Putative GTP-Binding Protein, Gtr1, Associated with the Function of the Pho84 Inorganic Phosphate Transporter in *Saccharomyces cerevisiae*

MASANORI BUN-YA, SATOSHI HARASHIMA, AND YASUJI OSHIMA*

*Department of Biotechnology, Faculty of Engineering, Osaka University,
2-1 Yamadaoka, Suita-shi, Osaka 565, Japan*

We have found an open reading frame which is 1.1 kb upstream of *PHO84* (which encodes a P_i transporter) and is transcribed from the opposite strand. In *Saccharomyces cerevisiae*, this gene is distal to the *TUB3* locus on the left arm of chromosome XIII and is named *GTR1*. *GTR1* encodes a protein consisting of 310 amino acid residues containing, in its N-terminal region, the characteristic tripartite consensus elements for binding GTP conserved in GTP-binding proteins, except for histidine in place of a widely conserved asparagine residue in element III. Disruption of the *GTR1* gene resulted in slow growth at 30°C and no growth at 15°C; other phenotypes resembled those of *pho84* mutants and included constitutive synthesis of repressible acid phosphatase, reduced P_i transport activity, and resistance to arsenate. The latter phenotypes were shown to be due to a defect in P_i uptake, and the Gtr1 protein was found to be functionally associated with the Pho84 P_i transporter. Recombination between chromosome V (at the *URA3* locus) and chromosome XIII (in the *GTR1-PHO84-TUB3* region) by using a plasmid-encoded site-specific recombination system indicated that the order of these genes was telomere-*TUB3-PHO84-GTR1-CEN*$_{XIII}$.

Two systems are known to be involved in active transport of P_i into *Saccharomyces cerevisiae* cells from the cultivation medium; one has a low K_m value (8.2 μM) for external P_i, and the other has a high K_m value (770 μM) (29). The low-K_m system is repressed by P_i through the same system as for regulation of P_i-repressible acid phosphatase (rAPase; EC 3.1.3.2) encoded by *PHO5* (37) and involves *PHO81*, one of the genes in the *PHO* regulatory system (37). The *PHO84* gene most probably encodes the P_i transporter of the low-K_m system (7). *pho84* mutant cells have pleiotropic phenotypes: severely reduced P_i uptake (29, 33), constitutive synthesis of rAPase (34), and resistance to 4.5 mM arsenate (our unpublished results). Expression of all these structural genes of the *PHO* regulon is stimulated directly by two positive regulatory proteins, Pho4 and Grf10, whereas expression of the *PHO8* gene encoding repressible alkaline phosphatase (EC 3.1.3.1) is stimulated by Pho4 only, independent of Grf10 (15, 37).

During sequence determination of *PHO84* (7), we found a previously undescribed open reading frame (ORF) on a cloned fragment carrying the *PHO84* ORF. This new ORF encoded a protein with significant similarities in its N-terminal amino acid sequence to GTP-binding proteins. A large number of genes encoding such GTP-binding proteins have been identified in eucaryotes and named collectively the *ras* superfamily. The best known members of this family are *ras* proto-oncogenes (for reviews, see references 3 and 13). The *ras* and *ras*-related proteins have similar structural and biochemical properties. A conformational change of the protein caused by transition from the GDP-bound to the GTP-bound form results in a change in the regulatory function of the protein, allowing it to interact with other proteins (22).

Here we report the characterization of this new gene,

GTR1. Disruption of *GTR1* conferred on the cells pleiotropic phenotypes similar to those of *pho84* mutants, i.e., constitutive synthesis of rAPase, reduced uptake of P_i, and arsenate resistance. In a *gtr1* disruptant, transcription of *PHO84*, like that of *PHO5*, was constitutive. Thus, the Gtr1 protein might be involved in the mechanism of P_i uptake in collaboration with the Pho84 P_i transporter. The Gtr1 protein is also important for cell growth, since *gtr1* disruptant cells showed slow growth at 30°C and no growth at 15°C.

MATERIALS AND METHODS

Organisms and plasmids. The *S. cerevisiae* strains used are listed in Table 1. All strains were selected from our stock culture or constructed in this study. *Escherichia coli* JA221 (10), MV1184 (35), and GM33 (19) were used for manipulation of DNA. Plasmids pUC118 and pUC119 (35) were used for preparation of single-stranded DNAs (ssDNAs) for DNA sequencing and of hybridization probes with a helper phage M13 KO7 (35). The plasmid vectors used in *S. cerevisiae* were YCp50 (25), YEp24 (25), and YIp5 (25). Plasmid pHM153, used for generation of R protein, a site-specific recombinase of plasmid pSR1, was constructed previously (20). Plasmid pMB201, used as a hybridization probe for *GTR1*, *PHO84*, and *URA3* transcripts, was constructed by ligating a 0.7-kb *Hin*dIII$_2$-*Hin*dIII$_3$ fragment containing the *GTR1* C-terminal region (Fig. 1A), prepared from plasmid pMB15 (7), into the *Hin*dIII site of a plasmid which was constructed by inserting the 0.7-kb *Cla*I$_3$-*Hpa*I fragment containing a portion of the *PHO84* ORF (Fig. 1A) from pMB15 into the *Cla*I-*Nru*I gap of YIp5. A 1.0-kb *Hin*dIII-*Xho*I fragment of the *S. cerevisiae* *ACT1* gene used as a hybridization probe was prepared from plasmid pYA301 (7). The other plasmids used in this study are shown in Fig. 1 and 8.

Media; genetic and biochemical methods. Nutrient YPAD (nutrient high-P_i), nutrient low-P_i, synthetic high-P_i (contain-

* Corresponding author.

TABLE 1. *S. cerevisiae* strains used

Strain	Genotype[a]
P-28-24C	*MATa pho3-1*
KA31	*MATa/MATα ade2/ade2 his3-532/his3-532 leu2-3,112/leu2-3,112 trp1/trp1 ura3-1,2/ura3-1,2*
NBW5	*MATα ade2 leu2-3,112 his3-532 trp1-289 ura3-1,2 can1*
MB200	*MATa ade2 his3-532 leu2-3,112 trp1 ura3-1,2 gtr1-1Δ*
MB203	*MATa ade2 his3-532 leu2-3,112 trp1 ura3-1,2 pho84-1Δ*
PP2	*MATα ade2 his3-532 leu2-3,112 trp1 ura3-1,2*
PP4	*MATα ade2 his3-532 leu2-3,112 trp1 ura3-1,2 gtr1-2Δ pho84-2Δ*
PP9	*MATα ade2 his3-532 leu2-3,112 trp1 ura3-1,2 gtr1-3Δ*

[a] The genetic symbols used are as described by Mortimer et al. (23).

ing 11.0 mM P$_i$), and low-P$_i$ (0.22 mM P$_i$) media, His, Leu, and Ura test media, and media for *E. coli*, as well as most genetic and analytical methods, were as described previously (7). Synthetic glucose (SGlu), galactose (SGal) (20), and YP (containing 1% yeast extract and 2% polypeptone [18]) media and the method for rAPase assay of a cell suspension with *p*-nitrophenylphosphate as the substrate (32) were as described previously. The uptake of P$_i$ (33), glucose (18), and sulfate (21) by *S. cerevisiae* cells was assayed as described previously. Sulfate-deficient medium, used in sulfate uptake experiments, was prepared by substitution of 2 g of MgCl$_2$ per liter for MgSO$_4$ · 7H$_2$O in synthetic high-P$_i$ medium. Three closely linked genes were mapped relative to the centromere by using a site-specific recombination system encoded by plasmid pSR1 as described previously (17, 20). *S. cerevisiae* chromosomes were separated by contour-clamped homogeneous electric field gel electrophoresis (CHEF) (9).

Nucleotide sequence accession number. The nucleotide sequence data reported have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D10018.

RESULTS

Nucleotide sequence of the *GTR1* gene. An ORF encoding a protein with an amino acid sequence similar to those of GTP-binding proteins was found in a DNA fragment of *PHO84* DNA (7) (Fig. 1A). This ORF, designated as *GTR1* (*GTP*-binding protein resemblance), encodes a protein consisting of 310 amino acids (Fig. 2). It is located 1,129 bp upstream of *PHO84* and is opposite in transcriptional direction to *PHO84* (Fig. 1A and 2). No TATA box was found in the 5' noncoding region, but there are two long poly(dA-dT) stretches, from −193 to −245 and from −328 to −399 (with 98 thymidine residues in the total region of 125 bp), which may serve as a promoter element for constitutive expression (28). Three copies of a putative polyadenylation sequence, AATAAA, were found at +1033, +1060, and +1189 in the 3' noncoding region (Fig. 2).

The calculated molecular size of the predicted Gtr1 protein is 35.8 kDa. Comparison of its amino acid sequence with those in the EMBL data base (release 15.0, August 1990) by using the GENETYX program (Software Development Co., Tokyo, Japan) revealed some similarities with the yeast *ras*-related protein Ypt1 (12) and human rho12 protein (36). The similarities were confined to the N-terminal half (Fig.
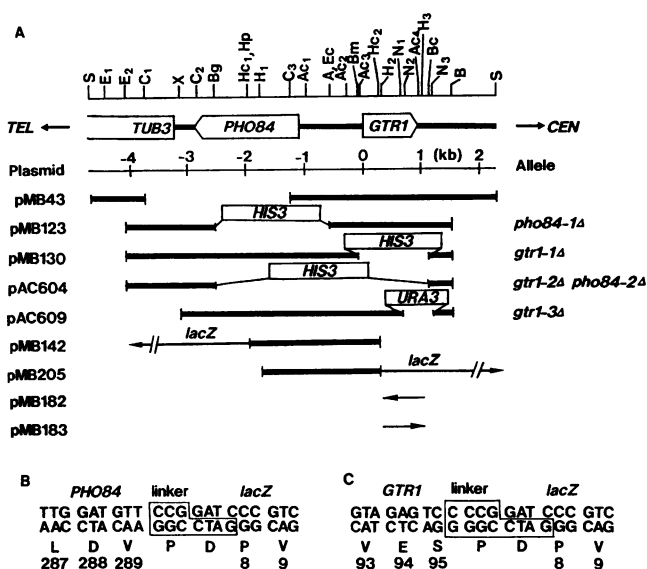

FIG. 1. Restriction maps of *GTR1* DNA and plasmids; structures of the ligation sites in the *PHO84-lacZ* and *GTR1-lacZ* fusion genes. (A) Restriction maps and structures of the original 7-kb *Sau*3AI fragment cloned on plasmid p373, a YCp50-based plasmid (7), and its derivatives. Open arrows indicate the ORFs of *TUB3*, *PHO84*, and *GTR1*. Plasmid pMB43 was constructed by deleting a 2.5-kb *Cla*I$_1$-*Cla*I$_3$ fragment from p373. Plasmid pMB123 was described previously (7). pMB130 was constructed by inserting a 1.7-kb *Bam*HI fragment of the *HIS3* gene prepared from YIp1 (25) into the 1.3-kb *Bsm*I-*Bcl*I gap of pMB15 (7) (detailed procedures for construction not shown). pAC604 was constructed by inserting the 1.7-kb *Bam*HI fragment of the *HIS3* gene into the *Bgl*II-*Bcl*I gap of pMB15. pAC609 was constructed as follows. A hybrid plasmid was constructed by cloning a 4.7-kb *Xho*I-*Bam*HI fragment of the *PHO84-GTR1* DNA into the *Sal*I-*Bam*HI gap of pUC118. Then a 1.2-kb *Hin*dIII fragment containing the *S. cerevisiae URA3* DNA prepared from YEp24 was blunt ended and inserted into the 541-bp *Nsp*(7524)V$_1$-*Nsp*(7524)V$_3$ gap of the *PHO84-GTR1* moiety of the hybrid plasmid. pMB142, bearing a *PHO84-lacZ* fused gene, was constructed previously (7). Plasmid pMB205, a YEp-type plasmid bearing a *GTR1-lacZ* fused gene, was constructed as follows. A 2.1-kb *Hin*dIII$_1$-*Hinc*II$_2$ fragment of the *PHO84-GTR1* DNA, prepared from pMB15, was connected with a 12-bp *Bam*HI linker (Takara Shuzo Co., Kyoto, Japan) at the *Hinc*II$_2$ end and filled in at the *Hin*dIII$_1$ end. Then the fragment was inserted into the *Sma*I-*Bam*HI gap of pMC1587 (8) to form pMB205. The arrows with "*lacZ*" on plasmids pMB142 and pMB205 represent the reading direction of the *lacZ* gene. pMB182 and pMB183 were constructed by cloning the 0.7-kb *Hin*dIII$_2$-*Hin*dIII$_3$ fragment into the *Hin*dIII site of pUC119 and were used to generate ssDNA in the indicated directions. Abbreviations of restriction sites: A, *Apa*I; Ac, *Acc*I; B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; Bm, *Bsm*I; C, *Cla*I; E, *Eco*RI; Ec, *Eco*0109I; H, *Hin*dIII; Hc, *Hinc*II; Hp, *Hpa*I; N, *Nsp*(7524)V; S, *Sau*3AI; X, *Xho*I. Two or more identical restriction sites in the map are distinguished by suffixes. The only *Sau*3AI sites indicated are those at the end of the 7-kb fragment. The recognition sites for *Bcl*I and *Cla*I indicated as Bc and C$_2$ overlap the methylation site of *dam*. (B and C) Nucleotide sequences of the ligation sites of the *PHO84-lacZ* (B) and *GTR1-lacZ* (C) fusion genes in plasmids pMB142 and pMB205. The numbers below the amino acid sequence represent the codon numbers relative to the ATG translation initiation codon of the *PHO84*, *GTR1*, and *lacZ* genes.

3A). The N-terminal half of Gtr1 showed 24.5 and 22.3% identity with those of Ypt1 and rho12 over sequences of 147 and 157 amino acid residues, respectively, and contains the tripartite consensus elements for binding GTP that are
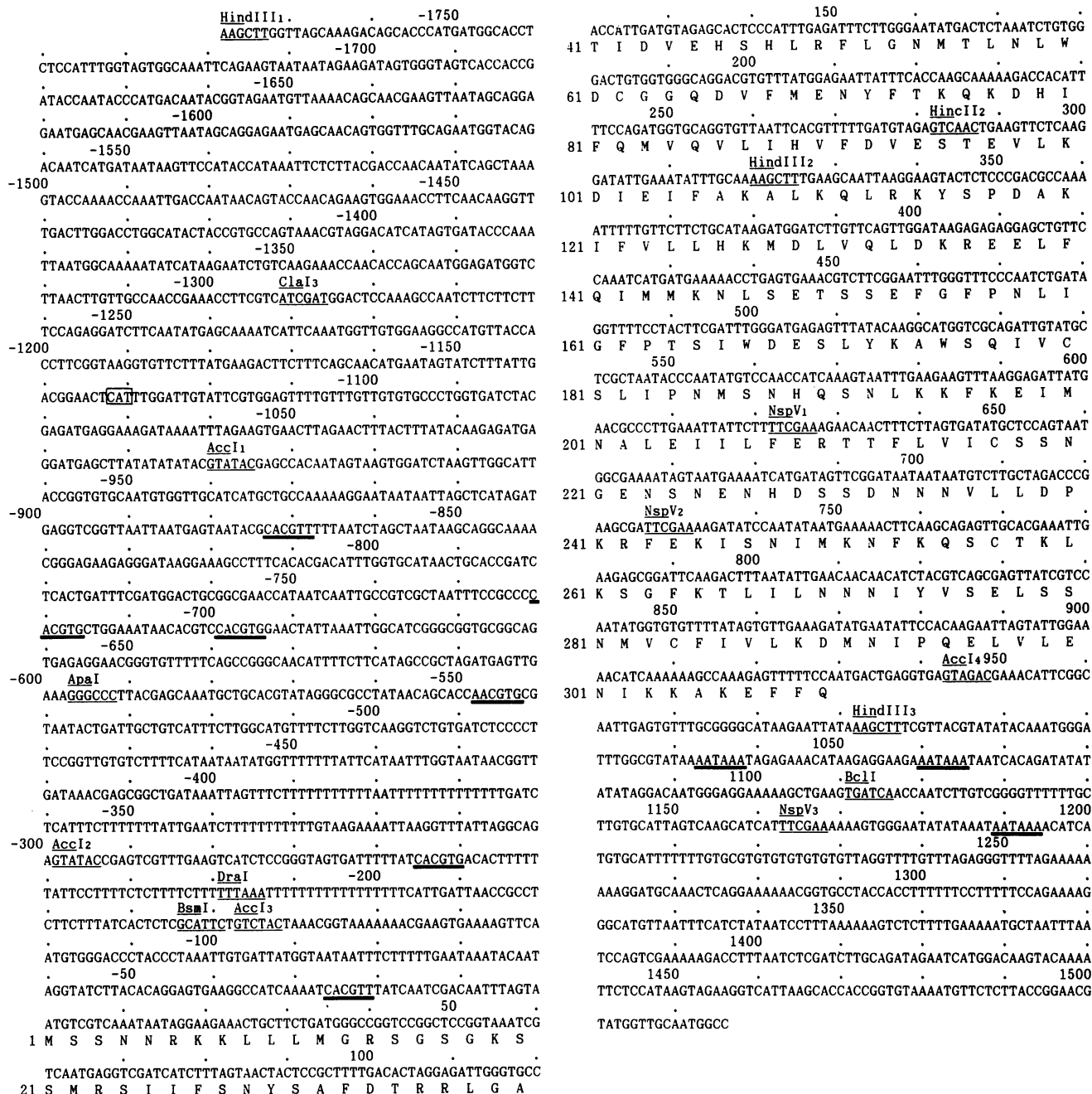
FIG. 2. Nucleotide sequence and deduced ORF of the *GTR1* gene. The indicated amino acid sequence is the longest ORF found in the sequenced region. The ATG initiation codon of the *PHO84* ORF is boxed (indicated as CAT, as the *PHO84* coding frame is in the opposite direction of that of *GTR1*). Sequences homologous to the potential polyadenylation sequence, AATAAA, and the 6-bp Pho4 binding motifs are underlined.

conserved in a large number of GTP-binding proteins (5). These elements have significant identity with those of Ypt1 and rho12 both in spacing and in sequence (Fig. 3B). The widely conserved asparagine residue in the third consensus element is, however, histidine in the Gtr1 protein. In the C-terminal region of a wide variety of GTP-binding proteins, cysteine residues are reported to function in anchorage to the membrane (14). Gtr1 protein does not have such a cysteine residue in its C-terminal region.

Expression of the *GTR1* gene. The upstream region of the

PHO84 ORF contains four direct copies and one reverse copy of the 5'-CACGT(G/T)-3' motif, which is proposed to be the binding site of the Pho4 protein, a positive regulatory factor of the *PHO* system (15). The presence of this structure is consistent with the fact that expression of *PHO84* is regulated by external P_i (7). In this work, we noticed an additional copy of the 6-bp motif, 5'-CACGTT-3', at nucleotide position -26 of the *GTR1* ORF (Fig. 2). Because these six copies of the 6-bp motif are in the 5' upstream region of *GTR1*, transcription of *GTR1* might also be regulated by

A

Gtr1	1	MSSNNRKKLLLMGRSGSGKSSMRSIIFSNYSAFDTRRLGATIDVEHS-HLRFLGNMT
Ypt1	1	MNSEYDYLFKLLLIGNSGVGKSCL-LLRFSDDTYTNDYISTIGVDFKIKTVELDGKTVK
rho12	1	MAAIRKKLVIVGDGACGKTCL-LIVFSK-DQFPEVYVPTVFENYVADIEVDGKQVE

Gtr1	57	LNLWDCGGQDVFMENYRTKQKDHIFQMVQVLIHVFDVVESTEVLKDUEIFAKALKQLRKYS
Ypt1	59	LQIWDTAGQE-----RFRTITSSYYRGSHGIUIMYDVTDQESFNGVKMW---LQEIDRYA
rho12	55	LALWDTAGQE----DYF-DRLRPLSYPDTDVILMCFSIDSPDSLENIIP--EKWTPEVKHFC

Gtr1	117	PDAKII-FVLLHKMDLVQLDKREELFQIMMKNLSETSSEFGFFNLIG...
Ypt1	111	TSTVLKLLVGNKDDL--KDKRVVEYDV-AKEFADANKMPFLETSAL...
rho12	108	ENVPII-ILVGNKKDLRNDEHTRRELAKMKQEPVKPEEGRDMANRIG...

B

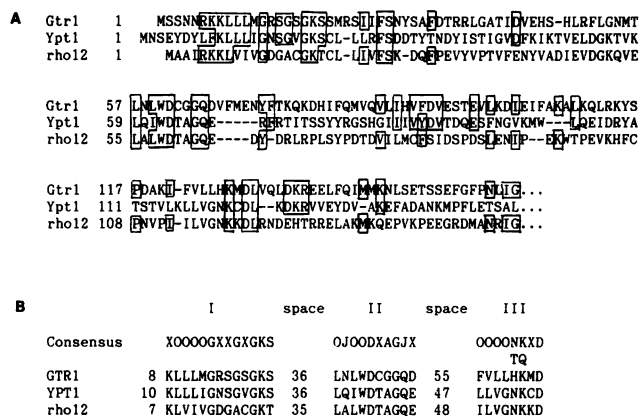|		I	space	II	space	III
Consensus		XOOOOGXXGXGKS		OJOODXAGJX		OOOONKXD TQ
GTR1	8	KLLLMGRSGSGKS	36	LNLWDCGGQD	55	FVLLHKMD
YPT1	10	KLLLIGNSGVGKS	36	LQIWDTAGQE	47	LLVGNKCD
rho12	7	KLVIVGDGACGKT	35	LALWDTAGQE	48	ILVGNKKD

FIG. 3. Homology of Gtr1 protein with GTP-binding proteins. (A) Amino acid sequence similarity of the predicted Gtr1 protein with other GTP-binding proteins. The amino acid sequences of the Ypt1 and rho12 proteins are aligned to obtain maximum fitting with the Gtr1 sequence. Identical residues of the Gtr1 protein and Ypt1 (12) and rho12 (36) proteins are boxed. Numbers at the left indicate positions of the first amino acid residues in the respective lines. Dashes indicate gaps introduced to optimize the alignment. (B) Comparison of the GTP binding motifs in the Gtr1, Ypt1, and rho12 proteins. The consensus sequence is that proposed by Bourne et al. (5). X indicates any amino acid; O and J represent hydrophobic and hydrophilic residues, respectively.

external P_i. To investigate this possibility and to determine the size and direction of the GTR1 transcript, we carried out Northern (RNA) hybridization using two ^{32}P-labeled ssDNA probes derived from pUC119 bearing a 672-bp HindIII$_2$-HindIII$_3$ fragment containing the C-terminal half of the GTR1 ORF (pMB182 and pMB183; Fig. 1A). Total RNA prepared from the wild-type cells, P-28-24C, cultivated in high-P_i medium gave a hybridization band of approximately 1.4 kb (Fig. 4, lane 1) with the pMB182 ssDNA bearing the antisense strand as a probe. The RNA sample from the cells cultivated in low-P_i medium gave a weak 1.6-kb band in addition to the 1.4-kb band (lane 2). These RNAs did not hybridize with the ssDNA probe of plasmid pMB183 (Fig. 1A) bearing the sense strand (data not shown). The hybridization signals were significantly lower than that of URA3 mRNA (Fig. 4, lanes 3 and 4), the transcription level of which is suggested to be the average for yeast genes when
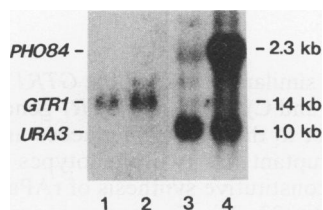

FIG. 4. Detection of the GTR1 transcript by Northern hybridization. Total RNAs were prepared from cells of the wild-type strain P-28-24C grown on nutrient high-P_i (lanes 1 and 3) or low-P_i (lanes 2 and 4) medium. After electrophoresis, the gel was blotted onto a nylon membrane and the filter was hybridized with a ^{32}P-labeled 0.7-kb HindIII$_2$-HindIII$_3$ antisense ssDNA of GTR1 (pMB182; lanes 1 and 2) or pMB201 bearing the GTR1, PHO84, and URA3 DNAs (lanes 3 and 4). Samples (10 μg each) of total RNA were used. The specific activities of probes were 10^8 cpm/μg of DNA. 25S and 18S rRNAs, visualized by staining with ethidium bromide (not shown), were used as size markers as described by Philippsen et al. (26).

the cells are cultivated in nutrient medium (1). The 1.4-kb transcript is consistent with the GTR1 ORF in size and reading direction. The amounts of the 1.4-kb mRNA in cells cultivated in high-P_i and low-P_i media indicated that the GTR1 gene is transcribed at an extremely low level even in low-P_i medium, independent of external P_i concentration. We do not know the significance of the 1.6-kb transcript.

To further examine regulation of GTR1 expression by P_i concentration, we studied the expression of a GTR1-lacZ fusion gene constructed by ligation of the 2,062-bp HindIII$_1$-HincII$_2$ fragment of the GTR1 DNA to lacZ DNA. This DNA construct has a 1,778-bp upstream region and a 284-bp ORF region encoding the N-terminal region of the Gtr1 protein. Since no β-galactosidase activity was detected in PP2 cells harboring a YCp-type (low-copy-number) plasmid bearing the GTR1-lacZ fused gene (data not shown), we examined the same DNA construct on a YEp multicopy plasmid, pMB205 (Fig. 1A and C). We determined the β-galactosidase activities of PP2 cells harboring plasmid pMB205 in the early-stationary-phase culture showing ca. 1 U of optical density at 660 nm (OD$_{660}$) in high-P_i and low-P_i versions of Leu test medium. The activities were 21.9 ± 5.6 U/mg of protein (mean and standard deviation for triplicate determinations) in low-P_i medium and 23.6 ± 4.2 U/mg of protein in high-P_i medium. These results indicated that the level of GTR1 expression is very low, as observed by mRNA analysis (Fig. 4), and may not be regulated by the external P_i concentration.

Slow growth but constitutive rAPase synthesis of gtr1 disruptant cells. To study the cellular function of GTR1, we constructed deletion mutants of the GTR1 and PHO84 genes by transformation of a diploid strain, KA31 (his3/his3 ura3/ura3 GTR1$^+$/GTR1$^+$), as described by Rothstein (27). For this analysis, we used BamHI-EcoRI$_2$ fragments prepared from plasmids pMB123, pMB130, and pAC604 (Fig. 1A), bearing HIS3 DNA, and the BamHI-EcoRI fragment (the EcoRI site is on the vector plasmid) prepared from plasmid pAC609 (Fig. 1A) bearing URA3 DNA. Several His$^+$ or Ura$^+$ transformants were picked at random and sporulated, and four-spored asci were dissected. We confirmed that these disruptions occurred as expected, by Southern blot analysis of genomic DNA of the transformants digested with appropriate restriction enzymes (data not shown). On tetrad analysis, one of the His$^+$ diploids, KA31-d1 (gtr1-1Δ), constructed with the BamHI-EcoRI$_2$ fragment of pMB130, showed a 2 His$^+$:2 His$^-$ segregation of the 12 asci tested. Similar 2 Ura$^+$:2 Ura$^-$ segregation was observed in eight asci from one of the Ura$^+$ transformants, KA31-d3 (gtr1-3Δ), constructed with the BamHI-EcoRI fragment of pAC609. The His$^+$ and Ura$^+$ segregants grew slowly, always forming tiny colonies on the dissection plate (data not shown), whereas the His$^-$ or Ura$^-$ segregants always formed colonies of normal size. The doubling times of the His$^+$ and Ura$^+$ segregants were about twice as long as those of the His$^-$ and Ura$^-$ segregants in both low-P_i and high-P_i media (Fig. 5a and b). These results indicated that GTR1 function is important, but not essential, for cell growth. Interestingly, His$^+$ segregants could not grow at 15°C but could grow at 30°C (data not shown).

We found that cells carrying the gtr1-1Δ null allele synthesized rAPase constitutively. The rAPase-constitutive phenotype, determined by a staining method (31), showed a segregation pattern of 2+:2− ratio on YPAD medium in the 12 asci of KA31-d1 tested, and the rAPase$^+$ clones all showed the His$^+$ phenotype. The rAPase activity of haploid
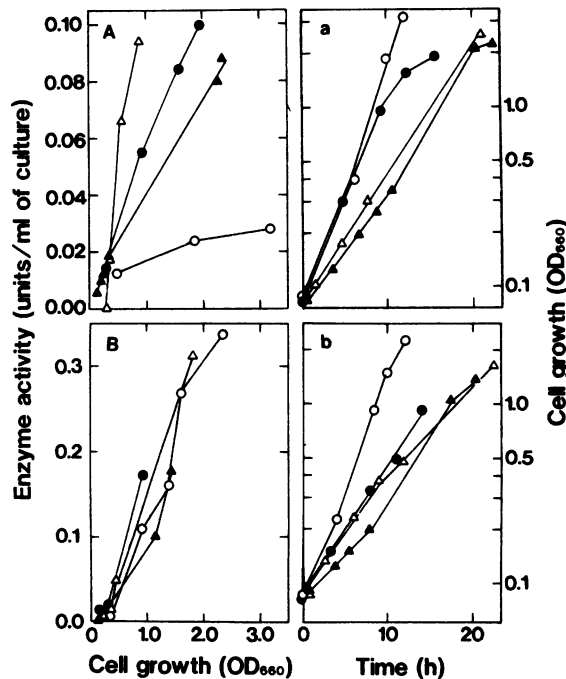
FIG. 5. Time courses of acid phosphatase synthesis in wild-type, *gtr1-3Δ, pho84-1Δ*, and *gtr1-2Δ pho84-2Δ* strains. Cells cultivated in nutrient high-P$_i$ medium with shaking at 30°C for 24 h were harvested, washed, and suspended in the same volume of sterilized water. Volumes of 1 ml of the cell suspension were inoculated into 100 ml of synthetic complete high-P$_i$ (A and a) or low-P$_i$ (B and b) medium and shaken at 30°C. Acid phosphatase activity (A and B) as a function of cell growth (a and b; optical density at 660 nm [OD$_{660}$] of the cultures) was determined with intact cell suspension as an enzyme source. Symbols and strains: ○, PP2 (*GTR1$^+$ PHO84$^+$*); ▲, PP9 (*gtr1-3Δ PHO84$^+$*); ●, MB203 (*GTR1$^+$ pho84-1Δ*); △, PP4 (*gtr1-2Δ pho84-2Δ*).

gtr1-3Δ clones segregated from KA31-d3 was higher than that of *GTR1$^+$* segregants in repressed conditions (data for typical clones are shown in Fig. 5A). Thus, *gtr1* disruption increased synthesis of rAPase under repressed conditions. All of the disruptants, however, showed a level of enzyme activity similar to that of the wild-type cells in derepressed conditions (Fig. 5B). Regardless of the external P$_i$ concentration, the growth rate of the *gtr1* disruptants was half that of the wild-type cells. In contrast, the growth rate of the *pho84-1Δ* mutant was the same as that of wild-type cells in high-P$_i$ medium. These results suggest that the decreased growth rate of the double mutant was due to *gtr1* disruption, not to *pho84* disruption.

Defect in P$_i$ uptake by *gtr1* disruptant cells. The low-K_m P$_i$ transport system is repressed by P$_i$ (29). Therefore, we investigated the P$_i$ transport activity of the *gtr1* disruptant. We found that MB200 cells (the *gtr1-1Δ* disruptant) grown in synthetic low-P$_i$ medium had significantly lower P$_i$ uptake activity than did *GTR1$^+$* cells (strain PP2) but substantially higher activity than did *pho84-1Δ* cells (strain MB203) (Fig. 6A). When the cells were cultivated in synthetic high-P$_i$ medium, all of these strains showed severely repressed P$_i$ uptake activity (data not shown). Since the GTP-binding protein may have a global function, we examined whether the uptake of glucose (an energy source) and sulfate (an essential element) was affected by *gtr1* disruption. The glucose and sulfate transport activities of the *gtr1* disruptant
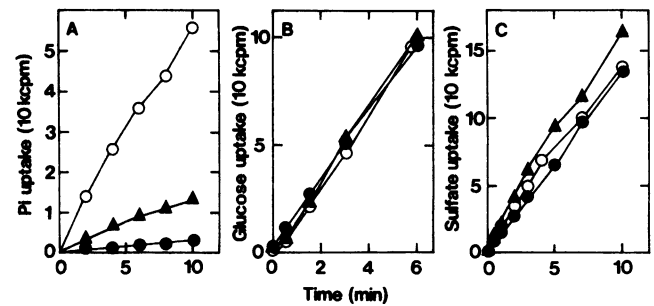

FIG. 6. P$_i$, glucose, and sulfate uptake by cells of the *gtr1* disruptant. (A) Defect in P$_i$ uptake of *gtr1-1Δ* cells. Cells to be tested were cultivated in YPAD to the stationary phase, washed, suspended in the same volume of sterilized water, inoculated into synthetic low-P$_i$ medium supplemented with appropriate nutrients, and shaken at 30°C. The cells were collected when they reached an OD$_{660}$ of 1, washed, and inoculated into synthetic low-P$_i$ medium at a cell concentration giving an OD$_{660}$ of 0.1. The radioactivity of the medium was adjusted to 3.4 × 10^5 cpm/ml with ^{32}P$_i$. Samples were taken from the reaction mixture at appropriate intervals, filtered through a nitrocellulose membrane filter, and washed. Then radioactivity on the filter was counted in a liquid scintillation counter (model LS 6000IC; Beckman Instrument, Inc., Fullerton, Calif.). The amount of P$_i$ absorbed by the cells was expressed as counts per minute of ^{32}P radioactivity per milliliter of cell suspension of OD$_{660}$ = 0.1. (B) Normal glucose uptake by *gtr1-1Δ* cells. Cells cultivated in YPAD to the stationary phase were suspended in the same volume of sterilized water, 1 ml of the suspension was added to 100 ml of YP medium supplemented with 0.05% glucose, and the cells were shaken for 14 h at 30°C. The cells were harvested, washed with 0.1 M potassium phosphate buffer (pH 6.5) at room temperature, and resuspended in the same buffer at a cell concentration of about OD$_{580}$ = 15 as described previously (18). The suspension was mixed with an appropriate amount of radioactive glucose (D-[U-^{14}C]glucose; Amersham International Plc, Amersham, England) and incubated at 30°C. Samples of 80 μl were taken at appropriate intervals and filtered. The radioactivity on the filter was measured by the same procedure as for assay of P$_i$ absorption. (C) Normal uptake of sulfate by *gtr1-1Δ* cells. Cells cultivated in synthetic high-P$_i$ medium to the stationary phase were washed twice with sulfate-deficient medium, resuspended in the same volume of sulfate-deficient medium, and shaken for 4 h at 30°C. The cells were harvested and resuspended in sulfate-deficient medium at a cell concentration of OD$_{660}$ = 1.0. The suspension was mixed with an appropriate amount of radioactive Na$_2$SO$_4$ (E. I. du Pont de Nemours & Co. Inc., Wilmington, Del.) and incubated at 30°C. Samples of 100 μl were taken at appropriate intervals, and radioactivity absorbed by the cells was determined as for assays of P$_i$ and glucose uptake. Symbols and strains: ○, PP2 (*GTR1$^+$ PHO84$^+$*); ▲, MB200 (*gtr1-1Δ PHO84$^+$*); ●, MB203 (*GTR1$^+$ pho84-1Δ*).

were, however, similar to those of the *GTR1$^+$* and *pho84-1Δ* strains (Fig. 6B and C). Thus, the *GTR1* gene contributes to P$_i$ uptake but not to the uptake of glucose and sulfate.

The *gtr1* disruptant has two phenotypes resembling the *pho84* mutant, constitutive synthesis of rAPase and reduced P$_i$ uptake activity (33, 34). Furthermore, we found that the *pho84* mutant is resistant to 4.5 mM arsenate, which inhibits growth of wild-type *S. cerevisiae* in nutrient medium (our unpublished results). The *gtr1* disruptants were also resistant to 4.5 mM arsenate. In contrast, the *pho80* and *pho85* mutations did not confer resistance to this concentration of arsenate, although these mutations resulted in the rAPase-constitutive phenotype.

The rAPase-constitutive phenotype of the *gtr1* disruptant might be caused by a defect in P$_i$ uptake in one of the following ways: (i) there may be a disruption of the promoter
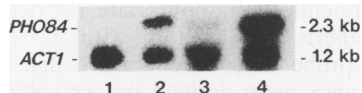
FIG. 7. Detection of *PHO84* transcript in the wild-type and *gtr1* disruptant by Northern hybridization. Samples of 10 μg of total RNAs prepared from cells of strains PP2 (*GTR1*$^+$; lanes 1 and 2) and MB200 (*gtr1-1*Δ; lanes 3 and 4) grown on nutrient high-P$_i$ (lanes 1 and 3) or low-P$_i$ (lanes 2 and 4) medium were charged in slots. The ^{32}P-labeled 564-bp *Bgl*II-*Xho*I fragment of *PHO84* DNA and a 1.0-kb *Hind*III-*Xho*I fragment of *S. cerevisiae* encoding the *ACT1* gene were used as probes for detection of the *PHO84* and *ACT1* transcripts, respectively. The specific activities of probes were 10^8 cpm/μg of DNA. 25S and 18S rRNAs visualized by staining with ethidium bromide were used as size markers as described by Philippsen et al. (26).

region of *PHO84* caused by insertion of *HIS3* or *URA3* DNA in construction of the *gtr1-1*Δ and *gtr1-3*Δ alleles; (ii) the Gtr1 protein may be necessary for transcription of *PHO84*; or (iii) the Gtr1 protein may be important for function of the Pho84 transporter. To examine these possibilities, we introduced plasmid pMB43 (Fig. 1A) bearing the *URA3* marker and intact *GTR1* ORF, but not *PHO84*, into *gtr1-1*Δ *ura3* cells (strain MB200). The finding that Ura$^+$ transformants, isolated at random, showed the wild-type phenotypes for both cell growth and rAPase synthesis (data not shown) excluded the first possibility. The other two possibilities were examined by studies on the *PHO84* transcript in the *gtr1-1*Δ disruptant. Total RNA prepared from *GTR1*$^+$ (PP2) and *gtr1-1*Δ (MB200) cells cultivated for 16 h (stationary phase) in nutrient low-P$_i$ or high-P$_i$ medium was examined by Northern hybridization with a ^{32}P-labeled 564-bp *Bgl*II-*Xho*I fragment of the *PHO84* DNA (Fig. 1A) as a probe. The RNA of *GTR1*$^+$ cells gave a hybridization band when cultivated in low-P$_i$ medium but not when cultivated in high-P$_i$ medium (Fig. 7, lanes 1 and 2). We detected weak *PHO84* transcription in the *gtr1-1*Δ disruptant cells cultivated in high-P$_i$ medium, but significant repression of *PHO84* transcription by P$_i$ in the medium was still observed.

We then examined the effect of *gtr1* disruption on expression of a *PHO84-lacZ* fusion gene. Strains PP2 (*GTR1*$^+$ *PHO84*$^+$), MB200 (*gtr1-1*Δ *PHO84*$^+$), MB203 (*GTR1*$^+$ *pho84-1*Δ), and PP4 (*gtr1-2*Δ *pho84-2*Δ) were transformed with the *URA3* marked YCp-type plasmid, pMB142 (7), bearing a *PHO84-lacZ* fusion gene (Fig. 1A and B). The promoter region of this fusion gene should be the same as that of *PHO84*. The Ura$^+$ transformants were cultivated in synthetic low-P$_i$ and high-P$_i$ media to the stationary phase, and then their β-galactosidase activities were determined (Table 2). Although β-galactosidase activities differed over a twofold range in the different host strains, activities were

TABLE 2. β-Galactosidase activities in transformants harboring a YCp plasmid, pMB142, bearing the *PHO84-lacZ* fused gene

Strain	Relevant genotype	β-Galactosidase activity[a] (U/mg of protein) in:	
		High P$_i$	Low P$_i$
PP2	*GTR1*$^+$ *PHO84*$^+$	3.3 ± 0.3	830 ± 25
MB200	*gtr1-1*Δ *PHO84*$^+$	455 ± 28	525 ± 73
MB203	*GTR1*$^+$ *pho84-1*Δ	1050 ± 69	1510 ± 95
PP4	*gtr1-2*Δ *pho84-2*Δ	509 ± 20	784 ± 54

[a] Values are means for triplicate determinations with standard deviations.

much higher in mutants with a disruption of the *GTR1* or/and *PHO84* gene than in the wild-type cells in high-P$_i$ medium. Activities were similar to those of the wild-type cells in low-P$_i$ medium. These observations are consistent with reduced P$_i$ transport activity in the *gtr1* disruptant, resulting in derepression of genes normally repressed by P$_i$, as observed in the *pho84* mutant (33). Although we observed only a trace of the *PHO84* transcript in the *gtr1-1*Δ cells in high-P$_i$ medium (Fig. 7), as in the *pho84* mutant (7), the low level of intracellular P$_i$ allowed transcription of *PHO84*, as well as *PHO5*, even in high-P$_i$ medium (Fig. 5). This trace amount of *PHO84* transcription resulted in significant activity of β-galactosidase from the *PHO84-lacZ* fusion gene (Table 2). We do not know the reason for the high enzyme activity. However, we conclude from these results that the Gtr1 protein is involved in the P$_i$ transport system in collaboration with the Pho84 transporter but is not directly involved in *PHO84* transcription.

Relative locus order of *PHO84* and *GTR1* on chromosome XIII. Sequence analysis revealed that the *GTR1*, *PHO84*, and *TUB3* (7) loci are located side by side in that order on the left arm of chromosome XIII (Fig. 1). To determine whether *GTR1* or *TUB3* is proximal to the centromere, we used the mapping method with a site-specific recombination system encoded by plasmid pSR1 (17, 20). Two haploid strains with inserts of a 2.1-kb *Sal*I fragment (RS fragment) bearing the specific recombination site of the pSR1 plasmid, obtained by using two YIp5-based plasmids marked with the *URA3* gene, were constructed from NBW5 by site-directed integration of the RS fragment at the *ura3* locus as described previously (20). The only difference between these two strains was that the inserted RS fragments were in opposite directions. These two strains were further transformed to the His$^+$ phenotype by insertion of a *Eco*RI-*Bam*HI fragment of pMB147 (Fig. 8A), which was constructed by ligation of the 2.1-kb RS fragment at the *Xho*I site of pMB93 constructed previously (7). The *Bam*HI-*Eco*RI fragment of pMB147 has the structure [whole *GTR1* DNA]::[5' half of the *PHO84* DNA]::[RS fragment]::[*HIS3* DNA]::[portion of the *TUB3* DNA]. This fragment should be inserted into the relevant region of chromosome XIII of the two Ura$^+$ transformant clones of NBW5. The His$^+$ Ura$^+$ transformants were then transformed with a plasmid, pHM153, bearing the *R* gene encoding specific recombinase of pSR1 and marked with the *LEU2* gene (20). The two resultant His$^+$ Ura$^+$ Leu$^+$ transformants, MB195 and MB197, were cultured overnight in SGal medium supplemented with appropriate nutrients to allow expression of the *R* gene connected with the *GAL1* promoter. They were then diluted appropriately and spread on SGlu plates supplemented with the nutrients. Several colonies that developed on the plates were isolated at random, and their chromosomal patterns were examined by CHEF. Three of five clones from MB195 examined showed new bands (Fig. 8B; only one of the three clones with new bands is shown). In contrast, none of five clones from MB197 examined showed a new band. If the RS fragment at the *ura3* locus on chromosome V is in the same direction relative to the centromere as that of the RS fragment in the *GTR1-PHO84-TUB3* region on chromosome XIII, interchromosomal recombination catalyzed by the R protein should give two monocentric recombinant chromosomes, whereas if the RS sites in the *ura3* locus and the *GTR1-PHO84-TUB3* region are inserted in opposite directions relative to the respective centromere, the specific recombination should give one acentric and one dicentric chromosome. In other words, no recombinant chromosomes should be detected if the config-
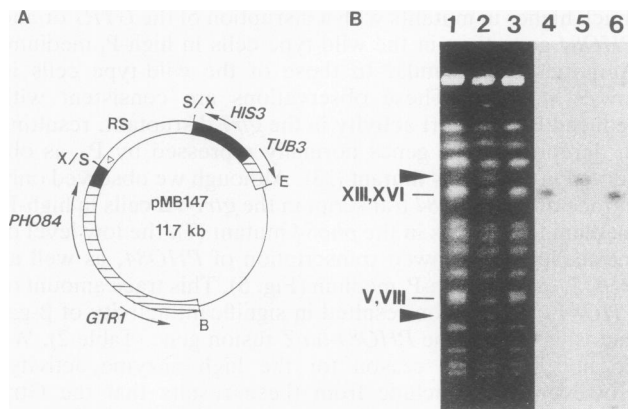
FIG. 8. Recombinant chromosome formation between chromosomes V (at the URA3 locus) and XIII (in the GTR1-PHO84-TUB3 region). (A) Structure of plasmid pMB147. The plasmid was constructed by ligation of the 2.1-kb RS fragment (20) into the XhoI gap of pMB93 (7). The triangle in the RS fragment shows the approximate site of the 58-bp specific recombination sequence (20). Abbreviations for restriction sites are as described in the legend to Fig. 1. (B) Detection of recombinant chromosomes by CHEF and Southern hybridization. Chromosomal samples were from MB195 cells without cultivation in SGal medium (lanes 1 and 4), from a clone showing the recombinant chromosomes after cultivation in SGal medium and curing of the pHM153 plasmid (lanes 2 and 5), and from a clone of MB197 cultivated in SGal medium (lanes 3 and 6). The gel was blotted onto a nylon filter and hybridized with the ^{32}P-labeled 4.7-kb BamHI-XhoI fragment of pMB15 containing the GTR1-PHO84 DNA as a probe. Arrowheads show the sites of new chromosome bands. Bands of chromosomes V and XIII are also indicated.

urations of the RS fragments are opposite, because these dicentric and acentric chromosomes should be lethal to the cells. Thus, the two RS fragments inserted into the ura3 locus and the GTR1-PHO84-TUB3 region in MB195 should be in the same direction relative to the respective centromere. On the basis of the direction of the RS fragment inserted at the ura3 locus, determined by Southern hybridization of the genomic DNA digested with appropriate restriction enzymes and probe DNAs (details not shown), and the fact that the transcriptional direction of the URA3 locus is toward its centromere (16), we concluded that the order of these three genes is telomere-TUB3-PHO84-GTR1-CEN_{XIII} (Fig. 1A).

Because several chromosomes comigrated on CHEF (for example, chromosomes V and VIII and chromosomes XIII and XVI comigrated in some of our strains), it was possible that one or two recombinant chromosomes comigrated with another chromosome(s). To overcome this difficulty, we blotted the chromosomal bands on the agarose gel onto a nylon membrane filter and hybridized them with a ^{32}P-labeled 4.7-kb BamHI-XhoI DNA fragment bearing the GTR1 and PHO84 ORFs but not the TUB3 ORF (Fig. 1A) as a probe. We found that the probe hybridized with the one of the two new chromosome bands that migrated behind chromosome XIII of MB195 cells grown in galactose medium (Fig. 8B, lanes 2 and 5). When the chromosome bands of the same transformant of MB195 without cultivation in SGal medium (lanes 1 and 4) and those of MB197 cells grown in SGal medium (lanes 3 and 6) were examined with the same probe DNA, hybridization signals were observed on the band of chromosome XIII. We examined the genomic DNAs of these MB195 and MB197 cells grown in SGal and SGlu

media by treatments with appropriate restriction enzymes and then Southern hybridization with a ^{32}P-labeled RS fragment as a probe. Results confirmed that the recombinant chromosomes were derived by recombination at the two RS-specific recombination sites on the fragments inserted at the ura3 and GTR1-PHO84-TUB3 loci (details not shown).

The molecular size of chromosomes V was calculated to be 590 kb (23), and the distance between URA3 and the telomere was calculated to be approximately 140 kb (17). The size of the shorter recombinant chromosome was estimated to be 500 kb from its migration distance on CHEF agar (Fig. 8B), i.e., 90 kb shorter than the original chromosome V. These findings, and the fact that the distance from the right-arm telomere to the URA3 locus of chromosome V is almost 450 kb (17), indicate that the GTR1-PHO84-TUB3 cluster is located at a site 50 kb from the left-arm telomere of chromosome XIII.

DISCUSSION

Three classes of mutations derepress rAPase synthesis in high-P_i medium (24). One class includes mutations in regulatory genes such as PHO4(Con), pho80, and pho85. The second is a promoter mutation of PHO5, i.e., the PHO83 mutation, which is caused by insertion of a Ty element into the 5' noncoding region of PHO5 (30). The third class includes the pho84 mutation, in which derepression of rAPase in high-P_i medium is thought to be due to a deficiency in intracellular P_i because of a defect in a P_i transport system. The gtr1 disruptant showed the same phenotypes as did the pho84 mutant—synthesis of rAPase in high-P_i medium (Fig. 5), arsenate resistance, and a defect in the P_i uptake system (Fig. 6)—and so falls in the same class.

The amino acid sequence of the N-terminal region of the Gtr1 protein, deduced from the nucleotide sequence, is homologous to those of ras and ras-related proteins (Fig. 3). The widely conserved asparagine (or threonine) residue in the third consensus region of GTP-binding proteins is, however, replaced by histidine in the Gtr1 protein. An H-ras mutant protein that also has histidine in place of this conserved asparagine shows reduced affinity to GTP (11). Thus, the GTP-binding activity of the Gtr1 protein may also be weak.

The gtr1 disruptant was found to have a defect in uptake of P_i but not of glucose and sulfate (Fig. 6). This observation suggests the following alternative roles for the Gtr1 protein. One possibility is that it is required for modulation of the PHO84-encoded P_i transporter. The G protein is known to be obligatory for opening the ionic channel or to have a modulatory effect with some other stimulus such as the membrane potential, which is obligatory for channel opening (6). The rAPase activity of the gtr1 pho84 double disruptant in high-P_i medium was higher than that of the pho84 or gtr1 single disruptant (Fig. 5). This finding is consistent with the idea that the Gtr1 protein is involved in the Pho84 P_i transporter system and also in the other P_i transport system. The gtr1 mutants showed a slow growth phenotype in both low-P_i and high-P_i media, whereas the pho84 mutants showed a slow-growth phenotype only in low P_i medium (Fig. 5). If the slow growth phenotype of the gtr1 mutant in high-P_i medium is caused by reduction of P_i transport activity, the Gtr1 protein might also be involved in the low affinity P_i transport system, which is thought to function in high-P_i medium (29).

A second possible role of the Gtr1 protein is in intracellular localization of the P$_i$ transporter, because some GTP-binding proteins have been shown to have this function (2). However, even if Gtr1 is involved in translocation of protein, it is not involved in a general secretion system, because *gtr1* disruption was not lethal.

There are six copies of a Pho4 binding motif between the initiation codons of the *GTR1* and *PHO84* genes. The alignment of these six copies of the 6-bp motif seems not to be significantly different for the *PHO84* and *GTR1* genes. However, the *GTR1* gene is under relaxed control or no control by P$_i$, whereas expression of the *PHO84* gene is strictly regulated by P$_i$ (Fig. 4 and 7). In addition, the amount of *GTR1* mRNA was extremely low, whereas the *PHO84* transcript was relatively abundant (Fig. 4). These differences might be caused by the existence of long poly(dA-dT) sequences in the common 5′ noncoding region of the *PHO84* and *GTR1* genes, but located closer to the *GTR1* ORF. These sequences are known to activate transcription constitutively, a longer one being more effective (4, 28). The more closely adjacent poly(dA-dT) sequences might release *GTR1* expression from strict regulation by the *PHO* regulatory system preferentially.

ACKNOWLEDGMENTS

REFERENCES

1. **Bach, M.-L., F. Lacroute, and D. Botstein.** 1979. Evidence for transcriptional regulation of orotidine-5′-phosphate decarboxylase in yeast by hybridization of mRNA to the yeast structural gene cloned in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **76:**386–390.
2. **Balch, W. E.** 1990. Small GTP-binding proteins in vesicular transport. Trends Biochem. Sci. **15:**473–477.
3. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **56:**779–827.
4. **Berger, S. L., W. D. Cress, A. Cress, S. J. Triezenberg, and L. Guarente.** 1990. Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. Cell **61:**1199–1208.
5. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature (London) **349:**117–127.
6. **Brown, A. M.** 1990. Ionic channels and their regulation by G protein subunits. Annu. Rev. Physiol. **52:**197–213.
7. **Bun-ya, M., M. Nishimura, S. Harashima, and Y. Oshima.** 1991. The *PHO84* gene of *Saccharomyces cerevisiae* encodes an inorganic phosphate transporter. Mol. Cell. Biol. **11:**3229–3238.
8. **Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou.** 1983. β-Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100:**293–308.
9. **Chu, G., D. Vollrath, and R. W. Davis.** 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science **234:**1582–1585.
10. **Clarke, L., and J. Carbon.** 1978. Functional expression of cloned yeast DNA in *Escherichia coli*: specific complementation of argininosuccinate lyase (*argH*) mutations. J. Mol. Biol. **120:**517–532.
11. **Der, C. J., B.-T. Pan, and G. M. Cooper.** 1986. *ras*^{H} mutants deficient in GTP binding. Mol. Cell. Biol. **6:**3291–3294.
12. **Gallwitz, D., C. Donath, and C. Sander.** 1983. A yeast gene encoding a protein homologous to the human c-*has/bas* proto-

13. **Gibbs, J. B., and M. S. Marshall.** 1989. The *ras* oncogene—an important regulatory element in lower eucaryotic organisms. Microbiol. Rev. **53:**171–185.
14. **Hancock, J. F., A. I. Magee, J. E. Childs, and C. J. Marshall.** 1989. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell **57:**1167–1177.
15. **Hayashi, N., and Y. Oshima.** 1991. Specific *cis*-acting sequence for *PHO8* expression interacts with PHO4 protein, a positive regulatory factor, in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**785–794.
16. **Jinks-Robertson, S., and T. D. Petes.** 1986. Chromosomal translocations generated by high-frequency meiotic recombination between repeated yeast genes. Genetics **114:**731–752.
17. **Kawasaki, H., H. Matsuzaki, R. Nakajima, and Y. Oshima.** 1991. The *PHO80/TUP7* locus in *Saccharomyces cerevisiae* is on the left arm of chromosome XV: mapping by chromosome engineering. Yeast **7:**859–865.
18. **Lewis, D. A., and L. F. Bisson.** 1991. The *HXT1* gene product of *Saccharomyces cerevisiae* is a new member of the family of hexose transporters. Mol. Cell. Biol. **11:**3804–3813.
19. **Marinus, M. G., and N. R. Morris.** 1975. Pleiotropic effects of a DNA adenine methylation mutation (*dam-3*) in *Escherichia coli* K12. Mutat. Res. **28:**15–26.
20. **Matsuzaki, H., R. Nakajima, J. Nishiyama, H. Araki, and Y. Oshima.** 1990. Chromosome engineering in *Saccharomyces cerevisiae* by using a site-specific recombination system of a yeast plasmid. J. Bacteriol. **172:**610–618.
21. **McCready, R. G. L., and G. A. Din.** 1974. Active sulfate transport in *Saccharomyces cerevisiae*. FEBS Lett. **38:**361–363.
22. **Milburn, M. V., L. Tong, A. M. DeVos, A. Brunger, Z. Yamaizumi, S. Nishimura, and S.-H. Kim.** 1990. Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. Science **247:**939–945.
23. **Mortimer, R. K., D. Schild, C. R. Contopoulou, and J. A. Kans.** 1989. Genetic map of *Saccharomyces cerevisiae*, edition 10. Yeast **5:**321–403.
24. **Oshima, Y.** 1982. Regulatory circuits for gene expression: the metabolism of galactose and of phosphate, p. 159–180. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast Saccharomyces: metabolism and gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Parent, S. A., C. M. Fenimore, and K. A. Bostian.** 1985. Vector systems for the expression, analysis and cloning of DNA sequences in *S. cerevisiae*. Yeast **1:**83–138.
26. **Philippsen, P., M. Thomas, R. A. Kramer, and R. W. Davis.** 1978. Unique arrangement of coding sequences for 5S, 5.8S, 18S and 25S ribosomal RNA in *Saccharomyces cerevisiae* as determined by R-loop and hybridization analysis. J. Mol. Biol. **123:**387–404.
27. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101:**202–211.
28. **Struhl, K.** 1985. Naturally occurring poly(dA-dT) sequences are upstream promoter elements for constitutive transcription in yeast. Proc. Natl. Acad. Sci. USA **82:**8419–8423.
29. **Tamai, Y., A. Toh-e, and Y. Oshima.** 1985. Regulation of inorganic phosphate transport systems in *Saccharomyces cerevisiae*. J. Bacteriol. **164:**964–968.
30. **Toh-e, A., Y. Kaneko, J. Akimaru, and Y. Oshima.** 1983. An insertion mutation associated with constitutive expression of repressible acid phosphatase in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **191:**339–346.
31. **Toh-e, A., and Y. Oshima.** 1974. Characterization of a dominant, constitutive mutation, *PHOO*, for the repressible acid phosphatase synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. **120:**608–617.
32. **Toh-e, A., Y. Ueda, S. Kakimoto, and Y. Oshima.** 1973. Isolation and characterization of acid phosphatase mutants in *Saccharomyces cerevisiae*. J. Bacteriol. **113:**727–738.
33. **Ueda, Y., and Y. Oshima.** 1975. A constitutive mutation, *phoT*, of the repressible acid phosphatase synthesis with inability to

transport inorganic phosphate in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **136:**255–259.

34. **Ueda, Y., A. Toh-e, and Y. Oshima.** 1975. Isolation and characterization of recessive, constitutive mutations for repressible acid phosphatase synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. **122:**911–922.

35. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.

36. **Yeramian, P., P. Chardin, P. Madaule, and A. Tavitian.** 1987. Nucleotide sequence of human rho cDNA clone 12. Nucleic Acids Res. **15:**1869.

37. **Yoshida, K., N. Ogawa, and Y. Oshima.** 1989. Function of *PHO* regulatory genes for repressible acid phosphatase synthesis in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **217:**40–46.