we synthesized oligonucleotides which carried 25 nucleotides from the genes of interest with the CTTCC motif under study located at the center of each oligonucleotide (see Table 1). These oligonucleotides were then tested by DNA band shift assay for their ability to interact with the DNA-binding domain of GCR1. We were unable to detect specific binding with any of these oligonucleotides (Fig. 6).

DISCUSSION

Recently, we provided evidence that *GCR1* encodes a DNA-binding protein and suggested that the CTTCC sequence found in front of many genes encoding glycolytic enzymes lay at the core of its binding site (5). In this communication, we extend our studies on the DNA-binding characteristics of GCR1 and further define the GCR1-binding site. Through the use of a series of MBP-GCR1 hybrid fusion proteins, we were able to map the DNA-binding domain of GCR1 to the carboxy-terminal 154 amino acids. We had previously suggested, on the basis of DNA sequence analysis, that this region may contain the DNA-binding domain of GCR1 since this region contains a possible helix-turn-helix motif from amino acids 784 to 803 (4). We were able to generate smaller hybrid polypeptides that contained the putative helix-turn-helix motif which were unable to bind DNA [Fig. 2B, pMAL-GCR1(706–844) and pMAL-GCR1 (783–844)]. The negative result obtained with these smaller polypeptides is difficult to interpret. Whereas SDS-PAGE analysis of the smaller polypeptides indicated that they were stable (data not shown), we have no data concerning the higher-order structure of these proteins. On the basis of this analysis, we conclude that all residues necessary and sufficient for GCR1 binding reside in the carboxy-terminal 154 amino acids of the native GCR1 polypeptide.

The CTTCC sequence motif found in front of many genes encoding glycolytic enzymes has long been proposed to be an important *cis*-acting element in their regulation (54). DNase I protection studies utilizing the purified MBP-GCR1 hybrid polypeptide revealed two areas of protection in the region of the *TPI* UAS element. One clear area of protection was centered over the CTTCC sequence motif at position −375, and an area of partial protection was observed over the related sequence CATCC at position −335, with respect to the translational start codon. Genomic footprinting experiments with wild-type strains showed that both of these sites are occupied in vivo. Similar experiments with a *gcr1*-deletion mutant strain showed that these sites were vacant. Adjacent REB1- and RAP1-binding sites were occupied in both the wild-type and *gcr1*-deletion mutant strains. We have previously shown that the *TPI* UAS has an absolute requirement for the RAP1-binding site, whereas the REB1-binding site is dispensable (64). From the results presented here, it is clear that *GCR1* function was not required for RAP1 or REB1 binding at the *TPI* UAS. However, RAP1 and REB1 may influence the binding of GCR1 at the *TPI* UAS element in vivo. The notion that RAP1 may facilitate factor binding at adjacent sites has been suggested previously (5, 27).

Santangelo and Tornow proposed that GCR1 acts through RAP1-binding sites (62). We have shown that GCR1 binds at two sites adjacent to the RAP1-binding site in the *TPI* UAS element, and we did not observe any protection over the RAP1-binding site in the in vitro DNase I protection assays that we carried out with purified MBP-GCR1 (Fig. 3). We showed previously that the binding of in vitro synthesized GCR1 to DNA was independent of the presence of a

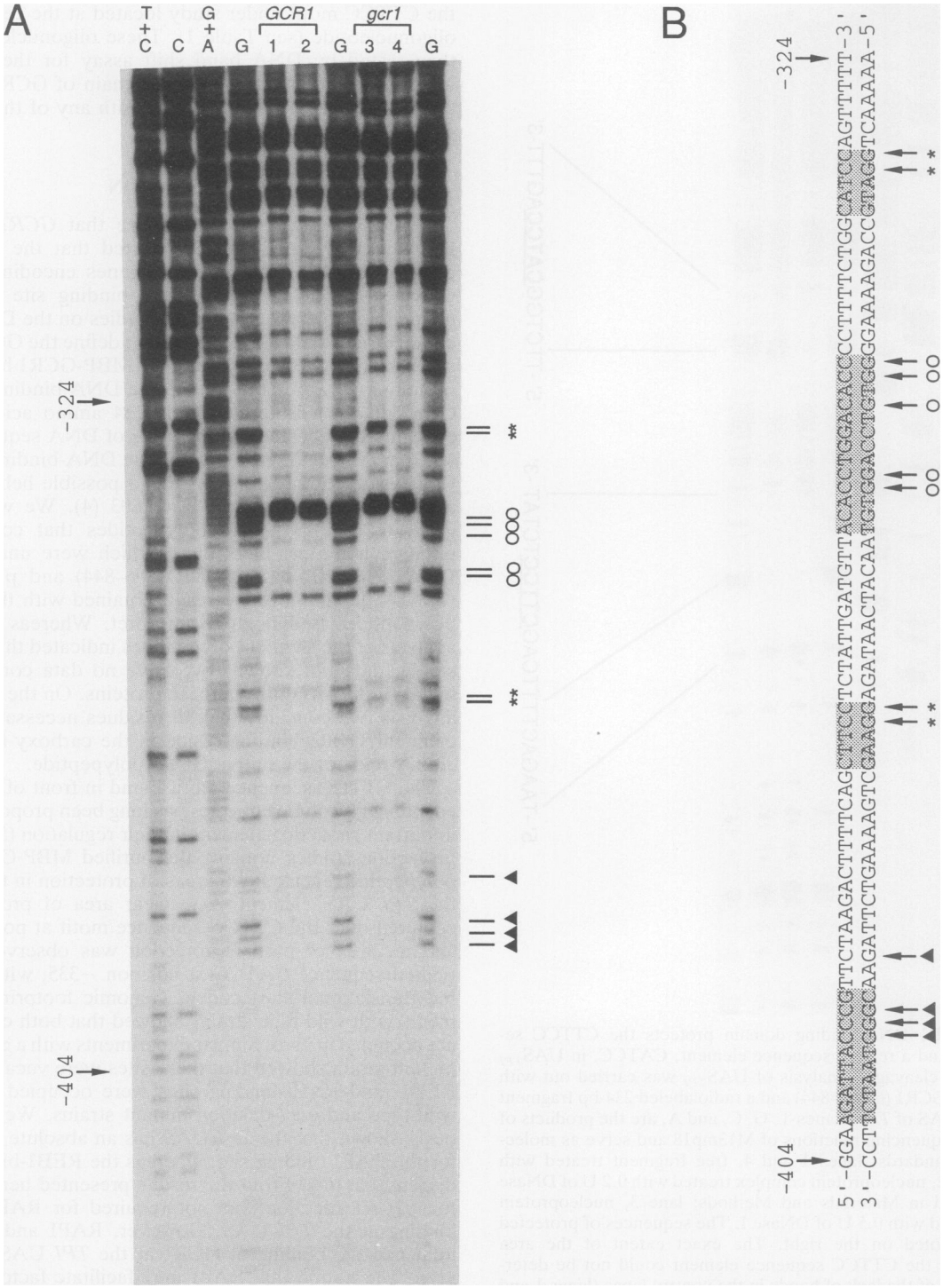

FIG. 4. (A) In vivo G methylation protection footprints of the *TPI* UAS in wild-type and *gcr1*-deletion mutant strains. Lanes T+C, C, G+A, and G, genomic sequencing reactions of UAS_{TPI} (the strand sequenced corresponds to the bottom strand shown in panel B); lanes 1 and 2, in vivo G methylation (3- and 4-min dimethyl sulfate treatment, respectively) of glucose-grown wild-type strain S150-2B; lanes 3 and 4, in vivo G methylation (4- and 5-min dimethyl sulfate treatment, respectively) of glycerol and lactic acid-grown *gcr1*-deletion mutant strain HBY4. (B) Summary map of *TPI* controlling region. The DNA sequence of the *TPI* 5′ noncoding region from positions −404 to −324 with respect to the translational start codon with protected G residues is denoted. The stippled areas denote the core binding sites for REB1, GCR1, and RAP1. *, G residues protected by GCR1; ○, G residues protected by RAP1 (64); ◀, G residues protected by REB1 (65).

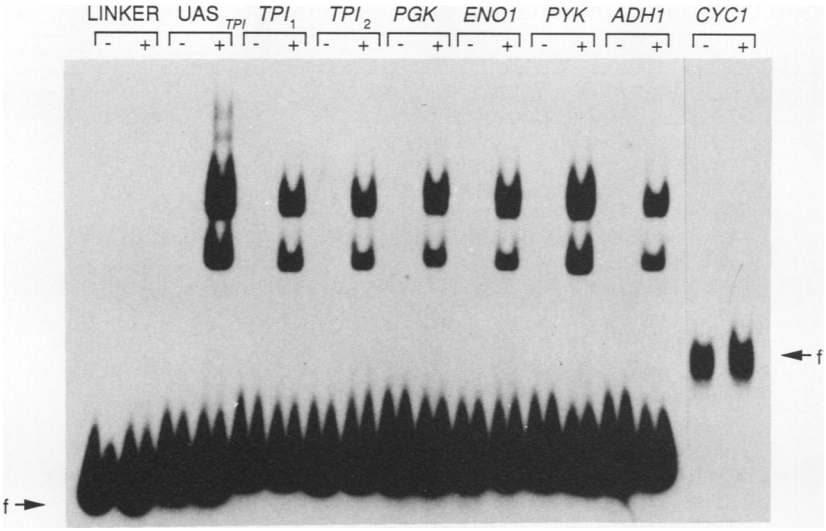

FIG. 5. GCR1 DNA-binding domain binds to CTTCC sequence elements found in front of other glycolytic genes. DNA band-shift analysis was carried out with purified MBP-GCR1 (690 to 844) and the radiolabeled oligonucleotides listed in Table 1. The absence (−) and presence (+) of 10 ng of MBP-GCR1 fusion protein in the DNA band-shift assay are indicated above each lane.

RAP1-binding site but was dependent on the presence of a CTTCC sequence element (5). We have provided evidence that GCR1 can interact directly with CTTCC sequence motifs found in front of *TPI*, *PGK*, *ENO1*, *PYK*, and *ADH1*. The experiments for which the results are shown in Fig. 5 and 6 have allowed us to propose the degenerate consensus GCR1-binding site, (T/A)N(T/C)N(G/A)NC(T/A)TCC (T/A)N(T/A)(T/A)(T/G), through which we propose that GCR1 acts. The extent of the proposed consensus GCR1-binding site (16 nucleotides) was chosen on the basis of the area of protection observed in the in vitro DNase I footprinting studies. We are currently in the process of refining and determining the optimal GCR1-binding site. We appreciate the fact that GCR1 may interact with sequences which contain several mismatches to the proposed consensus sequence. In fact, we consider it likely that such cases will be found. GCR1 may be aided at binding to sites in vivo which appear to be low-affinity sites in vitro because of the presence of cooperative interactions with proteins bound at adjacent sites, e.g., RAP1 or REB1 or vice versa. Table 2 presents a survey of CTTCC and CATCC sequence motifs and the positions of known or putative RAP1-binding sites in the 5′ noncoding region of genes encoding glycolytic enzymes.

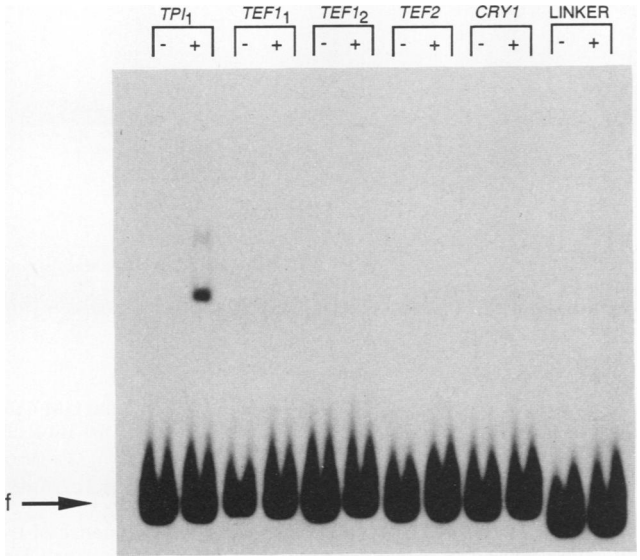

FIG. 6. GCR1 DNA-binding domain does not bind to CTTCC sequence elements found in front of translational component genes. DNA band-shift assays and symbols are as described in the legend to Fig. 5.

The core of the GCR1-binding site shares sequence similarity with the complement of the binding site of the *ets* family of DNA-binding proteins (43). Since it had been some time since the DNA sequence of *GCR1* was first reported (4), we repeated the searches of the GenBank and EMBL data bases with the deduced amino acid sequence of GCR1 to see whether we could find any significant sequence similarities between GCR1 and members of the *ets* family of proteins or any other DNA-binding proteins. The searches were carried out by using the algorithms of Pearson and Lipman (57) with a k-tup value of 1 for increased sensitivity (55, 56). We also carried out the comparison by using the BLAST algorithm of Altschul et al. (2). No obvious homologies between GCR1 and other sequences in the data bases were identified.

The expression of the genes encoding elongation factor EF-1α (*TEF1* and *TEF2*) and ribosomal protein 59 (*CRY1*) is reduced two- to fourfold in *gcr1* mutant strains (62). We were unable to identify GCR1-binding sites in the 5′ noncoding region of these genes. From the work of Kief and Warner (45), it is known that the cellular ribosomal content is proportional to growth rate; glucose-grown cells have 2.5-fold more ribosomes than do cells grown on ethanol. Herruer et al. (38) have shown in a series of nutritional upshift experiments (ethanol to glucose) that there is a coordinate fourfold increase in the levels of cellular ribosomal protein mRNAs within 30 min of the shift. *gcr1* lesions are known to result in severe growth defects when mutants are grown in the presence of glucose (22). In addition, the magnitude of the effect of *gcr1* mutations on the expression of glycolytic genes is greater than that observed for the genes encoding

TABLE 2. CTTCC and CATCC sequence elements found in front of glycolytic genes

Gene	Position[a]	Sequence	No. of misses[b]	RAP1 site[c]	Reference(s)
PGI	−363 R	GAGCGCGTTT CTTCC TATAATCCAA	4	−377[d]	34
	−334 R	AAATTACCAG CTTCC TGTAATTCCA	1		
PFK1	−870	GTTTTATCAG CTTCC ACTAATTTTT	1		36
	−364	TTATTAAATT CTTCC GCTCTTAATA	4		
	−318	CTTGTGTAAT CATCC TTTTTCTTTA	0		
	−298	CTTTAATTTT CTTCC TTTTCTTTTT	2		
PFK2	−1212	TCCTCCCCCT CTTCC TCCTGAATAG	3	−531[d]	36
	−582	TGATTTCTAG CTTCC ACTTTTTTCA	0		
	−342	CCTTTTTTTC CATCC GGTCTTTATC	3		
	−175	TTATTTATTA CTTCC TTGATTAAAA	3		
FBA	−793	CAATACCAGC CTTCC AACTTCTGTA	1	−478[d]	63
	−428	GAAAACGTGG CATCC TCTCTTTCGG	2		
	−386	CTGCCGTGAG CATCC TCTCTTTCCA	2		
	−84	AGTTATTGTT CTTCC TTGCGTTATT	3		
TPI	−414 R	AACGGGTAAT CTTCC ACCAACCTGA	3	−358	1, 64
	−385	GACTTTTCAG CTTCC TCTATTGATG	0		
	−345	CCTTTTCTGG CATCC AGTTTTTAAT	0		
TDH2	−517	ATGTTTTGGG CTTCC ACTAATTCAA	1	−463[d]	28, 40, 49
	−472	TTGTGAAGAG CATCC AGAAATAATG	3		
	−389 R	AAACGCGCGA CTTCC TGTAAATGCC	3		
	−151 R	TTTATATATT CTTCC AAACCTACAA	3		
TDH3	−493	TTATTCCTGG CATCC ACTAAATATA	1	−512	8, 9, 40, 49
	−455	TTTAAGCTGG CATCC AGAAAAAAAA	1		
PGK	−467	ACACGCTCGA CTTCC TGTCTTCCTA	2	−475	19, 67
	−459	GACTTCCTGT CTTCC TATTGATTGC	0		
	−442	TTGATTGCAG CTTCC AATTTCGTCA	1		
PGM	−714 R	TCTCCCTTTT CTTCC TGCTCACAAA	4	−396[d]	37
	−367 R	ATTTAGCGAG CTTCC TCTTTTCTTC	0		
	−116	AACTGATTCT CTTCC TCCTTCTTGT	3		
	−76	TTGTAATTAC CTTCC TTTGTAATTT	1		
ENO1	−545 R	CTTTTTTTTT CTTCC TTCTTTTTCG	2	−469	11, 25, 47
	−491	CTAATCCGAG CTTCC ACTAGGATAG	0		
ENO2	−544	GCATCTGCTT CTTCC CAAAGATGAA	4	−508	11, 24
	−462	CCAAAACTGG CATCC ACTAATTGAT	1		
	−178	AAAGCATTAT CTTCC TACCAGAGTT	4		
	−103	TGTATCTTTT CTTCC CTTGTCTCAA	3		
PYK	−786 R	CTATTTTTTT CTTCC TTAGTTATTT	2	−655	14, 50, 53
	−649	AGACATCGGG CTTCC ATAATTTCGG	0		
	−323	CTTGTGATGT CTTCC AAGTGATTTC	2		
	−303	ATTTCCTTTC CTTCC CATATGATGC	3		
	−272	CCTTTAGTGT CTTCC TAAAAAAAAA	2		
	−101	CATTCTTTTT CATCC TTTGGTTTTT	3		
PDC1	−630	GCACCCATAC CTTCC TTAAAAACGT	3	−630	15, 44
	−613	AAAAACGTAG CTTCC AGTTTTTGGT	1		
	−590	GTGGTTCTGG CTTCC TTCCGATTCG	2		
	−586	TTCTGGCTTC CTTCC GATTCGCCGC	4		
ADH1	−694	ACAATATGGA CTTCC TCTTTTCTGG	0	−664	7, 68
	−342 R	CCTTTAGTGT CTTCC ATCATTTTTT	2		
	−242	AAACTTTTTC CTTCC TTCATTCACG	2		
	−194	GGTATACGGC CTTCC TTCCAGTTAC	3		
	−190	TACGGCCTTC CTTCC AGTTACTTGA	3		
	−79	CGTTCCCTTT CTTCC TTGTTTCTTT	3		

[a] All positions are numbered with respect to the translational start of the indicated gene; R indicates that the sequence is present on the complementary strand.
[b] Number of misses indicates the number of differences from the proposed consensus GCR1-binding site.
[c] RAP1 site denotes the positions of known or putative RAP1-binding sites (based on consensus GRF1-binding sites [12] and consensus RPG sequences [70]).
[d] Putative RAP1-binding site.

the aforementioned translational components. Therefore, we suggest that the most likely effect of *gcr1* lesions on the expression of *TEF1*, *TEF2*, and *CRY1* is an indirect one resulting from differences in growth rate between *gcr1* mutants and wild-type strains.

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