JOURNAL OF BACTERIOLOGY, June 1992, p. 4148–4156
0021-9193/92/124148-09$02.00/0

Vol. 174, No. 12

Proline Biosynthesis in *Saccharomyces cerevisiae*: Molecular Analysis of the *PRO1* Gene, Which Encodes γ-Glutamyl Kinase

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Received 24 January 1992/Accepted 20 April 1992

The *PRO1* gene of *Saccharomyces cerevisiae* encodes the 428-amino-acid protein γ-glutamyl kinase (ATP:L-glutamate 5-phosphotransferase, EC 2.7.2.11), which catalyzes the first step in proline biosynthesis. Amino acid sequence comparison revealed significant homology between the yeast and *Escherichia coli* γ-glutamyl kinases throughout their lengths. Four close matches to the consensus sequence for GCN4 protein binding and one close match to the RAP1 protein-binding site were found in the *PRO1* upstream region. The response of the *PRO1* gene to changes in the growth medium was analyzed by measurement of steady-state mRNA levels and of β-galactosidase activity encoded by a *PRO1-lacZ* gene fusion. *PRO1* expression was not repressed by exogenous proline and was not induced by the presence of glutamate in the growth medium. Although expression of the *PRO1* gene did not change in response to histidine starvation, both steady-state *PRO1* mRNA levels and β-galactosidase activities were elevated in a *gcd1* strain and reduced in a *gcn4* strain. In addition, a *pro1* bradytrophic strain became completely auxotrophic for proline in a *gcn4* strain background. These results indicate that *PRO1* is regulated by the general amino acid control system.

The yeast *Saccharomyces cerevisiae* synthesizes proline from glutamate via the intermediates γ-glutamyl phosphate, γ-glutamyl semialdehyde, and Δ^1-pyrroline-5-carboxylic acid (P5C) (12, 13, 49, 59). The same pathway is found in members of the family *Enterobacteriaceae* (for a review, see reference 38) and mammals (56, 60). The three enzymes involved in this pathway are γ-glutamyl kinase (the product of the *PRO1* gene), γ-glutamyl phosphate reductase (the product of the *PRO2* gene), and P5C reductase (the product of the *PRO3* gene). The gene-enzyme relationships were demonstrated by interspecies complementation of *proB*, *proA*, and *proC* mutants of *Escherichia coli* and *Salmonella typhimurium* by the yeast *PRO1*, *PRO2*, and *PRO3* genes, respectively (59), and of a yeast *pro1* mutant by the *E. coli proB* gene (49). There is substantial deduced amino acid sequence similarity between the *E. coli* (23) and *S. cerevisiae* (this work) γ-glutamyl kinases, between the *E. coli* (23) and *S. cerevisiae* (10) γ-glutamyl phosphate reductases, and among the P5C reductases of bacteria (24, 30, 52), *S. cerevisiae* (13), plants (22), and mammals (27).

Proline-requiring *S. cerevisiae* strains were isolated long after auxotrophs for almost all of the other amino acids had been obtained (12). This was because of their peculiar property of being unable to grow in the standard rich medium in common use in yeast laboratories. Repression and inactivation of both the general amino acid and the proline-specific permeases in media rich in nitrogen sources (20) is believed to be responsible for this inability of proline auxotrophs to grow.

In this report, we present a molecular characterization of the *PRO1* gene encoding γ-glutamyl kinase, including its DNA sequence and amino acid sequence homology to the γ-glutamyl kinase of *E. coli*. Since this is the first committed step as well as the rate-limiting step (49) in this pathway, we examined the regulation of this gene to determine whether it is repressed by proline, induced by glutamate, or sensitive to the general control system for amino acid biosynthesis. Contrary to our expectations for the behavior of the first gene in this biosynthetic pathway, *PRO1* appears to be unresponsive to either proline or glutamate in the medium. However, it responds to the general control system which increases expression of many amino acid biosynthetic genes in cells undergoing amino acid starvation.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* and *E. coli* strains employed in this study are listed in Table 1. Strain DT1103 (*pro1::URA3*) is a derivative of wild-type strain MB1433, in which a 0.6-kb *Xba*I-*Pvu*II DNA fragment (nucleotides +878 to +1415) was deleted from the 3′ half of the *PRO1* coding region and replaced by a 1.3-kb *Xba*I-*Pvu*II fragment carrying the wild-type *URA3* gene. DT1003 (*pro1*$^{\pm}$) is a leaky *pro1* strain derived from strain MB1433 and has a doubling time of 2.5 h in minimal ammonia medium supplemented with proline and a doubling time of 5 to 6 h without proline supplementation. Wild-type strain MC1001 and its isogenic *gcd1* derivative strain F98, wild-type strain F113 and its isogenic *gcn4* derivative strain F194, and strains H1080 and H1081 were obtained from Alan Hinnebusch. Diploid strain WG9 was constructed by crossing strain H1081 to strain WG6-5A, which carries chromosome IV markers from strain X3271-1C (Yeast Genetics Stock Center) and the *pro1-59* allele from strain MB282-2D (15). *E. coli* strains HB101 and GM2929 were used for plasmid amplification.

Growth media. The media used for growth of *S. cerevisiae* strains contained glucose (2%), yeast nitrogen base without ammonium sulfate and amino acids (0.17%), combinations of ammonium sulfate (0.2%), proline (0.1%) and glutamate (0.1%) as nitrogen sources, and appropriate amino acid supplements when necessary. At these concentrations, both

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TABLE 1. Strain list

Strain	Genotype	Source or reference
S. cerevisiae		
MB1433	*MATα ura3-52 trp1*	11
DT1103	*MATα ura3-52 trp1 pro1::URA3*	59
DT1003	*MATα ura3-52 trp1 pro1*$^{\pm}$	59
MC1001	*MATα ura3-52*	A. G. Hinnebusch
F98	*MATα ura3-52 gcd1-101*	A. G. Hinnebusch
F113	*MATa ura3-52 ino1 can*^{r}	A. G. Hinnebusch
F194	*MATα ura3-52 gcn4-Δ1*	A. G. Hinnebusch
H1080	*MATa ura3-52 leu2-3,112 gcn4::LEU2*	A. G. Hinnebusch
H1081	*MATa ura3-52 leu2-3,112 gcn2::LEU2*	A. G. Hinnebusch
WG6-5A	*MATα pro1-59 leu2 trp4*	This work
WG9	*MATa/MATα leu2-3,112/leu2 ura3-52/URA3 gcn2::LEU2/GCN2 trp4/TRP4 pro1-59/PRO1*	This work
E. coli		
HB101	F^{-} *hsdR hsdM proA2 lacZ24 leuB thi-1 rpsL20 supE44 recA13*	41
GM2929	F^{-} *ara-14 leuB6 fhuA13 lacY1 tsx-78 supE44 (glnV44) galK2 galT22 λ^{-}mcrA dcm-6 hisG4 rfbD1 rpsL136 dam-13*::Tn*9 xyl-5 mtl-1 recF143 thi-1 mcrB*	*E. coli* Genetics Stock Center

proline and glutamate are transported into cells in the presence of ammonia (10, 12). 3-Amino-1,2,4-triazole (AT) was supplied at a final concentration of 10 mM. For DNA transformation of proline prototrophic yeast strains, cells were grown in a 1% yeast extract–2% peptone–2% glucose medium. Proline auxotrophs were grown in minimal medium supplemented with proline (0.1%). *E. coli* strains were grown in LB broth or agar supplemented with ampicillin (100 μg/ml) as required.

Reagents. Restriction endonucleases, T4 DNA ligase, DNA polymerase I (Klenow fragment), and calf intestinal phosphatase were obtained from New England BioLabs, Boehringer-Mannheim Biochemicals, or Bethesda Research Laboratories and used as recommended by the manufacturers. The *Exo*III/mung bean nuclease deletion kit was purchased from Stratagene. The Sequenase kit (version 2.0) was purchased from United States Biochemical Corp. AT and *o*-nitrophenyl-β-D-galactopyranoside were purchased from Sigma Chemical Co.

DNA and RNA preparation. Plasmid DNA from *E. coli* was isolated by the alkaline denaturation method (8) or by the cesium chloride gradient method (41). Isolation of plasmid and genomic DNA from *S. cerevisiae* was carried out by the method of Hoffman and Winston (35). Total RNA from *S. cerevisiae* was isolated by the method of Needleman et al. (47).

Transformation. *E. coli* transformation was done by the calcium chloride method (19). *S. cerevisiae* transformation was performed by the lithium acetate method (37).

Construction of plasmids for DNA sequencing. A 2.5-kb *Cla*I DNA fragment from plasmid pTB34 (59) was inserted in both orientations into the *Cla*I site of plasmid pBS-KS^{+} (Stratagene) to form plasmids pLB2 and pLB3. This fragment contained a functional *PRO1* gene, as determined by complementation testing (59).

A series of plasmids containing nested deletions of *PRO1* was constructed by using the *Exo*III/mung bean nuclease deletion kit (Stratagene). Plasmid pLB2 was linearized at the unique *Not*I site, and the ends were filled with thiodeoxynucleoside triphosphates by using the large fragment of DNA polymerase I (Klenow fragment). After digestion with *Bam*HI, the DNA was treated with *Exo*III at 30°C and the reaction was terminated at different time points as recommended by the manufacturer. Single-stranded DNA was cut by treatment with mung bean nuclease, and the blunt-ended DNA was ligated and transformed into *E. coli*. Fifteen plasmids carrying deletions that spanned the *PRO1* gene were chosen for sequencing. A second series of 15 nested deletions was constructed from plasmid pLB3 by using the same method.

DNA sequencing. Double-stranded plasmid DNA was sequenced by the dideoxynucleotide-chain termination method (51), using the Sequenase kit (United States Biochemical Corp.). Approximately 5 μg of plasmid DNA (denatured by alkali as described by the manufacturer) was used per sequencing reaction. The universal M13 primer (5′-GTTT TCCCAGTCACGAC-3′) was annealed to the denatured DNA template for initiation of the reaction. The newly synthesized DNA chains were labelled by using [α-^{32}P]dATP (Amersham Corp.) and terminated by addition of the dideoxynucleotides. The resulting DNA samples were denatured and run on prewarmed 8% polyacrylamide sequencing gels. Both strands of the *PRO1* inserts were sequenced in their entirety.

Quantitative DNA hybridization analysis. Approximately 10 μg of total DNA from plasmid-bearing yeast strains was digested with *Nde*I and *Hin*dIII. DNA fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell Corp.) according to the manufacturer's instructions. A 0.7-kb *Eco*RV-*Sma*I DNA fragment from the wild-type *URA3* gene on plasmid YEp24 (9) was radioactively labelled by nick translation using the Multiprime kit (Amersham Corp.) and served as the *URA3* probe. Hybridization was carried out by the method of Southern (57). Autoradiographic signals on several X-ray film exposures were quantitated with a computing densitometer (Molecular Dynamics) by using the volume integration method. The membranes were also cut, and the rates of emissions from the bands were counted in a Beckman scintillation counter. The plasmid copy numbers of strains carrying the plasmid-borne *PRO1-lacZ* fusion were determined by comparing the radioactive signal of the 1.2-kb *Nde*I wild-type *URA3* fragment from the plasmid with that of the 2.3-kb *Nde*I-*Hin*dIII fragment from the genomic *ura3-52* gene. The plasmid copy numbers of the strains carrying either the *HIS4-lacZ* or the *URA3-lacZ* gene fusion were not determined because these CEN plasmids are maintained at 1 to 2 copies per cell and the β-galactosidase activities from those plasmids are high compared with those from the *PRO1-lacZ* fusions.

RNA hybridization analysis. Approximately 7 to 12 μg of total RNA per lane was subjected to 1% agarose gel electrophoresis with methylmercury (5 mM) (5). The RNA was transferred to nitrocellulose membranes, and hybridization was carried out as described by Thomas (58). A 1.6-kb *Kpn*I-*Pvu*II *PRO1* DNA fragment (nucleotides −214 to +1415) from plasmid pLB2 and a 1.1-kb *Bam*HI-*Sac*I DNA fragment from plasmid pBlue-L29 carrying the yeast ribosomal protein *L29* gene (46) were labelled by using the

Multiprime kit and served as probes. Autoradiographic signals were quantitated by densitometry as described in the preceding section. In each lane, the *PRO1* signal was compared with the mature *L29* signal.

Construction of the *PRO1-lacZ* gene fusion. The high-copy-number plasmid pLB5, which contains the *PRO1-lacZ* gene fusion, was constructed as follows. A 0.8-kb *Bcl*I DNA fragment (−740 to +38 bp) carrying the *PRO1* promoter and the first 12 codons of the *PRO1* coding region was prepared from plasmid pLB2 and inserted into plasmid YEp358R (45) at the *Bam*HI site to form an in-frame fusion to the eighth codon of the *E. coli lacZ* open reading frame. Plasmid pLB5 carries *URA3*, 2μm, amp^R, and *ori* DNA for selection and maintenance in *S. cerevisiae* and *E. coli*.

The low-copy-number *PRO1-lacZ* plasmid pLB8 was constructed as follows. A 1.7-kb *Hin*dIII-*Cla*I DNA fragment from plasmid pLB5 containing the entire *PRO1* insert and the 5′ part of the *lacZ* open reading frame was ligated to a 9-kb *Hin*dIII-*Cla*I fragment of the yeast shuttle vector pABC1 (54). Plasmid pLB8 carries *URA3*, *CEN4*, amp^R, and *ori* DNA for selection and maintenance in *S. cerevisiae* and *E. coli*. The correct in-frame fusion of the *PRO1* and *lacZ* genes was confirmed by sequencing the junction region between them.

Cell extract preparation and β-galactosidase activity assay. *S. cerevisiae* strains carrying plasmids with the *PRO1-lacZ* gene fusion were inoculated to an initial density of 1 to 3 Klett units (Klett-Summerson colorimeter, blue filter) in 10 ml of minimal medium in 125-ml sidearm flasks and grown at 30°C. The cells were harvested at a density of 80 to 100 Klett units, and a crude extract was prepared by vortexing the cells with glass beads (14). β-Galactosidase was assayed as described by Miller (43). The specific activity of β-galactosidase is expressed as nanomoles of *o*-nitrophenol formed per minute per milligram of protein. The β-galactosidase activities from three to six individual transformants of each strain were measured, and the standard variation was less than 25% among replicate cultures. The normalized β-galactosidase activities were obtained by dividing the specific activity by the plasmid copy number determined from quantitative DNA hybridization analysis.

Computer analysis of DNA and amino acid sequences. The comparison of the *PRO1* and *PRO3* promoter sequences was performed by using the Altschul program (1) of the EuGene (molecular biology) software package (Baylor College of Medicine, Waco, Tex.). The comparison of the amino acid sequences was performed by using the GAP (48), COMPARE, and DOTPLOT (40) programs of the University of Wisconsin Genetics Computer Group.

***PRO1* DNA sequence accession number.** The *PRO1* DNA sequence has been entered in GenBank and was assigned the accession number M85293.

RESULTS

Sequence analysis of the *PRO1* gene. To determine the amino acid sequence of γ-glutamyl kinase and the nucleotide sequence of the upstream control region, the *PRO1* gene was sequenced as described in Materials and Methods. The complete sequence of 2,552 nucleotides, which covers the *PRO1* open reading frame and its 5′ and 3′ flanking regions, and the deduced amino acid sequence of the *PRO1* gene product are shown in Fig. 1.

The sequence analysis revealed a single long open reading frame (from nucleotide +1 through +1284) capable of encoding a 428-amino-acid-residue polypeptide. Four sequences with some relationship to the TATA box, consensus sequence TATA(A/T)A(A/T) (16), and TATAAA (18) were found in the upstream region. They are AATAAAT at −186, TATTTAT at −226 and −230, and AATATAT at −315 relative to the start site of the *PRO1* open reading frame. The TATTTA sequence was found to be functional in the *HIS3* promoter (31). Four sequences related to the upstream activation sequence for GCN4 protein binding (consensus sequence, RRTGACTCATTT) (34) were found: TATGA CACTATC at −222, TAAGACTCTCAC at −271, AGTGA ATCATAA at −326, and TATGATTCACTG at −315 (reverse complement). None contains an exact match to the core TGACTC, although the sequence at −315 matches a functional core sequence adjacent to the *ILV2* gene (34). A match to the consensus RAP1 recognition sequence, GCAC CCACACATC, was also found in the *PRO1* upstream region between nucleotide −178 and −166. RAP1 is a yeast transcriptional regulatory protein, and its binding to promoter DNA [consensus sequence, 5′-(A/G)(A/C)ACCCANNCA (T/C)(T/C)-3′] (17) can either activate or repress the transcription of the associated gene. RAP1 has been found to bind to the E boxes of mating type loci (53), telomeres (17), and the promoters of many genes, including *HIS4* (25).

Comparison of the *PRO1* upstream sequence with that of *PRO3* (13), which encodes P5C reductase, the third enzyme of the pathway, revealed four different highly homologous regions (69 to 82% identical) ranging from 11 to 26 nucleotides in length (Fig. 2). The *PRO1* sequence at −182 also contains the RAP1 recognition sequence. However, the *PRO3* sequence appears to lack significant homology to that sequence. The other homologous regions show no match to either the RAP1 or GCN4 consensus sequence (see above). Whether these sequences are involved in any coordinate regulation between *PRO1* and *PRO3* genes remains to be tested.

Analysis of the 414-bp DNA sequence downstream of the 3′ end of the *PRO1* open reading frame revealed multiple translational stop codons in all three frames in this region. A tripartite terminator, TAG.....TAGT.....TTT (62), was found at the region between nucleotides +1437 and +1491. A TAAATAATG sequence, which is related to the polyadenylation signal [consensus sequence TAAATAA(A/G) (7) or AATAAA (29)] was found at position +1361, 77 bp downstream from the 3′ end of the *PRO1* open reading frame. No terminator TTTTTATA (32) was found in the downstream region.

Another open reading frame was found in the *PRO1* 5′ upstream DNA on the opposite strand (nucleotide −561 through the 5′ end of the sequenced fragment, nucleotide −854). Five hundred sixty nucleotides separate the two open reading frames. This incomplete open reading frame is capable of encoding 98 amino acid residues and runs in the orientation opposite to that of *PRO1*. No homology was found between this deduced amino acid sequence and the protein sequences in the GenBank, PIR, or EMBL data bases. Transcription of this upstream region was not examined.

Deduced *PRO1* amino acid sequence and its homology to the *E. coli proB* gene product. The sequence of 428 amino acid residues of the yeast γ-glutamyl kinase deduced from the *PRO1* DNA sequence was aligned with the *E. coli* γ-glutamyl kinase amino acid sequence (Fig. 3). The two proteins are 38.4% identical and have 60.7% overall similarity. Their relatedness is also obvious in the dot matrix plot shown in Fig. 4.

Identification of *PRO1* mRNA. The *PRO1* mRNA was

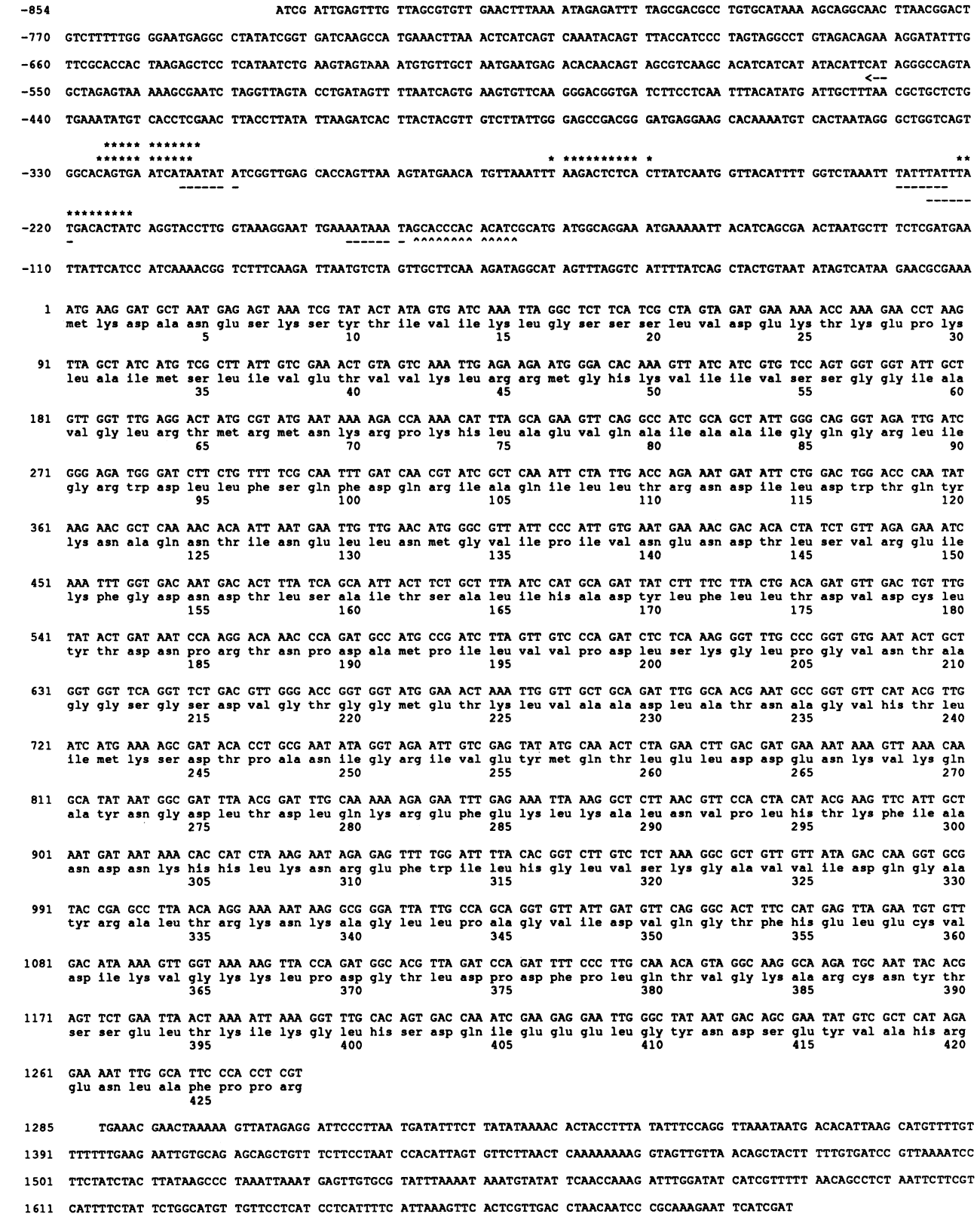

FIG. 1. Nucleotide and deduced amino acid sequences of the *PRO1* gene. DNA sequence analysis revealed one long open reading frame. Position +1 refers to the translational initiation site. Close matches to known consensus sequences are marked as follows: TATA box, dashed underline; GCN4 protein-binding site, asterisks above the sequence; RAP1-binding site, carets below the sequence. The arrow indicates the start site and direction of the incomplete open reading frame found in the upstream region.

POSITION		ALIGNMENT (5'-3')	NO. OF IDENTICAL NUCLEOTIDES	(%)
PRO1	-407	AGATCACTTACTACGTTGTCTTATTG		
		||| |||| |||| || | || | |	18/26	(69%)
PRO3	-255	AGAGGACTTCCTACATTTTATTGTCG		
PRO1	-382	GGGAGCCGACG		
		|| |||| |||	9/11	(82%)
PRO3	-306	GGAAGCCAACG		
PRO1	-276	AACATGTTCATACTTTAA		
		||||| ||| | |||||	14/18	(78%)
PRO3	-279	AACATTTTCTGATTTTAA		
PRO1	-182	AATAGCACCCACACA		
		||||| | |||||	11/15	(73%)
PRO3	-45	AATAGAAGTAACACA		

FIG. 2. Sequence homologies between the *PRO1* and *PRO3* promoters. The alignment was determined by the Altschul program of EuGene (1). The position numbers refer to the start site of translation. Identical nucleotides are indicated by vertical bars. The *PRO1* sequence at −182 contains the RAP1-binding sequence similarity. The *PRO1* sequence at −276 is the reverse complement.

identified by RNA hybridization analysis of total yeast RNA. In the wild-type strain MB1433, a single 1.5-kb transcript was detected (Fig. 5, lane 1). This band is reduced to a stable 1-kb species in strain DT1103, which carries a *PRO1* deletion of nucleotides +878 to +1415. In Fig. 5, lane 2, the shortened *PRO1* message comigrates with the pre-*L29* mRNA, but it was clearly visible in experiments in which only *PRO1* was used as the probe (data not shown).

Transcriptional regulation of the *PRO1* gene. To determine whether the *PRO1* gene was regulated by end product repression, substrate induction, or general control, the steady-state *PRO1* mRNA levels and the β-galactosidase activities from two plasmid-borne *PRO1-lacZ* gene fusions were measured in two wild-type strains under various growth conditions and in mutant yeast strains derived from the wild-type strains. Although the two wild-type strains are not isogenic, they express *PRO1* similarly under the conditions tested. Because the *PRO1* gene is expressed at a low level, β-galactosidase activities from a *PRO1-lacZ* CEN plasmid (1 to 2 copies/cell) were low. To confirm these results, β-galactosidase activities from the same *PRO1-lacZ* fusion on a high-copy-number plasmid (2 μm [10 to 20 copies/cell]) were measured, and plasmid copy number determinations were also made to control for differences in copy number. γ-Glutamyl kinase activity was not measured because a suitable assay for the yeast enzyme has not been developed.

```
PRO1    1 MKDANESKSYTIVIKLGSSSLVDEKTKEPKLAIMSLIVETVVKLRRMGHK 50
            |.| |:|:|||.| |.::. :  :  |:.|: : ...|:  ||:
ProB    1 .....MSDSQTLVVKLGTSVLTGGSRRLNRAHIVELVRQ.CAQLHAAGHR 44

         51 VIIVSSGGIAVGLRTMRMNKRPKHLAEVQAIAAIGQGRLIGRWDLLFSQF 100
            ::||.||:||.|   :   . |  :|. | :||:||:|||. |: ||| :
         45 IVIVTSGAIAAGREHLGYPELPATIASKQLLAAVGQSRLIQLWEQLFSIY 94

        101 DQRIAQILLTRNDILDWTQVKNAQNTINELLNMGVIPIVNENDTLSVREI 150
            : :::|:||||.|: |:..: ||.:|:..||: .::|::||||.:.. .|
         95 GIHVGQMLLTRADMEDRERFLNARDTLRALLDNNIVPVINENDAVATAAI 144

        151 KFGDNDTLSAITSALIHADYLFLLTDVDCLYTDNPRTNPDAMPILVVPDL 200
            |.||||.|||:.. |  || |:|||| ..|||.:||.||:|  |  | ::
        145 KVGDNDNLSALAAILAGADKLLLLTDQKGLYTADPRSNPQAELIKDVYGI 194

        201 SKGLPGVNTAGGSGSDVGTGGMETKLVAADLATNAGVHTLIMKSDTPANI 250
            ..:|.::  ||:|.|::|||||.||| |||:|..||:.|:|  :..|: |
        195 DDALRAI..AGDSVSGLGTGGMSTKLQAADVACRAGIDTIIAAGSKPGVI 242

        251 GRIVEYMQTLELDDENKVKQAYNGDLTDLQKREFEKLKALNVPLHTKFIA 300
            | ::|                                .:.: | | |
        243 GDVME.....................................GISVGTLFHA 257

        301 NDNKHHLKNREFWILHGLVSKGAVVIDQGAYAALTRKNKAGLLPAGVIDV 350
            ...  .|.||. ||: | .. |.:.:|:|| ||:  :. .:||| |: .|
        258 QAT..PLENRKRWIF.GAPPAGEITVDEGATAAILERG.SSLLPKGIKSV 303

        351 QGTFHELECVDIKVGKKLPDGTLDPDFPLQTVGKARCNYTSSELTKIKGL 400
            |.|   |.: |            .::. .:::.: :.|.|..| :| | |
        304 TGNFSRGEVIRI.............CNLEGRDIAHGVSRYNSDALRRIAGH 341

        401 HSDQIEEELGYNDSEYVAHRENLAFPPR  428
            ||::|:. |||: :. ..|||::  .
        342 HSQEIDAILGYEYGPVAVHRDDMITR..  367
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FIG. 3. Homology between the deduced *PRO1* amino acid sequence (top lines) and the *proB* polypeptide sequence of *E. coli* (bottom lines) (23). The alignment was performed by using the GAP program. Dotted lines represent gaps in the sequence.

End product repression of gene expression is a common regulatory mechanism in biosynthetic pathways of different organisms. In *S. cerevisiae*, *LEU1* and *LEU2* (2, 36), *ARG3* (21), and *GLN1* (44) are regulated in part by end product repression. RNA hybridization was performed on RNA isolated from wild-type strain MB1433 grown on minimal-ammonia medium in the presence or absence of proline. If the *PRO1* gene is repressible by proline, both steady-state RNA levels and β-galactosidase activities of the cultures grown in the presence of proline should be lower than those of cultures grown in the absence of proline. Figure 5 (lanes 4 and 5) shows that quantitatively similar levels of steady-state *PRO1* RNA (normalized to levels of mature ribosomal protein *L29* transcript) were found in cells grown under these two conditions. β-Galactosidase activities were measured in the wild-type strain MC1001 carrying a *PRO1-lacZ* gene fusion on either the CEN plasmid pLB8 or the 2 μm plasmid pLB5. As shown in Table 2, no significant decrease in β-galactosidase activity was seen in transformants carrying either plasmid. These results suggest that the transcription of the *PRO1* gene is not repressed by addition of proline to the growth medium.

Although substrate induction is not a common phenomenon in the transcriptional regulation of amino acid biosynthetic pathways in *S. cerevisiae*, it has been observed in the expression of *LEU1* and *LEU2*, genes that encode isopropylmalate isomerase and β-isopropylmalate dehydrogenase, respectively, of the leucine biosynthetic pathway. These genes are induced by α-isopropylmalate (4). To determine whether the expression of the *PRO1* gene is induced by glutamate, steady-state RNA levels and β-galactosidase activities were measured in the two wild-type strains. The results indicated that the presence of glutamate in the growth medium did not alter the expression of the *PRO1* gene (Fig. 5, lanes 3 and 4, and Table 2), suggesting that the transcription of the *PRO1* gene is not induced by glutamate.

General amino acid control is the regulatory system that controls many amino acid biosynthetic pathways in *S. cerevisiae* (34). To determine whether the *PRO1* gene is regulated by the general control system, two different conditions were examined. These were (i) the effect of known general control mutations on *PRO1* expression and (ii) amino acid starvation induced by the presence of the histidine analog AT. Steady-state *PRO1* RNA levels and β-galactosidase levels (normalized for copy number) were measured in *gcd1* (F98) and *gcn4* (F194) strains and their isogenic parent strains. The *gcd1* mutation causes constitutive derepression of the genes subject to general control, and the *gcn4* mutation eliminates derepression in response to amino acid starvation. *PRO1* mRNA levels increased 1.7-fold in strain F98 and decreased to one-half in strain F194 compared with the parental wild-type strains MC1001 and F113, respectively, under nonstarvation conditions (Fig. 5, lanes 6 through 9). The normalized β-galactosidase activities of the transformants carrying the *PRO1-lacZ* gene fusion increased

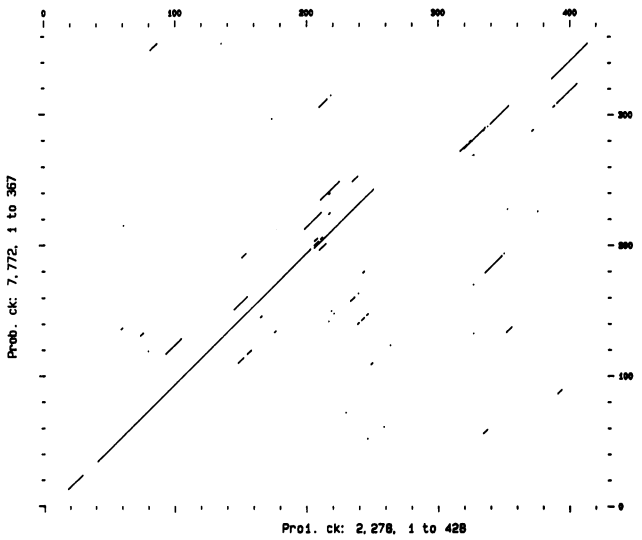

FIG. 4. Dot matrix plot of the γ-glutamyl kinases of *E. coli* (Prob., *y* axis) and *S. cerevisiae* (Prol., *x* axis). The COMPARE program was run with a window of 25 and a stringency of 13.

1.4-fold (CEN plasmid) and 1.8-fold (2 μm plasmid) in the *gcd1* strain, and were reduced to 88% (CEN plasmid) and 22% (2 μm plasmid) of the wild-type values in the *gcn4* strain (Table 3). Because the expression of single-copy *PRO1-lacZ* is low and there is typically variability in the assay, we consider the normalized β-galactosidase activities from high-copy-number plasmids to be more reliable than the lower values from CEN plasmids. The *HIS4* gene, which is known to be regulated by the general control system, behaved as expected in the *gcd1* and *gcn4* strains. The *URA3* gene is not regulated by the general control system and served as a negative control. Although the magnitude of the effect on *PRO1* is small, it is typical of genes under general control in *S. cerevisiae* and leads one to the conclusion that *PRO1* is under general control.

When histidine starvation was induced by the addition of AT to the growth medium, the *HIS4* gene behaved as expected. β-Galactosidase activity encoded by the *HIS4-lacZ* fusion increased fourfold in the wild-type strain when AT was added, and this increase was dependent on *GCN4*. Unexpectedly, both *PRO1* mRNA levels (not shown) and normalized β-galactosidase activity encoded by *PRO1-lacZ* (Table 3) dropped in the wild-type strain F113 when AT was added, and no *GCN4*-dependent derepression was seen.

The role of the GCN4 protein in controlling expression of the *PRO1* gene was confirmed by examining the behavior of a leaky *pro1* strain (DT1003). In the absence of proline, this strain has a twofold increase in doubling time compared to that when proline is present. β-Galactosidase activity from the *HIS4-lacZ* fusion carried on a plasmid in this strain increased 2.8-fold in the absence of proline (specific activity, 477), compared with that obtained in the presence of proline (specific activity, 173). In the parent strain MB1433, specific activity values were 122 in the absence of proline and 149 in the presence of proline. These results confirm that the *pro1*$^{\pm}$ cells were starving for proline. Double *pro1*$^{\pm}$ *gcn4* strains derived from repeated crossing of DT1003 (*pro1*$^{\pm}$) and H1080 (*gcn4*) derivatives failed to grow at all in the absence of proline. Introduction of a plasmid bearing the *GCN4* gene into the double-mutant strain restored slow growth in the absence of proline.

Chromosomal position of the *PRO1* gene. A *PRO1* probe was used by A. J. Link and M. V. Olson (39) to map the gene to chromosome IV with their collection of *Not*I-*Sfi*I yeast genomic restriction fragments. Preliminary crosses of a *pro1* strain to strain X3271-1C (*MATa pet14 aro1D trp4 ade8 leu2 rna3 mal gal4*) indicated that *PRO1* was linked to *TRP4* and unlinked to *ADE8*. To determine the location of *PRO1* more precisely, diploid strain WG9 (*MATa*/*MATα leu2-3,112*/*leu2 ura3-52*/*URA3 gcn2::LEU2*/*GCN2 pro1-59*/*PRO1 trp4*/*TRP4*) was constructed and sporulated. Eighty-four tetrads were dissected and analyzed. *GCN2* and *TRP4* are 63 centimorgans (cM) apart, and *PRO1* lies between them, about 24 cM from *GCN2* and 37 cM from *TRP4* (Table 4).

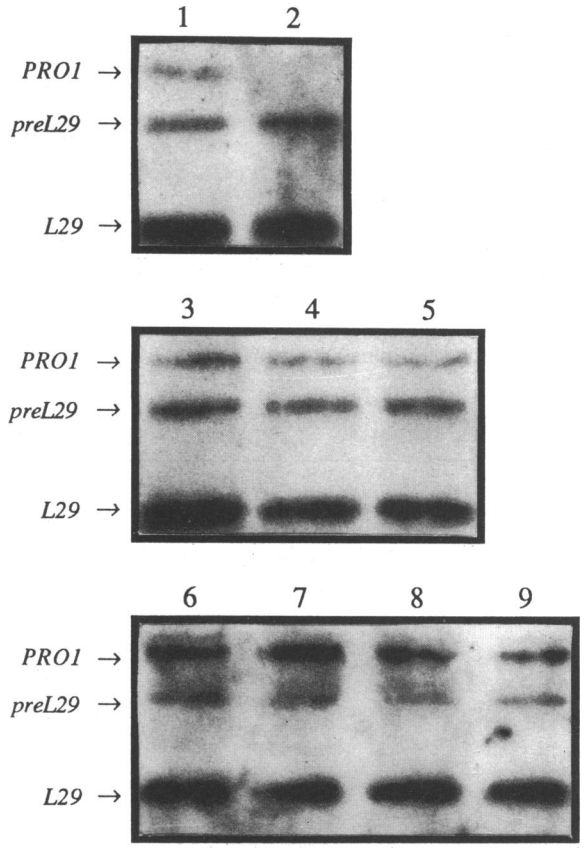

FIG. 5. RNA hybridization analysis of *PRO1*. Approximately 10 μg of total RNA per lane was hybridized to *PRO1* and *L29* probes as described in Materials and Methods. *PRO1* (1.5 kb), pre-*L29* (1 kb), and *L29* (0.6 kb) transcripts are indicated. All strains were grown in minimal medium, to which ammonium sulfate (Amm), proline (Pro), or glutamate (Glt) was added as indicated. Lanes: 1, wild-type strain (MB1433) grown on Amm + Pro; 2, *pro1* deletion strain (DT1103) grown on Amm + Pro; 3, MB1433 grown on Amm + Glt; 4, MB1433 grown on Amm; 5, MB1433 grown on Amm + Pro; 6, wild-type strain (MC1001) grown on Amm; 7, *gcd1* strain (F98) grown on Amm; 8, wild-type strain (F113) grown on Amm; 9, *gcn4* strain (F194) grown on Amm. The *PRO1*/*L29* ratios of autoradiographic signals in lanes 3 through 9 were 0.20, 0.21, 0.23, 0.55, 0.96, 0.62, and 0.31, respectively.

TABLE 2. Effect of proline and glutamate on the expression of *PRO1*

PRO1-lacZ plasmid	Nitrogen source[a]	β-Galactosidase sp act[b]
pLB8 (CEN)	Amm	14
	Amm + Glt	10
	Amm + Pro	15
pLB5 (2μm)	Amm	755
	Amm + Glt	617
	Amm + Pro	698

[a] All transformants of strain MC1001 were grown in minimal medium, to which ammonium sulfate (Amm) was added. Proline (Pro) or glutamate (Glt) was provided as indicated.

[b] Units of β-galactosidase specific activity are nanomoles of *o*-nitrophenol formed per minute per milligram of protein.

DISCUSSION

The *S. cerevisiae* and *E. coli* γ-glutamyl kinases display considerable sequence similarity. This result is not unexpected, since the *PRO1* gene complemented the corresponding mutants in *E. coli* and *S. typhimurium* (59) and the cloned *E. coli proB* gene functions in *S. cerevisiae* (49). Although the mammalian genes encoding γ-glutamyl kinase and γ-glutamyl phosphate reductase have not yet been identified, the cloned human P5C reductase gene can complement the corresponding yeast *pro3* mutant (27). These results confirm that diverse organisms share an identical proline biosynthetic pathway.

In *E. coli*, the first two enzymes of the proline biosynthetic pathway, γ-glutamyl kinase and γ-glutamyl phosphate reductase, form a complex to channel the unstable intermediate, γ-glutamyl phosphate (3). The γ-glutamyl kinase activity was undetectable unless the purified γ-glutamyl phosphate reductase was added to the in vitro assay system (55). Whether the first two yeast enzymes also form a complex in order to function is still unclear, since the yeast enzymes have not been purified and a method to measure the yeast γ-glutamyl kinase has not been developed. However, Tomenchok and Brandriss (59) reported that the *PRO1* gene complemented the bacterial *proB* strains carrying deletion mutations but did not complement those carrying point mutations. This suggests that the yeast γ-glutamyl kinase can complex with the bacterial γ-glutamyl phosphate reductase if it is not already complexed with a (defective) bacterial kinase polypeptide.

In the initial studies on the general control of amino acid biosynthesis, Wolfner et al. (61) predicted that proline biosynthesis should be included among the amino acid biosynthetic pathways regulated in this manner on the basis of the behavior of general control mutants in the presence of the proline analog azetidine carboxylic acid. We have shown in this report that the *PRO1* gene is regulated by the general control system as indicated by the changes in steady-state levels of its RNA in general control mutant strains and by the inability of a leaky *pro1*$^{\pm}$ mutant to grow in the absence of proline in a *gcn4* background. Preliminary results (10) indicate that *PRO2* is also regulated by general control, while *PRO3* is not (13).

Four close matches to the GCN4 protein-binding consensus sequence were found in the *PRO1* upstream region from 222 to 326 bp upstream of the translation initiation site. All of these sequences contain only a five-of-six match to the core consensus sequence, but the core sequence at −315 matches the core sequence known to be functional in the *ILV2* gene (28, 34). In other studies, some sequences containing one unmatched nucleotide are functional in the *HIS3* and *HIS4* genes (26, 33). Which of these sequences found in the *PRO1*

TABLE 3. Effect of general control mutations on *PRO1* expression

Gene fusion (plasmid type)	Genotype (strain)	AT (10 mM) addition	β-galactosidase sp act[a]	Plasmid copy no./cell[b]	Normalized β-galactosidase sp act[c]
PRO1-lacZ (CEN)	Wild type (MC1001)	−	14	1.2	12
	gcd1 (F98)	−	19	1.1	17
	Wild type (F113)	−	11	1.3	8
	gcn4 (F194)	−	9	1.3	7
	Wild type (F113)	+	9	ND[d]	ND
	gcn4 (F194)	+	8	ND	ND
PRO1-lacZ (2μm)	Wild type (MC1001)	−	755	14.5	52
	gcd1 (F98)	−	1,265	13.7	92
	Wild type (F113)	−	814	11.2	73
	gcn4 (F194)	−	251	15.6	16
	Wild type (F113)	+	334	ND	ND
	gcn4 (F194)	+	209	ND	ND
HIS4-lacZ (CEN)	Wild type (MC1001)	−	702		
	gcd1 (F98)	−	3,797		
	Wild type (F113)	−	517		
	gcn4 (F194)	−	94		
	Wild type (F113)	+	1,927		
	gcn4 (F194)	+	105		
URA3-lacZ (CEN)	Wild type (MC1001)	−	121		
	gcd1 (F98)	−	134		

[a] See Table 2, footnote *b*.

[b] Plasmid copy number per cell was determined by quantitative DNA hybridization.

[c] Normalized β-galactosidase specific activity was determined by dividing the specific activity by the plasmid copy number.

[d] ND, not determined.

TABLE 4. Chromosomal mapping of *PRO1*

Gene pair	No. of asci of type[a]:			Map distance (cM)[b]
	PD	NPD	T	
gcn2-pro1	48	1	35	24
pro1-trp4	37	3	44	37
gcn2-trp4	23	9	52	63

[a] PD, Parental ditype; NPD, nonparental ditype; T, tetratype. The genotype of diploid WG9 is *MATa/MATα leu2-3,112/leu2 ura3-52/URA3 gcn2::LEU2/GCN2 pro1-59/PRO1 trp4/TRP4*.

[b] Distance in centimorgans = [100(T + 6NPD)]/[2(PD + NPD + T)] (50).

promoter are actually GCN4-binding sites remains to be determined.

DNA sequence comparison of the *PRO1* and *PRO3* promoters revealed several highly homologous regions. These homologous sequences may be the binding sites for a common regulatory protein, perhaps one involved in a specific regulatory mechanism that coordinates expression of the yeast proline biosynthetic genes. Such pathway-specific regulation has been identified in other amino acid biosynthetic pathways in *S. cerevisiae*. In the leucine biosynthetic pathway, leucine-specific repression is mediated by a GC-rich sequence found in the promoters of the *LEU1*, *LEU2*, and *LEU4* genes (2, 6, 36). In the arginine biosynthetic pathway, the expression of the *ARG3* and *ARG5,6* genes is coordinately regulated by the ARGR proteins, and sequences homologous to those used for ARGR protein binding were identified in the promoters of both genes (42). Whether the *PRO* homologous sequences mediate any specific regulation remains to be determined, although in this study, expression of the *PRO1* gene in wild-type yeast strains was not reduced by the addition of proline to the growth medium. The *PRO3* gene encoding P5C reductase, the last enzyme in the proline biosynthetic pathway, is also not repressed by the presence of proline in the growth medium (13).

ACKNOWLEDGMENTS

We thank A. Hinnebusch and H. Fried for strains and plasmids and W. Gao and M. Kelley for strain construction.

This work was supported in part by the Graduate School of Biomedical Sciences of the University of Medicine and Dentistry of New Jersey and by grant 5 R01 GM40751 from the National Institutes of Health.

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